Exploration of putative endothelial progenitor cells in cells mobilised by granulocyte colony stimulating factor.

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Declaration

I declare that the work presented in this is my own unless otherwise stated. The material presented in the thesis has not been submitted for any other degree or professional qualification.

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Abstract

Identification of circulating endothelial progenitor cells (EPC), which share a common precursor with haematopoietic progenitor cells (HPC), the haemangioblast, has generated considerable interest in isolating, characterising and expanding them for clinical use. There is no definitive phenotype of EPC but there appears to be two main types, the CD14+ monocyte derived early EPC and the CD34+ derived endothelial outgrowth cell (EOC). These populations differ in their proliferative potential and appear quite distinct, though their function in vasculogenesis is debated. Potential sources of such cells include peripheral blood, bone marrow and umbilical cord blood. Peripheral blood stem cell (PBSC) transplantation is the paradigm of adult stem cell therapy. It relies on the use of granulocyte colony stimulating factor (G-CSF) to mobilise HPC from the bone marrow. With EPC and HPC sharing common origins it has been suggested that G-CSF mobilised peripheral blood would be an excellent source of EPC for clinical use. This work centres on the identification of EPC in G-CSF mobilised peripheral blood.

G-CSF mobilised and non mobilised peripheral blood samples were obtained at a number of time points from autologous and allogeneic donors referred for PBSC collection using G-CSF, given alone or sequentially with chemotherapy. We consistently demonstrated marked reductions in early EPC following the administration of G-CSF, using standard commercially available colony assays (CFU-EPC), which is reversible within a month of G-CSF treatment. We have also been unable to generate EOC from mobilised blood samples. Our goal has been to resolve why, when EPC are contained within the bone marrow, that we cannot find evidence of their mobilisation together with HPC following G-CSF.

A series of experiments were performed in order to exclude technical factors as potential influences on CFU-EPC formation in mobilised blood. Flow cytometric analysis showed clear changes in the proportions of leukocyte subpopulations in MNC obtained from whole blood samples following G-CSF. We have explored the influence of cellular factors on CFU-EPC formation and present evidence that CD66b+ granulocytes affect CFU-EPC. We have identified phenotypic differences between CD34 positive cells mobilised with G-CSF and CD34 positive cells present in umbilical cord blood, another potential source of CD34 positive cells for clinical use. We believe that these differences contribute to the failure of

EOC development in mobilised blood. We have yet to resolve why we are unable to generate CFU-EPC or EOC from mobilised blood but using these results we are moving to explore other areas including G-CSF induced alterations of cell adhesion molecules expressed by CD14+ monocytes.

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List of abbreviations

AMD3100	Plerixafor
BM	bone marrow
BMT	bone marrow transplantation
CEC	circulating endothelial cell
CSU	Cell Separator Unit
CFU-EPC	colony forming unit- endothelial progenitor cell
CFU-Hill	colony forming unit- endothelial progenitor cell (Hill method)
CFU-Hill(direct)	colony forming unit- endothelial progenitor cell (modified Hill method)
EOC	endothelial outgrowth colonies
EPC	endothelial progenitor cells
G-CSF	granulocyte colony stimulating factor
hESC	human embryonic stem cells
HL	Hodgkin lymphoma
HPC	haematopoietic progenitor cells
HSC	haematopoietic stem cells
HSCT	haematopoietic stem cell transplantation
LVEF	left ventricular ejection fraction
MM	multiple myeloma
MNC	mononuclear cells
NHL	non Hodgkin lymphoma
PB	peripheral blood
PBSC	peripheral blood stem cell
PBSCH	peripheral blood stem cell harvest
PBSCT	peripheral blood stem cell transplantation
PMN	polymorphonuclear cells
SNBTS	Scottish National Blood Transfusion Service
UCB	umbilical cord blood
WCC	white cell count

1. Introduction

Haematopoietic stem cell transplantation (HSCT), both from bone marrow (BM) or more recently from peripheral blood (PB) as peripheral blood stem cells (PBSC), has been a clinical treatment for patients with haematological disease for more than 30 years and is a paradigm for stem cell therapies. Over this period, our understanding of stem cell biology has improved and refinements have been made in transplantation techniques and supportive care. As the population ages, there is an increasing demand to be able to repair damaged tissue and restore organ function. Transplantation of tissues (blood, heart valves) or organs (liver, kidney, heart) is limited by donor organ shortages. Transplantation is also restricted by transplant-related issues including human leukocyte antigen incompatibility, the requirement for immunosuppression and the inability to transplant some organs and tissues (e.g. nervous system). This has led to increased efforts to find alternatives such as the use of stem cells or cell-based therapies for tissue regeneration.

Whilst HSCT has been used in the haematological setting for many years, recent clinical and laboratory research suggests that other tissues may be amenable to regenerative cell therapy. In particular, vascular regeneration has received much attention. It is believed that bone marrow and/or circulating stem/progenitor cells may contribute to vascular repair and that such cells share common precursors with haematopoietic progenitor cells (HPC), in which case they may be obtainable from bone marrow or by leukapheresis of cytokine mobilised peripheral blood.

Novel observations in our laboratory, employing a recently described but widely accepted test of endothelial progenitor cell activity, have indicated reduced endothelial progenitor cell activity in CD34-rich blood following mobilisation of HPC from the bone marrow with granulocyte colony stimulating factor (G-CSF). This could indicate that the preferred source of cells for vascular regeneration

should be bone marrow rather than mobilised peripheral blood. This study aims to further characterise this phenomenon and its implications for sourcing autologous cells for cell therapy for vascular repair.

1.1. Cells for tissue regeneration

Stem cells are defined by their unique ability to self-renew, their high proliferative potential and their capacity for multi-lineage differentiation into specialised cells. A number of different cell types may be used as sources of stem cells for cellular therapy (outlined below). Totipotent stem cells can generate all cells constituting the body as well as the extra-embryonic tissue that gives rise to the placenta. Pluripotent stem cells are capable of generating all cells found within an individual but cannot form an embryo, as they are incapable of producing extra-embryonic tissue. Multipotent stem cells give rise to cells of a single layer, while tissue-specific oligopotent stem cells are capable of differentiating into lineage restricted tissue-specific cell types.

1.1.1. Embryonic stem cells

Embryonic stem cells have the ability to proliferate indefinitely *in vitro* and to differentiate into any somatic cell type (1). The blastocyst stage of embryonic development, reached 3–5 days post fertilisation, consists of a hollow ball of 100–200 cells, the outer layer of which develops into the trophoblast/placenta, while the inner cell mass develops into the embryo. The inner cell mass can be extracted from the blastocyst and grown on a fibroblast (feeder) cell line to yield human embryonic stem cells (hESC), which display long-term self-renewal potential and retain pluripotency. Since the first reports of successful isolation of hESC in 1989, ethical concerns have surrounded the procurement of cell lines from blastocysts derived from *in vitro* fertilisation programmes. The clinical application of hESC or their products is currently limited by a number of issues, including how to control hESC proliferation and direct hESC differentiation to specific cell types. The

transplantation of hESC also raises immunological concerns with the potential for tissue incompatibility and the need for long-term immunosuppression and the potential complications associated with this (e.g. infection) (2, 3).

1.1.2. Somatic cell nuclear transfer

Somatic cell nuclear transfer (SCNT) (or 'cloning') involves the introduction of the nucleus of an adult donor cell into an enucleated oocyte (3). Cell stimulation gives rise to the fusion of the donor nucleus with the oocyte, which can then be cultured *in vitro* to form a blastocyst. If the blastocyst is successfully transferred into the uterus of a female recipient, the resulting animal will be genetically identical to the donor (reproductive cloning). Therapeutic cloning describes the process in which a cloned blastocyst derived as above is explanted in culture to give rise to embryonic stem cells. hESC derived in this way could, in principle, be differentiated *in vitro* into a homogenous population of syngeneic immune-compatible cells to regenerate tissue. In humans, SCNT remains limited by the availability of ova, the inefficiency of the process with the potential for errors (1) and it remains unclear whether epigenetic modifications affect the phenotype or functions of cloned cell lines (3).

1.1.3. Induced pluripotent stem (iPS) cells

Induced pluripotent stem (iPS) cells offer another means of reprogramming cells that compared to SCNT is technically easier to achieve, is more reproducible and does not require oocytes (4). Re-programming of mature lineage restricted somatic cells to a pluripotent state was initially achieved using a set of 4 transcription factors, Oct4, Sox2, Klf4 and c-Myc, in a murine model, shown later to be sufficient also for iPS generation in the adult (5), but other combinations of transcription factors have subsequently been described to achieve this (6). It is widely accepted that mouse and human iPS cells possess morphological, molecular and developmental features that closely resemble those of blastocyt embryonic stem cells but it is yet to be determined whether these cells are identical to embryonic

stem cells or how similar these cells need to be for their use in cellular therapy (7). Murine models of disease (8, 9) have already illustrated the potential of iPS cells as a means of generating patient-specific pluripotent stem cells for use in regenerative medicine and therapeutic purposes. However, for clinical application, a means of inducing the 'iPS state' other than using viruses would be preferable.

1.1.4. Adult stem cells

Adult stem cells are found within differentiated tissue and traditionally were considered to be oligopotent stem cells. Adult stem cells may remain quiescent for many years before being activated by inflammation or injury (10, 11), with cells then being directed to differentiate into specific cell types to replenish damaged tissue. Research has shown that adult stem cells are found in many organs, including the bone marrow, skin, muscle, gut and liver, and are thought to be present in most, if not all, tissues (10). Although constantly replenished tissues, such as blood or skin, may exhibit a continually active system with a constant rate of turnover, other tissues are noted for their self-renewal capacity (e.g. liver) whereas certain tissues have been thought of as not amenable to such renewal (e.g. heart) although these tissues are now under investigation for such mechanisms. There is evidence that adult stem cells display the ability to differentiate into cells of other germ lines (plasticity or transdifferentiation), rather than being restricted to tissue types present in a specific organ (12, 13). It is not clear whether, or to what extent, plasticity is a real phenomenon and to what extent an artefact of the experimental conditions. This potential plasticity of adult stem cells has increased interest in using bone marrow-derived cells as sources of material for a wide range of cellular therapies (1, 10). Non-haematopoietic stem cells found in the bone marrow compartment include mesenchymal stem cells (MSC) which can give rise to bone marrow stromal cells, and endothelial progenitor cells (EPC) which give rise to vascular structures. Differentiation of MSC into fibroblasts, bone, cartilage and fat has been demonstrated in an experimental context.

There is evidence that rather than being locally restricted, adult (tissue) stem cells can contribute to tissue repair in sites remote to their origin by entering the circulation and migrating to other sites (1). The factors that stimulate stem cells, promote stem cell circulation or direct homing of these cells to remote sites are not yet fully understood. Stem cells offer the hope of being useful in the treatment of many disease processes, including neurodegenerative disease, diabetes and myocardial ischaemia but at present the isolation and characterisation of the adult stem cell populations remain difficult and basic questions such as stem cell phenotype, the best source of material and optimal culture methods remain unanswered.

1.2. Clinical use of haematopoietic stem and progenitor cells

Haematopoietic stem cells (HSC), the best characterised adult stem cell, are rare cells, comprising one in 10^4–10^5 of bone marrow (BM) cells. Bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT) are established therapies for the treatment of haematological malignancies. Recombinant human granulocyte colony stimulating factor (G-CSF) is one of many cytokines capable of mobilising HSC and HPC from the bone marrow into the peripheral blood and it is the agent most commonly used (14). Since the feasibility of HPC harvesting by leukapheresis was demonstrated in 1980 (15) there has been rapid growth in the use of G-CSF to mobilise PBSC, making it the new international standard for HSCT (16). Since 1993, PBSC have been the preferred source of HPC (IBMTR data) with G-CSF mobilised PBSC accounting for 74% of unrelated donor HPC donations in 2008 (NMDP data *www.marrow.org*).

1.2.1. Mobilisation of HPC into the circulation

HPC, including HSC, are recruited into the blood (mobilised) following treatment with G-CSF or chemotherapy; mimicking the physiological release of HPC from the bone marrow that occurs in response to injury, inflammation or other stress signals

(17). Originally HPC were defined and quantitated by clonogenic haematopoietic colony assays with CFU-GM the most commonly used indicator of the haematopoietic reconstitutive capacity of transplanted cells (18). Since the standardisation of CD34 cell measurements, colony based assessments are no longer routinely performed and CD34 is used as a marker for HPC. Under steady state conditions less than 0.05% of blood leukocytes are $CD34^+$ cells but this increases substantially post G-CSF (19). Allogeneic PBSC donors are mobilised using 4-7 days of G-CSF alone (5-16μg/kg/d G-CSF, as 1-2 daily doses (16, 20) and 1-3 leukapheresis procedures are usually adequate to collect PBSC (14). Whilst the administration of chemotherapy results in a 5-15x increase in $CD34^+$ cells (21), administering it together with G-CSF to autologous PBSC patients increases $CD34^+$ numbers even further (22, 23).

A proportion of healthy adult donors (and higher proportions of patients with haematological disease) fail to mobilise sufficient PBSC into the peripheral blood. HPC mobilisation is affected by donor age, gender, cytokines used, patient diagnosis, patient treatment and radiotherapy (24-26). Various strategies to improve PBSC mobilisation can be used including repeated mobilisation with increased cytokine doses and use of alternative mobilisation agents (19). For autologous PBSC patients, the potential risks of PBSC mobilisation and transplant must be balanced against the potential benefits of PBSCT with regard to disease free survival, overall survival and quality of life (27).

1.2.2. Safety of G-CSF and PBSC collection by leukapheresis

G-CSF-related side effects are frequently experienced. They can be divided into expected or commonly experienced side effects which are usually of mild-moderate severity, and into uncommon or unexpected complications that are often more severe in nature. Common side effects include bone pain, myalgias, malaise, nausea and headache (16, 28). Uncommon side effects include splenic enlargement or rupture (1/5000-1/10000 donors) (29, 30). Serious adverse events and even death

have occurred as a result of G-CSF induced sickling crises (31). The rise in PB leukocyte counts following G-CSF is associated with a thrombosis risk (16). Patients with cardiovascular disease have an increased risk of unstable angina, acute myocardial infarction (MI) and stroke following G-CSF (16). Mild-moderate thrombocytopenia, as a result of G-CSF or the leukapheresis procedure, occurs in ~25% of healthy donors (28). The long-term safety of administering G-CSF to healthy donors was questioned after two cases of leukaemia were reported in this group (32). National Marrow Donor Program (NMDP) data following >4000 donors at least 12 months post donation showed no cases of leukaemia or lymphoma in donors and the incidence of cancer was the same as the age-adjusted population average (33). Whilst it is still debated whether short term G-CSF, as administered for HPC mobilisation, can increase the chance of haematological disease including leukaemia, the current view is that there is no excess long term leukaemia risk for allogeneic (normal) PBSC donors above that of aged-matched controls.

Procedure related side effects of PBSC collection for the donor include vascular access difficulties with the need for placement of a temporary collection catheter, anticoagulation related side effects (due to citrate sensitivity) and various other apheresis complications (red cell loss, thrombocytopenia, vasovagal episodes, air embolus, adverse venepuncture reactions and machine malfunctions). Donor counselling as to the known and potentially unknown risks of G-CSF and PBSC collection is therefore an essential part of donor assessments (33, 34).

1.2.3. Effects of G-CSF on haematopoietic elements

G-CSF receptors are found on pluripotent and myeloid-committed progenitors as well as on differentiated myeloid cells, the numbers of G-CSF receptors increasing with cell differentiation from myeloblast to neutrophil (35, 36). Binding of G-CSF to myeloid cells stimulates the proliferation of myeloid precursors and accelerates the release of neutrophils from the bone marrow resulting in markedly increased peripheral blood neutrophil counts. G-CSF also promotes the release of contents

from specific and azurophilic granules from neutrophils, activates secretory vesicles and enhances neutrophil phagocytic function (27, 37, 38).

G-CSF receptors are not restricted to myeloid cells and they have been demonstrated on other cell types including subpopulations of monocytes, lymphocytes, platelets and endothelial cells (35, 39-41). Administration of G-CSF increases PB monocyte counts ~3-fold, activates monocytes and alters the expression of effector molecules such as IL-10 (42, 43). There is some evidence that G-CSF enhances platelet aggregation (44). A prothrombotic state may develop in some healthy donors independent of leukocytosis induced changes, as evidenced by increased plasma markers of endothelial activation and activation of coagulation (37, 45, 46).

1.2.4. Effects of G-CSF on progenitor cell populations

1.2.4.1. SDF1α- CXCR4 interactions

Stromal cells create bone marrow niches in which HPC are retained for growth and differentiation (47). Stromal derived factor (SDF-1α, also known as CXCL12) is a homeostatic chemokine that is constitutively expressed on bone marrow stromal cells and binds to its receptor, CXCR4, located on $CD34^+$ cells. It plays a key regulatory role in trafficking of haematopoietic cells including $CD34^+$ HPC. Interactions between SDF-1α and CXCR4 are important for the bone marrow retention of $CD34^+$ HPC and in regulating their release into the peripheral circulation (48, 49). SDF-1α influences other cell types within the bone marrow and helps keep B cell precursors in close contact with bone marrow stromal cells (16). The SDF-1α-CXCR4 axis is responsive to HIF-1 (hypoxia inducible factor-1), a central mediator of tissue hypoxia, which induces SDF-1α expression in ischaemic tissues (including within the relatively hypoxic bone marrow environment) (50). The induction of endothelial cell SDF-1α expression by HIF-1 has a role in attracting circulating stem and progenitor cells to the area of tissue damage, providing a niche for CXCR4-mediated tissue repair (51, 52).

1.2.4.2. Effects of G-CSF to produce HPC mobilisation

The binding of G-CSF to G-CSF receptors on myeloid cells leads to the release of proteolytic enzymes (elastase, cathespin G, proteinase 3, CD26) from maturing myeloid cells as well as the release of matrix metalloproteinases (MMP2, MMP9) from neutrophils which then inactivate and degrade bone marrow components (adhesion molecules, trophic cytokines) (53). These elements disrupt the SDF-1α-CXCR4 axis leading to release of $CD34^+$ HPC from the bone marrow into the peripheral blood. Whatever the precise mechanism of G-CSF induced PBSC mobilisation, it can be implied that agents that disrupt SDF-1α-CXCR4 binding leading to loss of the bone marrow retention signal for HPC, result in HPC mobilisation (49). Re-establishment of interactions between HPC and their bone marrow environment is required for homing and retention of HPC in the bone marrow. Homing is a rapid process that relies on the activation of adhesion interactions to retain HPC that have crossed the blood endothelium barrier within the bone marrow space, a process that differs from short and long term engraftment of bone marrow progenitors (50).

G-CSF mobilisation is also accompanied by shedding of cell adhesion molecules. Studies have identified increased levels of sL-selectin (CD62L), sE-selectin, sCD44 but not sICAM-1 following G-CSF (43, 54) and demonstrated that G-CSF mobilised $CD34^+$ cells express lower levels of CXCR4, VLA-4 (CD49d) and L-selectin than BM $CD34^+$ or non-mobilised PB $CD34^+$ cells (55, 56). G-CSF induces down-regulation of SDF-1α in bone marrow stromal cells and reductions in plasma levels of SDF-1α (56). In the presence of proteases there is cleavage of CXCR4 leading to mobilisation of HSC (57, 58).

1.2.5. Alternative agents for HPC mobilisation

1.2.5.1. AMD3100 HSC/HPC mobilisation

AMD3100, a reversible antagonist of CXCR4, was developed as a treatment for HIV infection. Transient increases in peripheral blood leukocyte and CD34 counts observed during treatment of patients with HIV led to testing of AMD3100 as a mobiliser of $CD34^+$ HPC. Drug administration to healthy volunteers produced increases in peripheral blood leukocytes and CD34 counts comparable to 4 days of G-CSF (10μg/kg/d) (59) and it was seen to act synergistically with G-CSF. AMD3100 (given on day 5) significantly increased $CD34^+$ HPC mobilisation compared to use of G-CSF alone (49). AMD3100 has also been used to mobilise $CD34^+$ HPC in haematology patients (60, 61). AMD3100 rapidly antagonises CXCR4 to inhibit the SDF-1α -CXCR4 axis, and as a result $CD34^+$ HPC are mobilised into the peripheral blood (49). The rapidity of action of AMD3100 compared to G-CSF reflects its direct effect on the SDF-1α-CXCR4 axis. It has been proposed that higher CXCR4 expression on AMD3100 mobilised $CD34^+$ cells compared to G-CSF mobilised $CD34^+$ cells indicated reversible inhibition of CXCR4 without proteolytic cleavage of the receptor (62).

1.3. Embryonic haematopoietic and endothelial development

1.3.1. Early embryonic vascular development

The development of blood and vascular elements requires co-ordination of a number of cell types and processes and relies on the temporal and spatial proximity of these elements in the developing embryo (63). The embryonic vascular system develops by vasculogenesis; the *de novo* formation of blood vessels from the in situ differentiation of undifferentiated mesodermal cells into endothelial cells. Small clusters of undifferentiated mesodermal cells appear in the extraembryonic yolk sac and develop into blood islands. The organisation of blood islands, with haematopoietic stem cells located centrally and endothelial progenitors towards the periphery, illustrates the close relationship between these elements (63). With development, multiple blood islands fuse to form the capillary network of the

extra-embryonic yolk sac (63, 64). Once foetal liver and later bone marrow haematopoiesis is established the intraembryonic arteriovenous vascular system develops (65, 66).

1.3.2. Embryonic haematopoiesis

Developmental studies using different animal species have shown that cellular regulatory factors are conserved within vertebrates (67). The development of the blood system in vertebrates is characterised by the sequential switching of sites where HSC undergo renewal, expansion and differentiation (68). In humans, at approximately 6 weeks gestation, blood islands begin to regress and the foetal liver becomes the major site of haematopoiesis. Like yolk sac haematopoiesis, erythrocytes are the dominant blood cell but myeloid precursors can be seen. It was previously thought that yolk sac HSC migrated to the liver but pioneering work performed by Dieterlen-Lievre showed that this was not the case and that the embryo body and not the yolk sac was the source of cells for the adult blood system (69, 70). At around week 5 of human embryonic development, large populations of $CD34^+$ haematopoietic precursors, which are the main source of HSC in the foetal liver, can be identified in the ventral endothelium of the dorsal aorta (71). This region is equivalent to the para-aortic splanchnopleura demonstrated in animal models of haematopoietic development (72, 73). It contains both endothelial and haematopoietic progenitors, with the haematopoietic precursors migrating to the foetal liver and then the bone marrow (74). Although haematopoietic progenitors can be found in the bone marrow from about 8 weeks gestation the bone marrow does not become the major site of haematopoiesis until around the 5^{th} month gestation (75).

1.3.3. Embryonic haemangioblast

On the basis of the close relationship observed between embryonic haematopoietic and endothelial elements the existence of a common origin for these was proposed

(His, 1900). Early studies of chick embryos identified synchronous development of blood and endothelial cells (76, 77). Murray dissected out cells capable of differentiating into both blood and endothelial cells, and he referred to this common cell of origin as 'the haemangioblast'; a bipotential cell with the ability to differentiate along both haematopoietic and endothelial cell lines. Work has continued to define the cellular events and processes surrounding the differentiation of these two elements and results from a number of lines of investigations point towards the presence of this cell population.

A number of shared genes with important roles in haematopoietic and endothelial development have been described and mutations of these affect the development of both elements. Failure of blood island formation and vasculogenesis occurred in Flk-1 (also known as KDR/ VEGFR2) deficient mice (78). Disruption of the Flk-1 gene was associated with absence of yolk sac blood islands and organised blood vessels with much reduced numbers of haematopoietic precursors and *in utero* death of homozygous mice between 8.5 and 9.5 days (78). Clonal studies on chick embryos demonstrated that $VEGFR2^{+}$ mesodermal cells differentiated into haematopoietic and endothelial elements (79). CD34 has been identified on elements involved in embryonic murine vasculogenesis and on haematopoietic-like cells which were thought to represent blood stem cell progenitors (80).

Scl protein is a crucial regulator of early haematopoiesis (81, 82). It has been demonstrated in haematopoietic and endothelial elements of yolk sac, and embryonic Scl expression parallels the haematopoietic activity of different organs during murine development (83). Stainier identified a single cell in the ventral marginal zone of early zebrafish blastula that gave rise to both endothelial and haematopoietic elements. A zebrafish mutant, cloche, had absent endocardial development and severely reduced numbers of blood cells. Overexpression of Scl protein in this mutant rescued the defects in haematopoiesis and endocardial

development whilst in wild type zebrafish embryos, Scl over-expression led to expansion of haematopoietic and endothelial cells (84, 85).

Whilst supporting the concept of the haemangioblast these studies did not prove its existence. The development of a culture system whereby embryonic stem cells were cultured to establish the embryoid body assisted the pursuit of the haemangioblast (86) as previously embryonic cells could not be studied prior to the stage of blood island development (87). Embryoid bodies differentiated from embryonic stem cells for 3-3.5 days were demonstrated to contain a unique precursor population with definitive haematopoietic potential. When cultured in the presence of VEGF, c-kit ligand SCF and endothelial conditioned medium, these cells, termed blast colony forming cells (BL-CFC), expressed a number of genes common to both haematopoietic and endothelial cells (VEGFR2, CD34, Scl/Tal-1) (87, 88). BL-CFC were subsequently demonstrated to differentiate into haematopoietic (primitive erythrocytes and multilineage haematopoietic cells) and endothelial cells (88) and cultured embryonic stem cells were used to define a cell phenotype for cells at the divergence of endothelial and haematopoietic cell line (89).

Until recently the mechanisms for the development of haematopoietic cells from the haemangioblast were unclear. The identification of formation of a hemogenic endothelium from haemangioblasts as an intermediate stage in embryonic haematopoietic development provided a link between haematopoietic and endothelial precursors (90). Lancrin *et al* demonstrated that upregulation of c-kit in $Tie2^{+}$ cells by SCL/Tal-1 was required for the establishment of the hemogenic endothelium and Runx1 (AML-1) was essential for formation of haematopoietic progenitors (90).

1.4. Vascular development in the adult

It was previously held that new blood vessel formation in adulthood occurred by the processes of angiogenesis and arteriogenesis and that vasculogenesis did not

occur postnatally. Angiogenesis refers to the sprouting of endothelial cells from post capillary venules and encompasses the proliferation and migration of endothelial cells and remodelling of pre-existing vessels. Arteriogeneis is the development of new blood vessels from the maturation of collateral vessels. The discovery of endothelial progenitor cells (EPC) as a population of non-haematopoietic precursors in the bone marrow that can be mobilised into the peripheral circulation and then home to sites of neovascularisation (91) opened up many new possibilities in the field of regenerative medicine and created great interest in extending cellular therapy beyond the transplantation of haematopoietic progenitors.

1.4.1. Circulating endothelial cells and endothelial progenitor cells

Asahara *et al* proposed that peripheral blood contained a population of cells that could differentiate into blood vessels (91). This landmark study provided evidence that $CD34^+$ cells could differentiate into cells with endothelial characteristics and that $CD34^+$ (enriched to a purity of ~15%) or KDR^+ cells isolated from human peripheral blood could participate in vasculogenesis at sites of neovascularisation in a murine hindlimb ischaemia model, challenging the understanding of adult blood vessel development (91). These circulating cells displayed endothelial and progenitor properties and were termed endothelial progenitor cells (EPC). EPC display similar properties to the embryonic angioblast and have the capacity to migrate, proliferate and differentiate in response to physiological or pathological stimuli but have not yet acquired mature endothelial markers (92, 93). It is now thought that the processes of angiogenesis and vasculogenesis occur simultaneously at sites of neovascularisation and that both processes then contribute to vascular repair.

Since the identification of EPC in adult peripheral blood (91) extensive studies have been performed and we are now beginning to understand the complex relationships between adult haematopoietic and endothelial elements. The bipotential haemangioblast was thought to be a transient cell population restricted to yolk sac and early intra-embryonic vascular development. Recent evidence supports the

presence of a cell population that has properties similar to the embryonic haemangioblast in the adult. Pelosi *et al* demonstrated that $CD34^+/KDR^+$ cells from human bone marrow or umbilical cord blood (UCB), plated at the single cell level in long term culture were able to generate haematopoietic and endothelial colonies and hypothesised that haemangioblasts were contained within this cell population (94). Assuming 1% of cells in MNC are $CD34^+$ and 1% of $CD34^+$ cells are KDR^+ the calculated frequency of $CD34^+/KDR^+$ haemangioblasts was 5 per million MNC (94).

Circulating endothelial cells (CEC) are mature non-dividing endothelial cells that have been sloughed from the vessel intima (95, 96). Within this cell population rare vascular derived EPC are also found. CEC are present in low concentrations (~3 cells/mL of whole blood) and can be measured by flow cytometry using markers such as CD146 (S-endo) (95, 97). Their enumeration might be a useful adjunct in understanding vessel injury and disease processes (96). CEC numbers increase following vascular stressors including infection, metabolic stress and in disease states including sickle cell disease, vasculitis, following BMT or post solid organ transplantation (98-101). CEC are interpreted as an indicator of vascular integrity and their increase in the circulation is generally thought to indicate vascular damage.

Until the demonstration of colonisation of Dacron material, wholly suspended within an intrathoracic graft by circulating (endothelial) cells (102), the origin of endothelial cells on implanted vascular material had been assumed to be entirely from infiltrating vessels from the anastemoses or from microvessel penetration of the graft material (103, 104). Evidence of circulating endothelial cells had also been provided by reports of recipient-derived endothelial cells within the coronary arteries of a transplanted heart (105) and of colonisation of the luminal surface of left ventricular assist devices by endothelial cells (106). Shi *et al* provided evidence for the existence of bone marrow derived endothelial cells. Dacron thoracic aorta vascular grafts implanted six months after successful canine BMT (full donor

engraftment) showed endothelial cells of donor origin (PCR studies) when grafts were recovered 12 weeks later (107). Other transplantation studies, using gender mismatched stem cells, differentiated between donor bone marrow derived and host vessel derived endothelial cells in the circulation (108).

1.4.2. Identification of endothelial progenitor cells

EPC comprise a minor proportion of PB MNC and can also be isolated from bone marrow (107) and UCB (109). The development of methods for EPC isolation has been hampered by the low frequency of EPC events (96). The two main strategies to enumerate putative EPC are flow cytometric analysis for the presence of specific surface antigens and *in vitro* culture methods.

1.4.2.1. Flow cytometry analysis for the expression of surface antigens

Phenotypic analysis enables EPC to be identified prospectively using a hypothetical definition of EPC phenotype without the need for cell culture. EPC have been variously defined according to their expression of CD34, KDR and CD133 antigens, with expression of these antigens alone or in combination being used to define EPC. EPC numbers, defined using $CD34^+$, $CD34^+/KDR^+$ or $CD34^+/ 133^+/ KDR^+$ expression or variants thereof have all been correlated with disease states (110, 111). There is little debate about the existence of endothelial and haematopoietic progenitors within the $CD34^+$ fraction of bone marrow, UCB and PB MNC but it seems that KDR (VEGFR2) and CD133, both used to define EPC, are also expressed by HSC/HPC (93, 112, 113). Expression of surface antigens may change during progenitor mobilisation and maturation (114). There are also difficulties associated with the antigenic distinctions between CEC and EPC (115). The common leukocyte antigen, CD45, can be used to exclude differentiated haematopoietic cells from endothelial cell populations but it does not identify lineage negative HSC. Endothelial precursors have been identified as $CD45^+$ by some (116, 117) and $CD45^-$ by others (118) but in many studies CD45 expression by putative endothelial progenitors has not been assessed. The numbers of putative EPC identified within a sample are not

only affected by the choice of phenotypic definition but also by the gating methods used for calculation of the cell population. Van Craenenbroeck *et al* analysed the whole blood and MNC EPC content, defined by CD34 and KDR expression, of samples obtained from healthy volunteers and illustrated the huge variations in EPC numbers introduced by the use of different gating methods (including lymphocyte gating, CD3 negative selection, Draq 5 nuclear staining) (119).

CD133 (AC133) was found to be present on all non-committed $CD34^+/38^-$ cells and the majority of committed myeloid $CD34^+$ cells (120). Similar to CD34, expression of CD133 declined during differentiation and was not found on mature cells and although HPC expression of CD133 parallels that of CD34, the two antigens do not share any homology (121). The actual functional role of CD133, like CD34, has not been elucidated. It has been reported that CD133 is not expressed by mature endothelial cells but is present on EPC in foetal liver, UCB and peripheral blood (122). De Wynter *et al* studied $CD34^+$ subsets within peripheral blood, bone marrow, and G-CSF mobilised peripheral blood and found that significant proportions of $CD34^+/133^+$ were present in each (36% UCB, 51% bone marrow, 75% mobilised peripheral blood $CD34^+$ cells) and that these cells, and not $CD34^+/133^-$ cells, were capable of repopulating NOD/SCID mice (123). A study using UCB came to similar conclusions with $CD34^+/133^{bright}$ expression identifying early high proliferating/stem and early committed progenitor cells with late committed precursors being $CD34^+/133^{dim}$ (124). Using culture-based colony assessments and a murine cancer model, Gehling *et al* concluded that $CD34^+/133^+$ cells from bone marrow and peripheral blood contained progenitor cells that differentiated into both haematopoietic and endothelial cells (125).

The action of VEGF, mediated through 2 receptors, VEGFR-1 (Flt-1) or VEGFR2 (KDR/Flk-1), is one of the main inducers for vasculogenesis and angiogenesis (125, 126) and VEGF signalling is essential for haematopoietic development and vasculogenesis. A study by Zeigler *et al* examining the phenotypes of primitive HSC

in bone marrow, peripheral blood, mobilised peripheral blood and UCB reported that pluripotent HSC were contained within the $CD34^+/KDR^+$ fraction and lineage committed progenitor cells were restricted to $CD34^+/KDR^-$ cells (127). $CD34^+/KDR^+$ cells (lin^-) accounted for 0.5-1.0% of $CD34^+$ cells, findings similar to those of Pelosi *et al* who hypothesised that haemangioblasts were contained within $CD34^+/KDR^+$ cells (94, 127).

Although $CD34^+/133^+/KDR^+$ ($CD34^+/133^+/VEGFR2^+$) cells were proposed by some as defining EPC (94), this population comprises an exceedingly small proportion of MNC and in the absence of primary enrichment are very difficult to isolate. The frequencies of $CD34^+/133^+/KDR^+$ cells were determined to be $0.0084\%^+/-0.0052\%$ in whole blood, $0.021\%^+/-0.0098\%$ in UCB and $2.07\%^+/-0.15\%$ in mobilised PB $CD34^+$ cells (112). This phenotype had been used to hypothetically define EPC in a number of studies (122, 125, 128) but the direct contribution of these cells to vasculogenesis has not been reported (113). It now appears that the $CD34^+/133^+/VEGFR2^+$ cells identified by Piechev *et al* and others (122, 125, 128) represent committed haematopoietic cells with no endothelial potential (112).

Flow cytometric detection and enumeration of EPC is subject to technical difficulties, mainly due to the rarity of these cells. When defined phenotypically as $CD34^+/KDR^+$ or $CD34^+/133^+/KDR^+$ cells, EPC may not be identifiable by flow cytometry, even with the collection of very large numbers of events (128). The ability to culture these cells enables rare cells to be identified.

1.4.2.2. Culture based methods to identify EPC

Varying cell sources, cell separation methods and culture conditions have been used by different groups examining the generation of cells with an endothelial-like phenotype from haematopoietic sources and it is likely that different cell populations have been studied (129). Recent opinion is that two broad types of EPC exist. Early work by one group demonstrated the presence of two EPC populations

within a single culture of human peripheral blood, in the gelatin adherent MNC fraction cultured in EGM2, and coined the terms early EPC and late EPC (130, 131). Subsequent studies have concurred with the findings of 2 distinct EPC populations and although these share some features including lectin binding, ac-LDL uptake and CD31 expression, the cell types differ in their proliferative capacity, morphology and functional characteristics (93, 108, 131-134).

1.4.2.2.1. Short term culture – early EPC

A landmark study by Hill *et al* correlated vascular function and cardiovascular risk with circulating EPC. In this study MNC were plated onto fibronectin coated plates in Medium 199 supplemented with foetal calf serum (FCS) with replating of fibronectin non-adherent cells onto fresh fibronectin plates after 48 hours (110). Morphologically distinct colonies consisting of multiple thin flat cells emanating from a central cluster of rounded cells appear spontaneously within the fibronectin non-adherent cell fraction after 5-7 days of culture (110). Endothelial cell lineage was demonstrated by immunostaining for CD31, VEGFR2, ac-LDL and Ulex lectin. Although cell proliferation does occur within CFU-EPC, demonstrated using radiolabelled thymidine (135) and EDU (O Tura, personal communication), cell proliferation is limited and other processes such as cell migration are involved in colony formation. This assay was widely adopted as a measure of circulating EPC and a commercial CFU-EPC assay (now also referred to as CFU-Hill) was developed (Stem Cell Technologies, UK).

It is now believed that early EPC, as presented in publications by Asahara and Hill among others and identified using the commercially available CFU-EPC (CFU-Hill) assay (Stem Cell Technologies, UK), plating MNC onto fibronectin coated plates, are haematopoietic and either of myeloid lineage or derived from earlier haematopoietic precursors (96). Our group has worked extensively using CFU-Hill assays and identified colony forming cells in 2-hour plastic adherent MNC, shown to be enriched to 80% purity for $CD14^+$ monocytes, but not in plastic non-adherent

MNC (136). Purification of $CD14^+$ monocytes (>98% pure) by immunomagnetic separation further increased CFU-EPC. Subsequent publications, particularly by Rohde's group (137) but also later by van Beem *et al* concurred with the view that in MNC from normal adults $CD14^+$ monocytes are essential for CFU-EPC (135). $CD14^+$ monocyte and $CD3^+$ T cell interactions were proposed to be important for the initiation of CFU-EPC (135, 137). The assay was interpreted by one group as a reflection of the T cell-mediated induction of monocytes towards pro-angiogenic cells (135). CFU-EPC were influenced by alterations to $CD3^+/4^+$ T cell numbers and whilst $CD14^+$ monocytes were essential for CFU-EPC, interactions with $CD3^+/4^+$ T cells, including those cytokine-mediated, were required for colony initiation (135). A series of subtractive analyses by Rohde *et al* demonstrated the presence of T cells and monocytes in CFU-EPC, and similar to van Beem *et al,* suggested that interactions between these cells was essential for CFU-EPC (137)

As an alternative to the short term culture of MNC using CFU-Hill (or CFU-EPC) assays and identification of EPC within day 1 or 2 fibronectin non-adherent MNC, others have plated MNC on fibronectin coated plates with a variety of growth factors and identified EPC within the fibronectin adherent fraction (129, 132, 138, 138, 139). The role of $CD14^+$ monocytes in the development of early EPC in fibronectin adherent MNC, as opposed to the 2 day fibronectin non adherent MNC in the CFU-Hill assay, has also been reported by a number of groups and so it appears that this culture method also identifies haematopoietic cells mainly of monocytic origin (129, 132, 139). A number of studies using this method to identify EPC in adherent MNC have specifically demonstrated the formation of EPC in CD14-enriched fractions. During short term culture, $CD45^+/14^+$ monocytes developed into spindle shaped adherent cells and altered their phenotype to express endothelial-type markers (including VWF, KDR, CD105, CD144, CD146) but without E-selectin or AC133 expression (129, 132, 140). Schmeisser *et al* cultured $CD34^-/14^+$ PB monocytes on fibronectin. These cells continued to be $CD45^+$ but expressed a range of endothelial markers at 2 weeks which were not present on day

1, and developed cord like structures when plated into Matrigel (129). In our laboratory, cells derived from CFU-EPC culture have not formed tubules when plated alone in Matrigel and have not incorporated into tubules formed by HUVECS (in Matrigel). It might be that the relatively 'late' appearance of EPC within fibronectin adherent MNC in this early study by Schmeisser *et al* actually identified late EPC.

When culturing PB MNC on fibronectin, the majority of peripheral blood derived cultured Ulex$^+$/ac-LDL$^+$ cells expressed monocyte/macrophage markers and although the cells did not proliferate significantly they did secrete pro-angiogenic factors (132, 139). Freshly isolated CD14$^+$ monocytes expressed higher levels of CD31, CD144 and BS-1 lectin, slightly lower levels of CD105 and equivalent UEA-1 lectins than cultured monocytes (139) meaning that these feature fail to distinguish between monocytes and cells of endothelial differentiation. The relevance of monocytic features to the generation of EPC with the capacity for neovascularisation was questioned by Urbich *et al* who showed that the origin of EPC isolated from blood was not restricted to CD14$^+$ monocytes and that other cells could display endothelial features (141). Comparable expression of endothelial markers (Dil-ac-LDL and lectin) was found in day 4 adherent CD14$^+$ and CD14$^-$ MNC. Using a murine hind limb ischaemia model, the authors showed that infusion of uncultured CD14$^+$ or CD14$^-$ MNC failed to improve ischaemia whilst infusion of cultured cells (CD14$^+$, CD14$^-$ or unselected MNC) did (141).

Similar culture methods were used to identify early EPC in the fibronectin adherent fraction of MNC ($10x10^7$ MNC cultured in growth factor supplemented EGM2 media (Clonetics, UK) for >4 days) from healthy subjects, the number of which correlated with the number of peripheral blood 'angiogenic' T cells (142). Angiogenic T cells, defined as the CD3$^+$/31$^+$/CXCR4$^+$ T cell subpopulation, were found predominantly within the central cores of CFU-EPC and were demonstrated to show improved pro-angiogenic properties over CD3$^+$/31$^-$ T cells (142).

The cells identified in CFU-EPC assays based on short term short term MNC adherence to fibronectin appear to form heterogeneous collections of cells. Although $CD14^+$ monocytes are essential for CFU-EPC it is likely there are vital contributions made by other inflammatory cells including T cells in the initiation of colony formation. Whilst *in vitro* functional studies (Matrigel assay) show reduced incorporation of these cells into tubules, delivery of these cells to ischaemic areas in animal models have produced clinical benefits (130). It is proposed that the pro-angiogenic effects of these cells are due to the production and release of angiogenic factors (VEGF, IL-8,) that act on adjacent endothelial cells to stimulate vessel formation (130). Additionally, monocytes and macrophages have been shown to be important in neovessel formation through their cord formation, incorporation into structures and secretion of elastases that degrade the extracellular matrix, providing a scaffold on which endothelial cells can grow and invade fibrotic tissue (143, 144).

1.4.2.2.2. Endothelial outgrowth cells

Cells variously defined as late EPC, endothelial outgrowth cells (EOC), endothelial colony-forming cells (ECFC) or outgrowth endothelial cells (OEC) are rare cells that arise from $CD45^-/14^-$ non-haematopoietic cells, display robust proliferative potential and possess *in vivo* vessel forming abilities (93, 115). They are similar to the bone marrow-derived EPC described by Shi *et al* (107). After 2 weeks of culture (or less if using UCB) (133) in supplemented EGM-2 media on collagen coated plates, adherent cells form colonies with a cobblestone appearance. Single cell studies have confirmed a proportion of EOC displayed clonogenic expansion, stem-like properties and could be transplanted to yield *de novo* vessel formation (93).

The formation of EOC and early EPC from distinct cell populations has been demonstrated using modified culture conditions (108, 145), that support the development of both EOC and short-term fibronectin adherent EPC (130, 146, 147).

Independent culture of $CD14^+$ MNC and $CD14^-$ MNC showed that early EPC were generated by $CD14^+$ MNC and EOC by $CD14^-$ MNC (146). The cell morphology displayed by early and late EPC was in agreement with other studies (108). Late EPC showed capillary formation when plated alone on Matrigel and incorporated better with HUVEC on Matrigel than early EPC. In another study early EPC appeared from the end of the first week of culture (and had disappeared by 8 weeks), late EPC (EOC) appeared after 2 weeks and then expanded greatly (130). EOC but not early EPC incorporated into vascular networks in an *in vitro* angiogenic assay (147).

Peripheral blood samples from patients with the myeloproliferative disorder (MPD) polycythaemia vera (a disorder of haematopoietic progenitor cells) positive for the Janus Kinase 2 V617F mutation were used to demonstrate the haematopoietic origin of early EPC and to show that CFU-EPC (CFU-Hill) and EOC cells were not clonally related (93). Whilst all genotyped CFU-EPC colonies expressed the Jak2 mutation, only 3 of 89 EOC colonies contained mutant alleles (all from same patient, possibility of germline mutation; sample contamination or haematopoietic derivation of colonies possible explanations). Similar to other studies (133), EOC colonies expressed markers of endothelial differentiation and were $CD45^-$ and $CD14^-$ whilst CFU-EPC were $CD45^+$ and $CD14^+$. Another controlled study of patients with MPD showed that disease-specific mutations (Janus Kinase 2 V617F mutation or BCR-ABL rearrangement) could be identified in CFU-EPC but not in EOC, demonstrating the haematopoietic origin of CFU-EPC but not of EOC (148).

Peripheral blood MNC from recipients of gender mismatched allogeneic BMT recipients (100% donor bone marrow and peripheral blood genotype by RFLP) cultured for EOC were used to demonstrate the presence of bone marrow derived endothelial cells with stem/ progenitor properties in the peripheral blood and to distinguish these from recipient derived circulating endothelial cells (CEC) (108). Cell origins were determined using FISH for X and Y chromosomes. Whilst in fresh

samples, only minor proportions of CEC were either transplanted cells or derived from transplanted cells (95% recipient and 5% donor genotype) the proportions of cells exhibiting donor genotype increased over the culture period and EOC colonies were of donor karyotype. Donor derived EOC had extraordinary growth capacity, suggesting these to be of stem or progenitor origin (108). EOC arising from different sources (UCB MNC and peripheral blood MNC) are not identical. UCB EOC appeared earlier in culture, contained larger colonies and in single cell cultures were enriched in highly proliferative progenitor cells compared to peripheral blood EOC (133). A hierarchy for EPC, along the same lines as the haematopoietic progenitor hierarchy, has been proposed (133).

EOC are believed to arise from non-haematopoietic cells contained in the $CD14^-$ MNC fraction (93, 115), perhaps from the $CD34^+$ cells contained within the heterogeneous $CD14^-$ cell population (131). There are complex relationships between HPC, EOC and early EPC which are further confused by the overlap of CD34, CD133 and KDR antigen expression between endothelial and haematopoietic differentiated cells (120, 127). Conflicting data has been presented on the phenotype of EOC and the contributions of different subsets of $CD34^+$ cells to colony formation (125, 149). The phenotype of cells contributing to EOC formation has been investigated by a number of groups that concluded that EOC arose from $CD45^-/34^+$ cells that did not express CD133 and who refuted the belief that EPC can be defined phenotypically by co-expression of CD34, CD133 and KDR (112, 134, 150).

Untegasser *et al* plated PB MNC or UCB MNC in collagen type 1 coated plates in EGM2. Following MNC selection for CD133, CD34 or CD14, EOC colony formation was observed in the 24 hour adherent cell fraction after 7-12 days exclusively in $CD34^+/133^-$ MNC and these cells were shown to express CD31, VWF, bind Ulex lectin and take up ac-LDL. Cultured cells showed formation of capillary like structures in Matrigel and homed to ischaemic myocardium in a murine model of infarction (150). Using bone marrow and UCB as sources of $CD34^+$ cells,

Timmermans *et al* reliably generated EOC with typical cobblestone morphology and endothelial antigen expression (CD31, CD146 CD105, CD34, VEGFR2) from $CD34^+/45^-$ cells but not from $CD34^+/45^+$ cells (134). Similar to others (131, 151), EOC were found to be $CD45^-/14^-/133^-$, with lack of CD133 antigen expression confirmed by mRNA studies. $CD34^+/45^+$ cells failed to generate EOC but did develop CFU-EPC like colonies (134). UCB $CD34^+/45^-$ cells formed EOC but failed to generate CFU-EPC or haematopoietic activity whilst UCB $CD34^+/45^+$ cells were enriched for haematopoietic colony forming activity (112). $CD34^+/133^+/KDR^+$ cells isolated from UCB, normal peripheral blood or G-CSF mobilised PB $CD34^+$ cells were simultaneously plated into endothelial and haematopoietic clonogenic assays. $CD34^+/133^+/KDR^+$ cells from UCB and mobilised PB $CD34^+$ cells failed to generate CFU-EPC or EOC but produced LPP-CFC and HPP-CFC in haematopoietic assays. In keeping with this, >99% of $CD34^+/133^+/KDR^+$ cells co-expressed CD45 (112). Case *et al* recommended inclusion of CD45 as an exclusionary gate when identifying $CD34^+/KDR^+$ precursors to reduce the numbers of false positive events.

1.4.3. Interactions between early EPC and EOC in promoting neovascularisation

Murine hind limb ischaemia studies showed no differences in the vasculogenic potential of early and late EPC (130). The discrepancy between *in vitro* and *in vivo* function may be due to enhanced secretion of angiogenic cytokines (VEGF, IL-8) by early EPC that may activate adjacent endothelial cells and promote angiogenesis (130, 132). Heterogeneity in the cells comprising early EPC might also influence the *in vivo* function of these cells. Synergy between haematopoietic derived CFU-EPC and non-haematopoietic endothelial outgrowth cells is perhaps not surprising considering their common origin and the close relationship between haematopoietic and endothelial progenitor development. It may be that paracrine and autocrine networks of cytokines between the two types of endothelial progenitors might be an important mechanism of neovascularisation (131). With recognition that pro-angiogenic effects of cell therapy extend beyond incorporation of transplanted

cells into the vessel wall, the influence of cytokines and proteases, including matrix metalloproteinases (MMP) released by EPC as well as other functions of EPC on vessel formation is being investigated. It has been shown that unlike EOC, early EPC secrete VEGF and IL-8 (130). The same group found that MMP-2 was secreted by $CD14^-$ cells and EOC and that MMP-9 was secreted by $CD14^+$ cells and early EPC (131). MMP-9 is involved in the mobilisation of HPC and EPC from bone marrow, facilitates homing of EPC and has important roles in sprouting angiogenesis (152). Synergistic effects on vascularisation in a murine hind limb ischaemia model were produced by culturing early EPC together with EOC (131) and this was accompanied by enhanced release of MMP-9 from early EPC and greater invasion depth of early EPC (131). The role of MMP in neoangiogenesis may be relevant to the use of G-CSF for progenitor mobilisation as G-CSF induced MMP release is important for mobilisation of HPC from the bone marrow (53).

1.4.4. Difficulties with models of vasculogenesis

Regardless of the definitions used, putative EPC might only give rise to endothelial cells depending on the exact combination of growth factors to which the cells have been exposed to *in vitro*, the animal model used to assess vasculogenic activity and the nature, extent and method of delivery of the antigenic stimulus *in vivo* (153). Cultured cells may have acquired or lost properties during culture such that the function and phenotype of *in vitro* propagated cells may differ significantly from those of non-cultured cells (141, 153).

The use of animal models for the functional assessment of putative EPC is flawed as *in vivo* models of vasculogenesis do not identify vasculogenesis according to the generation of EC from circulating EPC. They rely on the localisation of a donor cell population to the site of vessel injury/neovascularisation as evidenced by the presence of positively stained cells, ac-LDL uptake or lectin binding, rather than demonstrating the actual incorporation of cells into neovessels. Additionally, there are no antigenic markers unique to endothelial cells and lectin binding and ac-LDL

uptake are both properties also shown by haematopoietic cells including monocytes (132, 139, 153). Mature haematopoietic elements are involved in vascular repair and given the expression of 'endothelial antigens' by haematopoietic cells, co-localisation of endothelial and haematopoietic cells might be incorrectly identified as evidence of vasculogenesis.

1.5. Clinical application of endothelial progenitor cells

The lack of a standardised accepted definition of EPC and lack of standardised methods for their identification, isolation or expansion has not restricted entrance of EPC into the clinical arena and their use in cell-based therapy. The treatment of patients with cardiovascular and vascular disease has become an area of intense study. The majority of cell therapy studies have used bone marrow as a source of EPC with transplantation of whole bone marrow or BM MNC (or fractions thereof) into animal or human subjects. Whilst there may be clinical benefits reported, such are the inconsistencies in the definitions and methodologies used that it remains difficult to compare study outcomes. The results of some studies, mainly in the setting of cardiovascular disease are discussed below.

1.5.1. Local delivery of bone marrow MNC

Many investigators have reported on the outcomes of locally delivered (intracoronary infusion, myocardial delivery) bone marrow derived or growth factor mobilised cells (HSC and EPC). In the great majority of these studies, the delivered cell product was evaluated by flow cytometry analysis for the cell expression of CD34, alone or in combination with CD133 and/or VEGFR2 and colony formation was not assessed.

Intracoronary infusion of BM MNC using an inflated balloon catheter to deliver cells to the infarct related artery has produced clinical benefits in a number of small trials (154-156). On the basis that several different MNC fractions, including bone

marrow haemangioblasts, HSC and EPC may contribute to tissue regeneration, unselected BM MNC were infused (154, 155). The study by Dimmeler's group was notable as outcomes following intracoronary unselected bone marrow derived cells were compared to infusion of peripheral blood-derived endothelial progenitors; day 3 fibronectin-adherent MNC cultured in VEGF, atorvastatin and serum supplemented media, with no differences found between treatment arms (155). Implantation of autologous BM MNC by intramyocardial injection following MI was reported to reduce infarct size, improve wall thickening and wall motion (157) whilst another (earlier) uncontrolled study failed to show significant benefit of intramyocardial $CD133^+$ BM MNC when administered following coronary artery bypass grafting (CABG) post MI (158). Delivery of autologous BM MNC to the ischaemic myocardium by the endocardial route in patients with chronic ischaemic heart disease (non-randomised study) showed some benefits in left ventricular volumes and left ventricular ejection fraction (LVEF) compared to controls (159).

A meta-analysis of the use of stem cell therapy in acute MI reviewed 14 studies (20-187 patients in each) using BM MNC (12 studies) or G-CSF mobilised MNC (2 studies) (160). Stem/progenitor cell therapy did not appear to be associated with an increase in adverse events. It concluded that although there were some general short term improvements in cardiac function in the treated group there was insufficient data to assess the long term clinical effects of stem/progenitor cell therapy (160).

1.5.2. Peripheral blood stem cells for cellular therapy

Collection of bone marrow requires the patient to undergo a collection procedure whereby bone marrow is aspirated from bilateral iliac crests under anaesthetic. In haematology, collection of bone marrow for HSCT has largely been replaced by PBSC collected from the circulation by leukapheresis, therefore avoiding the risks of general anaesthetic, the risks of bone marrow harvest and the unpleasantness of this procedure. The possibility of using mobilised peripheral blood progenitor cells for cellular therapy is an attractive option. Bussolino *et al* demonstrated dose

dependent increases in human endothelial (umbilical vein) cell migration in response to G-CSF and GM-CSF and identified high affinity binding sites for both these cytokines on human endothelial cells suggesting that cytokines could mobilise EPC into the circulation (161).

Although there have been no direct comparisons between the therapeutic benefits of bone marrow and cytokine mobilised EPC, studies have reported the effects of G-CSF (and GM-CSF) administration on putative EPC using phenotypic analysis or culture-based colony assays, with a number also reporting its clinical effects on myocardial regeneration and function. G-CSF may contribute to the revascularization of the infarcted area and could also facilitate the differentiation of HSC into cardiac myocytes or supportive cells.

1.5.2.1. Effects of G-CSF on putative EPC – phenotypic analysis

There is no doubt that G-CSF mobilises HPC into the peripheral blood, identified by their surface expression of CD45 together with CD34 and/or CD133, it remains unclear whether EPC are mobilised together with or independently of HSC/HPC and whether EPC can be quantified prospectively by flow cytometry.

Studies reporting on the effects of G-CSF mobilised progenitor cells on vascularisation have used varied phenotypic combinations to prospectively identify putative EPC within mobilised blood; $CD34^+$ cells (162-164), $CD34^+/45^+$ cells (165, 166), $CD34^+/133^+$ (164, 167, 168), $CD133^+/VEGFR2^+$ (167), $CD34^+/VEGFR2^+$ (163), $CD34^+/133^+/VEGFR2^+$ (162, 163, 166). Additional antigens have also been used to identify cells with an endothelial phenotype; $CD34^+/31^+$, $CD34^+/117^+$ (168) for example, making it more difficult to compare study outcomes. Despite the range of phenotypic definitions used these studies all reported that G-CSF increases peripheral blood EPC. However, in many, if not all cases the definitions used can be applied to HPC and may not define EPC at all, especially if, for example EPC are $CD133^-$ and/or $CD45^-$.

1.5.2.2. Effects of G-CSF on putative EPC - culture based colony assays

A number of other studies have been published whereby the effects of G-CSF on putative EPC contained in peripheral blood samples obtained prior to and following G-CSF were assessed by short term colony assays. These studies differed substantially in the doses of G-CSF administered and the culture methods used for *in vitro* assessment of EPC, with no two studies using identical methods.

A number of studies of G-CSF and peripheral blood EPC have used modified CFU-Hill assays to identify EPC within day 1 or 2 fibronectin non-adherent MNC (110). In 2005 it was noted within our laboratory that CFU-EPC were depressed in samples obtained from G-CSF mobilised peripheral blood. On account of the raised CD34-content of mobilised peripheral blood we had expected to see an increase in CFU-EPC in these samples and we began to examine this further. Initial findings were published (136, 169) and we have continued our study of this area principally using the CFU-EPC assay. Powell *et al* used a method similar to that described by Hill (110) to assess the effects of G-CSF on CFU-EPC formation in patients with coronary artery disease. In a modification from previous publications by the group, EPC colonies were enumerated at 9 days, 7 days after re-plating day 2 non-adherent MNC (167). Powell *et al* reported an increase in CFU-EPC following administration of G-CSF to healthy controls and to patients with coronary artery disease, although patients exhibited lower CFU-EPC than healthy controls. Mauro *et al* assessed EPC during HPC mobilisation by G-CSF+chemotherapy using MNC suspended in Medium 199 supplemented with FCS (changed every 3 days) in fibronectin coated plates. EPC were identified in the 2-day fibronectin non-adherent MNC fraction after a total of 7 days of culture (CFU-end) (162). Mauro *et al* reported an increase in $CD34^+/133^+/VEGFR2^+$ cells in peripheral blood and leukapheresis collections following G-CSF and identified so called CFU-end in leukapheresis collections. No evaluations of PB CFU-End were made and the effect of G-CSF on colony formation could therefore not be assessed (162). Another study similarly assessed EPC colonies

using Medium 199 supplemented with FCS and fibronectin coated plates and reported increases in CFU EPC following G-CSF (163). Korbling *et al* shortened the primary plating period and EPC were defined in the 24 hour fibronectin non-adherent MNC fraction after 7 days of culture. A study of autologous and allogeneic PBSC donors reported increased CFU-EPC (vascular progenitor cells or VPC in this publication) following G-CSF, as assessed using MNC plated into fibronectin coated wells suspended in RPMI, supplemented with FCS and endothelial growth factor. VPC (EPC) were identified in the 2 day fibronectin non-adherent cells after 9 days in culture (170).

Honold *et al* defined EPC by ac-LDL uptake in the 3-day fibronectin adherent fraction of MNC cultured in EBM2 medium supplemented with FCS and atorvastatin (165). These adherent cells were then cultured for a further 2 weeks in VEGF and assessed for colony formation on the basis of morphology alone. Using these methods, CFU-EC were reported to increase following the administration of G-CSF to a small number of patients with chronic ischaemia heart disease. Interestingly, migratory responses of day 3 fibronectin adherent cells to SDF-1 and VEGF were impaired in G-CSF treated MNC compared to untreated cells (165).

A single study has examined the mobilisation of vascular progenitor cells in the course of PBSC mobilisation for allogeneic as well as autologous PBSCT (170). In this study, G-CSF was administered to donors and patients according to standard regimens for HSC/HPC mobilisation with leukapheresis commenced when PB CD34 counts exceeded 10/μL. EPC were identified as vascular progenitor cells (VPC) by colony assay, discussed above. Peripheral blood and leukapheresis samples obtained from autologous PBSC patients had lower pre G-CSF VPC than allogeneic PBSC donors. Despite this, VPC increased in both patient groups following PBSC mobilisation with G-CSF (and chemotherapy). VPC numbers failed to correlate with CD34 counts or haematopoietic colony assays (total CFU and GM-CFU) leading the authors to conclude that VPC were differentially mobilised from HPC (170).

Although it is currently believed that 'true' EPC are identified in the endothelial outgrowth colony assay, to date there is a lack of published studies examining these cells in the setting of G-CSF PBSC mobilisation.

1.5.2.3. G-CSF administration without cell isolation – clinical outcomes

A number of studies have reported on the effects of G-CSF on clinical outcomes without any evaluation of the effects of G-CSF on putative EPC phenotypes or colony formation. Use of G-CSF mobilised progenitor cells for cardiovascular regeneration stalled once adverse findings of increased incidence of instent stenosis following intracoronary implantation of autologous G-CSF mobilised MNC in the MAGIC trial were reported (171). Subsequent to this, an uncontrolled study reported improved LVEF and LV volumes following intracoronary infusion of autologous G-CSF mobilised MNC (172) but this study failed to include a G-CSF only arm, preventing comparisons being made between outcomes following systemic mobilisation of progenitors by G-CSF and the local implantation of mobilised cells.

As an alternative to local (intra-coronary or intramyocardial) implantation of autologous BM MNC or G-CSF mobilised progenitors, and on the premise that G-CSF mobilises EPC, a number of trials have reported on the use of subcutaneous G-CSF in the post infarct period as an adjunct to standard medical care. Administration of G-CSF (10μg/kg/d for 5 days), commencing 5 days after acute MI and percutaneous intervention failed to show any improvements in LVEF or infarct size at assessment 4-6 months post randomisation (173). In this randomised double blind trial placebo controlled trial (REVIVAL-2) both treatment groups (each containing >50 patients) showed improvement during follow-up. A significant proportion of patients (27%) reported G-CSF related side effects (173). Similarly, there were no differences in LVEF and systolic wall thickness at 6 months follow-up between treatment groups randomised to receive 10μg/kg/d G-CSF or placebo for 6 days following acute MI with successful percutaneous coronary intervention (PCI)

(174). In addition, no differences in global or regional myocardial function were observed between randomised treatment groups following subacute MI with percutaneous coronary reperfusion (168). A small randomised blinded study using lower dose G-CSF (5μg/kg/d for 4 days) or placebo in the same clinical setting failed to show any difference in infarct size and LVEF at 3 and 6 months of follow-up (166). Ellis *et al* reported no benefit of 5 or 10μg/kg/d G-CSF over standard treatment with regard to LVEF at 3 months (175). A small open label study of patient post MI with PCI reported improvements in regional wall motion and perfusion of G-CSF treated ((10μg/kg/d for 7 days) patients compared to controls (176). One randomised study, that reported a clinical benefit of G-CSF (10μg/kg/d x 6 days) following acute MI and PCI at 6 months (177) was criticised for the poor performance of the control group. Failure to detect an expected clinical improvement in the control group contributed to the positive outcome of this study (177).

A meta-analysis on the use of G-CSF in patients with acute MI (178) identified 10 studies, including the ones discussed above, that reported on the effects of G-CSF on myocardial regeneration post MI, all in the context of reperfusion with PCI. G-CSF administered in doses of 2.5-10μg/kg/d for 4-10 days produced an increase in PB CD34 counts. Compared with conventional treatment, G-CSF failed to improve LVEF or infarct size and no overall benefits of G-CSF administration were demonstrated (178).

Early termination of the MAGIC trial, which studied the effects of intra-coronary infusion of G-CSF mobilised MNC on patients who underwent coronary stenting following MI, due to excessive rates of instent restenosis cast doubts over the safety of G-CSF in this setting (171). The issue of cytokine induced instent restenosis has been investigated by Cho *et al* using rabbit models of vascular injury, producing apparently contradictory results. Increased neointimal hyperplasia following vascular injury in G-CSF treated rabbits was largely overcome by use of a drug (paclitaxel) eluting stent (179), whilst an earlier study, using GM-CSF, concluded

that GM-CSF potentiated the effects of intravascular radiation in reducing neointimal hyperplasia by accelerating re-endothelialisation (180). The authors' commented that the specific choice of animal vascular injury models, which differed between the studies, contributed to their findings and that the use of a paclitaxel eluting stent resulted in delayed re-endothelialisation. G-CSF pre-treatment was reported to accelerate re-endothelialisation and reduce neointima formation in a rat carotid balloon injury model (181) and a canine study with G-CSF starting a week following carotid vascular graft placement, similarly reported accelerated endothelialisation and microvessel formation with G-CSF (182).

It may be that administration of G-CSF alone is insufficient to produce clinical benefits and that local delivery of mobilised (and harvested) PBSC is important (178). It may also be that the timing of G-CSF administration in relation to acute (myocardial or other) injury is critical and there may be differences in the homing signals expressed by damaged tissues according to the time elapsed post injury.

1.6. Cell contributions to vascular repair

The cells contributing to the repair of damaged tissue are not well defined. It may be that bone marrow derived endothelial cells incorporate at sites of neovasculogenesis (107). Vascular wall derived endothelial precursors may also be important (113). However, there is likely a large contribution made by bone marrow derived cells of haematopoietic origin. A number of recent studies suggest that the main contribution of transplanted $CD34^+$ or $CD133^+$ cells may be to provide a supportive microenvironment of tissue regeneration and vascular repair, achieved through autocrine and paracrine actions (183).

A mouse bone marrow transplant model using green fluorescent protein (GFP)-labelled bone marrow cells from littermates 6 weeks prior to the induction of hindlimb ischaemia or fibrosarcoma cell implantation failed to demonstrate the incorporation of GFP-labelled cells into the vasculature (183). GFP-labelled cells

were found in the adventitia of collateral arteries and in perivascular spaces of leg muscles from ischaemic limbs but no stained endothelial cells were identified. Similarly, no stained cells were detected in the endothelium or smooth muscle of the implanted fibrosarcoma cells (183). A functional link between monocyte concentration and enhancement of arteriogenesis (collateral vessel growth) was demonstrated using 5-fluorouracil to manipulate PB monocyte counts in a rabbit model of hindlimb ischaemia whereby monocytopaenia was accompanied by reduced limb blood flow that improved once monocyte levels rebounded (184).

Administration of G-CSF leads to an increase in the numbers of circulating $CD34^+$ and $CD133^+$ cells, the great majority of which are haematopoietic, as determined by their co-expression of CD45. A number of studies, using murine transplant models, have shown accelerated tissue regeneration and repair following infusion of human UCB or mobilised PB $CD34^+$ (185) or $CD133^+$ cells (186, 187). Similar to Ziegelhoeffer *et al* (183), human $CD133^+$ or $CD34^+$ cells were identified in the region of injury with little/no evidence of incorporation of these cells into vessel walls or of their direct contribution to vasculogenesis.

Recent reports from Asahara's group provide some interesting observations of the contribution of locally implanted G-CSF mobilised $CD34^+$ or $CD133^+$ cells to neovascularisation following ligament injury (mouse) (185), skeletal muscle injury (rat) (187) and in critical limb ischaemia (human) (188). In the two murine studies (185, 187), the clinical benefits observed following the local implantation of (purchased) G-CSF mobilised human $CD34^+$ or $CD133^+$ cells compared to human non-mobilised MNC were not delivered through incorporation of implanted cells into sites of neovascularisation but by enhancing the angiogenesis of recipient cells by creating a favourable microenvironment for tissue regeneration and producing pro-angiogenic cytokines. In these studies far fewer numbers of localised stained cells could be demonstrated following implantation of unselected MNC compared to mobilised $CD34^+$ or $CD133^+$ cells (185, 187).

Results of a phase I/IIa trial of locally implanted autologous G-CSF mobilised $CD34^+$ cells for treatment of critical limb ischaemia have recently been reported (188). The G-CSF schedule used in this dose escalation study was the same as used for PBSC mobilisation in allogeneic PBSC donors. $CD34^+$ cells were collected by leukapheresis and purified by immunomagnetic (CliniMACS) separation. The outcomes of cell therapy were measured clinically without assessments of cell fate or tissue histology and the contralateral leg, not injected with $CD34^+$ cells, functioned as an internal control. Whilst a positive effect of systemic G-CSF on patient symptoms could not be discounted, differences in measured parameters (toe brachial pressure index and transcutaneous partial oxygen pressures) between limbs supported an additional effect of the implanted (mobilised) cells. A non-statistically significant improvement in clinical outcome was reported when using the author's combined 'Efficacy score' but significant improvements were reported during the 12 weeks follow-up for a number of variables including pain rating, walk distance, ulcer size in treated patients (188). This study also illustrated some of the difficulties of G-CSF progenitor cell mobilisation (188). Similar to autologous PBSC mobilisation for PBSCT, CD34 yields were variable and failed to meet target yields over a third of cases. Failure to reliably collect $1x10^6$ CD34/kg following G-CSF led to termination of recruitment into the 'Hi cell dose' arm of the study. Although G-CSF and leukapheresis side effects of mild-moderate severity were frequent they did not affect cell collection.

1.7. Mobilisation of EPC by CXCR4 antagonists

The ability of AMD3100, a reversible CXCR4 antagonist, to mobilise EPC has been reported by Shepherd *et al* (189). Culture-based colony assays were used to identify early EPC and endothelial outgrowth cells (late EPC) in samples from healthy volunteers mobilised sequentially with AMD3100 alone and then G-CSF alone. Both AMD3100 and G-CSF were reported to mobilise early and late EPC. Colony frequency was calculated per mL PB rather than per 10^6 MNC plated. In the setting

of cytokine induced leukocytosis this is difficult to interpret. Early EPC were defined as $CD45^+/14^+$ day 4 fibronectin adherent spindle shaped cells, grown in EGM2, supplemented with VEGF and foetal calf serum. Late EPC formed after 2-4 weeks of culture in EBM2 supplemented with VEGF and foetal calf serum on collagen type 1 plates. Flow cytometry analysis of cultured cells was used to confirm cell phenotypes but EPC were not prospectively identified in non cultured cells. A murine hindlimb ischaemia model also used cultured rather than freshly isolated cells. Recovery of perfusion was assessed by Doppler ultrasound with no assessment of location or activity of transplanted cells performed. Despite their origin from within the $CD34^+$ fraction of MNC, EPC could not be identified in cryopreserved apheresis collections.

The differential mobilisation of murine HPC and EPC into the peripheral blood by G-CSF, AMD3100 and VEGF has been reported (190). Pre-treatment with G-CSF enhanced HPC but not EPC mobilisation. Progenitor cell numbers were assessed using colony assays with EPC assessed in day 21 fibronectin adherent cells, plated in supplemented EBM2, a mix of features from the more commonly used short term CFU-EPC and EOC colony assays. EPC were not prospectively identified by FACS analysis but were assessed as $CD34^+/KDR^+/146^+/VWF^+$ day 21 adherent MNC that also bound lectins, took up ac-LDL and formed tubules. BM EPC were identified as $CD34^+/KDR^+$ cells but PB EPC numbers were not assessed in similar fashion. The cell numbers plated into EOC colony assay (5×10^5 lysed peripheral blood rather than MNC) were exceedingly low (and colony frequency high) compared to our own work (unpublished data) and other studies enumerating EOC in human peripheral blood (108, 133). Nevertheless, the authors showed using their colony assay that cell cycle status is critical for G-CSF mobilisation of HPC and that VEGF, through its influence to increase the proportion of actively cycling cells, abrogates HPC mobilisation, features not found with EPC. Despite apparent increases in EPC following $G\text{-}CSF^+$/-AMD3100, due to increased bone marrow egress of EPC, analysis of BM EPC showed that EPC were significantly reduced following G-CSF, a finding

that is contrary to their other results (190). As yet, the differential mobilisation of HPC and EPC by G-CSF and AMD3100 has not been reported in humans. It is difficult to extrapolate these findings to human subjects as the cytokine doses used; 2.5μg/kg/d G-CSF and 5mg/kg AMD3100, both by intraperitonal injection, differ substantially from the intravenous delivery of 10μg/kg/d G-CSF and 240μg/kg AMD3100 administered for HPC mobilisation in humans.

1.8. Thesis background and aims

This body of work is an extension of observations made by O Tura that CFU-Hill numbers declined following administration of G-CSF to patients mobilised with G-CSF$^+$/-chemotherapy for PBSC collection (136, 169). At that time various trials had been conducted using bone marrow (with only a few using PBSC) but it seemed that PBSC were an attractive prospect for clinical use, based on the experiences with HSCT. These findings of reduced CFU-EPC in G-CSF mobilised blood were contrary to a number of studies examining the roles of G-CSF and G-CSF mobilised EPC, in patients with cardiovascular disease (163, 165, 167) that were published in 2005-2006 just prior to commencement of this work. Shortly after this, (2007) 2 studies reported on G-CSF induced mobilisation of EPC in patients undergoing PBSC mobilisation for the collection of HPC (162, 170). Our understanding of EPC and their relationship to HPC at this point is summarised in Figure 1.1.

The short term culture of MNC on fibronectin in Medium 199 and foetal calf serum published by Hill *et al* (2003) was linked with vascular function and cardiovascular risk (110) and a commercial assay (CFU-EPC (CFU-Hill), Stem Cell Technologies, UK) developed from this was adopted by many groups, including ours, as a functional assessment of EPC. At that time phenotypic definitions proposed for EPC included CD34$^+$/133$^+$/KDR$^+$ cells, though almost every combination of CD34, CD133 and KDR expression has been used.

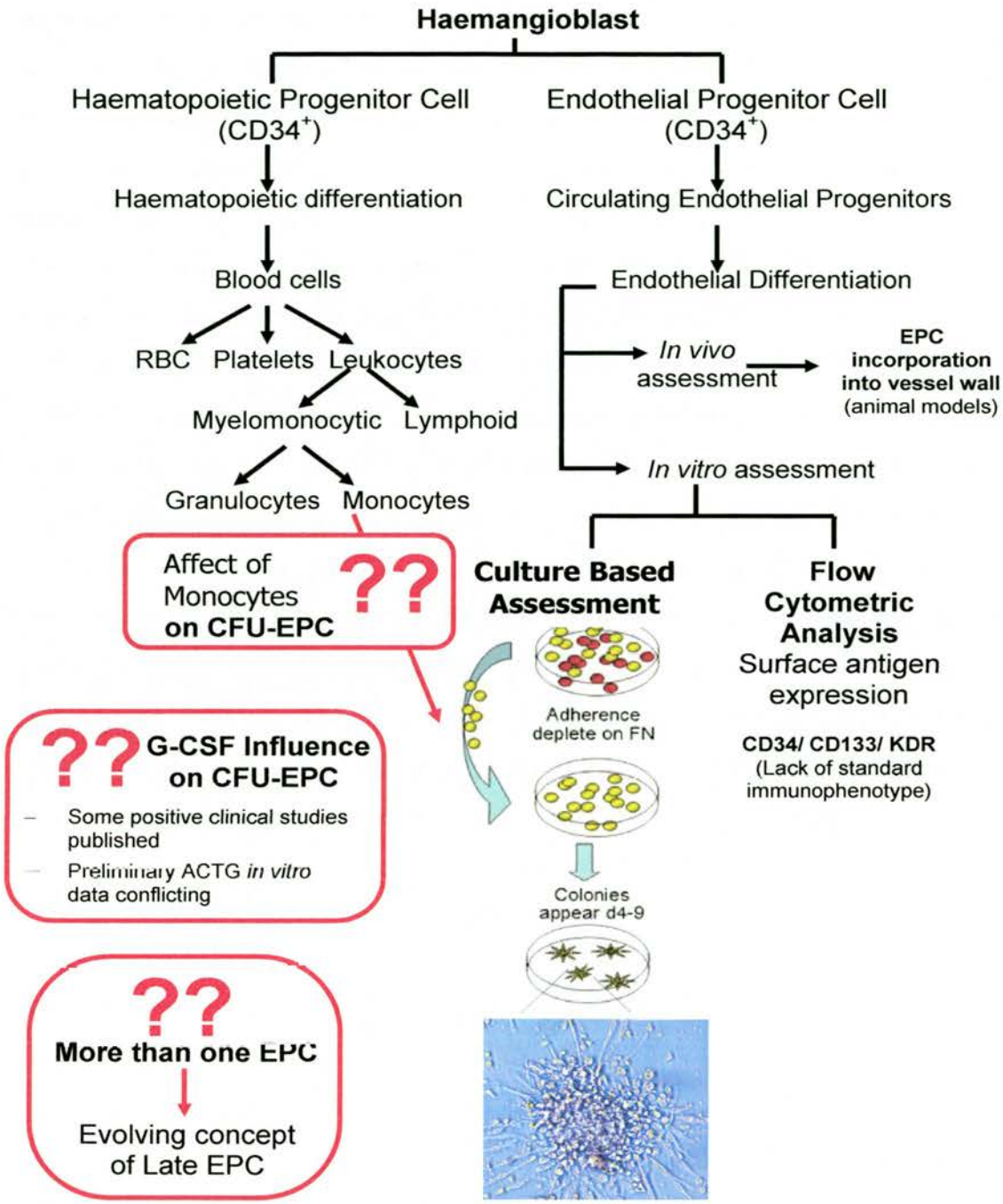


Figure 1.1 Illustration of relationship between haematopoetic and endothelial differentiation as understood in 2006.

There was strong laboratory evidence generated within our group that CFU-EPC were derived from $CD14^+$ monocytes (136) (oral presentation 2005 ISEH conference, Glasgow, O Tura) and were not dependent on $CD34^+$ or $CD133^+$ cells. Subtractive analysis performed to correlate the CFU-EPC assay with putative EPC phenotypes and the contributions of $CD34^+$ and $CD133^+$ cells to CFU-EPC, found selective enrichment for $CD34^+$ or $CD133^+$ MNC (MACS immunomagnetic separation) failed to generate CFU-EPC. The CD34 and CD133 depleted fractions had reduced CFU-EPC compared to unselected MNC and it was postulated that a proportion of colony-forming cells adhered to the MACS separation columns and were 'lost' from culture. Following on from this, 2-hour plastic-adherent cells from normal blood (enriched to ~80% purity for $CD14^+$ monocytes) were shown to contain all CFU-EPC. Immunomagnetic separations similarly showed that CFU-EPC formed in CD14-enriched (~98% purity) but not CD14-depleted MNC. Papers discussing the role of immune cells, in particular those by Rhode *et al*, had not been published. Other groups were proposing that many cells could be EPC including monocytes, especially the Dimmeler group who said there are many routes to an 'endothelial cell' some of which might be from normal MNC, backed up by a clinical study using peripheral blood MNC and bone marrow (155). Later the role of T cells in CFU-EPC was proposed (135, 139, 142). CD4, CD8 and CD19 positive cells could be detected in CFU-EPC generated from MNC but they were not detected in CFU-EPC generated from CD14-enriched MNC, leading us to question the role for T cells to CFU-EPC. At the time the group also took the view that CFU-EPC represented outgrowth from a single precursor cell, some form of 'proliferative monocyte'. Work using EDU demonstrated that cell proliferation did occur within CFU-EPC and was necessary for CFU-EPC as blocking cell proliferation with mitomycin C abrogated CFU-EPC formation. However, time lapse imaging also confirmed a role for cell migration in CFU-EPC development (G Padfield, personal communication). The work using G-CSF mobilised blood commenced in the midst of exploration of the role of $CD14^+$ monocytes in CFU-EPC. Although CFU-EPC were increased following CD14-enrichment, this could not be demonstrated for G-CSF mobilised samples. It

was found that in mobilised patient samples, 2-hour plastic adherence, performed to enrich for $CD14^+$ monocytes, failed to increase CFU-EPC in G-CSF mobilised samples as it did when using non-mobilised blood samples or UCB. The effects of G-CSF on CFU-EPC were therefore investigated. The changes that occurred in cell content plated into CFU-EPC assays following PBSC mobilisation were examined as it was evident that MNC recovered from density gradient centrifugation of mobilised blood differed from MNC recovered from non-mobilised blood. The presence of putative EPC phenotypes in mobilised samples was also assessed. In parallel with this it was questioned as to why normal peripheral blood contained the greatest number of CFU-EPC and that CD34-enriched mobilised blood failed to generate CFU-EPC (169).

In 2006, the CFU-EPC (CFU-Hill) assay and other short-term culture assays plating MNC onto fibronectin were used to functionally assess EPC. The lack of correlation between CFU-EPC and the putative EPC phenotypes was noted by our group (136, 169) and others. Subsequently, the concepts of early and late EPC evolved in the literature. Endothelial outgrowth cells were probably first described in experiments using bone marrow by Lin *et al* (2000) (108). The concept of late outgrowth endothelial cells and the presence of distinct populations of EPC within blood and bone marrow were identified (130, 146). More recently, the distinction between the haematopoietic (myelomonocytic) origin of CFU-EPC and the non-haematopoietic $CD34^+$ origin of endothelial outgrowth cells has been better defined (93, 115). The research of the group then moved to plate mobilised samples into endothelial outgrowth assays as well as CFU-EPC, to assess for the development of late outgrowth cells. The presence of putative EPC phenotypes were re-examined in light of this. Based on differences in 'stem cell' phenotype between UCB and mobilised blood samples (136, 169) the phenotypes of $CD34^+$ cells within UCB and mobilised blood were investigated further.

Whilst there are risks associated with G-CSF administration and leukapheresis, cell-based therapy would be less invasive if these cells could be procured from the peripheral blood following G-CSF administration rather than from the bone marrow. The improvements in disease free survival and overall survival for haematological malignancies made since the introduction of BMT and more recently PBSCT are testaments to the benefits of stem cell therapy and the prospect of using bone marrow-derived and blood-derived cells for applications other than HSCT is exciting. However, for G-CSF mobilised blood, use of the CFU-EPC assay, developed as an assessment of endothelial potential, failed to identify these cells, casting doubt on the suitability of cytokine (G-CSF) mobilised blood for cell-based (other than HSCT) or vascular therapy. This led us to pursue the evaluation of G-CSF mobilised blood further, drawing on these initial observations to move forward and explore the area further, possibly to shed light on the mechanism(s) behind the depression in CFU-EPC. This forms the basis of the work presented in this thesis.

The aims and objectives of this thesis were;

1. to extend the observations made by O Tura of the reduction in CFU-EPC frequency following the administration of G-CSF for PBSC mobilisation;
2. to establish whether any observed reductions in CFU-EPC could be attributed to technical factors/artefact;.
3. to establish the changes in peripheral blood and MNC leukocyte subpopulations and erythrocytes following PBSC mobilisation and to investigate how these might affect CFU-EPC development; and
4. to establish the frequency of putative EPC phenotypes in non-mobilised peripheral blood, mobilised peripheral blood and UCB by extended phenotypic definition and to observe how this changes following G-CSF mobilisation.

2. Materials and methods

2.1. Cell sources and sampling

2.1.1. General sample collection procedures

All cells were obtained from human sources. Appropriate ethics committee approval was obtained and institutional procedures were followed. Informed written consent was obtained from all patients who donated samples for this study.

2.1.1.1. Adult blood samples

The principal source of clinical samples was venous blood samples collected from adult patients referred to the SNBTS Cell Separator Unit in Edinburgh for autologous or allogeneic peripheral blood stem cell (PBSC) collection. Allogeneic PBSC donors were HLA-matched adults, donating PBSC for transplantation to their siblings who required a stem cell transplant. Autologous PBSC donors were largely haematology patients with multiple myeloma, non-Hodgkin lymphoma or Hodgkin lymphoma with smaller numbers of patients with other diseases including chronic myeloid leukaemia and acute lymphoblastic leukaemia. These patients were undergoing peripheral blood stem cell harvest (PBSCH) so that their own haematopoietic progenitor cells (HPC) could be used to intensify their (anti-cancer) therapy. HPC were 'mobilised' into the peripheral circulation from the bone marrow following administration of granulocyte colony stimulating factor (G-CSF) (Lenograstim) in a dose of 5-10μg/kg/day, given alone or sequentially following chemotherapy. Following HPC mobilisation, PBSC were collected from the patients' circulation by leukapheresis.

10mL of venous blood was collected into sampling tubes containing either lithium heparin or ethylenediaminetetraacetate (EDTA) anticoagulant. Samples were collected at the time of donor assessment within 30 days of planned PBSC collection (pre G-CSF sample), at the time of day 1 PBSC collection (Day 1 PBSCH) at the time

of blood sampling prior to commencement of leukapheresis (post G-CSF or mobilised sample) and 4-6 weeks following PBSC collection (follow-up sample).

Buffy coat leukocytes were obtained from SNBTS blood donations. Buffy coats are the leukocyte-rich layer which sediments out at the erythrocyte/platelet interface during the preparation of platelets from whole blood donations (buffy coat platelets).

2.1.1.2. Umbilical cord blood samples

Umbilical cord blood samples were collected from consenting women undergoing elective Caesarean section delivery. Umbilical cord blood was aspirated from the umbilical veins following uncomplicated, elective Caesarean deliveries and collected into heparinised 50mL containers.

2.1.2. Method of cell cryopreservation

Fresh MNC were suspended in PBS and counted. After the cell count was determined, an aliquot of cells was taken into a fresh tube following discard of the supernatant, cells were re-suspended in 500µL of complete culture media and transferred into 1.8mL DNAse and RNAse free tubes (Greiner, UK or NUNC) at a concentration of $1x10^6$ to 5×10^7/mL. Freezing medium; 400µL of foetal calf serum and then 100µL of DMSO; was added in drop-wise fashion to each vial. Samples were frozen in a temperature-controlled manner. Samples were placed in a minus 20°C freezer for 1-12 hours before being transferred to a minus 80°C freezer. In the majority of cases, samples remained in the minus 80°C freezer. A small number of samples were transferred to liquid nitrogen (minus 140°C) after being held at minus 80°C overnight.

2.1.3. Preservation of plasma by freezing

Whole blood samples were either allowed to sediment by gravity or centrifuged at 200g for 5 minutes. The plasma layer was removed by careful pipetting and

transferred to an Eppendorf tube. Platelet poor plasma was obtained by centrifuging the plasma in a microfuge (Biofuge 5000rpm for 5 minutes) and then reserving the supernatant. Plasma was frozen in aliquots in 1.8mL DNAse and RNAse free tubes (Greiner, UK), by being placed initially at minus 20°C for at least 1 hour before being transferred to a minus 80°C freezer.

2.1.4. Thawing of cells

Cryovials of cells were removed from the minus 80°C freezer and placed on ice. Aliquots of complete media (10mL) were placed in clean centrifuge tubes and warmed in water bath to reach 37°C. The cryovials were removed from the ice and placed in fumehood. Cells were thawed initially by being placed in a waterbath whilst being agitated. After spraying the cryovials with isopropanol, small amounts of warmed media were pipetted into the tube. The sample was then transferred to a clean centrifuge tube. Aliquots of warmed media were added to the cells up to a volume of 10mL, agitating tube throughout to promote mixing to dilute DMSO. Cell counts were determined using a Coulter counter.

2.2. Cell subpopulation isolation

2.2.1. Mononuclear cell isolation

Mononuclear cells (MNC) were isolated by buoyant density gradient centrifugation of blood samples over Histopaque (1.077g/mL; Sigma Diagnostics, UK). Samples were usually diluted 1:2 in sterile PBS and layered over Histopaque (5mL Histopaque in 15mL tubes and 15mL Histopaque in 50mL tubes) before being centrifuged at 400g for 20 minutes. Interface leukocytes were collected and washed twice in sterile PBS before being enumerated. Unless otherwise stated, these interface leukocytes were the basis of subsequent cell subpopulation isolations.

2.2.2. Polymorphonuclear cell isolation

Polymorphonuclear leukocytes (PMN) were isolated from human peripheral blood samples using Polymorphprep (PMP) (Axis-Shield PCAS, UK); a solution for the isolation of PMN (principally neutrophils) from whole blood. 5mL of freshly collected undiluted whole blood was layered over 5mL of PMP solution and the tube centrifuged at 250g for 30minutes. After centrifugation, the MNC and PMN are separated into two distinct bands of cells, with the erythrocytes sedimenting, with MNC in the upper band and PMN in the lower band. Both the upper and lower layers were collected by gentle pipetting. PMN (lower band of cells) were diluted by the addition of half strength PBS to restore normal osmolality. Cells were washed twice in sterile PBS and resuspended in Dulbecco's medium.

2.2.3. Mononuclear cell isolation using Leukosep centrifuge tubes

Leukosep centrifuge tubes contain an inert filter membrane. The tubes were prepared for MNC separation by the addition of 15mL Histopaque (1.077g/mL; Sigma Diagnostics, UK) to the tube (lying above membrane layer). The tubes were then centrifuged at 1000g for 1 minute to move the Histopaque through the membrane. The level of the membrane within the tube was adjusted so that it was positioned at the surface of the Histopaque solution. The diluted whole blood sample was placed in the tube over the membrane layer and the tube centrifuged at 400g for 40 minutes. Interface leukocytes were collected and washed twice in sterile PBS before being enumerated.

2.2.4. Purification of cells by immunomagnetic beads

2.2.4.1. Purification by antibody-conjugated microbeads

MACS® microbeads (Miltenyi Biotech) are superparamagnetic particles that are either coupled to specific monoclonal antibodies (used to label cells directly with microbeads) or to fluorochrome-conjugated antibodies (e.g. anti-FITC) (used for indirect cell labelling). Microbeads are used to label the target cell population which can then be separated from unlabelled cells by introducing them into a matrix

packed column held in a powerful magnet (magnetic cell separator). Where antibody conjugated microbeads were available for the antigen of interest (CD14, CD3), MACS immunomagnetic cell separation was performed using a direct labelling method.

MNC isolates were labelled using MACS® monoclonal antibody-conjugated microbeads (Miltenyi Biotech, UK). Up to 1 x 10^7 MNC isolated by buoyant density gradient centrifugation were incubated with 100μL of Fc-receptor blocking reagent (Miltenyi Biotech, UK) for 10 minutes at room temperature to inhibit non-specific or Fc-receptor mediated binding of the microbeads to non-target cells. The cells were then labelled with 10μL of microbeads, mixed well, and incubated for 30 minutes at 4°C. The cells were washed in sterile PBS and resuspended in 500μL of medium, ready for immunomagnetic separation. The purity of the cell populations were assessed by flow cytometry as outlined in section 2.3.1.

Immunomagnetic separation was carried out using MACS® MS separation columns (Miltenyi Biotech, UK). The separation column, held in the magnet, was fitted with a MACS® pre-separation filter (Miltenyi Biotech, UK) through which microbead labelled cells were introduced. Separation columns were prepared for use by washing with 3 x 500μL aliquots of buffer (PBS/0.2%EDTA/10%FCS), introduced into the pre-separation filter. The buffer was left to run down through filter and the column before the addition of the next aliquot of wash. Aliquots of labelled cells were then introduced into the MACS® pre-separation filter in volumes of 100μL. Cells were left to drip through the column before addition of the next cell aliquot. The microbead-labelled cells were retained in the column, and therefore enriched whilst the unlabelled cells were able to pass through the column. Both the positively and negatively selected cells were collected. After all labelled cells had been added to the column, at least 3 minutes was allowed to pass before washing was commenced. Three 500μL aliquots of buffer were used to wash columns (still placed in the separator), waiting for the buffer to clear from the column reservoir prior to

the addition of the next wash. The wash, together with any cells contained within it, were collected into the tube containing unlabelled cells. Positively selected cells were recovered by removing the column from the magnetic source, placing it over a clean collection tube and washing it with 3x 500µL aliquots of buffer (PBS/0.2% EDTA/10% FCS). A final wash with PBS/0.2%ETDA was followed immediately with use of the plunger supplied with the column to expel as many cells as possible from it. The purity of the cell populations were assessed by flow cytometry as outlined in section 2.3.1.

2.2.4.2. Purification by fluorochrome-conjugated microbeads

When specific monoclonal antibody-conjugated microbeads were not available, anti-fluorochrome conjugated microbeads were used and indirect cell labelling was carried out. Cells (up to 1 x 10^7 MNC recovered from buoyant density gradient centrifugation) were incubated with 100µL of Fc-receptor blocking reagent (Miltenyi Biotech, UK) for 10 minutes at room temperature to inhibit non-specific or Fc-receptor mediated binding of antibody and microbeads to non-target cells. MNC were then stained with the monoclonal antibody (e.g. anti-CD66b-FITC) and incubated at 4°C for 30 minutes in the dark. Cells were washed twice with sterile PBS and cells resuspended in medium. Anti-fluorochrome (e.g. anti-FITC) conjugated microbeads (Miltenyi Biotech, UK) were added to the washed antibody labelled cells and these were then incubated at 4°C for 30 minutes. The cells were washed in sterile PBS and resuspended in 500µL of medium, ready for immunomagnetic separation. The cell collection procedure was identical to that used for directly labelled cells.

2.3. Cell identification and phenotyping

2.3.1. Flow cytometry analysis

Peripheral blood samples were investigated by flow cytometric analysis prior to MNC isolation. Cell fractions obtained following MNC isolation using Histopaque density gradient centrifugation or cells isolated with the use of PMP density

gradient centrifugation were also phenotyped by flow cytometry. Four colour flow cytometric analysis was performed using a 488nm laser; used for phycoerythrin (PE), fluoroscein isothiocyanate (FITC) and peridin chlorophylla protein (PerCP) fluorochrome antibody conjugates; and a 633nm laser; for allophycocyanin (APC) fluorochrome antibody conjugates. Monoclonal antibodies used for analysis included anti-CD45-APC, anti-CD34-PerCP, anti-CD133-PE, anti-VEGFR2-FITC, anti-CD66b-FITC, anti-CD14-PerCP, anti-CD235-PE. See complete antibody list (section 2.6). When the nucleic acid stain SytoRed 59 was used, this required preparation of a working solution [5μL stock solution (supplied 5mM in DMSO) diluted in 500μL of CellWash (Becton Dickinson, UK]. SytoRed was assessed in channel 4 (FL4) on the same setting as used for APC.

2.3.2. Cell staining protocols

100μL aliquots of whole blood or MNC were placed in a Falcon-type 5mL tube. The sample was stained with the optimal concentration of the appropriate antibody or antibodies (normally 5μL of undiluted antibody) and then placed in the dark, either at 4C or at room temperature for 30 minutes. Unbound antibody was removed by dilution of stained cells in CellWash (Becton Dickinson, UK) and centrifugation. The mouths of the tubes were blotted on tissue in order to remove more of the decanted wash supernatants. Following centrifugation cells were resuspended in 500μL of CellFix (Becton Dickinson, UK).

Where whole blood was stained, removal of erythrocytes was required. 2.5mL of FACSLyse (Becton Dickinson, UK) was added to each tube after the initial 30 minute incubation that followed addition of antibody (or antibodies) and the tubes were then returned to the dark at room temperature for at least 20 minutes further incubation. Following erythrocyte lysis, unbound antibody and cell debris was removed by diluting cells in CellWash and washing them at least twice with centrifugation at 200g for 5 minutes. The washing step was repeated until the sample was free of traces of haemoglobin in the supernatant or cell pellet. Following

the final wash the washed were resuspended in 500µL of CellFix (Becton Dickinson, UK). Flow cytometry analysis was performed within 24 hours of antibody staining. When flow cytometry analysis was not performed immediately the stained cells, suspended in CellFix, were kept in the dark at 4°C. Unless specifically stated otherwise, whole blood (PB and UCB) flow cytometry was performed on lysed samples (using FACSLyse) and MNC flow cytometry was performed without erythrocyte lysis.

Events were collected on a FACS Sort flow cytometer linked to an Apple Macintosh computer equipped with CellQuestPro software (Becton Dickinson). The cytometer was calibrated and compensated using Becton Dickinson calibration beads and software for the fluorochromes used. Samples were all collected and analysed using identical stored instrument settings. Collection regions were set on forward scatter versus side scatter and for CD45 (or SytoRed59) versus side scatter to include leukocyte populations. Overall, for whole blood data, 100,000-300,000 events were collected in the relevant gate for 'stem cell' immunophenotyping and at least 50,000 events were collected for 'cell population' immunophenotyping. For MNC flow cytometry, 100,000-300,000 events were collected in the relevant gate for 'stem cell' immunophenotyping and at least 100,000 events collected for 'cell population' immunophenotyping. However, all events, whether falling within or outside of the collection gate, were recorded and saved for further analysis. Thus $CD45^-$ or $SytoRed59^-$ events (including erythrocytes and debris) could be examined. Flow cytometry data was saved to Listmode files and moved to PC (Windows) computers for detailed analysis. Flow cytometric analysis was performed using FCS Express software (De Novo software, www.denovosoftware.com).

2.3.2.1. Simple $CD34^+$ population analysis

Cells were stained with APC conjugated anti-human CD45 and PerCP conjugated anti-human CD34 and fixed with CellFix. $CD34^+$ population analysis was performed using a 'Stems CD45+R1 gate'; low forward and side scatter gated for CD45 and

CD34 according to the ISHAGE CD34 enumeration protocol (191) (Figure 2.1) and using a 'Stems R1' gate whereby $CD34^+$ events were assessed according to light scatter characteristics and CD34 expression without consideration of CD45 expression (Figure 2.2). The co-expression of other antigens by $CD34^+$ events was then measured.

For Stems CD45+R1 gating, stained and fixed cells were gated according to the ISHAGE CD34 enumeration protocol (191) using anti-CD34-PerCP and anti-CD45-APC antibodies (Figure 2.1).The same CD34 antibody (anti-CD34-PerCP) was used for Stems R1 gating (Figure 2.2).

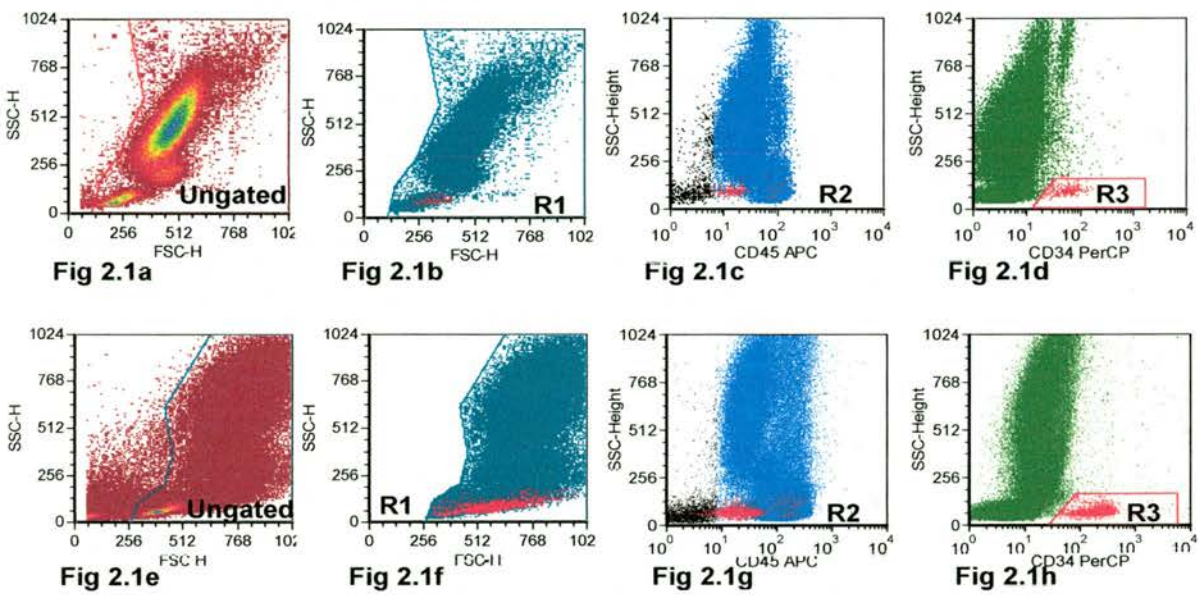


Figure 2.1 CD34 enumeration using ISHAGE guidelines in G-CSF mobilised samples. Samples are mobilised PB from an allogeneic PBSC donor (figures 2.1a-2.1d) and mobilised MNC from an autologous PBSC patient (figures 2.1e-2.1h) (Stems CD45+R1 gating).

Fig 2.1a and 2.1e Forward scatter versus side scatter (all events).
Fig 2.1b and 2.1f Primary gate (R1) set on forward scatter and side scatter.
Fig 2.1c and 2.1g Expression of CD45 by R1 gated events (R2).
Fig 2.1d and 2.1h Identification of $CD34^+$ cells in R2 gated cells (R3), displayed in red, as low side scatter events gating within R1 and R2 that were also $CD34^+$.
$CD34^+$ cells have been backgated (red) in fig 2.1b/2.1f and fig 2.1c/2.1g.

2.3.2.2. Defining stem cells

Stem cells were defined as low forward and side scatter cells gating within the live cell gate that expressed CD34 and/or CD133 (Stems R1 gate). CD45 was omitted

from this definition in order to detect haematopoietic ($CD45^+$) stem cells as well as any non-haematopoietic ($CD45^-$) stem cells, although the vast majority of stem cells were haematopoietic in origin.

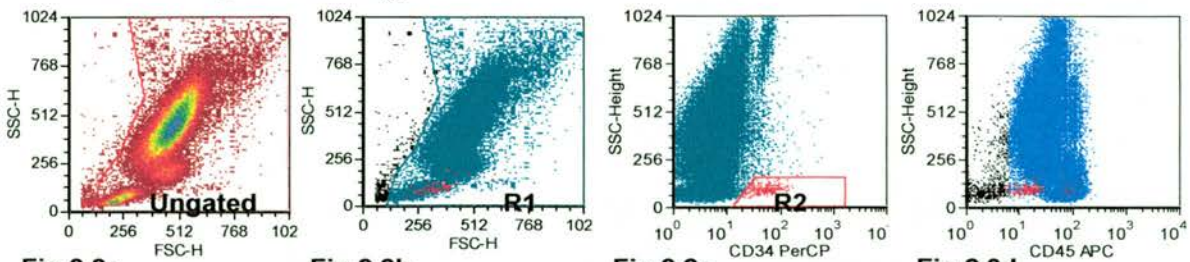


Fig 2.2a **Fig 2.2b** **Fig 2.2c** **Fig 2.2d**

Figure 2.2 CD34 enumeration using Stems R1 gating in a G-CSF mobilised PB sample. (allogeneic PBSC donor)

Fig 2.2a Forward scatter versus side scatter (all events).
Fig 2.2b Primary gate (R1) set on forward scatter and side scatter (cell size and granularity)
Fig 2.1c Expression of CD34 by low SSC events gated in R1 (R2), displayed in red.
Fig 2.1d Identification of $CD34^+$ cells $CD45^+$ events within R1 gate (blue).

In fig 2.2b the $CD34^+$ cells have been backgated (in red).
CD34 gating by ISHAGE guidelines for this sample was displayed in figures 2.1a-2.1d.

2.3.2.3. Complex Immunophenotyping

Essentially the same staining procedure was used as for $CD34^+$ population analysis (above) to stain whole blood samples and MNC samples with fluorochrome labelled monoclonal antibodies. The antibody staining protocol contained a larger panel of antibodies and tubes. The antibodies used included anti-CD34-PerCP, anti-CD45-APC, anti-CD45-FITC, anti-VEGFR2-FITC, anti-CD133-PE, anti-CD31-FITC, anti-CXCR4-PE, anti-CD3-PerCP, anti-CD19-PerCP, anti-CD66b-FITC, anti-CD33-FITC, anti-CD235a-PE and anti-CD14-PerCP (see complete antibody list, section 2.6). In most cases, lymphocytes were not identified using lymphocyte specific monoclonal antibodies (CD3 or CD19) but were identified as a population of low forward and side scatter $CD45^+$ events gating in the live cell gate (R1) that were $CD66b^-$, $CD14^-$ and $CD235a^-$ (Figure 5.1).

Unstained samples were used to establish negative staining boundaries. Isotype-matched negative control (non-binding) antibodies were not used. Of note, various isotype matched controls were used in earlier studies and compared to

unstained samples (O Tura). There were no differences found between strategies for setting the staining boundaries and I have continued with antibody staining protocols using a 'fluorescence minus one' strategy for multiparameter analysis, thus building up fluorochromes in a stepwise fashion (e.g. Tube 1 no antibody, Tube 2 CD45 only, Tube 3 CD45 and CD34, Tube 4 CD45, CD34 and CD133, Tube 5 CD45, CD34, CD133 and VEGFR2). The previous tube becomes the control for the next (192) (Figure 2.3).

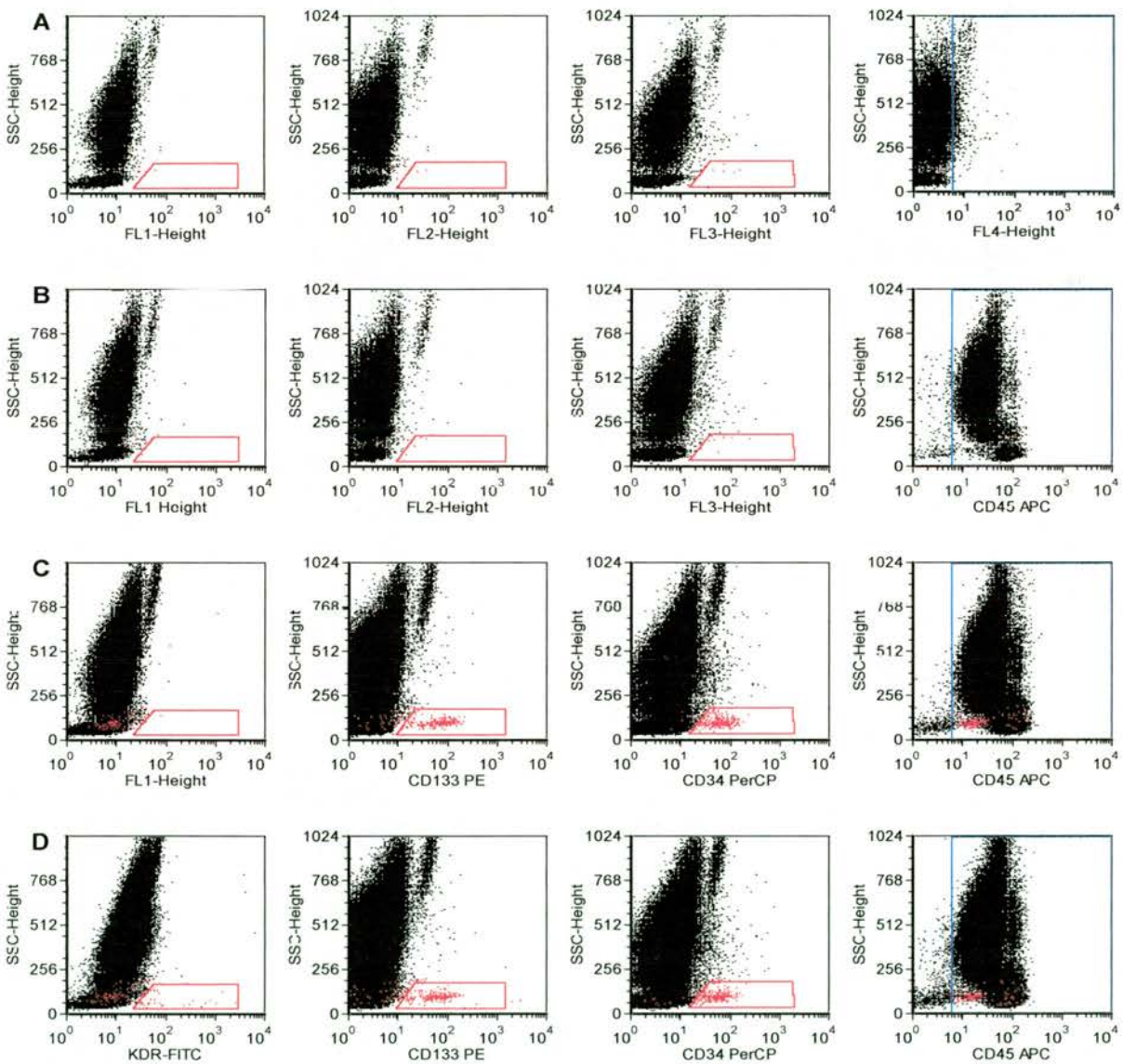


Figure 2.3 Fluorescence minus one gating strategy.

Illustrations of KDR-FITC, CD133-PE, CD34-PerCP and CD45-APC expression in an ungated whole blood sample from a mobilised PBSC donor using fluorescence minus one strategy whereby antigen staining is built up in a step-wise fashion and the previous tube becomes the control for the next.

A – no antigens, B – CD45-APC, C- CD133-PE, CD34-PerCP, D - KDR-FITD, CD133-PE, CD34-PerCP, CD45-APC.

2.4. Bioassays

2.4.1. Colony forming units – Endothelial Progenitor Cells (CFU-EPC) – CFU-Hill

This assay is based on that described by Hill *et al* (110) but it has been carried out using a commercially available kit, using the reagents supplied, in the manner recommended by the kit suppliers (Stem Cell Technologies), after Hill's published method.

MNC were isolated by buoyant density gradient centrifugation and washed twice with sterile PBS. Following enumeration, MNC were resuspended at a concentration of $2.5x10^6$/mL in Complete Endothelial Culture Medium (CECM) comprising Endocult Basal medium (Stem Cell Technologies, UK) supplemented with 1/5 dilution of Endocult supplements (Stem Cell Technologies, UK). Cells were plated at 2mL/well in fibronectin-coated 6-well plates (Becton Dickinson, UK) and incubated for 2 days at 37°C, 5% CO_2 with 95% humidity. After 2 days incubation, when mature monocytes and endothelial cells had adhered to the well surface(110), the non-adherent cells (containing EPC) were recovered from the well by gentle aspiration by pipette. These were enumerated and centrifuged before being resuspended at $0.8\text{-}1.0x10^6$ cells/mL in 1mL of fresh CECM and added to a fibronectin-coated 24-well plate (Becton Dickinson, UK). The cells were incubated for at least a further 3 days at 37°C, 5% CO_2 with 95% humidity. Depending on the experiment being performed, the wells were inspected from day 4 (after 2 days of culture in 24-well plates) for colony formation. Day 5 colony assessments were always performed. The number of CFU-Hill colonies per well were divided by the number of cells plated into the well of the fibronectin-coated 24-well plate on day 2 and expressed per 10^6 cells plated.

Colonies were defined according to the morphological criteria published by Hill *et al* (110) and the Stem Cell Technologies' technical manual. A colony was defined as having a central core of round cells with elongated cells 'sprouting' from the

periphery of the core. The core was arbitrarily defined as appearing to contain greater than 50 rounded cells. Three or more sprouting spindle-shaped cells were also required to be present at the periphery of the core in order for the cell collection to be scored as a CFU-EPC. CFU-EPC have now been reclassified as early-outgrowth CFU-EPC with the colonies arising when using the method of Hill *et al* (110) referred to as CFU-Hill (113). Tight collections of rounded cells (>50 cells) without sprouting peripheral spindle cells were graded as 'clusters', not colonies. A collection of less than 50 rounded cells, irrespective of whether spindle cells sprouted from its periphery was graded as a cell 'clump'. Average spindle cell number reflected the average number of spindle cells present per microscope field of (x20 power). Spindle cells were assessed according to their length, breadth and branching tendencies. In subsequent sections and chapters the abbreviation CFU-Hill refers specifically to CFU-EPC enumerated in fibronectin coated wells that contain day 2 fibronectin non-adherent MNC fraction.

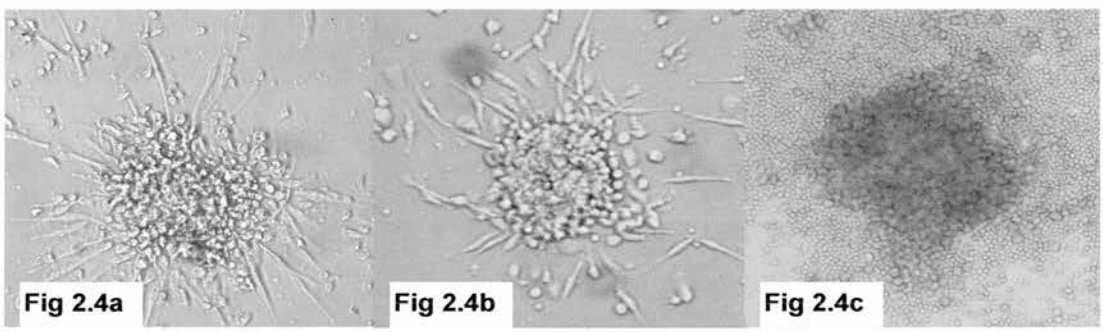


Figure 2.4 Assessment of CFU-EPC and cell clusters.
Illustration of 2 cell aggregates (Fig 2.4a and Fig 2.4b) scored as CFU-EPC and 1 aggregate not scored as CFU-EPC (Fig 2.4c).

2.4.2. Modifications to CFU-Hill assay

2.4.2.1. Fibronectin-coated 12-well plates

This assay is based on that described in section 2.4.1 (above) with the only difference from that method is in the use of fibronectin-coated 12-well plates rather than fibronectin-coated 6-well plates in the initial 2 day culture period. MNC isolated by buoyant density gradient centrifugation were plated at a concentration of $2.0x10^6$/mL in 1.5mL of CECM into fibronectin-coated 12-well plates (Becton

Dickinson, UK) and incubated for 2 days at 37°C, 5% CO_2 with 95% humidity. After 2 days incubation, the non-adherent cells were recovered and replated into a well of a 24 well fibronectin coated plate as per the CFU-Hill assay.

2.4.2.2. Directly plated CFU-EPC (Omission of culture on fibronectin-coated 6 or 12 well plates) - CFU-Hill(direct)

This assay is based on that described in section 2.4.1 (above) with the only difference from that method being that cell culture was performed entirely in fibronectin-coated 24-well plates and the initial plating of cells into 6 (or 12) well fibronectin coated plates was omitted. MNC isolated by buoyant density gradient centrifugation were resuspended at a concentration of $1.0x10^6$/mL in 1mL CECM and plated into a well of a 24 well fibronectin-coated plate (Becton Dickinson, UK) and incubated at 37°C, 5% CO_2 with 95% humidity. The wells were inspected from day 4 (after 4 days of culture in the 24-well plate) for colony formation with day 5 colony assessments always performed. In subsequent sections and chapters, the abbreviation CFU-Hill(direct) refers to freshly isolated (day 0) MNC directly plated for CFU-EPC assay into 24 well fibronectin coated plates.

2.4.2.3. Assessment of CFU-EPC potential of day 2 adherent MNC

500μL fresh CECM was added to the original well of a 6 or 12 well fibronectin coated plate immediately following the aspiration of non-adherent MNC on day 2. The plate was returned to the incubator and examined for CFU-EPC development in the same manner that the wells containing day 2 non-adherent MNC were handled.

2.4.2.4. Refreshing CECM during culture period

Alternate daily refreshment of CECM was carried out following the transfer of $1x10^6$ day 2 fibronectin non-adherent MNC in 1mL CECM into a well of a 24 well fibronectin coated plate (Becton Dickinson, UK) (CFU-Hill assay). On every other day during the culture period, 50% (500μL) of media was aspirated from the culture well and replaced with 500μL of fresh CECM.

2.4.2.5. Co-culture of cells using direct mixing of cells or transwell plates

Co-culture experiments were performed to assess the indirect/paracrine influence of cells on colony formation. MNC were obtained from standard Histopaque or PMP separation of whole blood. Granulocytes were obtained from either Polymorphprep (PMP) separation of whole blood or CD66b MACS separations of MNC. Cells were cultured, using a transwell set-up and/or allowing direct mixing of cells in the well. Either a CFU-Hill assay with re-plating of non-adherent cells on day 2 or, more commonly, a CFU-Hill(direct) assay (section 2.4.2.2) using 24-well fibronectin coated plates (Becton Dickinson, UK), was set up to assess CFU-EPC.

Experiments were carried out using cells from the same PBSC donor (autologous mixing experiments) or using cells from a PBSC donor together with buffy coat MNC or volunteer samples (allogeneic mixing experiments). Between 0.25 $x10^6$ and 0.50$x10^6$ 'secondary cells' (MNC or granulocytes) were added to 1$x10^6$ 'primary cells'/MNC; these figures chosen to reflect the degree of granulocytic contamination of G-CSF mobilised MNC preparations.

For transwell mixing studies, MNC (or other cell fraction) were placed in the well suspended in CECM as the base layer or primary cells. ThinCert™ inserts (24 well size, 0.4μm pore diameter ThinCert™ Greiner Bio-One, UK) were placed in the well, with care taken to ensure that the media volume rose to a level above the base of the ThinCert™ insert. Cells suspended in CECM were added to the ThinCert™ insert as the upper layer of the culture system. The volume of media used varied with the relative number of cells in the upper and lower levels and was adjusted within the recommended volumes recommended by the manufacturer (24 well ThinCert™ maximum working volumes - 0.4-1.0mL well volume and 0.1-0.35mL ThinCert™ volume) so that culture densities were very similar between the two cell populations. In general, for co-plating experiments using 24 well fibronectin coated plates and ThinCert™ inserts, base layer cells (primary cells) were suspended in 800μL CECM and upper layer cells (secondary cells) were suspended in 100μL

CECM before being added to the insert suspended in the well. When cells were mixed together in the well, cells were suspended in 900μL CECM. ThinCert™ inserts were removed from the wells and placed into clean wells containing sterile PBS during well inspection and colony enumeration with the inserts returned to the culture plate immediately following this.

2.4.3. Endothelial Outgrowth Cell (EOC) colony assays

MNC were isolated by buoyant density gradient centrifugation and washed twice with media. The cell pellet was resuspended and cells were counted using a Coulter counter. MNC, suspended in EBM2 media (Clonetics, UK) with EGM2 SingleQuots supplements were plated in a well of a collagen (collagen type 1) coated 6 or 12 well plate (Becton Dickinson, UK) up to a maximum density of $20x10^6$ MNC/well. Media volumes of 2mL and 1.5mL were used for wells of 6 and 12 well collagen coated plates respectively. Cells were cultured in 5% CO_2 at 37C. After 48 hours culture plates were removed from the incubator. The well contents were gently agitated using a pastette prior to the aspiration (and discard) of media, together with any collagen non-adherent MNC. The wells were washed with media (discarding the wash) and the fresh EBM2 with supplements is then added to the well and the plate returned to the incubator. On days 3-7 half (50%) of the media was replaced with fresh media (EBM2 with supplements) each day. From day 8 onwards 50% of the media was changed every 2-3 days. The wells were inspected for colony formation from week 2 of culture.

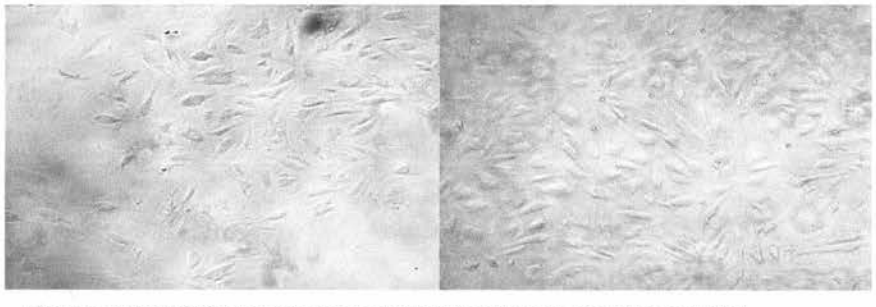


Figure 2.5 EOC development (umbilical cord blood sample).
(Courtesy of O Tura Ceide)

After culturing cells in EBM-2 medium for 2 weeks or more on collagen plates clusters of spindle-shapes cells begin to appear. These cells exhibit high proliferative capacity, can grow to confluence within the well and can be passaged at least 4-6 times (Figure 2.4).

2.4.4. Matrigel tube formation

Matrigel® Matrix (Becton Dickinson) is an endothelial cell matrix (ECM) preparation composed primarily of laminin, collagen type IV and a number of growth factors. Matrigel® is liquid at 4C and sets between room temperature and 37C so it needs to be handled cold using pre-cooled culture plates and equipment. Matrigel solution was thawed slowly overnight on ice/water at 4°C. Pipettes and plates were pre-cooled. 300μL of Matrigel was added to 24 well plates (Greiner, UK), with care being taken to avoid air bubbles and to ensure that the well surface was covered. The plate was placed in a 37°C, 5% CO_2, 95% humidity incubator for 45-60 minutes in order for the Matrigel to solidify. Pelleted cells (3-5x10^4cells/well) were resuspended in 500μL of EBM2 medium and cultured for 5-22 hours at 37°C with 5% CO_2 and 95% humidity. Capillary-like structures and endothelial cell networks were examined by phase contrast microscopy.

2.5. Statistical Analysis

Sets of data were compared using non-parametric statistical analysis, Mann-Whitney test for unpaired data and Wilcoxon matched pairs tests for paired data. The GraphPad Instat 3 statistical package was used for data analysis. All tests were two-sided and p values of less than 0.05 were considered to indicate statistical significance.

2.6. Reagents, culture mediums and antibodies used

Reagents list

CellFix (Becton Dickinson, UK)

CellWash (Becton Dickinson, UK)

EDTA/PBS (0.2%) Fisher, UK and Oxoid, UK)

FACSLyse (Becton Dickinson, UK)

Foetal calf serum (FCS) (Biowhittaker, UK)

Histopaque (1.077g/mL; Sigma Diagnostics, UK)

MACS® anti-FITC microbeads (Miiltenyi Biotech, UK)

MACS® anti-CD3 microbeads (Miltenyi Biotech, UK)

MACS® anti-CD14 microbeads (Miltenyi Biotech, UK)

MACS® Fc-R blocking reagent (Miltenyi Biotech, UK)

Matrigel Basement Membrane Matrix (BD Biosciences, UK)

Penicillin/Streptomycin (10000units/mL (penicillin) and 10μg/mL (streptomycin) (Sigma, UK)

Phosphate buffered saline (PBS) tablets (Oxoid, UK)

Polymorphprep (Axis-Shield PCAS, UK)

SytoRed59 fluorescent nuclear acid stain (Invitrogen, UK)

Culture mediums

Freezing medium – (10%dimethylsuphoxide (DMSO) (Sigma, UK) and 90% foetal calf serum (FCS)).

Iscove's Modified Dulbecco Media (IMDM) with 10% FCS (Biowhittaker, UK), 1% antibiotic (penicillin/streptomycin) (10000 units/mL and 10μg/mL respectively) (Sigma, UK).

Colony assay mediums

Complete Endothelial Culture Medium (CECM) comprising Endocult Basal Medium (Stem Cell Technologies, UK) supplemented with 1/5 dilution of Endocult supplements (Stem Cell Technologies, UK).

Endothelial Cell Basal Medium-2 (EBM®-2) (500 mL) (Clonetics, UK) with added growth supplements (EGM2™SingleQuots® containing FBS, hydrocortisone, hFGF, VEGF, R3-IGF-1, Ascorbic Acid, HEGF, GA-1000, heparin) (Clonetics, UK).

List of antibodies used (all anti-human specificity)

Anti-CD3-PerCP (BD Biosciences, UK)

Anti-CD14-PE (BD Biosciences, UK)

Anti-CD14-PerCP (BD Biosciences, UK))

Anti-CD66b-FITC (AbD Serotec, UK)

Anti-CD235a (Glycophorin A)-PE (Abcam, UK)

Anti-CD13-APC (BD Pharmingen, UK)

Anti-CD15-PE (BD Pharmingen, UK)

Anti-CD33-FITC (BD Pharmingen, UK)

Ant-CD64-FITC (BD Pharmingen, UK)

Anti-CD31-FITC (eBioscience, UK)

Anti-CD38-FITC (RD systems, UK)

Anti-VEGFR2 (KDR)-FITC (RD systems, UK)

Anti-CXCR4-PE (RD systems, UK)

Anti-CD133-PE (Mitenyi Biotech, UK)

Anti-CD45-FITC (BD Biosciences, UK)

Anti-CD45-APC (BD Biosciences, UK)

Anti-CD34-PerCP (BD Biosciences, UK)

Anti-CD146-PE (eBioscience, UK)

3. Review of peripheral blood stem cell harvest activities in Edinburgh

3.1. Introduction

The data presented in this chapter is a review of the collection of peripheral blood stem cells (PBSC) by the Cell Separator Unit (CSU) of the Scottish National Blood Transfusion Service (SNBTS), Edinburgh.

3.1.1. Aims of review

This review was performed in order to record the number of allogeneic and autologous peripheral blood stem cell harvests (PBSCH) performed by the CSU. The haematological diagnoses of patients referred for PBSCH and the PBSC mobilisation regimens prescribed were reviewed and the influences of these factors on the success of PBSC collection were examined. Similarities and differences between patient and treatment groups were considered in order to determine whether patients could be considered together in comparative studies and if there were any differences between patient groups which could be explored. Finally, the suitability of samples from autologous PBSC patients for inclusion in studies of the influence of G-CSF on mobilisation of haematopoietic progenitor cells (HPC) and non-haematopoietic progenitor cells were considered.

3.1.2. Methods

All referrals made for autologous PBSC collection between January 2004 and October 2006 and all healthy adults referred as potential allogeneic PBSC donors between January 2002 and October 2006 were identified. The collection period for allogeneic PBSC donor referrals was set 2 years greater than autologous PBSC referrals in order to increase the number of allogeneic PBSC donors reviewed. The data considers autologous PBSC patients and allogeneic PBSC donors referred for PBSC collection during the review periods who were assessed as fit to proceed with PBSCH and who then received the prescribed PBSC mobilisation regimen. The

medical records of patients referred for consideration of PBSC collection during this period were examined and the information contained within these were correlated with any additional written correspondence, laboratory results and clinical information that related to the PBSC mobilisation process.

Data was analysed using Mann-Whitney tests for non-parametric data with the probability value considered significant if it was $p<0.05$. In this review, the terms 'pre G-CSF' and 'pre mobilisation' refer to assessments made on donor review within 30 days of planned PBSC mobilisation with 'post mobilisation', 'mobilised' and 'post G-CSF' referring to assessments made on the first day of stem cell collection (day 1 PBSCH).

3.2. Allogeneic PBSC donors

3.2.1. Donors referred for allogeneic PBSC collection

Over the review period, 43 referrals for allogeneic PBSC mobilisation and collection were received (21 male and 22 female donors). There was considerable variation in the number of potential allogeneic PBSC donors referred for donor assessment and the number of allogeneic PBSC collections performed each year by the CSU. Thirty-five potential allogeneic PBSC donors were mobilised for PBSCH. Reasons for failure to proceed with PBSC mobilisation (n=8) included recipient ill-health/death (n=3), donor deferral for medical reasons (n=2), elective bone marrow harvest rather than PBSCH (n=2), PBSCH performed by another centre (n=2), uncertain reasons (n=2) (Table 3.1).

Year of referral**	2002	2003	2004	2005	2006$	Total
Donors referred	5	7*	8	14	9	43
PBSC Collected	5	3	7	12	8	35
Not collected	0	4*	1	2	1	8

Table 3.1 Allogeneic PBSC donor referrals and PBSC collections (2002-2006).
* Includes 2 patients referred for assessment with no intention of local PBSC collection.
** Referral year refers to date (year) patient was reviewed in CSU prior to PBSC collection.
$ January – October 2006 (10 months).

Allogeneic donors underwent PBSC mobilisation following the administration of G-CSF alone as the mobilising regimen. Lenograstim (non-glycosylated G-CSF) was administered in all cases. Donors generally received 10μg/kg/d G-CSF for 4 days (days 1-4) (n=33) before commencing leukapheresis (day 1 PBSCH) on day 5 (Figure 3.1). Alternative PBSC mobilisation regimens used were 10μg/kg/d G-CSF for 3 days (n=1) and 5μg/kg/d G-CSF for 4 days (n=1). Adequate numbers of $CD34^+$ HPC were mobilised into the peripheral blood (PB) to enable collection of sufficient PBSC for allogeneic peripheral blood stem cell transplant (PBSCT) in all but 1 case (34/35 donors, 97.1%).

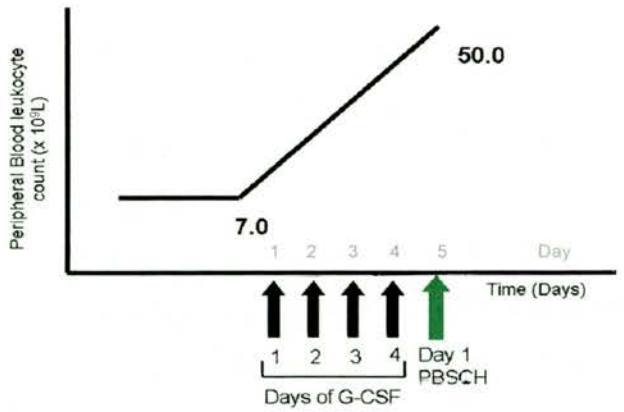


Figure 3.1 G-CSF PBSC mobilisation schedule for allogeneic donors.

3.2.2. Haematology parameters

There was no difference in donor age between female and male allogeneic PBSC donors (mean donor age 42.2 years for female and male donors) or in pre mobilisation PB leukocyte counts (means $6.4x10^9$/L and $7.4x10^9$/L in female and male donors respectively, p=ns). On day 1 PBSCH, following 4 days of G-CSF, there were no gender differences in PB leukocyte counts (white cell count (WCC) $47.8x10^9$/L and $49.0x10^9$/L respectively, p=ns) or CD34 counts (CD34 counts $58.6x10^6$/L and $52.6x10^6$/L respectively, p=ns) meaning there were no differences in the relative increases in PB leukocytes post G-CSF (7.4 and 7.1 in male and female donors respectively, p=ns).

3.2.2.1. Days of apheresis required to reach target CD34 numbers

The average number of days of leukapheresis required to reach the CD34 targets for allogeneic PBSCT was higher for female donors than male donors (2.1 and 1.4 days respectively), most likely a reflection of greater differences between donor and recipient body weights (and with recipient exceeding donor weight) when female allogeneic PBSC donors were used.

Ten of 17 male donors (58.8%) underwent a single leukapheresis with the remaining 7 (41.2%) requiring 2 days of PBSCH to reach the collection targets. Five of 18 female donors (27.7%) underwent a single leukapheresis. Seven female donors (38.9%) required 2 days of PBSCH and 6 (33.3%) required 3 collection days to reach minimum targets for CD34 yields. In excess of $3.0x10^6$/kg $CD34^+$ HPC, (based on recipient body weight) were collected from 20 of 35 allogeneic PBSC donors (Table 3.2).

Allogeneic PBSC donor details			Post mobilisation (Day 1 PBSCH) parameters (mean & (range))		
Gender	PBSCH days	Patients	WCC ($x10^9$/L)	CD34 count ($x10^6$/L)	Day 1 PBSCH CD34 yield ($CD34x10^6$/kg body weight)
Male	1	10	52.4 (32.4-90.4)	71.5 (45.2-106.8)	6.3 (4.5-9.8)
	2	7	41.9 (23.7-54.2)	41.5 (22.0-75.9)	2.7 (1.42-4.8)
Female	1	5	56.0 (48.6-80.1)	90.7 (34.0-126.0)	5.5 (4.1-9.4)
	2	7	53.9 (28.9-76.9)	35.8 (0.8-58.2)	1.7* (0.8-2.2)
	3	6	37.4 (18.4-53.2)	28.5 (15.4-53.2)	1.3 (1.0-1.8)

Table 3.2 Post mobilisation (day 1 PBSCH) PB leukocyte and CD34 counts.
Figures are displayed according to donor gender and number of days of leukapheresis required to attain CD34 targets (mean and range presented).
* Includes 1 donor who had insufficient $CD34^+$ cells collected over 2 leukapheresis and who proceeded to undergo a bone marrow harvest.

3.3. Autologous PBSC patients

3.3.1. Patients referred for autologous PBSC collection

Over the review period, 136 referrals were received for autologous PBSC collection; 126 adult haematology patients and 10 medical oncology patients (Table 3.3). Adult haematology patients with multiple myeloma (MM), non-Hodgkin lymphoma (NHL) or Hodgkin lymphoma (HL) made up the bulk of referrals for autologous PBSC collection and this section considers the PBSC mobilisation of these 120 patients (Table 3.3).

Patient Diagnosis	PBSC collection details	
	PBSC Referrals	Patients Mobilised
Haematology patients	**126**	**121**
Multiple Myeloma	55	53
Non Hodgkin Lymphoma	50	48
Hodgkin Lymphoma	15	14
Other Haematology patients*	6	6
Medical Oncology patients	**10**	**10**
Paediatric referrals[$]	5	5
Adult referral[£]	5	5
Total donor referrals	**136**	**131**

Table 3.3 Diagnoses of all patients referred for autologous PBSC collection.
* Other Haematology patients- 3 CML, 2 AML, 1 ALL (all in remission).
[$] Paediatric Oncology patients- 2 Ewings sarcoma, 1 Medulloblastoma, 1 Neuroblastoma, 1 HL.
[£] Adult oncology patients- 2 Ewings sarcoma, 2 Teratoma, 1 Rhabdomyosarcoma.

3.3.1.1. Haematology Patients (MM, NHL, HL)

All but 5 patients with MM, NHL or HL referred for autologous PBSCH (115 of 120 patients, 95.8%) proceeded to receive the prescribed PBSC mobilisation regimen. Overall, the PBSC mobilisation rate was 73.9% (85 of 115 patients) for autologous PBSC patients, meaning that these patients underwent leukapheresis to yield sufficient $CD34^+$ HPC for autologous PBSCT (Table 3.4).

Patient Diagnosis	MM	NHL	HL	MM/NHL/HL
Patients mobilised	53	48	14	115
PBSC harvested	45	28	13	85
Mobilisation rate	84.9%	58.3%	92.9%	73.9%

Table 3.4 Rates of successful PBSC mobilisation according to patient diagnosis.

There were no gender differences in the proportions of autologous PBSC patients who successfully mobilised PBSC; 49 of 64 male patients (76.6%) and 36 of 51 female patients (70.6%). Similarly there were no differences in pre mobilisation WCC, post mobilisation WCC and CD34 counts between male and female patients (Table 3.5).

Patients who failed to mobilise PBSC (inadequate CD34 numbers despite PB leukocyte counts >2.0x10^9/L following PBSC mobilisation) had WCC comparable to those patients who mobilised PBSC (Table 3.5).

Details	Mobilised PBSC			Failed PBSC Mobilisation		
Patient Group	All	Male	Female	All	Male	Female
Number of Patients	85	49	36	30	15	15
Non-mobilised WCC (x10^9/L)	5.2	5.2	5.1	5.0	4.8	5.3
Mobilised WCC (x10^9/L)	10.1	10.2	10.0	11.3	7.4	15.3
CD34 count (x10^6/L)	69.5	75.6	61.3	5.4	5.0	5.9

Table 3.5 Pre G-CSF and mobilised WCC and CD34 counts of autologous PBSC patients. Data includes patients who mobilised and who failed to mobilise PBSC.

3.3.2. Multiple Myeloma patients

Fifty-five referrals were received for autologous PBSC collections on MM patients. These were patients who had responded to initial chemotherapy, frequently oral CTD (comprising cyclophosphamide, thalidomide and dexamethasone) or intravenous combination chemotherapy (VAD (vincristine, doxorubicin, dexamethasone) or C-VAMP (cyclophosphamide, vincristine, doxorubicin, methylprednisolone) for example).

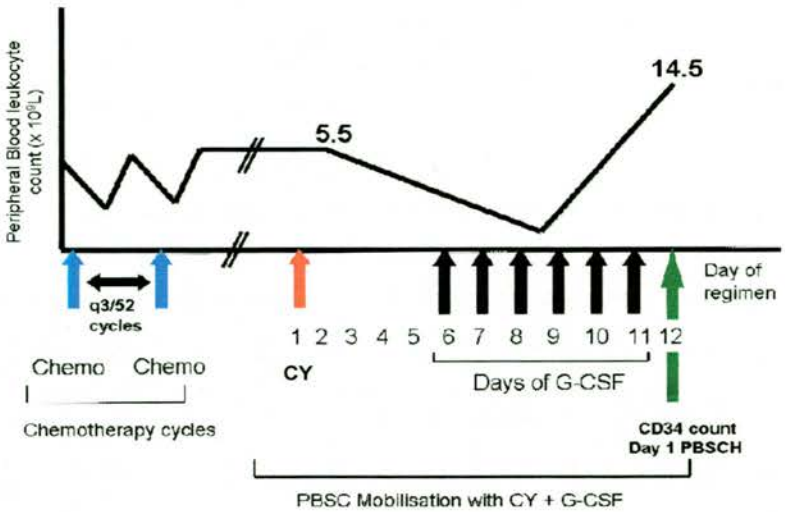


Figure 3.2 G-CSF PBSC mobilisation schedule for autologous MM PBSC patients.

Two patients died from disease progression prior to PBSC mobilisation. In general, MM patients received high dose cyclophosphamide (CY) (3g/m² body surface area (BSA)) (on day 1) followed by at least 6 days of G-CSF (days 6-11) with PB leukocyte and CD34 counts monitored daily from day 12 (Figure 3.2). PBSCH commenced when PB CD34 counts exceeded 10/μL.

PBSC mobilisation regimen	All Patients		Female Patients		Male Patients	
	Number	Sufficient PBSC	Number	Sufficient PBSC	Number	Sufficient PBSC
CY+5μg/kg G-CSF	17	12 (70.5%)	7	4 (57.1%)	10	8 (80%)
CY+10μg/kg G-CSF	35	30 (88.3%)	17	14 (82.4%)	18	16 (88.8%)

Table 3.6 Rates of PBSC mobilisation: MM patients.
Patients mobilised with CY 3g/m^2 BSA and 5-10μg/kg/d G-CSF.

MM patients were mobilised with CY+10μg/kg/d G-CSF (n=35) or CY+5μg/kg/d G-CSF (n=17) with 1 patient mobilised with 10μg/kg/d G-CSF for 4 days. Greater proportions of MM patents mobilised with sequential CY+10μg/kg/d G-CSF successfully mobilised PBSC and had sufficient CD34⁺ HPC collected for autologous PBSCT (>3.0x10⁶/kg CD34⁺ cells) (30 of 35 patients, 88.3%) compared to those MM patients who received CY+5μg/kg/d G-CSF (12 of 17 patients, 70.5%) (Table 3.6).

G-CSF Days	Patient numbers	PBSC mobilisation regimen and patient gender					
		CY+10μg/kg/d G-CSF			CY+5μg/kg/d G-CSF		
		All	Female	Male	All	Female	Male
6	35 (83.3%)	24 (80%)	12 (75%)	12 (85.7%)	11 (91.7%)	7 (87.5%)	4 (100%)
7	5 (11.9%)	5 (16.7%)	3 (18.75%)	2 (14.3%)	0	0	0
8	2 (4.8%)	1 (3.33%)	1 (6.25%)	0	1 (8.3%)	1 (12.5%)	0
Total	42	30	16	14	12	8	4

Table 3.7 Days of G-CSF administered prior to commencement of PBSCH: MM patients.

The great majority of MM patients (35 of 42 patients, 83.3%) commenced leukapheresis on day 12, after receiving 6 days of G-CSF, unaffected by G-CSF dose or patient gender (Figure 3.2 and Table 3.7). There were no gender differences in non-mobilised or mobilised WCC and CD34 counts between MM patients who received the same G-CSF dose (p=ns). MM patients mobilised with CY+10μg/kg/d G-CSF had higher WCC pre mobilisation and on day 1 PBSCH compared to those who received 5μg/kg/d G-CSF (p=ns) (Table 3.8). Although MM patients mobilised with CY+5μg/kg/d G-CSF had significantly greater proportions of PB $CD34^+$ HPC than CY+10μg/kg/d G-CSF patients (1.75% and 0.72% respectively, $p<0.04$), the higher post mobilisation WCC in CY+10μg/kg/d G-CSF MM patients meant that numbers of $CD34^+$ HPC did not differ between MM patient groups ($p>0.8$)) (Table 3.8). Use of higher dose G-CSF (10μg/kg/d) in MM PBSC mobilisation regimens has not improved day 1 PBSCH $CD34^+$ HPC yields and has not reduced the number of days of leukapheresis required to obtain sufficient PBSC for a single autologous PBSCT (Table 3.9).

Mobilisation Regimen	Details	Successful PBSC Mobilisation				Failed to mobilise	
		All	Female	Male	p value*	Female	Male
CY+ 10μg/kg/d G-CSF	Patient numbers	30	14	16	—	3	2
	Non-mobilised WCC ($x10^9$/L)	6.7	5.7	7.5	ns	3.8	2.5
	Mobilised WCC ($x10^9$/L)	14.5	11.7	17.0	ns	9.3	3.8
	CD34 count ($x10^6$/L)	76.1	61.7	88.8	ns	6.9	2.2
	CD34 (% WCC)	0.72	0.70	0.77	ns	0.10	0.05
CY+ 5μg/kg/d G-CSF	Patient numbers	12	4	8	—	3	2
	Non-mobilised WCC ($x10^9$/L)	4.8	3.9	5.3	ns	5.0	4.0
	Mobilised WCC ($x10^9$/L)	9.9	11.1	9.2	ns	15.8	9.5
	CD34 count ($x10^6$/L)	69.2	66.7	70.4	ns	6.9	2.5
	CD34 (% WCC)	1.75	0.6	2.3	ns	0.07	0.03

Table 3.8 Pre and post mobilisation WCC and CD34 counts: MM patients.
Figures are displayed according to patient gender and mobilisation regimen.
*comparison between males and females receiving same G-CSF dose (Mann-Whitney test).
ns = no significant difference

Use of higher dose G-CSF has resulted in 46.4% of successfully mobilised MM patients (13 of 28) having sufficient $CD34^+$ HPC collected for a tandem autologous PBSCT. More than half of MM patients mobilised with CY+10μg/kg/d G-CSF failed to reach this CD34 collection target as PBSCH was stopped after collection of sufficient $CD34^+$ HPC for a single PBSCT (>$3.0x10^6$/kg) once it was evident that even with additional days of G-CSF and further leukapheresis, original target CD34 yields (>$6.0x10^6$/kg) would not be achieved. The day 1 PBSCH CD34 yields following CY+10μg/kg/d G-CSF ranged from 0.8-$13.4x10^6$ CD34/kg.

G-CSF dose (μg /kg/day)	Patient Group	n	CD34 count ($x10^6$/L)	CD34 Day 1 PBSCH ($x10^6$/kg)	Number PBSCH Days	CD34 yield ($x10^6$/kg)	CD34 Yield >6 $x10^6$/kg
5	Female	4	66.7	3.3	1.7	5.7	1
	Male	8	70.4	4.8 ^	1.4	5.5	3
	All	12	69.2	4.3	—	5.5	4 (33.3%)
10	Female	14	61.7	3.9	1.7	6.3	5
	Male	16	88.8	4.4	1.9	6.7	8
	All	30	76.1	4.2	—	6.5	13 (46.4%)

Table 3.9 Day 1 PBSCH CD34 counts and day 1 CD34 collection yields: MM patients.
Mean values are displayed.
^ Result improved by day 1 PBSCH CD34 yield of $14x10^6$/kg (1 patient).

3.3.3. Non Hodgkin Lymphoma patients

Patients with a diagnosis of NHL were the second largest patient group referred for autologous PBSC collection (n=50). All but 2 of these underwent PBSC mobilisation with sequential chemotherapy and G-CSF (19 female and 29 male patients). Thirty-six patients (75%) were mobilised for PBSCH 'off the back' of a cycle of salvage chemotherapy, usually either IVE (ifosphamide, etoposide, and epirubicin) or DHAP (cisplatin, cytosine arabinoside, and dexamethasone) (Figure 3.3). This cycle of chemotherapy was administered in standard doses, with at least 6 days of G-CSF (5μg/kg/d) commencing a few (usually 4) days following chemotherapy. Three chemotherapy regimens were commonly used; IVE, DHAP or CY (44 of 48 patients) (Table 3.10). Four patients received alternate PBSC mobilisation regimens; IVE+10μg/kg/d G-CSF (n=1), CVP+5μg/kg/d G-CSF (n=1), 10μg/kg/d G-CSF alone (n=2). The highest PBSC mobilisation rates were observed following IVE+5μg/kg/d G-CSF (16 of 21 patients, 76%) with 54% of patients receiving DHAP+5μg/kg/d G-CSF and 50% of patients receiving CY+5μg/kg/d G-CSF mobilising sufficient PBSC (PB CD34 count >10/μL) to commence leukapheresis (Table 3.10).

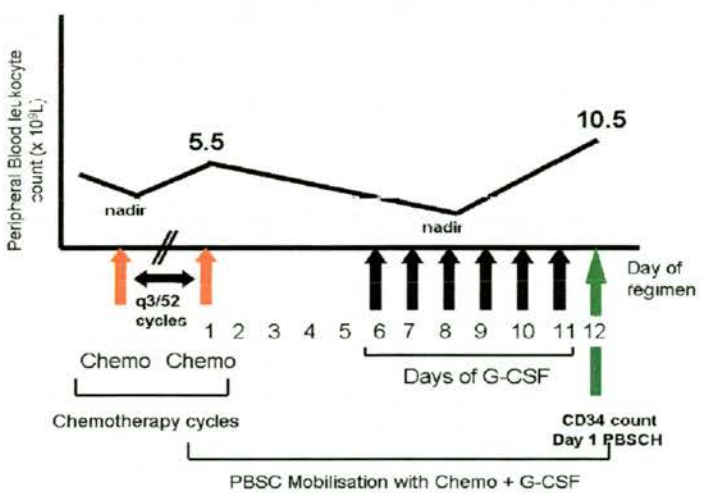


Figure 3.3 G-CSF PBSC mobilisation schedule for autologous lymphoma PBSC patients.

Not only did greater proportions of NHL patients mobilised 'off the back of IVE' undergo PBSCH, these patients had higher post mobilisation CD34 counts than NHL patients receiving alternate mobilisation regimens ($p<0.0001$), remaining

statistically significant when figures were adjusted to either include or exclude patients who failed to mobilise PBSC. The higher post mobilisation CD34 counts of these patients were not attributable to any differences in post mobilisation PB leukocyte counts between NHL patients mobilised with IVE+G-CSF and those who received alternate regimens (p>0.9) (Table 3.11).

PBSC Mobilisation	Total		Female		Males	
	#	Sufficient PBSC	#	Sufficient PBSC	#	Sufficient PBSC
IVE+5μg/kg/d G-CSF	21	16 (76.2%)	8	6 (75%)	13	10 (76.9%)
DHAP+5μg/kg/d G-CSF	11	6 (54.5%)	2	2 (100%)	9	4 (44.4%)
CY+5μg/kg/d G-CSF	12	6 (50.0%)	7	3 (42.9%)	5	3 (60.0%)

Table 3.10 Common PBSC mobilisation regimens used: NHL patients.
Numbers and proportions of patients are presented (n=44).

PBSC Mobilisation	Details	Mobilised PBSC		Failed mobilisation	
		Female	Male	Female	Male
IVE+ G-CSF (n=21)	Patient Number	6 (75%)	10 (76.9%)	2 (25%)	3 (23.1%)
	Mobilised WCC ($x10^9$/L)	6.7	4.7	1.6	7.7
	CD34 count ($x10^6$/L)	82.3	86.4	5.2	4.6
DHAP+ G-CSF (n=11)	Patient Number	2 (100%)	4 (44.4%)	0 (0%)	5 (55.6%)
	Mobilised WCC ($x10^9$/L)	6.2	4.9	—	5.7
	CD34 count ($x10^6$/L)	25.0	35.1	—	6.12
CY+ G-CSF (n=12)	Patient Number	3 (42.9%)	3 (60%)	4 (57.1%)	2 (40%)
	Mobilised WCC ($x10^9$/L)	5.5	6.8	6.3	13.3
	CD34 count ($x10^6$/L)	36.7	21.0	5.0	9.4

Table 3.11 Pre and post mobilisation WCC/CD34 counts: NHL patients.
Mean values are presented for 44 NHL patients.

The majority of NHL patients who mobilised PBSC following IVE or DHAP+G-CSF, required more than 6 days of G-CSF and did not commence PBSCH on day 12 (Table 3.12). There were no gender differences in pre G-CSF WCC or mobilised WCC and CD34 counts for NHL patients with sequential IVE+5μg/kg/d G-CSF (p=ns). For future comparisons, NHL patients mobilised by IVE+G-CSF were considered as a single group. Too few NHL patients were mobilised with DHAP for gender comparisons to be performed.

G-CSF Days	Chemotherapy regimen		All NHL patients
	IVE	DHAP	
6	8 (50.0%)	2 (33.3%)	10 (45.5%)
7	6 (37.5%)	3 (50.0%)	9 (40.9%)
8	1 (6.3%)	0	1 (4.5%)
9	1 (6.3%)	1 (16.7%)	2 (9.1%)
Total	16	6	22

Table 3.12 Days of G-CSF given prior to commencement of PBSCH: NHL patients. Figures are displayed according to PBSC mobilisation regimen (IVE or DHAP) used.

3.3.4. Hodgkin Lymphoma patients

Fifteen patients with HL (8 female and 7 male) were referred for autologous PBSC collection during the period reviewed and all but 1 underwent PBSC mobilisation. Two mobilisation regimens were commonly used; IVE+5μg/kg/d G-CSF (n=9) or DHAP+5μg/kg/d G-CSF (n=3) which were identical to the IVE and DHAP regimens administered to NHL patients (section 3.4.3 and Figure 3.3). Two patients were mobilised using G-CSF alone (10μg/kg/d G-CSF for 4 days). All 12 HL patients mobilised with sequential IVE or DHAP+5μg/kg/d G-CSF had successful PBSC collections performed (Table 3.13).

PBSC Mobilisation	Details	Mobilised PBSC	
		Female	Male
IVE+ 5μg/kg/d G-CSF	Patient numbers	4 (100%)	5 (100%)
	Total WCC ($x10^9$/L) (mean)	6.30	5.30
	CD34 count ($x10^6$/L) (mean)	87.7	78.8
DHAP+ 5μg/kg/d G-CSF	Patient numbers	1 (100%)	2 (100%)
	Total WCC ($x10^9$/L) (mean)	3.1	4.9
	CD34 count ($x10^6$/L) (mean)	16.7	58.5

Table 3.13 PB leukocyte and CD34 counts following PBSC mobilisation: HL patients.

No gender differences were noted in the pre G-CSF WCC, mobilised WCC or day 1 PBSCH CD34 counts for HL patients mobilised with IVE+G-CSF (p=ns). Too few HL patients were mobilised with DHAP for gender comparisons to be made.

Similar to our experience of NHL patients, the majority of HL patients mobilised with IVE or DHAP+G-CSF did not commence leukapheresis on day 12 as most

patients required at least 7 days of G-CSF to achieve sufficient PB CD34 counts to commence PBSC collection and PBSCH commenced on day 13 or later (Table 3.14).

Days G-CSF 5μg/kg/d	Chemotherapy regimen		All HL patients
	IVE	DHAP	
6	3 (33.3%)	0	3 (25%)
7	4 (44.4%)	3 (100.0%)	7 (58.3%)
8	2 (22.2%)	0	2 (16.7%)
Total	9	3	12

Table 3.14 Days of G-CSF administered prior to commencement of PBSCH: HL patients.

3.3.5. Lymphoma patients mobilised with IVE+5μg/kg/d G-CSF

There were no differences in pre G-CSF WCC or mobilised WCC and CD34 counts between the 16 NHL and 9 HL patients mobilised with sequential IVE+5μg/kg/d G-CSF (p=ns). All lymphoma patients mobilised using IVE+5μg/kg/d G-CSF were considered as a single patient group in subsequent comparisons. Too few patients were mobilised with sequential DHAP+G-CSF for comparisons to be made between lymphoma patients (Table 3.15).

Measured parameter	NHL patients (n=16) (Mean & (Median))	HL patients (n=9) (Mean & (Median))	P value
Non-mobilised WCC ($x10^9$/L)	4.7 (3.3)	3.8 (3.0)	ns
Mobilised WCC ($x10^9$/L)	5.4 (4.0)	5.8 (5.5)	ns
CD34 count ($x10^6$/L)	84.8 (68.0)	87.3 (41.4)	ns
CD34 percent (% WCC)	2.3 (1.65)	1.47 (1.16)	ns

Table 3.15 Haematology parameters in lymphoma patients treated with IVE+G-CSF. Figures are presented for 16 NHL & 9 HL patients with comparisons made between NHL and HL patients; ns = no significant difference.

3.3.6. Differences in PBSC mobilisation between autologous PBSC patient groups

Our data confirmed that two most frequently used regimens for autologous PBSC mobilisation at our institution, sequential CY+10μg/kg/d G-CSF and sequential IVE+5μg/kg/d G-CSF, were both moderately successful in mobilising sufficient $CD34^+$ HPC for PBSCH and subsequent autologous PBSCT (Table 3.16).

Mobilisation regimen	CY+10μg/kg/d G-CSF	IVE+5μg/kg/d G-CSF
Total number	36 (35 MM & 1 NHL)	30 (21 NHL & 9 HL)
PBSC Collection	30 (30 MM)	25 (16 NHL & 9 HL)
Successful mobilisation	83.3% (88.3% MM, 0% NHL)	83.3% (76.2% NHL & 100% HL)

Table 3.16 PBSC mobilisation with CY or IVE and G-CSF (autologous PBSC patients). Figures are displayed according to mobilisation regimen used and haematological diagnosis.

There were no gender differences observed in non-mobilised or mobilised WCC and CD34 counts (counts and proportions of leukocytes) for MM patients mobilised with CY+10μg/kg/d G-CSF (p=ns, n=30). Similarly there were no gender differences in the same 4 parameters for lymphoma patients (p=ns, n=25, 16 NHL and 9 HL patients) mobilised with IVE+5μg/kg/d G-CSF (p=ns). Autologous PBSC patients were therefore considered as belonging to 1 of 2 main treatment groups; MM patients mobilised with CY+10μg/kg/d G-CSF and lymphoma patients mobilised with IVE+5μg/kg/d G-CSF (Table 3.16).

There were differences in the mobilisation kinetics between these two treatment groups. MM patients had significantly higher pre mobilisation WCC on donor review than lymphoma patients ($6.7x10^9$/L and $4.5x10^9$/L respectively, $p<0.02$). This may reflect differences in the timing of PBSC referrals between patient groups. MM patients have low tumour burdens prior to PBSCH as patients are referred following completion of initial chemotherapy and confirmation of response of MM to treatment. These patients usually have had a chemotherapy-free period (of weeks rather than months) prior to being referred for PBSC collection. In contrast, lymphoma patients were typically referred for PBSC mobilisation once salvage chemotherapy (IVE) had been commenced for their relapsed lymphoma (usually following discharge from hospital after receiving first or second cycle of IVE) and their leukocyte counts had often not fully recovered at the time of donor review. Patients with MM were more likely to commence leukapheresis on day 12, after 6 days of G-CSF than lymphoma patients (Table 3.17).

Measured parameter (Mean & (Median))	MM patients (n=30)	Lymphoma IVE patients (n=25)	p value
Non-mobilised WCC ($x10^9$/L)	6.7 (5.9)	4.4 (3.15)	<0.02
Mobilised WCC ($x10^9$/L)	14.5 (10.9)	5.6 (4.2)	<0.0001
CD34 count ($x10^6$/L)	76.1 (57.2)	85.7 (63.0)	ns
CD34 percent (% WCC)	0.72 (0.49)	2.01 (1.51)	0.0002

Table 3.17 Haematology parameters taken prior to and following PBSC mobilisation.
Comparisons between MM and lymphoma patients (mean values presented).
MM patients were mobilised with CY+10μg/kg/d.
Lymphoma patients were NHL or HL patients mobilised with IVE+5μg/kg/d G-CSF.

MM patients had significantly higher post mobilisation (day 1 PBSCH) WCC (p=0.001) but lower proportions of PB $CD34^+$ HPC (p=0.0002) than lymphoma patients. The higher proportions of PB $CD34^+$ HPC in lymphoma patients might be a reflection of the administration of more myelosuppressive chemotherapy during PBSC mobilisation (with more rapid WCC recovery from lower nadirs) and their later commencement of PBSCH compared to MM patients (meaning more days of G-CSF therapy). The CD34 counts did not differ between patient groups (p=ns).

Lymphoma patients had higher yields of $CD34^+$ HPC from day 1 PBSCH (mean $6.0x10^6$/kg, median $4.5x10^6$/kg) than MM patients (mean $4.2x10^6$/kg, median $3.2x10^6$/kg) (p=ns). Although not statistically significant, the difference in $CD34^+$ HPC yields between patient groups meant that sufficient $CD34^+$ HPC were collected from a single leukapheresis for an autologous PBSCT in greater proportions of lymphoma PBSC patients; 18 of 24 lymphoma patients (75%; 7 of 9 HL patients and 11 of 15 NHL patients) and 16 of 29 MM patients (55.2%).

3.4. Autologous PBSC patients compared to allogeneic PBSC donors

Autologous PBSC patients had significantly lower PB WCC than allogeneic PBSC donors (means $5.2x10^9$/L and $6.9x10^9$/L respectively, p=0.0001) at donor review prior to PBSC mobilisation. Following PBSC mobilisation with G-CSF, administered alone or sequentially with chemotherapy, autologous PBSC patients continued to have lower PB WCC than allogeneic donors (means $10.1x10^9$/L and $48.4x10^9$/L respectively, p<0.0001) (Table 3.19). The proportions of $CD34^+$ HPC in mobilised PB samples were significantly greater in autologous PBSC patients than allogeneic PBSC donors (p<0.0001). However, due to the higher PB WCC counts in mobilised samples from allogeneic PBSC donors there were no significant differences in CD34 counts between mobilised autologous and allogeneic PBSC donors ($69.5x10^6$/L and $55.7x10^6$/L respectively, p>0.3) (Table 3.18).

Parameter	Allogeneic PBSC donors		Autologous PBSC patients		P value
	n	Mean	n	Mean	
Non-mobilised WCC ($x10^9$/L)	35	6.9	82	5.2	0.0001
Mobilised WCC ($x10^9$/L)	34	48.4	85	10.1	<0.0001
CD34 count ($x10^6$/L))	27	55.7	85	69.5	ns
CD34 (Proportion of WCC)	27	0.12%	84	1.22%	<0.0001

Table 3.18 Pre and post mobilisation WCC and CD34 counts in PBSC donors. Mean values are presented for allogeneic and autologous PBSC donors.

Comparisons were made between allogeneic donors (n=34) and the 2 main treatment groups of autologous patients (with MM or lymphoma, n=55) (section 3.3.6) who successfully mobilised PBSC (Table 3.19).

PBSC	Allogeneic PBSC Donor	Autologous PBSC Patient	
Diagnosis		MM	Lymphoma
Chemotherapy	Nil	CY	IVE
G-CSF	10μg/kg/d x 4	10μg/kg/d x 6	5μg/kg/d x 6
Patient numbers	34	30	25
CD34 (% leukocytes)	0.12%	0.72%	2.01%
CD34 counts ($x10^6$/L)	55.7	76.1	85.7
WCC ($x10^9$/L)	48.8	14.5	5.6
Neutrophils (%)	40.2 (82.5%)	12.1 (81.5%)	4.1 (66.6%)
Lymphocytes (%)	3.9 (8.0%)	0.7 (6.6%)	0.7 (16.2%)
Monocytes (%)	2.8 (5.8%)	1.1 (8.0%)	0.7 (16.2%)

Table 3.19 Haematology parameters following PBSC mobilisation.
Comparison between allogeneic and 2 groups of autologous PBSC donors. Figures are expressed as mean values with proportions of leukocytes in parentheses.

3.4.1. Allogeneic donors compared to CY+10μg/kg/d G-CSF mobilised MM patients

For reasons relating to the timing of PBSC collection and the mobilisation regimens used, the group of autologous PBSC patients most readily comparable to allogeneic PBSC donors was MM patients. MM patients are referred for PBSC collection early in the course of their disease (less than 12 months from diagnosis, often much earlier), after maximal disease response has been achieved with initial chemotherapy (assessed by plateau of serum paraprotein levels and reductions in bone marrow plasma cell load). The intention is to intensify therapy (with more myelosuppressive chemotherapy and autologous PBSCT) whilst the MM remains quiescent. At the time of PBSC mobilisation, MM patients have completed induction chemotherapy, have low disease bulk and a specific regimen of sequential chemotherapy (CY) and G-CSF is administered for solely to mobilise PBSC. In contrast, lymphoma patients referred for autologous PBSC collection may be many years post diagnosis. They are usually receiving salvage chemotherapy for relapsed or refractory disease and still have detectable lymphoma present when referred for PBSC collection.

MM patients were the only patient group referred for autologous PBSC collection who routinely received the same daily dose of G-CSF as used for allogeneic PBSC mobilisation, 10μg/kg/day. However, unlike allogeneic PBSC donors who received 4 days of G-CSF, MM patients received intravenous CY ($3g/m^2$ BSA) followed by a minimum of 6 days of G-CSF. Over the period reviewed, 30 MM patients were successfully mobilised following CY+10μg/kg/d G-CSF. There were no gender differences in non-mobilised and mobilised WCC and CD34 counts in MM patients (p=ns) or allogeneic PBSC donors (p=ns) (Table 3.19).

Allogeneic PBSC donors and autologous MM PBSC differed significantly in post-mobilisation PB leukocyte counts and proportions of PB $CD34^+$ HPC (assessed on day 1 PBSCH); (mean PB WCC $48.4x10^9$/L and $14.5x10^9$/L in mobilised allogeneic PBSC donors and autologous MM PBSC patients respectively, $p<0.0001$, and mean proportions of PB $CD34^+$ HPC 0.12% and 0.72%, $p<0.0001$). CD34 counts did not differ between these patient groups ($55.7x10^6$/L and $76.1 x10^6$/L, p=ns) (Table 3.19).

3.4.2. Conclusions

Whilst there are obvious differences between a patient with lymphoma or MM and a healthy adult donor, autologous PBSC patients treated at the CSU received a narrow range of chemotherapy and were mobilised for PBSCH with a similarly narrow range of mobilisation regimens. Mobilised PB from autologous PBSC patients is more CD34-rich (as proportions of total leukocytes) than allogeneic PBSC donor samples but the numbers of $CD34^+$ cells in mobilised PB samples do not differ between allogeneic and autologous PBSC donors. Autologous PBSC patients with MM were the most closely comparable group of autologous PBSC patients to healthy adult allogeneic PBSC donors.

3.5. Rationale for use of autologous PBSC donors as sources of G-CSF mobilised peripheral blood samples

Allogeneic PBSC donors (healthy adult subjects) remain an ideal source of G-CSF-mobilised PB samples for study of the influence of growth factors on cell populations. These PBSC donors are relatively young (most are 55 years old or younger), meet blood donor criteria and are free from co-morbidities that would preclude growth factor (G-CSF) administration or leukapheresis. However, only about 10 healthy donors are referred to the CSU annually for allogeneic PBSC collection and the bulk of patients treated with G-CSF are patients with haematological malignancies undergoing autologous PBSC mobilisation. Autologous PBSC donors are generally older than allogeneic PBSC donors (up to 70 years of age), and they may have co-morbidities including cardiopulmonary disease or vascular disease in addition to their haematological diagnosis. Unlike allogeneic PBSC donors who are mobilised using G-CSF alone, autologous PBSC donors are usually mobilised using sequential chemotherapy and G-CSF.

Review of autologous PBSC harvests performed by our centre confirmed that patients with MM and lymphoma (NHL or HL) formed the bulk of patients referred for autologous PBSC collection and that referrals numbers have remained fairly constant. Our data confirmed that autologous PBSC patient populations treated at the CSU fell into distinct groups and that these patient groups could be readily compared. Three PBSC mobilisation regimens were used for autologous PBSC mobilisation; sequential CY, IVE or DHAP chemotherapy and G-CSF, with CY and IVE the most commonly used. There were no gender differences within treatment groups, facilitating data analysis and inter-group comparisons. MM patients were the group of autologous patients undergoing PBSC mobilisation that were most readily comparable to healthy allogeneic PBSC donors.

There are no differences between PBSC mobilisation occurring in healthy allogeneic PBSC donors and that occurring in patients with haematological malignancies

undergoing autologous PBSC mobilisation. The disruption of the SDF-1α–CXCR4 bone marrow stem cell retention signal is central to the mobilisation of HPC, regardless of whether cytokines (G-CSF) are used alone or in combination with chemotherapy (57, 58). However, PBSC mobilisation occurs at lower PB leukocyte counts following sequential chemotherapy and G-CSF compared to those patients mobilised with G-CSF.

The same mechanisms operate to mobilise HPC in allogeneic PBSC donors treated solely with G-CSF and autologous patients undergoing PBSC mobilisation with G-CSF and chemotherapy. It is therefore likely that similar influences are being exerted on other bone marrow cell populations (including EPC and mesenchymal stem cells) in allogeneic PBSC donors and autologous PBSC patients following G-CSF administration. Clinical regenerative medicine research has largely used BM as a source of progenitor cells for revascularisation procedures but there is increasing interest in the use of PB-derived progenitor cells to regenerate damaged tissues. The use of established cytokines (G-CSF or GM-CSF) to mobilise progenitor cells into the circulation is being explored by a number of research groups including our own.

Administration of G-CSF for the mobilisation of PBSC is standard practice in haematology. Patients who receive G-CSF as part of routine care may be used as a starting point in the evaluation of the G-CSF effect on cell populations and the role of G-CSF mobilised blood for regenerative medicine purposes. Ultimately the clinical application of regenerative medicine techniques will not be within a healthy population but be used to benefit patients with significant organ dysfunction, including those with cardiovascular disease or other major organ injury. For this reason it is suggested that autologous PBSC donors, with their older age and co-morbidities, may be a more readily comparable group to future patients undergoing cell-based therapy than healthy adults. Blood samples obtained from allogeneic PBSC donors and autologous PBSC patients will therefore be studied.

Samples obtained from allogeneic PBSC donors can be used to validate observations made using autologous PBSC patient samples.

4. Investigation of the effect of *in vivo* G-CSF administration on CFU-EPC colony development

4.1. Introduction

In this chapter the effect of *in vivo* administration of G-CSF, given for the routine clinical mobilisation of HPC in haematology patients undergoing PBSC harvest (PBSCH), on CFU-EPC frequency will be investigated. Whole blood samples were collected from allogeneic and autologous PBSC patients on three occasions; (1) at donor review, prior to G-CSF administration and within 30 days of PBSCH (pre G-CSF), (2) on day 1 PBSCH following G-CSF administration (mobilised sample) and (3) at donor follow-up, usually 4-6 weeks following PBSC collection.

In the initial sections (sections 4.2-4.5) CFU-EPC formation was assessed using the CFU-Hill assay (as described in section 2.4.1). MNC obtained from Histopaque density gradient centrifugation of diluted whole blood samples were resuspended in CECM and plated into fibronectin-coated 6 or 12-well plates (Becton Dickinson, UK). After 2 days incubation at 37°C, 5% CO_2 with 95% humidity, the non-adherent cells (containing EPC) were recovered and enumerated. Between 0.8 $x10^6$ cells and $1.0x10^6$ cells were transferred in 1mL of fresh CECM to a fibronectin-coated 24-well plate (Becton Dickinson, UK) and then returned to the incubator. Culture wells were inspected for colony development daily from day 4 until at least day 8, with day 5 assessments always performed. CFU-EPC (CFU-Hill) were defined as a central core of >50 round cells with at least 3 elongated cells 'sprouting' from the periphery of the core. The number of CFU-Hill colonies per well were divided by the number of cells plated in the well of the fibronectin-coated 24-well plate and expressed per 10^6 cells plated.

In the latter half of this chapter a number of factors that might possibly influence the results of the CFU-EPC colony assay were identified. These have been examined to exclude whether the observed difference in CFU-EPC development following PBSC

mobilisation with G-CSF could be attributed to an introduced artefact rather than to real differences between patient samples.

4.2. CFU-EPC development in allogeneic PBSC donors

4.2.1. Patient samples

Anticoagulated venous blood samples were obtained from 21 individuals referred to the CSU as potential allogeneic PBSC donors (see section 2.1.1.1). Samples were collected from donors on the 3 occasions specified in section 4.1. PBSC were mobilised into the peripheral circulation with G-CSF alone, administered in doses of 10μg/kg/d for 4 days. Five potential allogeneic PBSC donors did not undergo PBSC mobilisation meaning that only pre G-CSF samples were available from these donors. PBSC were collected by leukapheresis following 4 days of G-CSF in the remaining 16 cases. Paired pre G-CSF and mobilised samples were available for 16 allogeneic PBSC donors with 14 of these 16 also having donor follow-up samples taken (matched pre G-CSF, mobilised and donor follow-up data available).

4.2.2. CFU-EPC formation

4.2.2.1. Pre G-CSF samples

CFU-EPC formation occurred in 16 of 21 (76.2%) allogeneic PBSC donor samples with 5 samples failing to generate CFU-EPC. Overall, the mean colony development was 8.9 CFU-EPC/10^6 MNC (median 6.0 CFU-EPC/10^6 MNC, range 0-35, n=21) (Table 4.2).

4.2.2.2. G-CSF Mobilised samples

CFU-EPC formation occurred in 6 of 16 (37.5%) allogeneic PBSC donor samples with 10 samples failing to generate CFU-EPC. The mean colony development in G-CSF mobilised samples was 1.6 CFU-EPC/10^6 MNC (median 0 CFU-EPC/10^6 MNC, range 0-10, n=16) (Table 4.2).

4.2.2.3. Donor follow-up samples

CFU-EPC formation occurred in 9 of 14 (64.3%) allogeneic PBSC donor follow-up samples with 5 samples failing to generate CFU-EPC. Overall, the mean colony development in donor follow-up samples was 11.0 CFU-EPC/10^6 MNC (median 7.0 CFU-EPC/10^6 MNC, range 0-40, n=14) (Table 4.2).

4.2.3. Paired non-mobilised and G-CSF mobilised samples

When only the patient samples with paired non-mobilised and G-CSF mobilised data were examined, the CFU-EPC frequencies were adjusted to be 11.0 CFU-EPC/10^6 MNC (n=16), 1.5 CFU-EPC/10^6 MNC (n=16) and 11.0 CFU-EPC/10^6 MNC (n=14) for pre G-CSF, mobilised and donor follow-up samples respectively (Table 4.3).

4.2.4. Differences in CFU-EPC formation following G-CSF administration

There were significant differences in the frequency of CFU-EPC between pre G-CSF and mobilised donor samples ($p<0.001$, paired data, n=16) and between mobilised and donor follow-up samples ($p<0.005$, paired data, n=14). No difference in CFU-EPC formation was observed between the donor samples taken pre G-CSF mobilisation and at donor follow-up ($p>0.8$, paired data, n=14). Sequential CFU-EPC formation in the allogeneic PBSC donor samples is displayed in Figure 4.1.

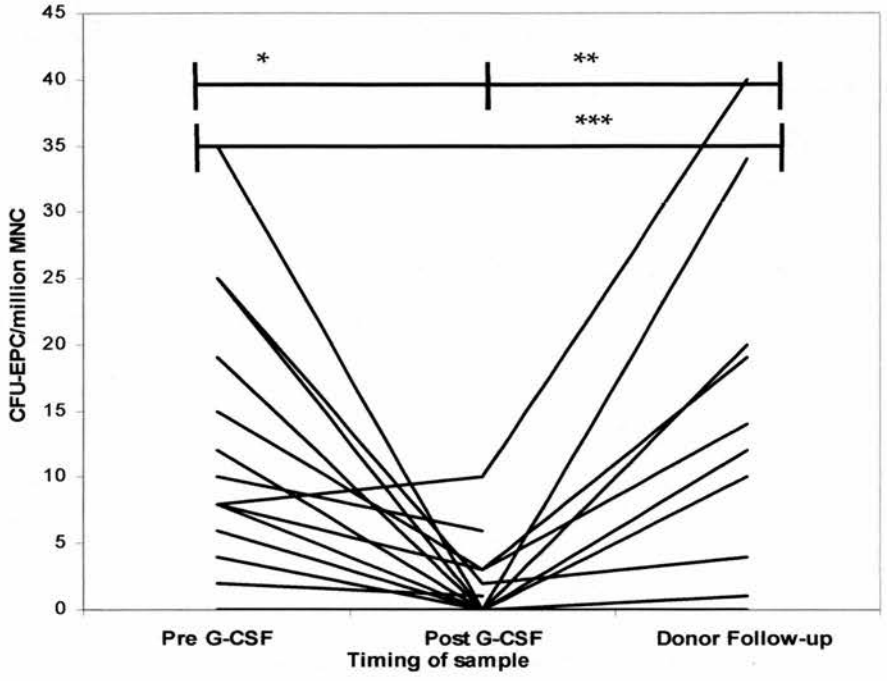


Figure 4.1 CFU-EPC in allogeneic PBSC donors.
Samples collected at 3 time points; pre G-CSF, day 1 PBSCH and at donor follow-up.

* p=0.0007; ** p= 0.0039; ***p>0.8, Wilcoxon matched pairs test.

4.3. CFU-EPC development in autologous PBSC patients

4.3.1. Patient samples

Anticoagulated venous blood samples were obtained from 55 patients referred to the CSU as potential autologous PBSC patients. Patient samples were collected on the same 3 occasions specified in section 4.1. Patient samples consisted of 54 pre G-CSF samples, 43 mobilised patient samples and 9 samples collected at patient follow-up. Patient follow-up samples were collected on admission for autologous PBSCT, usually 4-6 weeks post G-CSF administration and PBSC collection.

Paired pre G-CSF and mobilised patient samples were available for 36 patients. Six of these also had CFU-EPC performed on patient follow-up samples. An additional 3 patients had paired mobilised and donor follow-up samples collected (but no pre G-CSF sample available). PBSC were mobilised into the peripheral blood with sequential chemotherapy and G-CSF, administered in doses of 5-10μg/kg/d G-CSF for at least 6 days.

4.3.2. CFU-EPC formation

4.3.2.1. Pre G-CSF samples

CFU-EPC formation occurred in 30 of 54 (55.6%) pre G-CSF MNC. Overall, the mean CFU-EPC formation was 13.2 CFU-EPC/10^6 MNC (median 3.0 CFU-EPC/10^6 MNC, range 0-120, n=54) (Table 4.2).

4.3.2.2. Mobilised samples

CFU-EPC formation declined following the administration of G-CSF (and chemotherapy). CFU-EPC formation was observed in 24 of 43 (55.8%) mobilised patient samples with 19 failing to generate any CFU-EPC. The mean CFU-EPC formation was 2.2 CFU-EPC/10^6 MNC (median 0 CFU-EPC/10^6 MNC, range 0-39, n=43) (Table 4.2).

4.3.2.3. Patient follow-up samples

CFU-EPC formation was observed in 7 of 9 (77.8%) patient follow-up samples with 2 failing to develop CFU-EPC. Overall, the mean colony formation was 10.0 CFU-EPC/10^6 MNC (median 3.0 CFU-EPC/10^6 MNC, range 0-48, n=9) (Table 4.2).

4.3.3. Paired non-mobilised and G-CSF mobilised samples

When the CFU-EPC results from patients who had paired non-mobilised (pre G-CSF or donor follow-up) and mobilised patient samples collected were reviewed the mean rates of CFU-EPC formation were adjusted to be 18.6 CFU-EPC/10^6 MNC (n=36), 2.5 CFU-EPC/10^6 MNC (n=39) and 10.0 CFU-EPC/10^6 MNC (n=9) for pre G-CSF, mobilised and patient follow-up samples respectively with median CFU-EPC formation (per 10^6 MNC) being 5.0, 0.0 and 3.0 on the same 3 occasions (Table 4.3).

4.3.4. Differences in CFU-EPC formation following G-CSF administration

There were significant differences in the frequency of CFU-EPC between pre G-CSF and mobilised patient samples ($p<0.0001$, paired data, n=36) and between mobilised and patient follow-up samples ($p<0.02$, paired data, n=9). No difference in CFU-EPC formation was observed between donor samples taken pre G-CSF mobilisation and at patient follow-up ($p=0.25$, paired data, n=6). Sequential CFU-EPC formation in samples obtained from autologous PBSC patients is displayed in Figure 4.2.

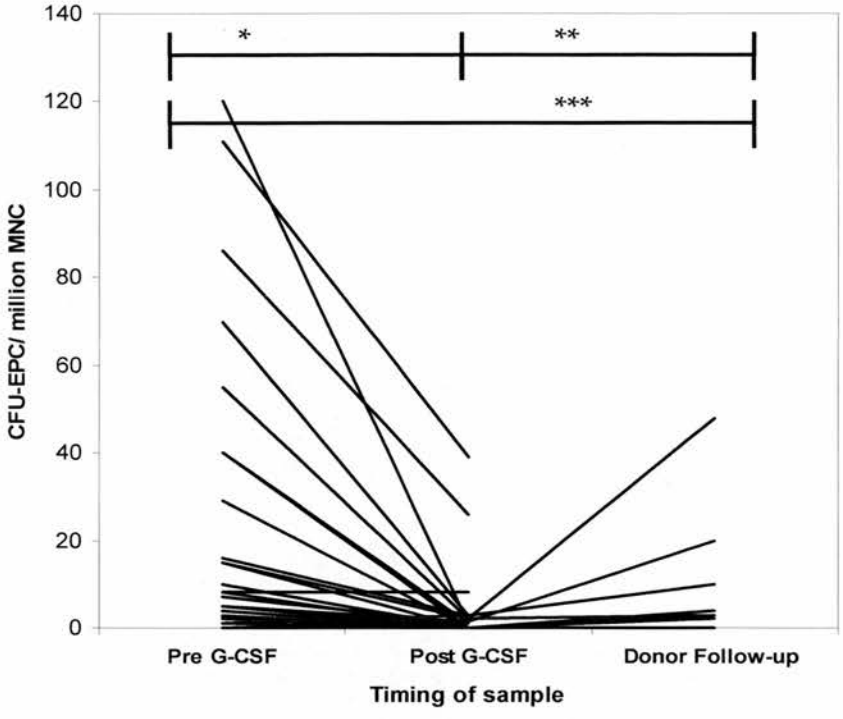


Figure 4.2 CFU-EPC in autologous PBSC patients.
Samples collected at 3 time points; pre G-CSF, day 1 PBSCH and at donor follow-up.

* $p<0.0001$; ** $p<0.02$; ***$p=0.25$, Wilcoxon matched pairs test.

4.3.5. Comparison of CFU-EPC between autologous PBSC patient groups

CFU-EPC formation in autologous PBSC patients was considered according to their haematological diagnoses. Of 39 patients with CFU-EPC formation assessed in paired non-mobilised and G-CSF mobilised samples, 21 had a diagnosis of multiple myeloma (MM) and 18 had a diagnosis of lymphoma (15 non Hodgkin lymphoma and 3 Hodgkin lymphoma patients). Analysis of the CFU-EPC formation of pre G-CSF and mobilised samples showed no differences between MM and lymphoma patients ($p>0.8$ and >0.6 in pre G-CSF and mobilised samples respectively) (Table 4.1).

MM patients showed mean CFU-EPC formation of 19.4 CFU-EPC/10^6 MNC, 2.6 CFU-EPC/10^6 MNC and 10.0 CFU-EPC/10^6 MNC from pre G-CSF, mobilised and patient follow-up samples respectively. The decline in mean CFU-EPC frequency following G-CSF was statistically significant ($p=0.0008$, paired data, $n=18$) as was the change (increase) in CFU-EPC formation between mobilised and patient follow-up samples ($p<0.02$, paired data, $n=9$) (Table 4.1).

Lymphoma patients showed mean rates of CFU-EPC formation of 17.8 CFU-EPC/10^6 MNC, 2.4 CFU-EPC/10^6 MNC from pre G-CSF and mobilised samples respectively. A statistically significant decline in mean CFU-EPC occurred following G-CSF administration ($p=0.005$, paired data, $n=18$) (Table 4.1).

CFU-EPC/10^6 MNC	n	Pre G-CSF	Mobilised	Follow-up
Multiple Myeloma	21	19.4 (4)	2.6 (1)	10.0 (3)
Lymphoma	18	17.8 (6)	2.4 (0)	No samples

Table 4.1 Comparison of CFU-EPC in autologous PBSC patient groups.
Paired non-mobilised and mobilised data displayed for MM and lymphoma (NHL+HL) patients with samples obtained pre G-CSF, on day 1 PBSCH and at donor follow-up. Figures are presented as mean and median (in parentheses) CFU-EPC per 10^6 MNC.

4.4. Comparison of CFU-EPC formation in allogeneic and autologous PBSC patients mobilised with G-CSF

The changes in CFU-EPC frequency following G-CSF administration observed in samples obtained from autologous PBSC patients were identical to the changes in CFU-EPC formation following administration of G-CSF to allogeneic PBSC donors. There were no significant differences in the frequency of CFU-EPC in pre G-CSF, mobilised and follow-up samples between autologous patient groups ($p=0.37$, $p>0.99$ and $p>0.99$ respectively) (Table 4.2).

CFU-EPC/10^6 MNC	Pre G-CSF	Mobilised	Follow-up
Allogeneic PBSC donors	8.9 (6)	1.6 (0)	11.0 (7)
Autologous PBSC patients	13.2 (3)	2.2 (0)	10.0 (3)

Table 4.2 CFU-EPC in allogeneic and autologous PBSC patients.
Pre G-CSF, mobilised and donor follow-up samples displayed.
Figures are presented as mean and median (in parentheses) CFU-EPC per 10^6 MNC.

Similar changes in CFU-EPC were observed when only samples from patients that had paired non-mobilised and mobilised data available were examined. Analysis of the mean frequencies of CFU-EPC in pre G-CSF, mobilised and donor follow-up samples obtained from PBSC patients showed that there were no differences between allogeneic and autologous PBSC patients ($p=0.56$, $p>0.8$ and $p>0.99$ respectively) (Table 4.3).

CFU-EPC/10^6 MNC	Pre G-CSF	Mobilised	Follow-up
Allogeneic PBSC donors	11.1 (8)	1.5 (0)	11.0 (7)
Autologous PBSC patients	18.6 (5)	2.5 (0)	10.0 (3)

Table 4.3 CFU-EPC in allogeneic and autologous PBSC patients (paired data).
Paired non-mobilised and mobilised CFU-EPC assessments are displayed. Samples were obtained pre G-CSF, following PBSC mobilisation and at donor follow-up.
Figures are presented as mean and median (in parentheses) CFU-EPC per 10^6 MNC.

For all PBSC donors (allogeneic and autologous PBSC donors combined) CFU-EPC activity decreased significantly following the administration of G-CSF for PBSC mobilisation ($p<0.0001$, paired data, n=52) and increased between G-CSF mobilisation and donor follow-up ($p<0.0001$, paired data, n=23).

4.4.1. Conclusions

The results presented in this section have established that when using a standard, commercially available, validated colony assay (CFU-Hill), the administration of G-CSF for PBSC mobilisation produces statistically significant reductions in CFU-EPC concentration. There were no differences in the reduction in CFU-EPC activity induced by G-CSF between allogeneic PBSC donors mobilised with G-CSF alone and autologous PBSC patients mobilised with G-CSF and chemotherapy (Figure 4.3).

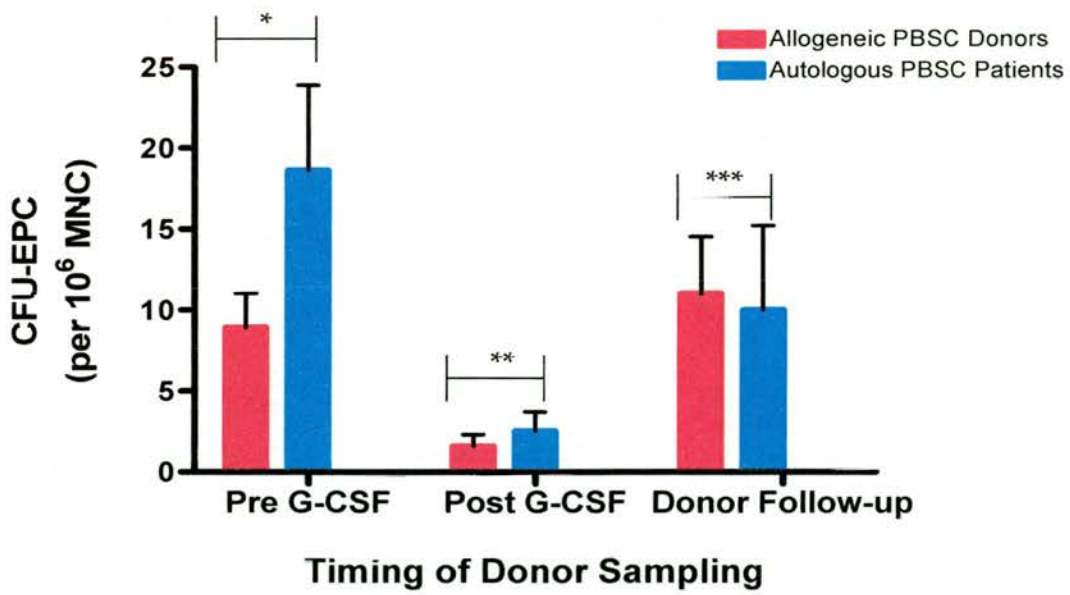


Figure 4.3 CFU-EPC formation in allogeneic and autologous PBSC patients. Samples obtained prior to G-CSF, following mobilisation and at donor follow-up. Paired non-mobilised and mobilised CFU-EPC data presented.

* p=0.37, **p>0.99, ***p>0.99, Mann-Whitney test

4.5. CFU-EPC development in autologous PBSC patients mobilised with G-CSF+AMD3100

Autologous PBSC patients were eligible to receive G-CSF+AMD3100 for the mobilisation of PBSC on the basis of their failure to mobilise PBSC following G-CSF, usually given sequentially with chemotherapy, on a previous occasion. AMD3100 (Plerixafor), a reversible CXCR4 antagonist, was administered with G-CSF (Lenograstim) (G-CSF+AMD3100), following a strict protocol to ensure that PBSC collections occurred during periods of peak drug activity. Patients received a total of 5 days of G-CSF (10μg/kg/d) and a single dose of AMD3100 (240μg/kg). AMD3100 was administered on the evening of day 4 after 4 doses of G-CSF, prior to commencing leukapheresis the next morning; 10-11 hours post AMD3100 and at least 1 hour after the 5th dose of G-CSF. Similar to allogeneic PBSC donors, patients mobilised with G-CSF+AMD3100 commenced leukapheresis without confirmation of their PB leukocyte or CD34 counts.

4.5.1. Patient samples

Anticoagulated venous blood samples were obtained from 6 patients undergoing 7 cycles of G-CSF+AMD3100 PBSC mobilisation, with 1 male patient undergoing 2 cycles of G-CSF+AMD3100 as the first cycle of PBSC collection was terminated prematurely due to intolerable AMD3100-induced gastrointestinal side effects. Blood samples were collected on the same 3 occasions as autologous PBSC patients mobilised with G-CSF+chemotherapy; with non-mobilised samples collected pre G-CSF+AMD3100 (n=5) or on donor follow-up (n=2) and mobilised samples collected on day 1 PBSCH following G-CSF+AMD3100 (n=6).

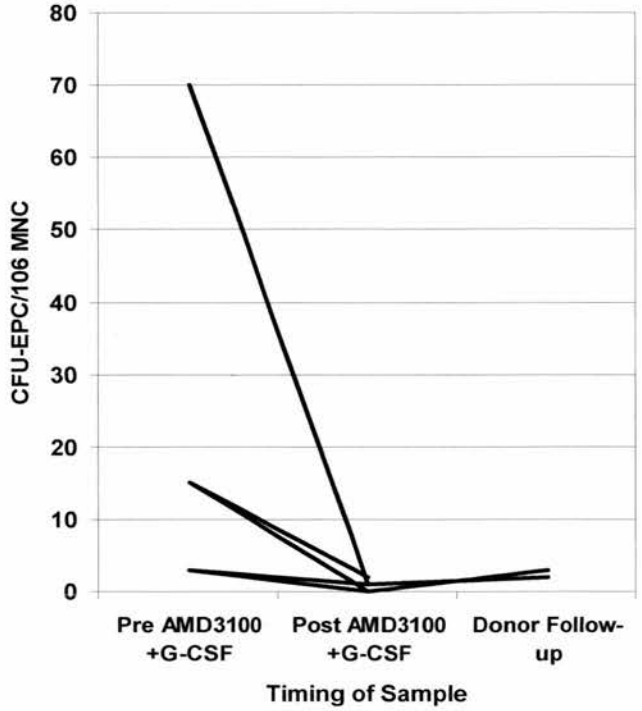
MNC were separated from whole blood samples by Histopaque density gradient centrifugation, using identical methods as employed for samples from patients receiving G-CSF^{+}/-chemotherapy for PBSC mobilisation (see section 4.2.1). The CFU-EPC culture methods used were identical to those used for G-CSF mobilised

patient samples (section 2.4.1) with colonies assessed daily from day 4 until at least day 8.

4.5.2. CFU-EPC formation

4.5.2.1. Pre G-CSF+AMD3100 samples

CFU-EPC formation was observed in all non-mobilised MNC. Pre G-CSF samples generated greater numbers of CFU-EPC than donor follow-up samples. The mean CFU-EPC frequency in pre G-CSF samples was 21.2 CFU-EPC/10^6 MNC (median 15 CFU-EPC/10^6 MNC, range 3-70, n=5) (Figure 4.4).



Timing of patient sample	CFU-Hill formation (CFU-EPC/10^6MNC)					
	298	299	299	313	316	321
Non-mobilised	70	3	3	15	—	15
G-CSF+AMD3100 mobilised	1	0	1	0	12	2
Donor follow-up	—	3	2	—	—	—

Figure 4.4 CFU-EPC in autologous PBSC patients mobilised with G-CSF+AMD3100. Samples collected pre G-CSF, day 1 PBSCH and at donor follow-up.

4.5.2.2. G-CSF+AMD3100 mobilised samples

CFU-EPC colony development declined from pre mobilisation levels following administration of G-CSF+AMD3100, to a mean of 0.8 CFU-EPC/10^6 MNC (median 1 CFU-EPC/10^6 MNC, range 0-12, n=6). In this small group of patients, the reduction in CFU-EPC formation post G-CSF+AMD3100 did not reach statistical significance (p=0.0625, paired data, n=5) (Figure 4.4).

4.5.2.3. Donor follow-up samples

Donor follow-up samples, obtained 4-6 weeks after PBSC mobilisation, showed relatively low CFU-EPC development (2.5 CFU-EPC/10^6 MNC, n=2). This was similar to the rate of CFU-EPC formation observed in the corresponding pre G-CSF+AMD3100 samples from these patients (3 CFU-EPC/10^6 MNC for both) and was higher than the levels of CFU-EPC generated by the same patients' mobilised blood samples (0 and 1 CFU-EPC/10^6 MNC) (Figure 4.4).

4.5.2.4. Parallels with patients mobilised with G-CSF$^+$/-chemotherapy

This pattern of CFU-EPC activity following the administration of G-CSF+AMD3100 paralleled that seen in autologous and allogeneic PBSC donors undergoing PBSC mobilisation with G-CSF alone or sequentially with chemotherapy.

4.6. CFU-EPC Colony morphology

CFU-EPC were defined as collections of cells consisting of a central core estimated to contain greater than 50 round cells with at least 3 spindle-shaped cells sprouting/radiating from the periphery of the core. Cell clusters of round cells without radiating spindle-shaped cells were not counted as colonies.

4.6.1. Non-mobilised samples

In non-mobilised samples that formed colonies, CFU-EPC displayed typical morphology as described by Hill (110) with CFU-EPC having tight central cores of round cells and spindle cells sprouting from the margins of these. Isolated cells (background cells) were heterogeneous in appearance and included a mixture of round and spindle cells. Although CFU-EPC all shared key characteristics there were considerable variations in colony morphology between patients (Figure 4.5). Whilst inter-patient variation in colony morphology was evident, there was less variation in CFU-EPC morphology in different samples from an individual. Consistent CFU-EPC morphology was observed in pre G-CSF and follow-up samples obtained from the same patient and from healthy volunteers sampled on more than one occasion.

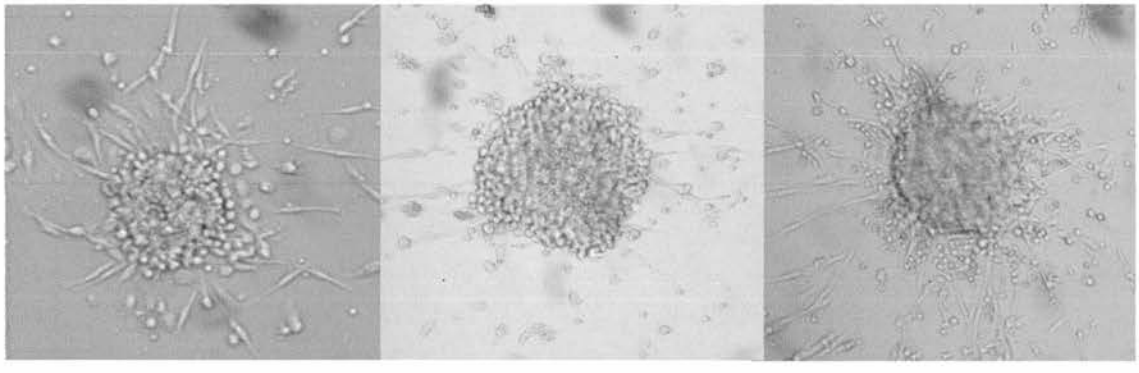


Figure 4.5 CFU-EPC in non-mobilised PBSC donor samples.
These images demonstrate the range of CFU-EPC morphology observed.

The presence of significant numbers of erythrocytes in some non-mobilised autologous PBSC samples made morphological assessments of CFU-EPC more

challenging as clumps of red cells were frequently positioned at the edge of cell cores/clusters, partially obscuring spindle cells from view. Nevertheless, CFU-EPC could still be assessed (Figure 4.6).

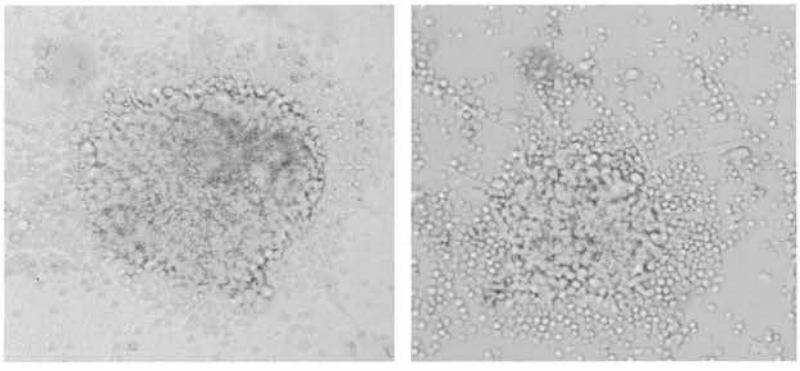


Figure 4.6 CFU-EPC in non-mobilised autologous PBSC patient samples.
The difficulty in assessment of core-associated spindle cells in the presence of erythrocytes (individual cells and clumps) is demonstrated.

4.6.2. Mobilised samples

The majority of G-CSF+/-chemotherapy or G-CSF+AMD3100 mobilised samples failed to develop any CFU-EPC and the formation of spindle cells was severely curtailed compared to that seen in non-mobilised samples from the same patient. In wells containing mobilised MNC, the great majority of cells were round in shape and were present either as single cells in small groups (<50 cell clumps) of cells, with larger cell clusters (>50 cells) rarely seen (Figure 4.7).

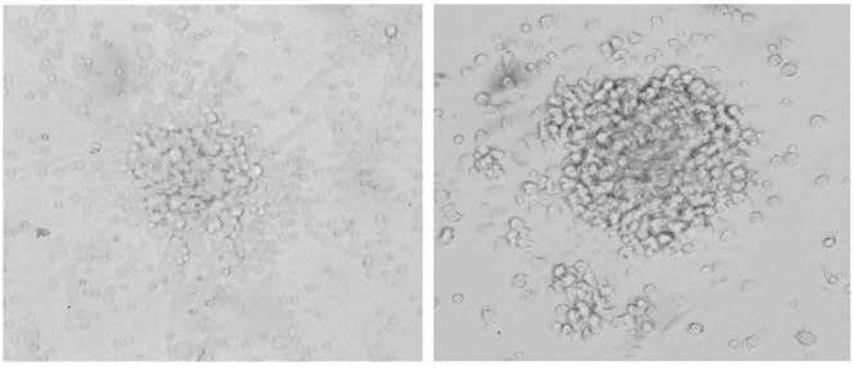


Figure 4.7 CFU-EPC/clusters in non-mobilised and mobilised samples (1).
CFU-EPC in non-mobilised sample (left) and cell cluster without core-associated spindle or background spindle in a mobilised sample (right) obtained from an allogeneic PBSC donor.

When CFU-EPC developed in mobilised patient samples their morphology differed from the comparative EPC-CFU from non-mobilised sample. CFU-EPC in mobilised samples was more ragged in appearance. Central cores of cells were less tightly aggregated and spindle formation was reduced (quantitatively and qualitatively) in that reduced numbers of core-associated spindles were present and these spindle cells were shorter and wider than those seen in non-mobilised samples (Figure 4.7 and Figure 4.8). Similar stunted spindle cells were present as part of the background cells, though most background cells were round (Figure 4.8).

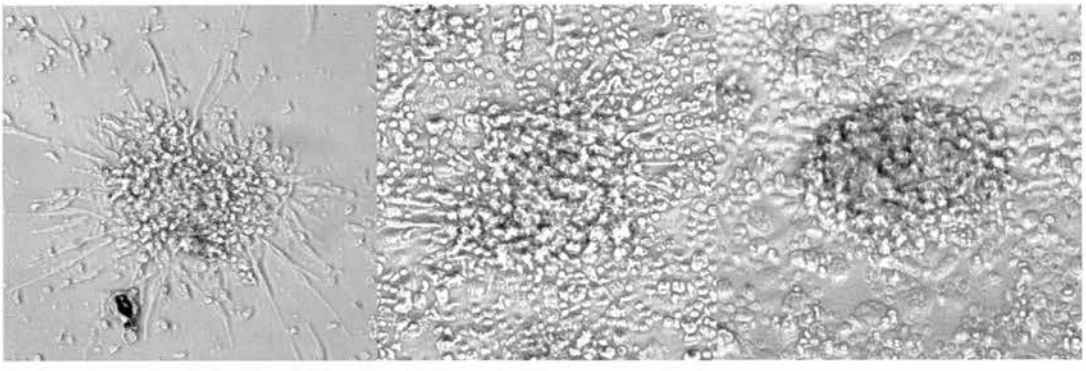


Figure 4.8 CFU-EPC/clusters in non-mobilised and mobilised samples (2).
CFU EPC in pre G-CSF (left) and G-CSF mobilised (centre) samples obtained from an allogeneic PBSC donor showing the reduction in spindle number and their stunted appearance following G-CSF. The great majority of cell clusters in mobilised samples consisted of round cells without colony-associated or background spindle cells (right).

4.7. Failure of CFU-EPC formation pre G-CSF in autologous PBSC patients may be a result of disease state or therapy

A patient's disease state or chemotherapy treatment might impact on CFU-EPC formation in the absence of G-CSF exposure, as outlined below. Follow-up samples were obtained from autologous PBSC patients, at the time of their hospital admission for autologous PBSCT, 4-6 weeks following PBSCH (n=9, all with MM). In contrast to pre G-CSF samples usually collected whilst patients were receiving chemotherapy, patient follow-up samples were obtained during the chemotherapy free period between PBSCH and induction chemotherapy for autologous PBSCT.

In most cases the frequency of CFU-EPC in donor follow-up samples approximated that of the patient's pre G-CSF sample but on a couple of occasions the follow-up sample generated considerably greater CFU-EPC than the comparative pre G-CSF sample. If these follow-up samples had not been available this might have suggested a reversal or reduction in the effect of G-CSF on CFU-EPC (Table 4.4).

Patient ID	Timing of patient sample		
	Pre G-CSF	Mobilised	Follow-up
277	—	2	3
278	0	1	20
283	1	0	4
284	0	0	0
291	3	0	3
299	3	0	2
302	1	3	10
279	—	1	48
314	—	0	0

Table 4.4 CFU-EPC in autologous PBSC patients.
Samples obtained pre G-CSF, after G-CSF mobilisation and donor follow-up.

Similar G-CSF effects on CFU-EPC occurred in samples from autologous PBSC patients as observed in samples from allogeneic PBSC donors. The patients' colony-forming potential and the effects of G-CSF on CFU-EPC might be underestimated if only the pre G-CSF and G-CSF mobilised autologous patient samples are compared and patient follow-up testing is omitted.

4.8. Investigation of technical factors that might influence CFU-EPC assay

In the earlier part of this chapter data was presented that illustrated the profound effect that *in vivo* G-CSF (given alone or in combination with chemotherapy or AMD3100) administration had on CFU-EPC frequency. These results are contrary to a number of studies that report enhanced CFU-EPC following G-CSF (162, 163, 165, 167, 170). In this section (section 4.8) a number of technical factors that were identified as possible influences on the CFU-EPC results obtained in the previous section are explored.

4.8.1. Reduction in CFU-EPC as a result of delayed MNC separation

In earlier studies, performed by O Tura and other group members, geographical separation of the laboratory from the patient review area led to significant delays (not infrequently 24 hours) between sample collection and MNC separation. This was solved by the laboratory's relocation to the same site as the stem cell collection facility (CSU). An assessment of whether results of earlier investigations were adversely affected by these delays in MNC separation was undertaken. It is important to note that all work presented in this thesis, unless specifically stated, refers to fresh samples that underwent MNC separation within 3 hours of collection.

4.8.1.1. Sample collection

Seven blood samples were collected from 6 patients; 2 volunteers, 1 allogeneic peripheral blood stem cell (PBSC) donor and 3 autologous PBSC patients, consisting of 4 non-mobilised and 3 mobilised samples. Venous blood samples collected into anticoagulated (lithium heparin) tubes were divided into two. One half of the sample underwent MNC separation within 3 hours of collection (T0) whilst the remainder was stored in a specimen bag in the laboratory on the bench-top at room temperature for 24 hours prior to MNC separation (T24). The time elapsing between

sample collection and commencement of MNC separation was the only difference between specimens.

4.8.1.2. Macroscopic differences between samples

T0 samples had clearer separation of the MNC layer post Histopaque density gradient centrifugation than T24 samples. Regardless of whether the samples were non-mobilised or mobilised samples, T0 samples usually had white/opaque MNC interface cells but T24 interface cells were frequently discoloured by the many red cells within them. Despite thorough washing of the reserved T24 interface cells, centrifuged cell pellets remained red and the resulting cell suspension pink. It was also noted that T0 whole blood samples had more complete red cell lysis during whole blood antibody staining for flow cytometry than T24 samples.

4.8.1.3. CFU-EPC colony formation

MNC were plated into CFU-Hill assays. Colony formation and spindle cell development were assessed from day 4 or day 5 and up to day 15 (range day 8-15). When the volume of media in a well of a 24-well fibronectin-coated plate declined to less than half of the original volume, additional Compete Endothelial Culture Medium (CECM) (200μL) was added to the well. Incubator malfunction rendered one post G-CSF sample (patient 215) unsuitable for further review, results are therefore available for 6 patient samples (Table 4.5).

Pt ID	G-CSF	CFU-EPC (CFU-EPC/10^6MNC)	
		T0	T24
JC	No	2.9	0
KS	No	25.3	3.75
208	Yes	4	4
212	Yes	10	0
215	No	0	0
215	Yes	—	—
218	Yes	0	0

Table 4.5 CFU-EPC formation in T0 and T24 samples.

CFU-EPC colony formation was adversely affected by delays in MNC separation and commencement of cell culture. Use of T24 rather than T0 non-mobilised samples resulted in a clear decrease in CFU-EPC. For mobilised samples the use of T0 rather than T24 samples failed to 'recover' CFU-EPC activity. The effect of G-CSF on CFU-EPC formation cannot be solely attributed to any delays in MNC separation and plating cells into culture.

The results of experiments suggest that use of blood samples stored overnight prior to MNC separation (T24 samples) reduced the magnitude of difference in CFU-EPC activity between non-mobilised and G-CSF mobilised samples, principally due to the reduction in CFU-EPC activity in T24 compared to T0 non-mobilised samples. This would have underestimated the possible effects of G-CSF on CFU-EPC. All analysed data, including all CFU-EPC results in section 4.2-4.5, and other results presented in this thesis refer to freshly collected samples with MNC separation commencing within 3 hours of blood collection (equivalent to T0 samples).

4.8.2. Potential for loss of CFU-EPC forming cells in the CFU-Hill assay

In the CFU-Hill assay, primary plating of MNC onto fibronectin with harvest of fibronectin non-adherent cells after 48 hours incubation aims to remove mature macrophages and endothelial cells from the specimen. The assay relies on the non-adherent MNC fraction to contain all colony-forming cells and for mature endothelial cells and macrophages to be retained in the fibronectin-adherent cell fraction. We have observed a consistent reduction in CFU-EPC following G-CSF administration (CFU-Hill assay). One concern is that the harvest and re-plating of day 2 non-adherent MNC fails to transfer all colony-forming cells to the secondary culture plate; i.e. the EPC forming cells may have become adherent. Omission of the day 2 re-plating step in the CFU-Hill(direct) assay (section 2.4.2.2) overcomes the possibility that a reduction of CFU-EPC in mobilised samples is attributable to the loss of colony forming cells during cell culture.

It has been found in this study that non-mobilised samples and some autologous PBSC patients' mobilised samples contain low MNC numbers, a result of the low PB WCC in these patients (Chapter 3). Plating cells into the CFU-Hill assay, thereby committing $5x10^6$ MNC to one assay, restricted the scope of experiments performed and limited the amount of comparative data that could be generated. An additional benefit of using CFU-Hill(direct) assays would be in enabling additional experiments to be performed using small volume/low cellularity patient samples. Previous studies from our group (O Tura) suggested that the initial plating step could be omitted in the CFU-EPC assay without affecting CFU-EPC formation. This section addresses whether CFU-EPC assessment could be simplified and if the CFU-Hill colony assay could be replaced by the CFU-Hill(direct) assay.

4.8.2.1. Sample Characteristics

CFU-EPC formation was assessed by plating MNC into CFU-Hill(direct) assays in parallel with the CFU-Hill assay. Forty patient samples from 28 patients were examined; 27 PBSC patients (10 allogeneic and 17 autologous PBSC donors) and 1 healthy volunteer. This included 12 G-CSF$^+$/-chemotherapy mobilised samples and 28 non-mobilised samples (20 pre G-CSF, 7 follow-up samples and 1 non G-CSF). Paired pre and post ($^+$/-follow-up) G-CSF samples were available for 6 patients (5 allogeneic and 1 autologous PBSC patient).

4.8.2.2. CFU-EPC assessment

CFU-EPC are expressed per 10^6 MNC plated into 24 well fibronectin coated plates. In CFU-Hill assays, colony formation is assessed in day 2 MNC that are non-adherent to fibronectin. Whilst a calculated number of MNC (3-$5x10^6$ MNC) are plated into culture on day 0, the primary plating step removes a variable proportion of fibronectin adherent MNC. Following harvest of non-adherent MNC on day 2, CFU-Hill formation is assessed within a defined number of MNC plated into 24 well fibronectin coated plates. In contrast, CFU-EPC formation in CFU-Hill(direct) assays is assessed in day 0 MNC, which represent the total population of MNC obtained

following density gradient centrifugation of whole blood. In this section (section 4.8.2) and subsequent sections colonies forming in the standard CFU-EPC assay (CFU-Hill) are referred to as CFU-Hill and those forming in MNC plated directly into 24 well fibronectin coated wells are referred to as CFU-Hill(direct) colonies.

4.8.2.3. CFU-EPC in non-mobilised samples

CFU-EPC formation was assessed in 28 non-mobilised patient samples. CFU-Hill were generated in 18 of 28 (64.3%) day 2 non-adherent non-mobilised MNC and CFU-Hill(direct) were generated in 19/28 (67.9%) day 0 non-mobilised MNC. Overall, comparable results were obtained between the culture methods (Table 4.6). Non-mobilised samples that generated no/low, medium or high level of CFU-Hill colonies generated the same level of CFU-Hill(direct) colonies. CFU-EPC morphology of CFU-Hill and CFU-Hill(direct) colonies were comparable for the same patient sample (Figure 4.11).

Slightly disparate results were seen on 4 occasions; development of CFU-Hill in day 2 non-adherent MNC but no CFU-Hill(direct) in day 0 MNC (n=1) and CFU-Hill(direct) formation but not CFU-Hill formation(n=3). Samples from 2 allogeneic PBSC patients that failed to generate CFU-EPC from pre G-CSF samples (CFU-Hill or CFU-Hill(direct)) also failed to generate CFU-EPC from mobilised blood or at donor follow-up by either culture method, suggesting that lack of CFU-EPC could be attributed largely to patient rather than to assay factors.

4.8.2.4. CFU-EPC in G-CSF mobilised samples

When CFU-EPC were observed in mobilised samples, colonies were reduced in frequency compared to non-mobilised samples. Following G-CSF, CFU-Hill were generated in 4/12 (33.3%) mobilised samples and CFU-Hill(direct) in 2/12 (16.7%). Details of CFU-EPC are presented in Table 4.6.

Disparate results between culture methods were seen in 5 cases. Three samples generated CFU-Hill but not CFU-Hill(direct). These showed 2 or less CFU-Hill/10^6

MNC. Two samples formed CFU-Hill(direct) but not CFU-Hill colonies. No pre G-CSF data was available for one of these patients who generated CFU-Hill(direct) colonies from mobilised MNC whilst the other patient's pre G-CSF sample also generated greater numbers of CFU-Hill(direct) than CFU-Hill per million MNC.

All samples	Time Point	Pre G-CSF	Mobilised	Follow-up	Non-mobilised
	CFU-Hill	6.6	0.8	14.0	8.1
	CFU-Hill(direct)	8.5	1.1	6.0	7.6
	Patient number	20	12	7	28

Allogeneic PBSC donors	Time Point	Pre G-CSF	Mobilised	Follow-up	Non-mobilised
	CFU-Hill	8.3	1.2	14.8	10.6
	CFU-Hill(direct)	13.7	0.4	6.4	11.1
	Patient number	9	5	5	14

Autologous PBSC patients	Time Point	Pre G-CSF	Mobilised	Follow-up	Non-mobilised
	CFU-Hill	5.5	0.6	11.0	6.3
	CFU-Hill(direct)	5.5	1.5	4.5	5.3
	Patient number	11	7	2	13

Table 4.6 CFU-EPC frequencies assessed using CFU-Hill(direct) and CFU-Hill assays. Samples obtained at assessments pre G-CSF, G-CSF mobilised and at donor follow-up.

Results (mean values) are presented for all samples, allogeneic and autologous PBSC patients. 'All samples' includes 1 healthy volunteer sample. CFU-EPC (expressed per 10^6 MNC) were quantified in day 2 fibronectin non adherent MNC as CFU-Hill or in day 0 MNC as CFU-Hill(direct).

4.8.2.5. CFU-EPC in paired non-mobilised and mobilised samples

Paired patient samples show that a marked reduction (virtual abolition) of CFU-EPC was recorded in all (CFU-EPC forming) patient samples following G-CSF, regardless of whether CFU-EPC were assessed using CFU-Hill (Figure 4.9) or CFU-Hill(direct) assays (Figure 4.10).

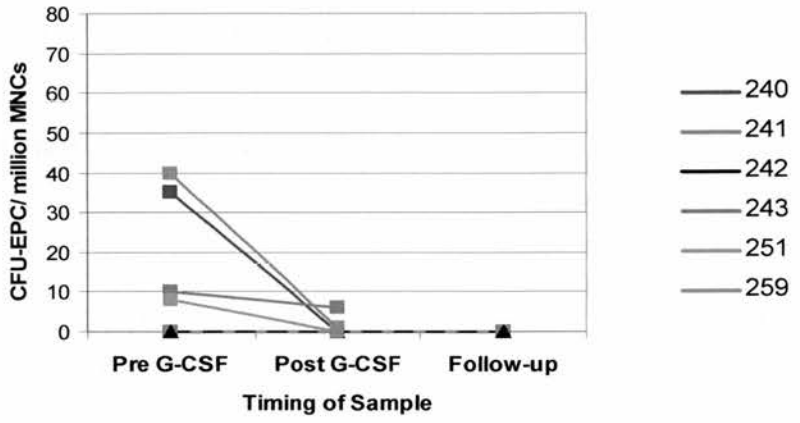


Figure 4.9 CFU-EPC formation assessed using the CFU-Hill assay.
Paired pre G-CSF and post G-CSF ($^{+}$/- donor follow-up) samples.

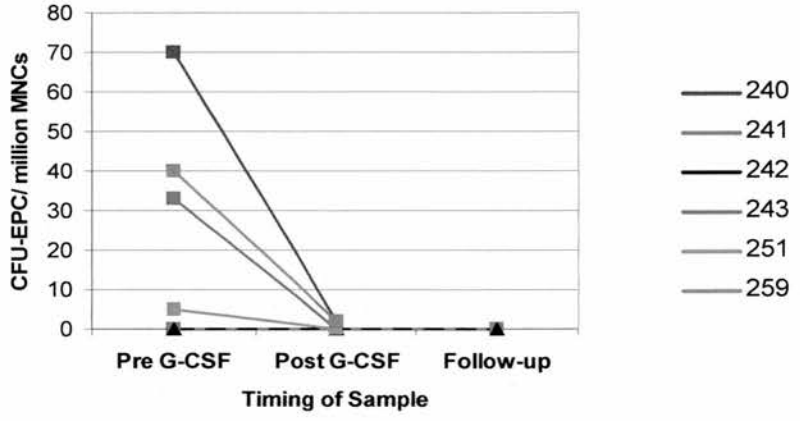


Figure 4.10 CFU-EPC formation using the CFU-Hill(direct) assay.
Paired pre G-CSF and post G-CSF ($^{+}$/- donor follow-up) samples.

4.8.2.6. Conclusions

Culture results using the CFU-Hill(direct) assay were equivalent to the results obtained using in the standard CFU-Hill assay. Although colony-forming samples showed some minor discrepancies between the culture methods, samples with low, medium or high CFU-EPC activity according to one method had consistent activity with the alternate method. Re-plating of day 2 non-adherent MNC did not select out non colony forming cells. CFU-EPC morphology was also equivalent in CFU-Hill and CFU-Hill(direct) colonies (Figure 4.11). Extending the period of colony review

out to day 15 failed to detect additional CFU-EPC (CFU-Hill or CFU-Hill(direct) that were not already present by day 7.

By virtue of the reduced cell numbers required, the abbreviated, directly plated CFU-EPC (CFU-Hill(direct)) assay does offer a reasonable means of expanding the breadth of investigations performed on non-mobilised (pre or follow-up) and mobilised patient samples. This is especially valuable when MNC numbers are limited. However, where CFU-EPC forming cells are scarce or inactive, it may be that plating only $1x10^6$ MNC is inferior to plating greater numbers of cells, and perhaps in this situation CFU-EPC assays might be better performed using greater MNC numbers.

For subsequent experiments, when MNC numbers were limited or when a large number of separate wells were being plated using a single patient sample, CFU-Hill(direct) assays with day 0 MNC suspended in CECM and plated into 24 well fibronectin coated plates were set up. Where possible a CFU-Hill assay was also performed.

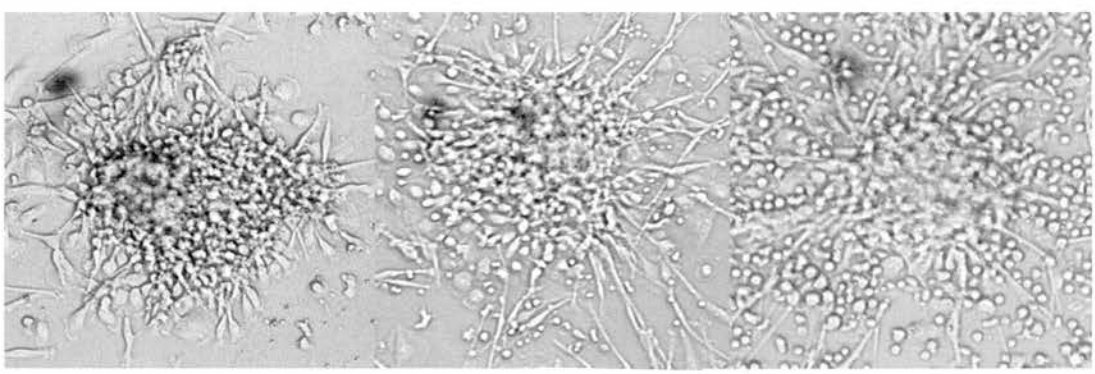


Figure 4.11 CFU-EPC in a non-mobilised sample (autologous PBSC patient).
Similar CFU-EPC morphology is demonstrated in CFU-EPC generated within day 2 adherent MNC (left), day 2 non adherent MNC (CFU-Hill) (centre) and day 0 MNC (CFU-Hill(direct) (right).

In addition to using CFU-Hill(direct) it was decided that due to the limited numbers of MNC available from many patient samples to reduce the well-size of the plates used for primary plating (day 0 MNC) in the CFU-Hill assay. The assay was modified so that $3x10^6$ MNC, suspended in 1.5mL of CECM, were plated in to a well of a 12 well fibronectin coated plate (rather than $5x10^6$ MNC being plated in 2mL CECM into a 6 well plate) (section 2.4.2.1). The transfer of $1x10^6$ day 2 non-adherent MNC in 1mL CECM into a well of a 24-well fibronectin-coated plate was carried out unmodified.

4.8.3. CFU-EPC development within day 2 adherent MNC

It is possible that treatment with G-CSF affects the ability of target/colony-forming cells to adhere to the fibronectin surface, and through this alter CFU-EPC formation. If cell adhesion to fibronectin was enhanced following G-CSF exposure this might result in failure to transfer colony forming cells within the day 2 fibronectin non-adherent MNC fraction on day 2 to 24 well plates (CFU-Hill assay). Colony forming cells would therefore be retained in the initial well, within the day 2 adherent MNC fraction, and not be counted within the CFU-EPC. CFU-Hill(direct) colonies would be unaffected.

Following transfer of non-adherent MNC into 24-well fibronectin coated plates on day 2 fresh CECM was added to the original 6 or 12 well fibronectin plate. These 'day 2 adherent' MNC were returned to the incubator and then cultured and assessed in identical fashion as the day 2 fibronectin non-adherent MNC in the CFU-Hill assay.

4.8.3.1. Sample population

Eighty-six patient samples (24 allogeneic and 62 autologous PBSC patient samples) comprising 51 non-mobilised samples (35 pre G-CSF and 16 follow-up samples) and 35 mobilised samples were examined for CFU-EPC within the day 2 adherent MNC fraction. For 80 of 86 samples the CFU- Hill assay was performed using 12 well and

then 24 well fibronectin coated plates. The CFU-Hill assay was performed using 6 well and 24 well fibronectin coated plates in the remaining 6 cases.

4.8.3.2. CFU-EPC in non-mobilised samples

Within day 2 non-adherent MNC (CFU-Hill), CFU-EPC were generated in 13/19 (68.4%) allogeneic PBSC donor samples and 24/32 (75.0%) autologous PBSC patient samples. CFU-EPC development in day 2 adherent MNC was observed more rarely with 4/19 (21.1%) allogeneic and 12/32 (37.5%) autologous PBSC patient samples generating colonies from day 2 adherent MNC. Mean CFU-EPC were 7.3 CFU-EPC/10^6 MNC and 2.5 colonies/well in day 2 non-adherent and adherent MNC respectively for allogeneic PBSC donors and 10.5 CFU-EPC/10^6 MNC and 3.2 colonies/well for autologous PBSC patients.

With 2 exceptions, the level of CFU-EPC in day 2 adherent MNC was low. One autologous PBSC patient who had florid CFU-EPC development in the CFU-Hill assay (>100 CFU-EPC/10^6 MNC) also had prolific colonies within day 2 adherent MNC (50 CFU-EPC/well). There was an error in cell transfer in one allogeneic PBSC donor as minimal cells were plated into the 24 well fibronectin-coated plate on day 2; 0 CFU-EPC/10^6 MNC and 40 CFU-EPC/well in day 2 non-adherent and day 2 adherent MNC respectively. The frequencies of CFU-EPC in day 2 non-adherent and day 2 adherent fractions are displayed in Table 4.7.

4.8.3.3. CFU-EPC formation in G-CSF mobilised samples

CFU-EPC development was reduced in mobilised samples. One of 5 (20%) allogeneic and 12/30 (40%) autologous PBSC patients showed some degree of CFU-EPC development within day 2 non-adherent MNC. CFU-EPC activity was observed within day 2 adherent MNC in 0/5 allogeneic and 2/30 (6.7%) autologous PBSC patients.

Patient Group	G-CSF	Day 2 non-adherent MNC (CFU-EPC/10^6 MNC)	Day 2 adherent MNC (CFU-EPC/well)	n
Autologous PBSC patients	No	7.6	1.7	31*
	Yes	1.1	0.1	30
Allogeneic PBSC donors	No	7.7	0.4	18**
	Yes	0.6	0	5
All PBSC donors	No	7.7	1.2	49***
	Yes	1.1	0.1	35

Table 4.7 Mean CFU-EPC development in day 2 non-adherent and day 2 adherent MNC.
* Excludes 1 outlying patient (CFU-EPC 10.5/10^6 and 3.2/well in D2 NA & AD MNC; n=32).
** Excludes 1 outlying patient (CFU-EPC 7.3/10^6 and 2.5/well in D2 NA & AD MNC; n=19).
*** Excludes 2 outlying patients (CFU-EPC 9.3/10^6 and 2.9/well in D2 NA & AD MNC; n=51).

4.8.3.4. Changes in CFU-EPC from day 2 adherent MNC and day 2 non-adherent MNC are comparable in mobilised samples

Eleven of 16 non G-CSF samples (pre G-CSF or donor follow-up samples) that generated CFU-EPC within day 2 adherent MNC had similar changes in CFU-EPC frequency in day 2 adherent cells following G-CSF PBSC mobilisation.

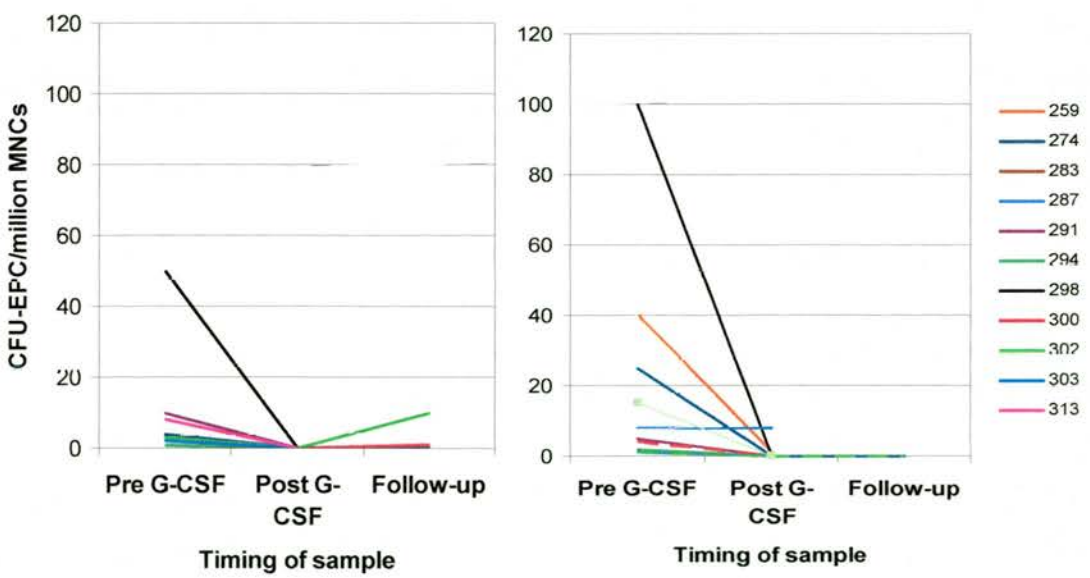


Figure 4.12 CFU-EPC frequencies in day 2 adherent MNC.
Pre G-CSF, G-CSF mobilised and donor follow-up samples

Figure 4.13 CFU-EPC in day 2 non-adherent MNC (CFU-Hill).
Same pre G-CSF, G-CSF mobilised and donor follow-up samples as used for figure 4.12.

When the CFU-EPC frequencies in day 2 adherent and day 2 non-adherent MNC from paired patient samples were examined, we saw that the reduction in CFU-EPC

following G-CSF administration observed in day 2 adherent MNC (Figure 4.12) almost mirrored the reduction in CFU-EPC following G-CSF in day 2 non-adherent MNC (CFU-Hill assay) (Figure 4.13).

4.8.3.5. CFU-EPC morphology

When CFU-EPC formed in day 2 adherent cells the colony morphology was very similar to that of CFU-EPC generated in day 2 non-adherent MNC (Figure 4.11 and 4.14).

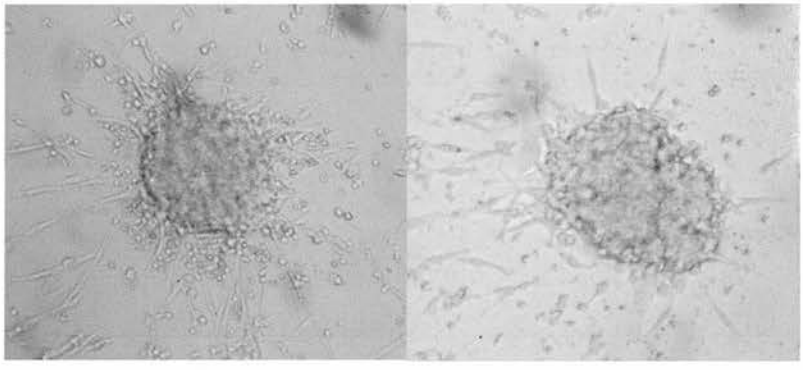


Figure 4.14 CFU-EPC in day 2 non-adherent MNC (left) and adherent MNC (right). Non-mobilised sample obtained from an autologous PBSC patient.

4.8.3.6. Conclusions

The addition of fresh CECM to the adherent MNC remaining following recovery of non-adherent cells on day 2 with continuation of cell culture did not reveal CFU-EPC activity within the adherent cell fraction at the expense of CFU-EPC formation in day 2 non-adherent MNC. On some occasions, colony forming cells were retained within the adherent cells. This is particularly important in specimens containing few colony forming cells. Care should be taken to ensure that all non-adherent MNC are transferred and wells should be examined for cell content following aspiration of the non-adherent cells to confirm that there has been no error in collection of cells.

The failure of CFU-EPC development post G-CSF administration cannot be attributed to the retention of colony forming cells within adherent MNC at the time of re-plating non-adherent MNC on day 2. When CFU-EPC were present in day 2 adherent MNC pre G-CSF, a decline in CFU-EPC formation within day 2 adherent cells was also noted following G-CSF. This decline indicates that CFU-EPC forming cells within day 2 adherent and non-adherent MNC are affected similarly by G-CSF. It should be identified whether these colonies developing within day 2 adherent MNC are identical to those developing in day 2 non-adherent MNC and if they arise from haematopoietic elements. It should also be considered whether these colonies should be included in colony numbers for a sample and whether CFU-EPC can be assessed using a single continuous culture.

4.8.4. Influence of changing CECM during cell culture on CFU-EPC

Two mobilised autologous PBSC patient samples were plated into CFU-Hill assays. 1 x10^6 non-adherent MNC were transferred in duplicate into wells of a 24 well fibronectin-coated plate on day 2. One well received no further intervention. The other well had 50% of its media refreshed on alternate days; 500µL media was aspirated and replaced with 500µL of fresh CECM. Wells were inspected daily from day 4 for CFU-EPC.

4.8.4.1. Results

CFU-EPC activity in these mobilised patient samples was minimal (1 CFU/10^6 MNC and 0 CFU-EPC/10^6 MNC, patients 218 and 221 respectively). Refreshing CECM during CFU-EPC culture did not increase CFU-EPC (1 CFU/10^6 MNC and 0 CFU-EPC/10^6 MNC, patients 218 and 221 respectively). In addition, there were no differences observed in the numbers of cell clusters, cell clumps or spindle cells between the culture wells.

4.8.4.2. Conclusions

Changing 50% of CECM on alternate days did not influence CFU-EPC formation or alter the cell morphology seen in G-CSF mobilised MNC plated into CFU-EPC assays.

4.8.5. Influence of plating increased numbers of mobilised MNC into CFU-EPC assays

If the decline in CFU-EPC activity seen in mobilised samples is attributable solely to reduced proportions of CFU-EPC colony forming cells within these samples then plating increased cell numbers into CFU-EPC assays should increase CFU-EPC.

A series of mobilised patient samples (n=7) were plated into CFU-EPC assays (CFU-Hill(direct) (n=5) or CFU-Hill (n=2)) using increasing cell doses; $1x10^6$, $2x10^6$ or $4x10^6$ day 0 MNC in CFU-Hill(direct) and 1 $x10^6$ or $2x10^6$ day 2 fibronectin non-adherent MNC in CFU-Hill assays. Wells were inspected for CFU-EPC from day 4.

4.8.5.1. CFU-EPC in CFU-Hill(direct) assay

Three of 5 patient samples plated into CFU-Hill(direct) assays failed to show any CFU-EPC in wells containing $1x10^6$, $2x10^6$ or $4x10^6$ day 0 MNC. One patient sample, with MNC plated into CFU-Hill(direct) assays, showed no (0 CFU-EPC/10^6 MNC) CFU-EPC in wells containing $1x10^6$ and $2x10^6$ day 0 MNC but a single CFU-EPC colony was seen in the well containing $4x10^6$ day 0 MNC (0.25 CFU-EPC/10^6 MNC). The paired non-mobilised sample from this patient generated 7 CFU-EPC/10^6 MNC. The second patient had more impressive CFU-EPC generation with plating increased numbers of mobilised MNC into culture. G-CSF mobilised MNC plated in CFU-Hill(direct) assays generated 0 CFU-EPC when $1x10^6$ MNC were plated into culture but 9 CFU-EPC were generated from the well containing $2x10^6$ day MNC (4.5 CFU-EPC/10^6 MNC). The corresponding non-mobilised sample from this patient generated 19 CFU-EPC/10^6 MNC.

4.8.5.2. CFU-EPC in CFU-Hill assay

Both mobilised samples plated into CFU-Hill assays at increasing concentrations showed a dose effect. Mobilised MNC from one patient generated 0 CFU-EPC when standard cell numbers were plated ($3x10^6$ day 0 MNC into 12 well fibronectin plate with replating of $1x10^6$ non-adherent MNC on day 2) but 10 CFU-EPC when $10x10^6$ cells were plated on day 0 and $2x10^6$ cells were transferred on day 2 (5 CFU-EPC/10^6 MNC). The non-mobilised sample from the second patient had high levels of colony formation 70 CFU-EPC/10^6 (CFU-Hill assay). Following G-CSF, the standard CFU-Hill assay generated 1 CFU-EPC colony (1 CFU-EPC/10^6 MNC) and plating $10x10^6$ MNC on day 0 with transfer of $2x10^6$ cells on day 2 generated 3 CFU-EPC (1.5 CFU-EPC/10^6 MNC).

4.8.5.3. Conclusions

There is some, albeit inconsistent, evidence to supporting a cell dose effect for CFU-EPC formation in mobilised samples. However, CFU-EPC activity fails to recover to levels approaching that shown by the corresponding non-mobilised sample. If it is postulated that formation of CFU-EPC is a consequence of a single cell and that G-CSF administration reduces the frequency of these cells then plating increased cell numbers into CFU-EPC assay should detect CFU-EPC. However, if G-CSF has resulted in making responsive cells inert then increasing cell numbers will not increase CFU-EPC. Even greater cell numbers should be plated into CFU-EPC culture to detect lower frequency events before we draw any conclusions along those lines.

In addition to performing CFU-EPC using standard cell numbers, MNC should be plated in CFU-EPC in higher cell doses where possible. In healthy adult allogeneic PBSC donors, PB leukocyte counts increase ~7-fold following G-CSF administration and MNC numbers obtained from density gradient centrifugation of whole blood are also increased compared to non-mobilised samples, meaning that MNC are available for this. However MNC numbers may be insufficient to support plating higher doses of MNC from autologous PBSC patients into CFU-EPC cultures as

MNC numbers were found to be low in many mobilised samples from these patients.

4.8.6. Erythrocytes as a possible cause of reduction in CFU-EPC in mobilised samples

Significant erythrocyte contamination of the MNC interface, which could then be seen throughout the CFU-EPC assay, has been noted in many but not all specimens obtained from mobilised PBSC patients. Erythrocyte contamination was seen as macroscopic red or pink discolouration of the interface layer and then as redness of MNC pellets after centrifugation. Erythrocytes were also evident on microscopic and on later macroscopic inspection of culture wells after plating MNC into the CFU-EPC assay. In contrast, non-mobilised samples did not usually have significant erythrocyte contamination of MNC. No differences could be identified between those mobilised MNC samples that displayed high or low levels of erythrocyte contamination in how these specimens were collected and handled (including anticoagulant used in the collection tubes, sample volume, time lapsing between sample collection and MNC density gradient separation, centrifugation speeds, and the presence of any clotted material in the specimens).

The presence of erythrocytes could be reducing the accuracy of day 0 and day 2 MNC enumerations and therefore be affecting the number of MNC plated into cell culture. Erythrocytes might also be having direct negative effects on CFU-EPC formation. As erythrocyte content of mobilised samples was higher than non-mobilised samples (section 5.2) it might contribute to the 'G-CSF effect' on CFU-EPC formation. A number of areas where the influence of erythrocytes on cell culture could be assessed were identified; the use of acetic acid lysis of cell suspensions when counting MNC and use of alternative centrifuge tubes for density gradient centrifugation.

4.8.6.1. Acetic acid erythrocyte lysis prior to MNC enumeration

MNC concentrations of day 0 or day 2 MNC (n=13) were counted by haemocytometer prior to and following the addition of 3% acetic acid. Except when there was extremely heavy erythrocyte contamination observed, a 1:1 acetic acid dilution was performed (20μL 3% acetic acid added to 20μL MNC suspension).

MNC concentrations were consistently lower following acetic acid dilution of the specimen prior to counting with mean reductions in MNC concentrations of 25% observed. This most likely reflected better distinction of small lymphocytes (whose diameter of 7-8μm approximated the average diameter of erythrocytes). Additionally, use of acetic acid improved nuclear detail and allowed easier distinction of lymphocytes and monocytes from granulocytes and myeloid precursors. The magnitude of differences between MNC counts made prior to and following acetic acid is displayed in Table 4.8.

The magnitude of change in MNC counts when counting using acetic acid lysis were greater for mobilised samples. Following on from these results, dilution of MNC 1:1 with acetic acid prior to MNC enumeration by haemocytometer was adopted as standard practice.

Patient ID	Type	G-CSF	Day 0 or Day 2 MNC	[MNC] (x10^6/mL)		Percent Change in value
				Pre Dilution	Post Dilution	
227	Auto	Post	0	3.1	2.5	17.6
227	Auto	Post	2	1.8	1.3	29.2
226	Auto	Post	0	RBC ++	1.4	—
226	Auto	Post	2	RBC ++	2.6	—
228	Auto	Post	0	7.9	3.9	51.0
228	Auto	Post	2	4.5	3.4	24.4
225	Auto	Post	0	2.1	0.7	65.4
225	Auto	Post	2	1.4	1.0	26.6
229	Allo	Post	0	2.9	2.7	6.2
232	Allo	Pre	0	1.9	1.7	10.3
232	Allo	Pre	2	2.0	1.9	5.9
234	Allo	Pre	0	1.7	1.2	25.3
230	Auto	Post	0	6.4	5.2	19.4

Table 4.8 Change in MNC counts following acetic acid dilution.
RBC+++ indicates that MNC sample was heavily contaminated by erythrocytes.

4.8.6.1.1. CFU-EPC activity

CFU-EPC formation pre and post G-CSF was evaluated using 7 paired non-mobilised and mobilised patient samples, where MNC enumeration was performed using acetic acid dilution. A consistent reduction in CFU-EPC was noted following G-CSF administration. This suggested that although there might have been inaccuracies in cell plating generated from counting errors made as a result of failure to exclude erythrocytes from MNC enumeration, the 'G-CSF effect' on CFU-EPC can not be attributed to this (Figure 4.15).

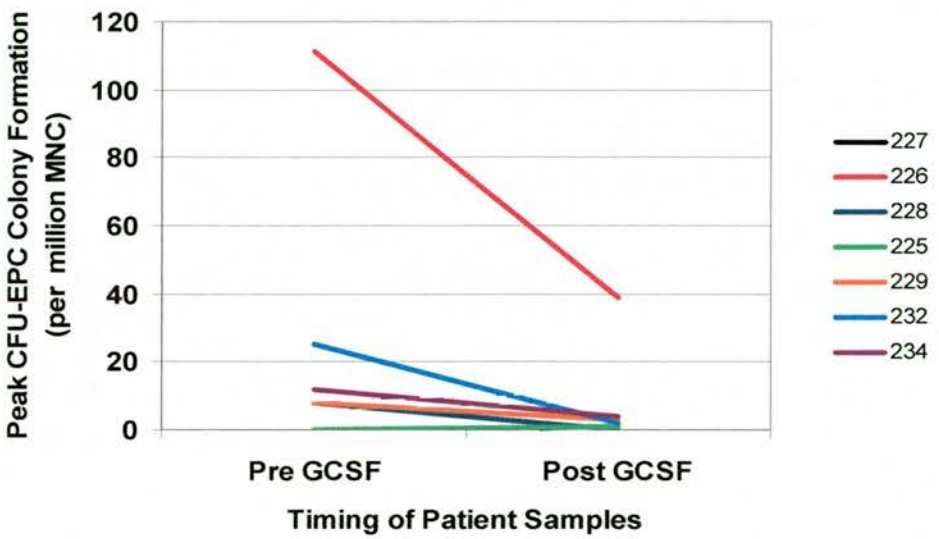


Figure 4.15 CFU-EPC generated from non-mobilised and mobilised patient samples. MNC enumeration using acetic acid erythrocyte lysis.

4.8.6.2. Use of Leucosep centrifuge tubes

Leucosep tubes (Greiner Bio-one, UK) appeared to be an option for MNC separation as the fixed porous membrane provided a barrier between the pelleted erythrocytes and granulocytes and the MNC layer. The Histopaque is forced below the porous barrier by a brief period of centrifugation prior to the addition of the diluted whole blood sample. This ensures that the specimen is layered adequately when it is loaded into the centrifuge tube. Additionally, it was hoped that the barrier would not inhibit the passage of RBC clumps through the membrane and that a cleaner interface layer would be produced.

Leucosep centrifuge tubes were used for MNC separation on 5 occasions (for 4 mobilised samples and 1 non-mobilised sample). For the non-mobilised sample, the MNC interface layer was positioned close to the porous membrane and was not clearly separated from it. Additionally the MNC interface from the Leucosep tube contained greater numbers of erythrocytes, evident macroscopically and confirmed by flow cytometry (25.3% versus 3.3% $CD235a^+$ erythrocytes).

Use of Leucosep centrifuge tubes failed to improve MNC separation of mobilised samples and in fact worsened the erythrocyte contamination. Red cell clumps were caught above the porous membrane. These clumps extended into the interface layers which were positioned close to the membrane. Flow cytometry showed increased proportions of $CD235a^+$ erythrocytes with Leucosep tubes (29.4% versus 24.6% $CD235a^+$ erythrocytes).

4.8.6.3. Conclusions

The finding of erythrocyte contamination of MNC obtained from Histopaque mobilised and some non-mobilised patient samples (principally those obtained from autologous PBSC patients who were recovering from chemotherapy) suggested that there is a population of (probably immature) erythrocytes in the peripheral blood which have similar density properties to MNC. These therefore co-separate with MNC during conventional buoyant density gradient centrifugation. These erythrocytes also appear to be more robust and 'lysis resistant' than other erythrocytes. The level of erythrocyte contamination has not been satisfactorily reduced using the above methods. The reduction in erythrocyte levels was not pursued further as CFU-EPC formation did not appear to be adversely affected by their presence (Chapter 5).

4.8.7. Influence of altered day 2 CECM volume on CFU-EPC activity

Previous CFU-EPC colony assay experiments performed within our laboratory had frequently been performed using 500µL rather than 1000µL CECM when re-plating day 2 MNC. This was a minor deviation from the CFU-EPC colony assay method published by Stem Cell Technologies.

The reduced CFU-EPC formation of mobilised samples might have been influenced by altered CECM volumes during cell culture. Use of reduced CECM volumes results in increased cell densities within the culture well. This might have influenced intercellular interactions and paracrine actions within the well. A reduction in media volume will also increase the concentrations of any cytokine, chemokine or any other soluble factor produced, with the potential of this to affect cell activity. A reduction in media volume might also affect cell behaviour and CFU-EPC formation due to relative nutrient deficiencies within the well.

4.8.7.1.1. Method

This experiment was performed in order to assess the influence of increasing cell concentrations, as a result of reducing CECM volume when re-plating day 2 MNC, on CFU-EPC development and colony morphology. Eight patient samples (4 non-mobilised and 4 mobilised samples) from 5 patients were assessed, with paired non-mobilised and mobilised samples available for 3 patients. Patient samples were collected into anticoagulated tubes and underwent MNC separation by buoyant density gradient centrifugation within 3 hours of collection. MNC interface cells were recovered, washed and counted.

CFU-EPC development was assessed using the CFU-Hill assay. $5x10^6$ MNC (or maximum available MNC) were re-suspended in 2mL of CECM ($2.5x10^6$ cells/mL) and placed in a well of a 6-well fibronectin coated plate. Non-adherent MNC were recovered on day 2. After counting, 2 aliquots, each containing $1x10^6$ cells, were transferred into clean centrifuge tubes and pelleted. MNC were resuspended in

either 500μL or 1000μL of fresh CECM and transferred to wells of a 24-well fibronectin coated plate. Wells were inspected daily from day 4 for CFU-EPC. The cells in all wells appeared healthy throughout the observation period.

In addition to colony enumeration, assessments were made of the numbers of cell clusters and spindle cells present. Clusters were defined as collections of rounded cells, estimated to contain >50 cells, distinguished from colonies by the lack of spindle cells sprouting from their peripheries. Average spindle cell numbers were the mean of the number of spindle cells present in 4 randomly selected x20 microscope fields not containing CFU-EPC colonies, reducing the influence of colony-associated spindle cells on the background spindle cell count.

Patient ID	GCSF	Day 2 non-adherent cells			
		500μL CECM		1000μL CECM	
		CFU-EPC (/10^6 MNC)	Clusters (/10^6 MNC)	CFU-EPC (/10^6 MNC)	Clusters (/10^6 MNC)
219	Post	2	3	1	5
220	Pre	55	35	20	72
220	Post	10	100	3	110
223	Pre	67	65	86	82
223	Post	23	63	26	58
224	Pre	7	36	4	28
224	Post	0	30	0	24
225	Pre	0	13	0	19

Table 4.9 Influence of altered day 2 media volume on CFU-EPC.

4.8.7.2. CFU-EPC in non-mobilised samples

Three of 4 non-mobilised samples generated CFU-EPC (Table 4.9). There was no consistent change in the number of CFU-EPC according to volume of CECM used on day 2; lower CFU-EPC formation with 500μL CECM in 2 samples and increased in 1. Spindle cells were more common in the wells containing 500μL CECM. Earlier dispersal of colonies and also of clusters occurred in the 500μL wells. This may have resulted in the appearance of a more cellular background containing more spindle cells and rounded cells.

4.8.7.3. CFU-EPC in G-CSF mobilised samples

Three of four mobilised samples generated CFU-EPC, but in lower frequency than non-mobilised samples (Table 4.9). No consistent change in CFU-EPC numbers according to the day 2 volume of CECM used was observed. See table. Use of the lower media volume was accompanied by a reduction in CFU-EPC activity in 2 mobilised samples and a very slight increase in CFU-EPC in 1 case. Spindle morphology was stunted in mobilised samples with shortened and thickened spindle cells seen. Similar to non-mobilised samples, spindle cells were more prominent in the wells containing 500µL CECM and dispersal of colonies and clusters occurred earlier in these wells.

4.8.7.4. CFU-EPC in paired 500µL and 1000µL CECM volumes

Pairing of CFU-EPC results when plating day 2 non-adherent MNC into 500µL or 1000µL CECM showed no consistent influence of media volume on CFU-EPC (Figure 4.16). The reduced CFU-EPC in mobilised samples should not be attributed to plating day 2 MNC into lower volumes of CECM.

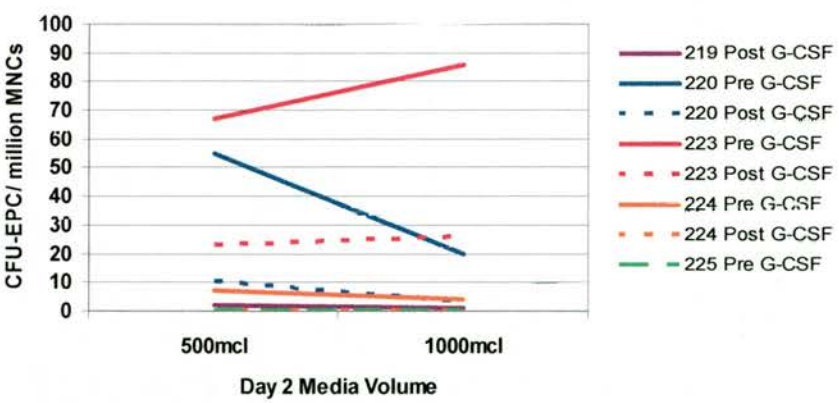


Figure 4.16 Influence of day 2 CECM volume on CFU-EPC formation.

4.8.7.5. CFU-EPC in paired non-mobilised and mobilised samples

CFU-EPC formation was assessed in 3 paired non-mobilised and mobilised patient samples, with day 2 MNC plated into both 500µL and 1000µL of CECM. Regardless

of whether day 2 MNC were re-plated into 500μL or 1000μL CECM CFU-EPC formation declined following G-CSF PBSC mobilisation (Figure 4.17).

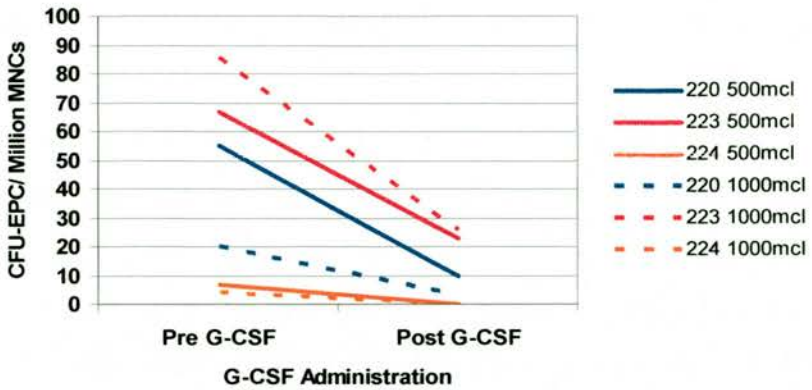


Figure 4.17 CFU-EPC with day 2 MNC plated into either 500μL or 1000μL CECM. Paired non-mobilised and mobilised PBSC samples.

4.8.7.5.1. Conclusions

We found no evidence that re-plating day 2 MNC into a lower volume of CECM curtailed or enhanced CFU-EPC activity. A similar 'G-CSF effect' on CFU-EPC was observed when day 2 MNC were plated into either 500μL or 1000μL CECM volumes. Increasing day 2 MNC CECM volumes did not improve CFU-EPC formation in samples that failed to generate CFU-EPC using 500μL CECM. Reductions in CFU-EPC formation in mobilised samples could not be attributed to plating cells into lower CECM volumes on day 2.

4.9. Effects of technical factors on CFU-EPC from G-CSF+AMD3100 mobilised PBSC patients

4.9.1. CFU-EPC formation in day 2 adherent MNC

The rate of CFU-EPC formation in day 2 fibronectin adherent MNC was assessed in 4 pre mobilisation samples, 2 donor follow-up samples and 6 G-CSF+AMD3100 mobilised samples. CFU-EPC formation was observed in the day 2 adherent fraction in 2 of 6 non-mobilised samples. In both cases the day 2 non-adherent MNC generated moderate-high CFU-EPC (70 and 15 CFU-EPC/10^6 MNC). The day 2 adherent MNC contained 50 and 8 CFU-EPC/well respectively. Day 2 adherent MNC from mobilised samples failed to generate CFU-EPC (0 CFU-EPC/well, n=6).

4.9.2. CFU-Hill(direct) assay

CFU-EPC formation was also assessed by both CFU-Hill and CFU-Hill(direct) assays in 4 non-mobilised samples. The rates of CFU-EPC generated in the CFU-Hill(direct) assays were comparable to that observed with the CFU-Hill assay (mean 19.5 CFU-EPC/10^6 MNC and 6.3 CFU-EPC/10^6 MNC and median 3 CFU-EPC/10^6 MNC and 3.5 CFU-EPC/10^6 MNC for CFU-Hill and CFU-Hill(direct) assays respectively, n=4). Mean CFU-EPC differed substantially between culture methods for 1 patient sample; 70 CFU-EPC/10^6 MNC were generated in the CFU-Hill assay and 12 CFU-EPC/10^6 MNC in the CFU-Hill(direct) assay.

4.9.3. Increasing cell numbers at stage 2 of CFU-Hill assay

Increased cell concentrations of G-CSF+AMD3100 mobilised MNC were plated into CFU-Hill assays (n=2). $10x10^6$ MNC were plated into 12 well fibronectin coated plates on day 0 and $2.0x10^6$ day 2 non-adherent MNC were transferred into 24 well fibronectin coated plates. Plating greater cell numbers failed to increase CFU-EPC in 1 patient sample (0 CFU-EPC/10^6 MNC) but plating greater cell numbers into CFU-EPC generated 3 CFU-EPC (1.5 CFU-EPC/10^6 MNC) in the other sample, marginally higher than that of standard cell concentrations (1 CFU-EPC/10^6 MNC).

4.10. Endothelial outgrowth colony formation of PBSC donor samples

4.10.1. Introduction

It is currently thought that two broad groups of EPC exist and that the cells identified in endothelial outgrowth colony assessments represent 'true EPC' (93, 131, 133). There is evidence that EOC are derived from $CD34^+$ cells (131)and more recent published evidence supporting a $CD34^+/133^-$ origin of these cells (112, 134, 150). Other members of our group have cultured EOC from unselected umbilical cord blood (UCB) MNC. We sought to establish whether EOC could be generated from another CD34-rich blood source, G-CSF mobilised peripheral blood.

MNC were obtained following Histopaque buoyant density gradient centrifugation of whole blood samples. MNC were suspended in EBM2 media (with supplements) and then plated onto plastic plates coated with type 1 collagen (Becton Dickinson, UK)). Six or 12 well collagen type 1 plates were used. A maximum of $20x10^6$ MNC were plated into each well for EOC culture. MNC were cultured according to the method described in section 2.4. Wells were regularly inspected for EOC development from the second week of culture.

4.10.2. Patient samples

PBSC samples from 8 PBSC donors (4 allogeneic PBSC donors and 4 autologous PBSC patients) were plated into EOC assays. The allogeneic PBSC donor samples consisted of 1 donor follow-up sample, 1 pre G-CSF sample and 2 G-CSF mobilised samples. The autologous PBSC patient samples consisted of 3 pre G-CSF samples and 1 G-CSF+chemotherapy mobilised sample. When MNC numbers permitted, multiple wells were plated into the EOC assay, enabling multiple cell concentrations to be plated for a single sample. This was more likely to be achievable with MNC

from allogeneic PBSC donors than autologous PBSC patients due to their higher peripheral blood WCC and therefore higher MNC yields.

4.10.3. EOC formation

Cells in the culture wells remained healthy throughout the culture period. No infection was noted in any of the culture wells. Few cells remained in the culture wells following aspiration of the non-adherent cells on day 2. This cell loss was more pronounced in wells containing G-CSF mobilised cells.

No EOC formation was observed in any well over the review period. No other proliferating cell populations were observed in the culture wells. However, some differences in cell morphology between wells containing non-mobilised and G-CSF mobilised MNC were noted. Wells containing G-CSF mobilised MNC appeared to contain larger, bulkier-looking cells that had an ovoid shape. Non-mobilised MNC were small and round. No cobblestone appearances were observed in the culture wells and no significant cell proliferation was observed. No change in cell morphology was noted when the culture period was extended beyond four weeks and delayed colony formation did not occur.

4.10.3.1. Matrigel tubule formation

After approximately 4 weeks of culture (mean 26 days, range 16-36 days, median 30 days) no EOC formation was observed in any culture well, regardless of the density of MNC initially plated. The morphologic differences between culture wells containing non-mobilised and G-CSF mobilised MNC continued to be observed. It was questioned whether the altered appearances of mobilised compared to non-mobilised MNC might be the result of atypical EOC formation and that these plumper mobilised cells might be a form of 'cobble-stoning', albeit with an unusual morphology.

Representative culture wells containing MNC from 6 patients were selected for the Matrigel tubule formation assay; 3 non-mobilised MNC (1 allogeneic and 2 autologous PBSC patients) and 3 mobilised MNC (1 autologous and 2 allogeneic PBSC donors). Where multiple wells had been plated for the same specimen, only the well plated with the greatest cell density was selected for the Matrigel assay. The method for Matrigel tubule formation was described in section 2.4. Non-adherent cells were aspirated from the culture wells and discarded and the adherent MNC were washed with PBS and incubated with trypsin/EDTA for a total of 10 minutes. On examination there appeared to be a relatively high proportion of adherent cells at this point and the cells were incubated with trypsin for a further 10 minutes. Trypsinised cells were recovered from the culture wells and cells were counted using a Coulter cell counter. Matrigel was thawed overnight in a 4C refrigerator. A pre-cooled 24 well plastic plate was prepared with Matrigel and then 0.7×10^4 cells suspended in EBM2 media (with supplements) were added to each prepared culture well. The plate was placed in incubator overnight and the wells were inspected for tubule formation the next morning.

No evidence of tubule formation was detected in any culture well. Cells were sparsely placed in the wells. One limitation of this assay was the lack of a positive control (no HUVEC were available).

4.10.4. Conclusions

No EOC development in MNC prepared from peripheral blood samples from PBSC donors was demonstrated whether or not the samples were collected following G-CSF administration. The lack of EOC development in CD34-rich G-CSF mobilised samples was surprising considering the CD34-dependence of EOC. EOC have been generated from another CD34-rich source, umbilical cord blood. The difference in EOC development between 2 CD34-rich sources led to an investigation of the subsets of $CD34^+$ cells within these samples. This is presented in Chapter 6.

4.11. Discussion

Evaluation of CFU-EPC in samples obtained from allogeneic and autologous PBSC patients has shown that CFU-EPC frequency varies considerably, both in healthy donors and in patients with haematological malignancies. Absence of CFU-EPC formation in non-mobilised samples was more likely to occur in samples obtained from autologous PBSC patients than from healthy allogeneic PBSC donors. However, a significant proportion of healthy donors failed to develop CFU-EPC in their pre G-CSF samples in the absence of either chemotherapy or G-CSF exposure. The huge variations in CFU-EPC between individuals made it more difficult to interpret the results of CFU-EPC based experiments.

G-CSF administration, either given alone, sequentially with chemotherapy or sequentially with AMD3100, resulted in a marked reduction (and abolition in some cases) in CFU-EPC. This was consistently demonstrated for both allogeneic and autologous PBSC donors regardless of the mobilisation regimen used. The decline in CFU-EPC following G-CSF was temporary, as follow-up samples taken 4-6 weeks after PBSC mobilisation showed increased CFU-EPC towards pre mobilisation levels. Differential effects of G-CSF and AMD3100 on CFU-EPC could not be assessed as no patients underwent PBSC mobilisation with AMD3100 alone. AMD3100 was available for a brief period, for PBSC mobilisation in autologous PBSC patients when it was administered sequentially with G-CSF, but its use is not currently being funded.

There may have been differences in CFU-EPC between allogeneic and autologous patient groups caused by possible effects of chemotherapy on CFU-EPC. CFU-EPC frequency was comparable in both non-mobilised samples (pre G-CSF and donor follow-up samples). There were no significant differences in the frequency of CFU-EPC between samples obtained from allogeneic donors and autologous PBSC patients. However, pre G-CSF samples from autologous PBSC patients displayed higher mean CFU-EPC than comparable samples from allogeneic PBSC donors (13.2

and 8.9 CFU-EPC/10^6 MNC for autologous and allogeneic PBSC donors respectively, increasing to 18.6 and 11.1 CFU-EPC/10^6 MNC when only those donors with both non-mobilised and mobilised samples were considered), affected by 5 autologous PBSC patient samples that showed >50 CFU-EPC/10^6 MNC and by a greater proportion of patients failing to generate CFU-EPC (44.4%). Median CFU-EPC for pre G-CSF samples was higher for allogeneic PBSC donors. There was no difference in mean CFU-EPC between donor follow-up and mobilised samples obtained from allogeneic or autologous PBSC donors.

The administration of myelosuppressive chemotherapy increases the numbers of $CD34^+$ HPC present in the circulation during the recovery phase. It might be that CFU-EPC forming cells are similarly increased in this period. As autologous PBSC patients were reviewed at varying times (but within a month) prior to PBSC collection it was possible that some patients could have mobilised colony-forming cells as a result of chemotherapy, producing a wider range of CFU-EPC frequency in this patient group. However, a disparity in CFU-EPC between pre G-CSF and patient follow-up samples obtained from some autologous PBSC patients was noted, with some patient follow-up samples demonstrating considerably greater CFU-EPC than their pre G-CSF samples. No additional G-CSF or chemotherapy had been administered between the time of PBSC mobilisation and collection of the follow-up sample. As there was no difference in CFU-EPC frequency in follow-up samples obtained from autologous PBSC patients and allogeneic PBSC donors this would suggest that instead of increasing CFU-EPC, administration of chemotherapy overall reduces CFU-EPC.

A number of technical issues that might have affected the CFU-EPC assay were identified. It was demonstrated that none of these substantially affected the CFU-EPC results obtained from PBSC donors, and that the effect of G-CSF of reducing or abolishing CFU-EPC is real and not an artefact.

Delays in MNC separation might have underestimated the differences in CFU-EPC between non-mobilised and mobilised samples as delayed MNC separation reduced CFU-EPC in non-mobilised samples. Prompt MNC separation of mobilised samples however, failed to improve CFU-EPC in these samples. Colony forming cells were not retained within the day 2 fibronectin adherent MNC in the CFU-Hill assay at the expense of CFU-EPC formation in the day 2 non-adherent MNC. Use of CFU-Hill(direct) assays failed to increase CFU-EPC in day 0 MNC from mobilised patient samples. Alterations in the media volume used to culture day 2 non-adherent cells failed to increase CFU-EPC in mobilised samples. Similarly, changing the medium during cell culture failed to increase CFU-EPC in mobilised MNC. Many MNC contained significant proportions of $CD235a^+$ erythrocytes and MNC were better enumerated using a Coulter counter, but as it appeared that $CD235a^+$ erythrocytes were not adversely affecting CFU-EPC formation (Chapter 5), we did not pursue reduction of erythrocyte levels in CFU-EPC.

One possible interpretation of the decline in CFU-Hill following G-CSF would be that the reduction in CFU-Hill is caused by the expansion in MNC that occurs after G-CSF administration, with rare colony forming cells diluted in amongst increased PB leukocytes. We have observed very similar effects (reductions) of CFU-EPC frequency in allogeneic PBSC donors and PBSC patients following G-CSF PBSC mobilisation and think that it is unlikely that a dilution effect explains these findings. In allogeneic donors, PB leukocyte counts do increase several fold following G-CSF, from $6.9x10^9$/L to $48.8x10^9$/L, (data obtained from section 3.2) and it is plausible that this might contribute in part to our findings. However, the decline in CFU-EPC following G-CSF administration to autologous PBSC patients cannot be attributed to dilution of colony forming cells as PB leukocytes counts remained within the normal range following G-CSF; PB leukocyte counts increasing slightly from $5.1x10^9$/L to $10.7x10^9$/L during PBSC mobilisation (data obtained from section 3.3).

The change in CFU-EPC following PBSC mobilisation and the administration of G-CSF has been observed independently by a number of members of our group. Photographs were taken of CFU-EPC cultures to record the colonies and clusters assessed. These have been reviewed by other group members and similar conclusions were reached as how to 'score' cell aggregates, based on descriptions by Hill *et al* and the Stem Cell Technologies manual. It may be that other research groups are relying too heavily on immunostaining and cell uptake of ac-LDL or Ulex Lectin binding, all non specific characteristics, to score a cell cluster as CFU-EPC rather than to first examine 'colony' morphology to ascertain whether it meets CFU-EPC definitions. Representative images published in a paper by one group (163) would not be scored as CFU-EPC as the colonies shown did not have tight central cores of rounded cells with spindle cells emanating from the edges of these, described by Hill *et al* as a CFU-EPC(110). The images instead show rather loosely aggregated central cores of rounded cells without spindle cells sprouting from the periphery. Regardless of whether the cells in these colonies stained for CD133, VE-Cadherin and VWF, these would not have been scored as CFU-EPC.

These results showing lack of EOC formation in non-mobilised and mobilised MNC from autologous and allogeneic PBSC donors should be considered preliminary. Further work is being performed in this area. The ability to generate EOC from UCB MNC samples that contain lower proportions of $CD34^+$ cells than mobilised MNC led us to postulate that differences within the $CD34^+$/progenitor cell fraction between UCB and G-CSF mobilised MNC could be important in EOC formation.

5. Phenotypic investigation of the alterations in cell populations present in CFU-EPC culture following G-CSF administration

5.1. Introduction

The CFU-Hill assay is a commercially available bioassay that has gained widespread use for the assessment of putative CFU-EPC. Whilst the administration of G-CSF to promote endothelial regeneration/vasculogenesis has been reported to produce clinical benefits we have been unable to demonstrate increased (or even adequate) CFU-EPC formation using G-CSF mobilised peripheral blood samples and instead have consistently observed a decline in CFU-EPC following G-CSF (+/-chemotherapy or AMD3100). No technical factors that might have influenced the results of this study were identified.

G-CSF stimulates myeloid cells to proliferate, causing marked leukocytosis in normal donors, and to release a variety of enzymes including elastases, proteases and metalloproteinases, but it also effects on other cell populations including monocytes. It was hypothesised that G-CSF, through alterations in the subpopulations of leukocytes in mobilised blood, may have contributed to the reduction in CFU-EPC. Previous work by our group (136) and others (129, 132, 139, 140) explored and commented on the importance of $CD14^+$ monocytes to early EPC formation. Based on the increase in PB monocyte counts (in the region of a 3-fold increase (42, 43)) it would be reasonable to suppose that CD14-dependent CFU-EPC might also increase in mobilised samples. However, in chapter 4 data was presented that demonstrated a consistent decline in CFU-EPC following G-CSF. Earlier studies by our group (136) found that in mobilised patient samples, 2-hour plastic adherence, performed to enrich for $CD14^+$ monocytes, failed to increase CFU-EPC in G-CSF mobilised samples, as it did when using non-mobilised samples or UCB. Studies published on the effects of G-CSF on CFU-EPC (early EPC) formation have not presented phenotypic data on MNC actually plated into cell culture (162, 163,

165, 167, 170, 189), and despite evidence that short term culture of MNC on fibronectin results in colonies arising from myelomonocytic cells of haematopoietic origin (93, 96, 130, 131) it is the $CD34^+$ fractions the authors' have quantified and correlated with CFU-EPC.

The influence of $CD14^+$ monocytes and other leukocyte subpopulations on CFU-EPC in mobilised MNC was explored by phenotypic analysis of whole blood and MNC (section 5.2). It was found that the reduction in CFU-EPC in mobilised samples could not be attributed to any reductions in the proportions of $CD14^+$ monocytes in MNC following the administration of G-CSF (alone or sequentially with either chemotherapy or AMD3100) (section 5.2). It has been reported that CFU-EPC may require, or be influenced by T cells (135, 137, 142) and by a subpopulation of T cells, termed 'angiogenic' T cells, which have a phenotype $CD3^+/31^+/CXCR4^+$ (142). The numbers of angiogenic T cells in healthy subjects were reported to correlate with early EPC frequency (142). The proportions of whole blood T cells and angiogenic T cells were measured in PBSC donors to determine whether the decline in CFU-EPC following G-CSF could be linked to a fall in these cells (section 5.3). The influence of $CD66b^+$ granulocytes, the leukocyte population most stimulated by G-CSF, on CFU-EPC was then considered, with the effects on CFU-EPC of manipulating $CD66b^+$ granulocyte content of MNC examined (sections 5.4-5.6).

5.1.1. Patient samples

Peripheral blood samples were obtained from allogeneic and autologous PBSC patients on the same 3 occasions (at donor review pre G-CSF, on day 1 PBSC collection after G-CSF and at donor follow-up 4-6 weeks post G-CSF) as assessed in chapter 4. The cellular contents of non-mobilised and G-CSF mobilised whole blood and MNC samples were examined by flow cytometry. MNC were examined at two time points. The first time point for analysis of MNC was following Histopaque buoyant density gradient centrifugation of whole blood to obtain MNC (day 0 MNC). After enumeration, day 0 MNC were suspended in CECM and plated into

the first stage of the CFU-Hill assay or plated into the CFU-Hill(direct) assay. The second time point for analysis of MNC was on transfer of MNC (usually $1x10^6$ MNC in 1mL CECM) that were non-adherent after 2 days (48 hours) incubation on 6 or 12 well fibronectin coated plates (day 2 MNC). In this section, day 2 MNC refers specifically to MNC that were non-adherent to fibronectin after 2 days of incubation. These cells are identical to the cells re-plated into 24 well fibronectin plates as part of the CFU-Hill assay. MNC in these wells were inspected for CFU-EPC development. When plating MNC into CFU-EPC cultures, MNC were enumerated (cell concentrations) using a haemocytometer counting chamber (early samples) or a Coulter cell counter (virtually all data presented in this section). Both cell counting methods excluded erythrocytes from the cell count. When cells were counted using a haemocytometer, samples (whole blood and MNC) were mixed with 3% acetic acid to lyse erythrocytes prior to enumeration. Coulter cell counter settings were set to exclude erythrocytes (and smaller events) from the cell count.

5.1.2. Phenotypic definitions of cell populations

A live cell gate was established using forward and side light scatter to include all viable cells. Leukocytes were defined using a combination of light scatter characteristics, the expression of the pan-leukocyte marker CD45 and expression of lineage-specific antigens. $CD66b^+$ granulocytes were defined as events gating within the live cell gate co-expressing CD45 and CD66b (Figure 5.1). $CD14^+$ monocytes were defined as events gating within the live cell gate co-expressing CD45 and CD14 (Figure 5.1). $CD235a^+$ erythrocytes (red blood cells) were defined as low forward and side light scatter $CD45^-$ events expressing CD235a (Glycophorin A) but not CD14 or CD66b (Figure 5.2). Lymphocytes were small (low forward and side light scatter) $CD45^+$ events gating within the live cell gate and not expressing CD66b, CD14 or CD235a (Figure 5.1). Where events did not fall into one of these 4 defined populations (unclassified events) they were excluded from the analysis.

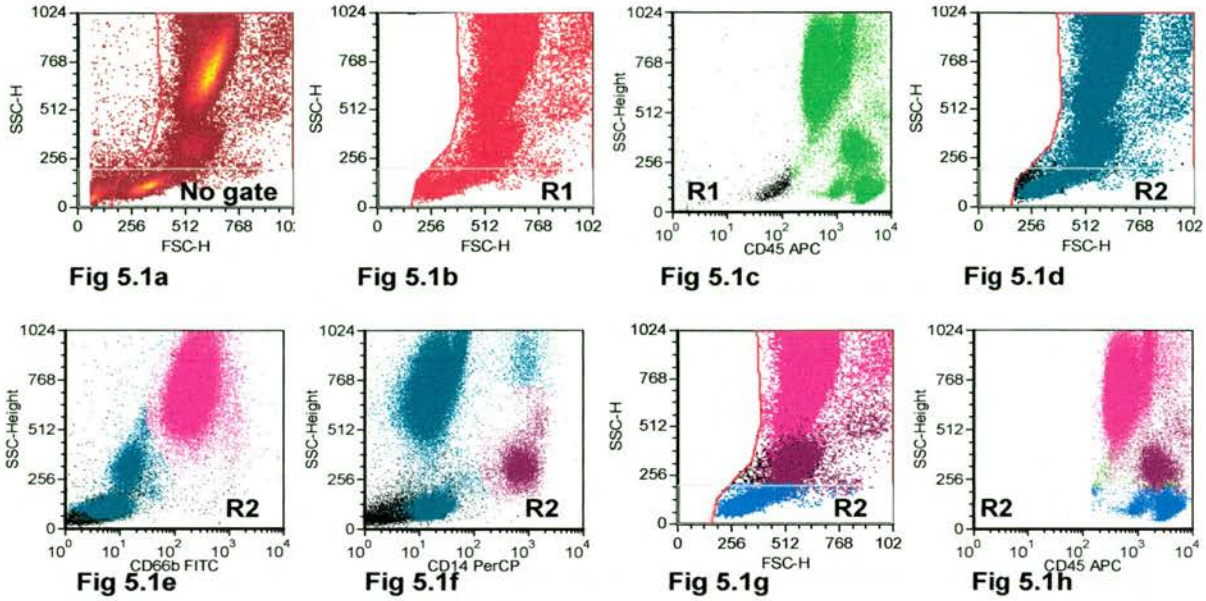


Fig 5.1a Fig 5.1b Fig 5.1c Fig 5.1d

Fig 5.1e Fig 5.1f Fig 5.1g Fig 5.1h

Figure 5.1 Phenotyping strategy used to enumerate leukocyte proportions in whole blood. Non-mobilised PB sample.

Fig 5.1a Forward (FSC) versus side scatter (SSC) (all events), live cell gate (R1) indicated.
Fig 5.1b FSC versus SSC (gated on R1) (indicated in red).
Fig 5.1c CD45 expression (light green) by events gating in R1 region.
Fig 5.1d FSC versus SSC of events defined by R1+CD45 (R2, teal population).
Fig 5.1c $CD66b^+$ granulocytes (pink) ($CD66b^+$ R2 gated events).
Fig 5.1f $CD14^+$ monocytes (purple) ($CD14^+$ R2 gated events).
Fig 5.1g Lymphocytes (blue) defined by low SSC R2 gated events not expressing CD66b or CD14.
Fig 5.1h CD45 expression of $CD66b^+$ granulocytes (pink), $CD14^+$ monocytes (purple) and lymphocytes (blue).

Whole blood samples were analysed to provide information on changes actually occurring in patients as a result of PBSC mobilisation. MNC samples were analysed to provide information on the phenotypes of cells, isolated under a specific set of conditions that were used to assess CFU-EPC formation of the sample. The MNC transferred into CFU-EPC assays were enumerated using methods that excluded erythrocytes from the cell count.

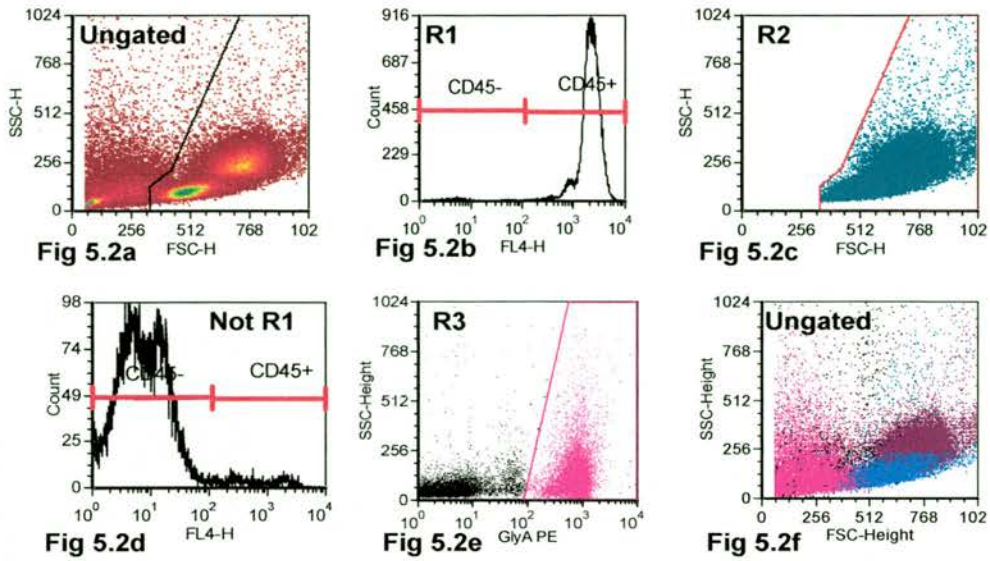


Figure 5.2 Phenotyping strategy used to enumerate CD235a$^+$ erythrocytes in MNC. Non-mobilised PB sample.

Fig 5.2a Forward (FSC) versus side scatter (SSC) (all events), live cell gate (R1) indicated.
Fig 5.2b CD45 expression (marker) by events gating in R1 region.
Fig 5.2c FSC versus SSC of events defined by R1+CD45 (R2, teal population).
Fig 5.2d CD45 expression of all events (marker) not included in live cell gate (Not R1) (R3 gated events).
Fig 5.2e CD235a$^+$ erythrocytes within R3 gated events (pink).
Fig 5.2f FSC versus SSC (all events) showing CD235a+ erythrocytes (pink), CD14$^+$ monocytes (purple) and lymphocytes (blue). In this figure no population of CD66b$^+$ granulocytes is visible (accounted for <2% leukocytes).

5.1.3. Data presentation

In the following section (section 5.2) the cell contents of non-mobilised (pre G-CSF) and mobilised (post G-CSF) samples are compared, with data presented first as proportions of all events (Table 5.1) and then as proportions of CD45$^+$ leukocytes (Table 5.2). By expressing data as proportions of CD45$^+$ leukocytes, erythrocytes are excluded from analysis. Unless otherwise stated, the figures presented refer to cells expressed as proportions of all events.

5.2. Changes in leukocyte subpopulations and erythrocytes following G-CSF$^+$/-chemotherapy

Exposure to G-CSF, given alone or sequentially with chemotherapy, produced predictable and reversible changes in the cell content of whole blood and MNC samples (Table 5.1 and Figure 5.3). Overall, autologous and allogeneic PBSC donors displayed similar changes in the composition of whole blood and MNC samples following G-CSF but there were differences noted between patient groups.

5.2.1. CD66b$^+$ granulocytes

5.2.1.1. CD66b$^+$ granulocytes in whole blood samples

Analysis of the proportions of CD66b$^+$ granulocytes contained in whole blood samples from non-mobilised PBSC donors showed that samples obtained from autologous PBSC patients contained significantly more CD66b$^+$ granulocytes (65.8%) than allogeneic PBSC donor samples (60.7%, $p<0.05$) (Table 5.1).

Following G-CSF treatment, the proportions of CD66b$^+$ granulocytes increased significantly in allogeneic PBSC donor samples (78.3%, $p<0.0005$) whilst no alterations in the proportions of whole blood CD66b$^+$ granulocytes were observed in autologous PBSC patients (65.1%, p=ns) (Table 5.1).

5.2.1.2. CD66b$^+$ granulocytes in MNC samples

Analysis of the proportions of CD66b$^+$ granulocytes contained in day 0 MNC and in day 2 MNC from non-mobilised blood samples was not significantly different for allogeneic PBSC donors (2.86% and 2.73% respectively) or autologous PBSC patients (5.05% and 4.22% respectively). There was no significant difference between the patient groups at either time point (day 0 MNC p= 0.25, day 2 MNC p=0.48) (Table 5.1).

Allogeneic PBSC Donors						
Sample timing	Pre G-CSF			G-CSF mobilised		
Cells examined	Whole Blood	Day 0 MNC	Day 2 MNC	Whole Blood	Day 0 MNC	Day 2 MNC
Number	13	16	12	11	13	11
$CD66b^+$ granulocytes	60.7%	2.9%	2.7%	78.3%	31.1%	31.3%
$CD14^+$ monocytes	5.2%	10.8%	7.7%	7.5%	20.6%	11.2%
lymphocytes	30.3%	61.9%	71.4%	15.1%	37.3%	38.8%
$CD235a^+$ erythrocytes	—	11.5%	10.9%	—	9.0%	6.7%

Autologous PBSC Patients						
Sample timing	Pre G-CSF			G-CSF mobilised		
Cells examined	Whole Blood	Day 0 MNC	Day 2 MNC	Whole Blood	Day 0 MNC	Day 2 MNC
Number	38	45	33	35	36	33
$CD66b^+$ granulocytes	65.8%	5.1%	4.2%	65.1%	27.7%	31.2%
$CD14^+$ monocytes	8.4%	13.1%	12.8%	11.3%	11.0%	9.5%
lymphocytes	16.9%	34.4%	44.4%	14.1%	19.2%	21.1%
$CD235a^+$ erythrocytes	—	31.3%	25.8%	—	34.6%	27.8%

Table 5.1 Distribution of major cell populations within pre G-CSF and mobilised blood. Samples obtained from allogeneic PBSC donors and autologous PBSC patients undergoing CFU-EPC culture. Figures are expressed as mean proportions of all events.

The major changes observed following G-CSF were marked increases in the proportions of $CD66b^+$ granulocytes contained in day 0 MNC and day 2 MNC (5.5-fold and 6.6-fold increases respectively (all PBSC patients)). Although there was no change in the proportions of $CD66b^+$ granulocytes in whole blood samples from autologous PBSC patients following PBSC mobilisation with G-CSF+chemotherapy compared to non-mobilised samples, significant changes were observed in the proportions of $CD66b^+$ granulocytes in day 0 and day 2 MNC following PBSC mobilisation.

The proportions of $CD66b^+$ granulocytes in day 0 MNC increased significantly from pre G-CSF levels following PBSC mobilisation with G-CSF alone in allogeneic PBSC donors ($p<0.0001$) and in autologous PBSC patients mobilised with G-CSF+chemotherapy ($p<0.0001$). MNC from mobilised allogeneic PBSC donors contained 31.1% and 31.3% $CD66b^+$ granulocytes in day 0 and day 2 MNC respectively, marginally higher than the 27.7% and 31.2% $CD66b^+$ granulocytes

observed in day 0 MNC and day 2 MNC from mobilised autologous PBSC patient samples (p=ns for comparisons between PBSC patient groups) (Table 5.1).

5.2.1.3. Changes in proportions of $CD66b^+$ granulocytes between day 0 and day 2 MNC

No reduction in the proportions of $CD66b^+$ granulocytes contained in MNC from non-mobilised samples occurred between the time points examined (p=ns for both allogeneic PBSC donors and autologous PBSC patients). Similarly, there was no reduction in $CD66b^+$ granulocytes between day 0 MNC and day 2 MNC for G-CSF mobilised samples from allogeneic PBSC donors (p=ns) or autologous PBSC patients (p=ns).

5.2.1.4. $CD66b^+$ granulocytes as proportions of $CD45^+$ leukocytes

Analysis of $CD66b^+$ granulocytes as proportions of $CD45^+$ leukocytes in non-mobilised and G-CSF mobilised MNC showed no statistically significant differences in proportions of $CD66b^+$ granulocytes between allogeneic and autologous PBSC donors. For non-mobilised or mobilised allogeneic PBSC donor samples, analysis of $CD66b^+$ granulocytes as proportions of $CD45^+$ events did not differ significantly from the analysis as proportions of all events in day 0 and day 2 MNC (p=ns for all comparisons). Analysis showed significant differences in the proportions of MNC $CD66b^+$ granulocytes from autologous PBSC patient samples when expressed as proportions of $CD45^+$ leukocytes or proportions of all events ($p<0.03$ non-mobilised day 0 MNC, $p<0.001$ mobilised day 0 MNC, $p<0.02$ mobilised day 2 MNC) without a significant difference in the proportions of $CD66b^+$ granulocytes in non-mobilised day 2 MNC (Table 5.2).

5.2.1.5. Comment on differences in $CD66b^+$ granulocytes between autologous and allogeneic PBSC samples

Non-mobilised samples from autologous PBSC patients contained higher levels of $CD66b^+$ granulocytes than non-mobilised samples from allogeneic donors. This could result from patient exposure to chemotherapy/treatment during the patient referral and review process. At PBSC assessment, most autologous PBSC patients

were receiving active treatment and patients' leukocyte counts were often recovering following their most recent chemotherapy. These patients might have had stimulated and left shifted bone marrows, with the appearance of immature myeloid (and erythroid) cells in the peripheral blood. These features are similar to the changes the observed in peripheral blood and morphology following PBSC mobilisation with G-CSF.

Despite the higher proportions of $CD66b^+$ granulocytes contained in autologous PBSC patient samples, CFU-EPC were generated by non-mobilised samples from both allogeneic PBSC donors and autologous PBSC patients. In contrast, G-CSF mobilised samples from both allogeneic and autologous PBSC patients had severely reduced (virtually abolished) CFU-EPC. This suggests that if $CD66b^+$ granulocytes do affect CFU-EPC formation in G-CSF mobilised blood samples that their influence on CFU-EPC extends beyond being merely quantitative and they affect CFU-EPC by additional means.

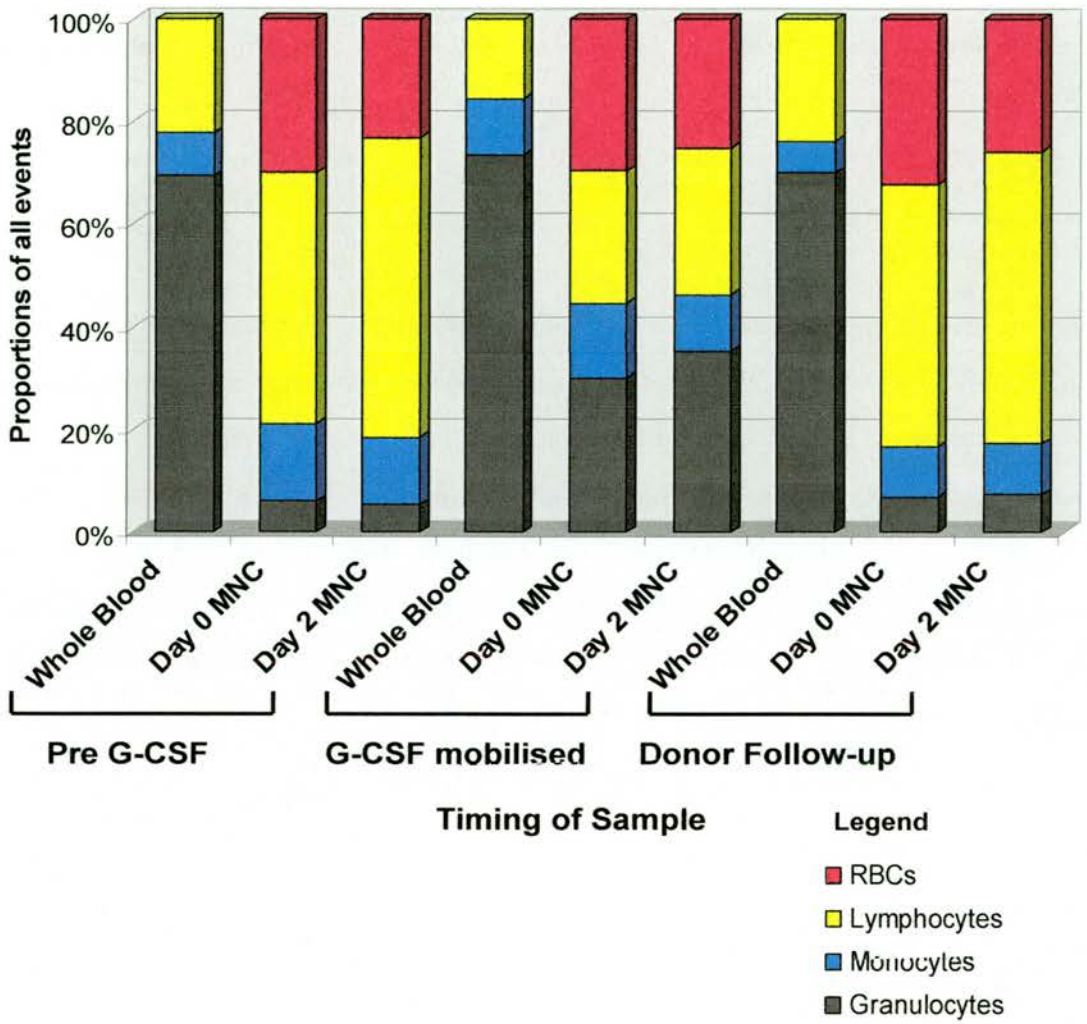


Figure 5.3 Proportions of cells present in whole blood, day 0 and day 2 MNC. Samples obtained from PBSC donors (allogeneic and autologous donors combined) pre G-CSF, following G-CSF mobilisation and at donor follow-up. Figures are presented as mean proportions of all events.

5.2.2. $CD14^+$ monocytes

5.2.2.1. $CD14^+$ monocytes in whole blood samples

Analysis of the proportions of $CD14^+$ monocytes in non-mobilised and G-CSF mobilised whole blood samples showed statistically significant increases in $CD14^+$ monocytes in mobilised samples for both patient groups compared to non-mobilised samples (allogeneic PBSC donors $p<0.02$ and autologous PBSC patients $p<0.002$). The proportions of $CD14^+$ monocytes increased from 5.2% to 7.5% post G-CSF in allogeneic donor samples and from 8.4% to 11.3% in autologous PBSC patient samples (Table 5.1).

5.2.2.2. $CD14^+$ monocytes in MNC samples

Analysis of the proportions of $CD14^+$ monocytes in day 0 and day 2 MNC in non-mobilised donors showed no significant difference for allogeneic PBSC donors (10.8% and 7.7%, p=ns) or autologous PBSC patients (13.1% and 12.8%, p=ns). There were no significant differences in the proportions of $CD14^+$ monocytes between non-mobilised samples from allogeneic and autologous donor MNC (day 0 MNC p=ns and day 2 MNC p=ns) (Table 5.1).

Following G-CSF mobilisation, day 0 MNC from allogeneic PBSC donors contained significantly higher proportions of $CD14^+$ monocytes (20.6%) than non-mobilised day 0 MNC (10.8%, $p<0.005$) or G-CSF mobilised day 2 MNC (11.2%, $p<0.02$). There were no significant differences in the proportions of $CD14^+$ monocytes in G-CSF mobilised autologous PBSC patient day 0 MNC (11.0%) compared to non-mobilised day 0 MNC (13.1%, p=ns) or to mobilised day 2 MNC (9.5%, p=ns) (Table 5.1). Day 0 MNC from mobilised allogeneic PBSC donors contained significantly greater proportions of $CD14^+$ monocytes than autologous PBSC patient samples (20.6% and 11.0% in allogeneic and autologous PBSC patient samples respectively, $p<0.0005$). This difference was not maintained in mobilised day 2 MNC (11.2% and 9.5% respectively, p=ns).

5.2.2.3. Changes in $CD14^+$ monocytes between day 0 and day 2 MNC

The proportions of $CD14^+$ monocytes in mobilised allogeneic PBSC MNC decreased significantly between day 0 and day 2 ($p<0.02$). This was not noted in non-mobilised allogeneic PBSC donor samples (p=ns). This could be a reflection of fibronectin adherent $CD14^+$ monocytes in mobilised allogeneic PBSC donor samples not present in other patient groups. The reduction in proportions of MNC $CD14^+$ monocytes between day 0 and day 2 MNC from autologous PBSC patients were not statistically significant (non-mobilised samples p=ns and mobilised samples p=ns).

5.2.2.4. $CD14^+$ monocytes as proportions of $CD45^+$ leukocytes

When considered as proportions of $CD45^+$ leukocytes rather than as proportions of all events, the proportions of $CD14^+$ monocytes increased significantly in non-mobilised day 0 MNC (14.3% versus 10.7% for allogeneic day 0 MNC ($p<0.05$) and 24.0% versus 13.1% for autologous day 0 MNC ($p<0.002$)) but not in non-mobilised day 2 MNC (9.3% versus 7.7% for allogeneic (p=ns) and 17.6% versus 12.8% for autologous PBSC samples (p=ns)) (Table 5.2). There were no significant changes in the proportions of $CD14^+$ monocytes in mobilised allogeneic PBSC donor MNC when considered as proportions of $CD45^+$ leukocytes (23.8% versus 20.6% day 0 MNC, p=ns and 12.5% versus 11.2% day 2 MNC, p=ns). The proportions of $CD14^+$ monocytes in mobilised autologous PBSC patient MNC increased significantly when analysed as proportions of $CD45^+$ leukocytes (19.7% versus 11.0% day 0 MNC ($p<0.0002$) and day 2 MNC 15.2% versus 9.5% ($p<0.005$) (Table 5.2).

Non-mobilised samples from autologous PBSC patients contained significantly greater proportions of $CD14^+$ monocytes as proportions of $CD45^+$ leukocytes than allogeneic PBSC donor samples (day 0 MNC 24.0% and 14.3% in autologous and allogeneic donor samples respectively, $p<0.005$ and day 2 MNC 17.6% and 9.3%, $p<0.02$). The difference between sample groups was not maintained in mobilised samples. There were no differences in $CD14^+$ monocytes as proportions of $CD45^+$ leukocytes between donor groups for mobilised PBSC in day 0 MNC (23.8% and

19.7% in allogeneic and autologous patient samples respectively, p=ns) or day 2 MNC (12.5% and 15.2% respectively, p=ns).

Significant reductions in the proportions $CD14^+$ monocytes between day 0 and day 2 MNC occurred in non-mobilised and mobilised allogeneic PBSC donor samples ($p<0.02$ and $p<0.01$ respectively) and non-mobilised autologous patient samples ($p<0.02$) but not in mobilised autologous PBSC patient samples (19.7% versus 15.2% in day 0 and day 2 MNC respectively, $p<0.06$) (Table 5.2).

Allogeneic PBSC donors				
Sample timing	Pre G-CSF		G-CSF mobilised	
Cells examined	Day 0 MNC	Day 2 MNC	Day 0 MNC	Day 2 MNC
$CD66b^+$ Granulocytes	4.0%	3.5%	35.8%	34.7%
$CD14^+$ Monocytes	14.3%	9.3%	23.8%	12.5%
Lymphocytes	81.3%	84.1%	40.4%	44.5%

Autologous PBSC patients				
Sample timing	Pre G-CSF		G-CSF mobilised	
Cells examined	Day 0 MNC	Day 2 MNC	Day 0 MNC	Day 2 MNC
$CD66b^+$ Granulocytes	9.7%	6.4%	44.5%	45.1%
$CD14^+$ Monocytes	24.0%	17.6%	19.7%	15.2%
Lymphocytes	63.6%	68.8%	34.6%	33.1%

Table 5.2 Leukocyte subpopulations in non-mobilised and mobilised day 0 and 2 MNC. Samples obtained from allogeneic and autologous PBSC patients undergoing CFU-EPC culture. Figures are expressed as mean proportions of $CD45^+$ leukocytes.

5.2.2.4.1. Paired analyses

When the proportion of $CD14^+$ monocytes was assessed using paired analysis there were significant reductions in $CD14^+$ monocytes as proportions of $CD45^+$ leukocytes between day 0 and day 2 MNC in non-mobilised samples from autologous PBSC patients (24.0% versus 17.6%, $p<0.02$) and allogeneic PBSC donor samples (14.3% versus 9.3%, $p<0.02$). A statistically significant reduction in $CD14^+$ monocytes as proportions of $CD45^+$ leukocytes occurred between day 0 and day 2 MNC in

mobilised patient samples (autologous 19.7% versus 15.2%, $p<0.05$ and allogeneic 23.8% versus 12.5%, $p<0.005$).

5.2.3. CD235a$^+$ erythrocytes

CD235a$^+$ erythrocyte populations, defined as CD45$^-$/235a$^+$ events not expressing CD14 or CD66b (Figure 5.2), were always detected in MNC samples. Whilst these populations contained some nucleated erythroid precursors, the majority of CD235a$^+$/45$^-$ events were mature erythrocytes that contaminated recovered MNC.

5.2.3.1. CD235a$^+$ erythrocytes in MNC samples

Analysis of the proportions of CD235a$^+$ erythrocytes as proportions of all events showed that non-mobilised autologous PBSC patient samples contained significantly greater proportions of CD235a$^+$ erythrocytes (31.3% and 25.8% in day 0 and day 2 MNC respectively) than comparative samples from allogeneic PBSC donors (11.5% and 10.9% respectively, $p<0.0001$ and $p<0.01$). This difference in CD235a$^+$ erythrocyte content between sample groups was maintained in mobilised day 0 MNC (9.0% and 34.6% CD235a$^+$ erythrocytes in mobilised day 0 MNC from allogeneic and autologous PBSC patients respectively, $p<0.0001$) and day 2 MNC (6.7% and 27.8% respectively, $p<0.005$). Within patient groups there was no difference in the day 0 and day 2 MNC CD235a$^+$ erythrocyte content of non-mobilised and mobilised samples (p=ns for all comparisons).

5.2.3.2. Changes in CD235a$^+$ erythrocytes between day 0 and day 2 MNC

Both patient groups showed little or no reduction in the proportions of CD235a$^+$ erythrocytes between day 0 and day 2 MNC in non-mobilised and G-CSF mobilised samples. The proportions of CD235a$^+$ erythrocytes in day 0 and day 2 MNC allogeneic PBSC donors decreased from 11.5% to 10.9% in non-mobilised MNC (p=ns) and from 9.0% to 6.7% in mobilised MNC (p=ns). Similar changes were seen in autologous PBSC patients; CD235a$^+$ erythrocytes decreased from 31.1%, to 25.8% in non-mobilised MNC (p=ns) and from 34.6% to 27.8% in mobilised MNC (p=ns) (Table 5.1).

5.2.3.2.1. Paired analyses

When the differences between the proportions of CD235a$^+$ erythrocytes in day 0 MNC and day 2 MNC were assessed using paired analysis, there were significant overall reductions in CD235a$^+$ erythrocyte content between day 0 and day 2 MNC in non-mobilised and G-CSF mobilised autologous PBSC patient samples (non-mobilised $p<0.005$ and mobilised $p<0.001$, n=31). There were no significant reductions in the proportions of CD235a$^+$ erythrocytes between day 0 and day 2 MNC in allogeneic donor samples (p=ns for non-mobilised and mobilised samples).

5.2.3.3. CD235a$^+$ erythrocytes as proportions of CD45$^-$ events

Overall, CD235a$^+$ erythrocytes accounted for between 48% (non-mobilised allogeneic PBSC day 0 MNC) and 84% (G-CSF mobilised autologous PBSC day 2 MNC) of CD45$^-$ events. Significant proportions of CD45$^-$ events remained 'unidentified.' These CD45$^-$/235a$^-$ events might have included platelet clumps, cell debris, necrotic cells or apoptotic cells. The largest populations of CD45$^-$/235a$^-$ events were present in non-mobilised day 0 MNC (allogeneic PBSC donors 12.5% and autologous PBSC patients 13.2%) (Table 5.3).

G-CSF	PBSC donor	MNC	CD45$^-$ (% all events)	CD45$^-$/235a$^-$ (% all events)
Non-mobilised	Allogeneic	Day 0	24.0%	12.5%
		Day 2	15.6%	4.7%
	Autologous	Day 0	44.6%	13.3%
		Day 2	34.7%	8.9%
G-CSF mobilised	Allogeneic	Day 0	13.6%	4.6%
		Day 2	11.2%	4.5%
	Autologous	Day 0	41.4%	6.8%
		Day 2	33.2%	5.4%

Table 5.3 Mean proportions of CD45$^-$/235a$^-$ events detected in day 0 and day 2 MNC. Samples obtained from non-mobilised and mobilised PBSC donors. Figures are expressed as mean proportions of all events.

5.2.3.4. Comment on differences in proportions of CD235a$^+$ erythrocytes between autologous and allogeneic PBSC samples

It is unclear why greater proportions of CD235a$^+$ erythrocytes were present in autologous PBSC patient MNC compared to allogeneic PBSC donor MNC for all

time points examined. It might be a result of active treatment for their haematological malignancy or may due to disease-related bone marrow changes. On microscopic examination erythrocytic elements consisted mainly of mature erythrocytes with rare nucleated erythrocyte precursors present.

Although autologous PBSC patient samples contained greater numbers of $CD235a^+$ erythrocytes it is probably on balance unlikely that this contributed to the reduction in CFU-EPC formation observed following G-CSF administration. There were no significant differences in $CD235a^+$ erythrocytes between non-mobilised and mobilised samples from autologous PBSC patients (day 0 MNC 31.3% and 34.6% in non-mobilised and mobilised samples respectively, p=ns, day 2 MNC 25.8% versus 27.8%, p=ns). Similarly, there were no differences in the MNC $CD235a^+$ erythrocyte content between non-mobilised and mobilised allogeneic PBSC donor samples (day 0 MNC 11.5% versus 9.0%, p=ns, and day 2 MNC 10.9% versus 6.7%, p=ns). Similar changes (reductions) in $CD235a^+$ erythrocyte content between day 0 and day 2 MNC were noted for non-mobilised and mobilised samples from both patient groups. Changes in $CD235a^+$ erythrocyte content alone would not account for the decline in CFU-EPC formation following G-CSF.

5.2.4. Lymphocytes

5.2.4.1. Lymphocytes in whole blood samples

Analysis of the proportions of lymphocytes in whole blood showed that non-mobilised samples from allogeneic PBSC donors contained significantly greater proportions of lymphocytes (30.3%) than comparative samples from autologous PBSC patients (16.9%, $p<0.0001$). There was no significant difference between patient groups in the proportions of lymphocytes contained in G-CSF mobilised whole blood samples (15.1% and 14.1%, p=ns) (Table 5.1).

5.2.4.2. Lymphocytes in MNC samples

The proportions of lymphocytes in non-mobilised day 0 and day 2 MNC were significantly greater in allogeneic PBSC donor MNC (61.9% and 71.4% in day 0 and day 2 MNC respectively) than autologous PBSC patient MNC (34.4% and 44.4% respectively, $p<0.0001$ and $p<0.0001$). Similarly, mobilised allogeneic donor MNC contained greater proportions of lymphocytes (37.3% and 38.8% in day 0 and day 2 MNC respectively) than autologous PBSC patient samples (19.2% and 21.1% respectively, $p<0.0001$ and $p<0.003$).

The proportions of lymphocytes in day 0 and day 2 MNC significantly decreased in allogeneic PBSC donor MNC following G-CSF mobilisation ($p<0.0001$ for days 0 and 2). Day 0 MNC lymphocytes fell from 61.9% to 37.3% and day 2 MNC lymphocytes decreased from 71.4 to 38.8% following G-CSF (Table 5.1). The proportions of lymphocytes in day 0 and day 2 autologous PBSC patient MNC declined significantly following G-CSF. Compared to non-mobilised autologous patient samples, mobilised day 0 MNC lymphocytes fell from 34.4% to 19.2% ($p<0.0001$) and mobilised day 2 MNC lymphocytes fell from 44.4% to 21.1% ($p<0.0001$) (Table 5.1).

5.2.4.3. Changes in lymphocytes between day 0 and day 2 MNC

Analysis of the proportions of lymphocytes in non-mobilised day 0 and day 2 MNC showed a significant increase in lymphocytes for autologous PBSC patient MNC ($p<0.005$) but this failed to reach statistical significance for allogeneic PBSC donor MNC ($p<0.06$). There were no changes in the proportions of lymphocytes between day 0 and day 2 MNC in mobilised MNC from either patient group (p=ns).

5.2.4.4. Lymphocytes as proportions of $CD45^+$ leukocytes

There were significant increases in the proportions of lymphocytes in autologous PBSC patient MNC ($p<0.0001$ for day 0 and 2 non-mobilised and mobilised MNC) and allogeneic donor MNC ($p<0.0005$ for day 0 and day 2 non-mobilised and mobilised MNC) when lymphocytes were expressed as proportions of $CD45^+$

leukocytes rather than of all events (Table 5.2). Non-mobilised allogeneic PBSC donor MNC contained significantly greater proportions of lymphocytes than non-mobilised autologous PBSC patient MNC ($p<0.0005$ for day 0 and day 2 MNC) with no differences in lymphocytes expressed as proportions of $CD45^+$ leukocytes in mobilised samples (day 0 MNC, p=ns and day 2 MNC, p=ns) (Table 5.2). Analysis of lymphocytes as proportions of $CD45^+$ leukocytes showed no significant changes between day 0 and day 2 MNC in non-mobilised and mobilised MNC from either patient group.

5.2.5. Comparisons between pre G-CSF and donor follow-up samples

There were differences observed in the proportions leukocytes and erythrocytes present in pre mobilisation samples obtained from autologous PBSC patients and allogeneic PBSC donors. These differences could be due to a number of factors including the patients' haematological diagnoses and the effects of chemotherapy on bone marrow haematopoietic activity. If differences in the proportions of leukocytes and erythrocytes between sample groups became less marked when patient follow-up samples were compared then this might indicate the influence of treatment related factors on pre mobilisation samples.

Donor follow-up samples were available for 16 allogeneic PBSC donors and 9 autologous PBSC patients. Follow-up samples from autologous PBSC patients were obtained after a chemotherapy-free period of 4-6 weeks immediately following PBSC mobilisation. Analysis of the proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes in whole blood and MNC showed little differences between non-mobilised patient samples. There were no significant differences in the pre mobilisation and donor follow-up samples from allogeneic PBSC donors (p=ns). The only statistically significant difference between pre mobilisation samples and donor follow-up samples from autologous PBSC patients was the higher proportions of $CD66b^+$ granulocytes in follow-up samples (11.2% and 11.0% in day 0 and day 2

MNC respectively) than pre mobilisation samples (5.1% and 4.2% respectively, $p<0.01$ and $p<0.01$) (Table 5.4 and Figure 5.4). It appears that the differences noted between pre mobilisation samples from allogeneic and autologous PBSC patients also exists in follow-up samples, suggesting that even in treatment free periods there are some differences between these patient groups.

Allogeneic PBSC donors						
Time point	Whole Blood		Day 0 MNC		Day 2 MNC	
Sample	Pre G-CSF	Follow-up	Pre G-CSF	Follow-up	Pre G-CSF	Follow-up
CD66b+ Granulocytes	60.7%	60.4%	2.9%	1.8%	2.7%	1.7%
CD14+ Monocytes	5.2%	4.9%	10.8%	7.3%	7.7%	5.9%
Lymphocytes	30.3%	27.7%	61.9%	53.3%	71.4%	65.7%
CD235a+ Erythrocytes	–	–	11.5%	23.0%	10.9%	12.7%

Autologous PBSC patients						
Time point	Whole Blood		Day 0 MNC		Day 2 MNC	
Sample	Pre G-CSF	Follow-up	Pre G-CSF	Follow-up	Pre G-CSF	Follow-up
CD66b+ Granulocytes	65.8%	73.4%	5.1%	11.2%	4.2%	11.0%
CD14+ Monocytes	8.3%	6.7%	13.1%	10.4%	12.8%	11.6%
Lymphocytes	16.9%	17.7%	34.3%	35.4%	44.4%	39.2%
CD235a+ Erythrocytes	–	–	31.3%	35.2%	25.8%	32.4%

Table 5.4 Distribution of major cell populations in non-mobilised patient samples. Pre G-CSF and donor follow-up samples obtained from allogeneic and autologous PBSC patients. Figures are presented as mean proportions of all events.

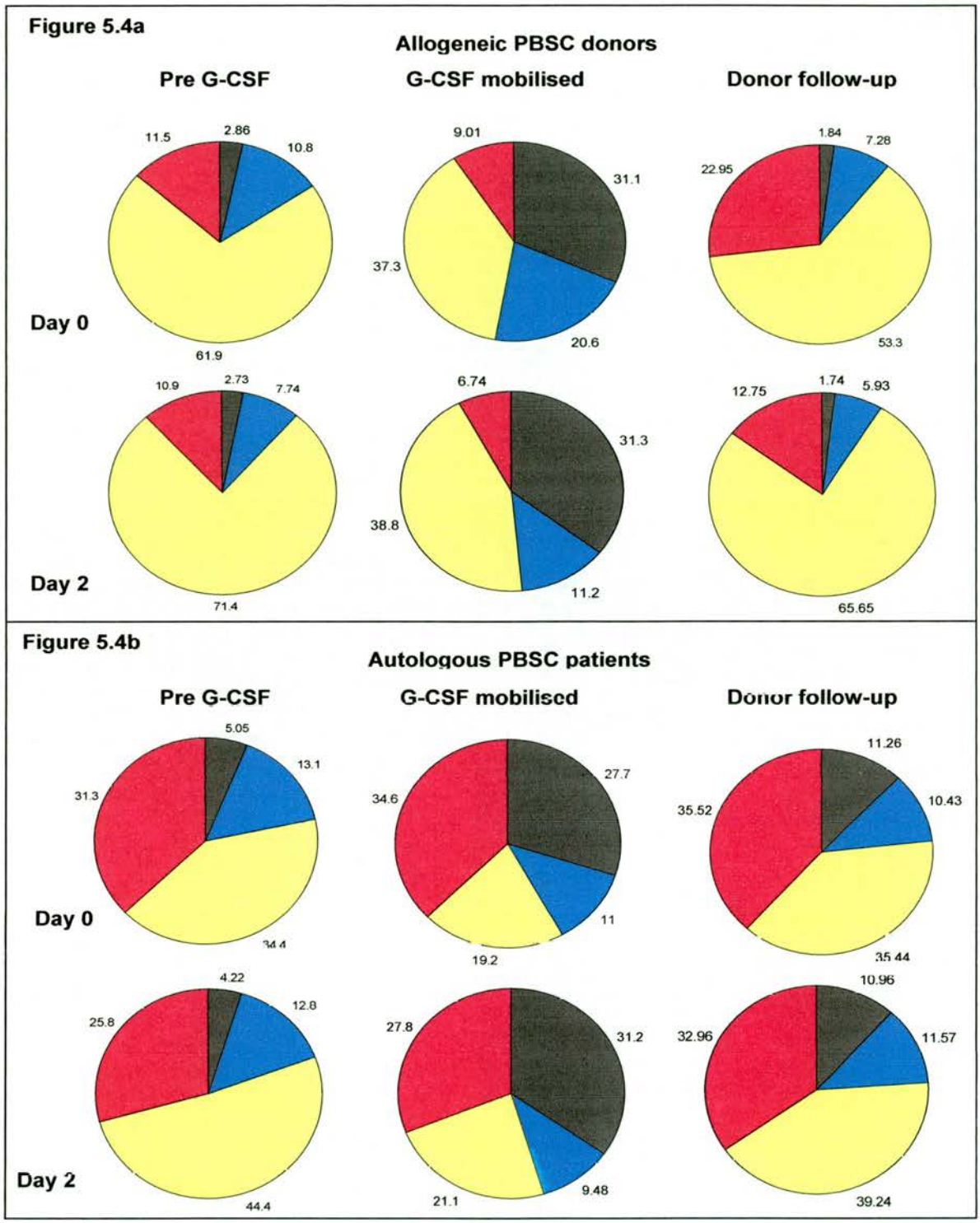


Figure 5.4 Changes in cell proportions between day 0 and 2 MNC during CFU-EPC.
Pre G-CSF, mobilised and donor follow-up samples.
Figures are presented as mean proportions of all events.

Fig 5.4a Allogeneic PBSC donors.
Fig 5.4b Autologous PBSC patients.

5.2.6. Changes in leukocyte subpopulations and erythrocytes following G-CSF+AMD3100

Samples from autologous PBSC patients mobilised with G-CSF+AMD3100 were analysed to determine whether mobilised and non-mobilised samples from these patients contained similar proportions of $CD66b^+$ granulocytes, $CD14^+$ monocytes, lymphocytes and $CD235a^+$ erythrocytes as those obtained from $G\text{-}CSF^+$/-chemotherapy mobilised autologous PBSC patients (section 5.2.1-5.2.5). The proportions of leukocyte subpopulations and erythrocytes contained in peripheral blood and MNC were determined at the same time points, using the methods and phenotypic definitions outlined in section 5.1.2 (Figure 5.1 and Figure 5.2).

5.2.6.1. Non-mobilised samples

Analysis of the proportions of leukocyte subsets in peripheral blood samples from non-mobilised patients showed that on average they consisted of 70.0% $CD66b^+$ granulocytes, 9.6% $CD14^+$ monocytes and 16.5% lymphocytes (Table 5.4). Day 0 and day 2 MNC from non-mobilised patients contained relatively low levels of $CD66b^+$ granulocytes (2.3% and 3.1% in day 0 and day 2 MNC respectively) as well as considerable $CD235a^+$ erythrocyte contamination (30.3% and 29.5% in day 0 and day 2 MNC respectively). There were little changes in proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes in non-mobilised samples between day 0 and day 2 MNC (Table 5.5).

The proportions of cells in non-mobilised samples from G-CSF+AMD3100 patients were not dissimilar to the proportions of cells in non-mobilised samples from G-CSF autologous PBSC patients and were midway between that seen in non-mobilised samples from autologous G-CSF+chemotherapy mobilised PBSC patients and G-CSF mobilised allogeneic PBSC donors. This might reflect the lack of recent chemotherapy given to these G-CSF+AMD3100 PBSC patients at the time of PBSC referral.

5.2.6.2. G-CSF+AMD3100 mobilised samples

Analysis of the proportions of leukocytes subpopulations in G-CSF+AMD3100 mobilised samples showed that peripheral blood samples contained greater proportions of CD66b$^+$ granulocytes (78.8%) with marginally-reduced proportions of CD14$^+$ monocytes (8.9%) and lymphocytes (10.4%) compared to non-mobilised samples (Table 5.5). Mobilised day 0 MNC contained significantly greater proportions of CD66b$^+$ granulocytes than non-mobilised day 0 MNC (20.2% and 2.3% respectively, $p<0.02$). There was no difference in CD235a$^+$ erythrocyte content of day 0 mobilised MNC (29.5%) compared to day 0 non-mobilised MNC (29.5%, p=ns), but mobilised day 0 MNC contained slightly lower proportions of CD14$^+$ monocytes (17.4% and 15.6%, p=ns) and especially lymphocytes than non-mobilised MNC (44.8% and 28.4%, p=ns) (Table 5.5).

PBSC	Cells	Unclassified	CD66b$^+$ Granulocytes	CD14$^+$ Monocytes	Lymphocytes	CD235$^+$ Erythrocytes
Non-mobilised samples	Whole Blood	4.0	70.0% (72.4)	9.6% (9.8)	16.5% (17.0)	—
	Day 0 MNC	5.2	2.3% (5.1)	17.4% (23.8)	44.8% (65.0)	30.3%
	Day 2 MNC	11.9	3.1% (5.9)	13.0% (19.0)	42.4% (64.7)	29.5%
Mobilised samples	Whole Blood	1.9	78.8% (79.2)	8.9% (8.9)	10.4% (10.5)	—
	Day 0 MNC	6.3	20.2% (29.4)	15.6% (23.9)	28.4% (42.3)	29.5%
	Day 2 MNC	19.3	9.0% (10.8)	32.1% (39.7)	26.0% (31.9)	13.6%

Table 5.5 Leukocytes and erythrocytes in G-CSF+AMD3100 PBSC patient samples. Non-mobilised and mobilised samples obtained from autologous G-CSF+AMD3100 mobilised PBSC patients. Figures are expressed as mean proportions of all events; proportions of CD45$^+$ leukocytes in parentheses.

Day 0 and day 2 mobilised MNC differed in their proportions of leukocytes and CD235a$^+$ erythrocytes. Day 2 MNC contained substantially lower proportions of CD66b$^+$ granulocytes and CD235$^+$ erythrocytes than day 0 MNC whilst the proportions of CD14$^+$ monocytes increased (p=ns for all comparisons). The reduction in proportions of CD66b$^+$ granulocytes and CD235a$^+$ erythrocytes was accompanied by an increase in 'unclassified events'. The proportions of cells in G-CSF+AMD3100 mobilised samples were similar to G-CSF mobilised samples from autologous PBSC patients mobilised with G-CSF+chemotherapy. Mobilised samples

from G-CSF+AMD3100 patients contained cell proportions mid-way between those of mobilised autologous and allogeneic G-CSF mobilised donors (Table 5.6).

Non-mobilised autologous PBSC Patients						
PBSC Group	G-CSF+chemotherapy			G-CSF+AMD3100		
Time point	Whole Blood	Day 0 MNC	Day 2 MNC	Whole Blood	Day 0 MNC	Day 2 MNC
$CD66b^+$ Granulocytes	65.8%	5.1%	4.2%	70.0%	2.3%	3.1%
$CD14^+$ Monocytes	8.4%	13.1%	12.8%	9.6%	17.4%	13.0%
Lymphocytes	16.9%	34.4%	44.4%	16.5%	44.8%	42.4%
$CD235a^+$ Erythrocytes	—	31.3%	25.8%	—	30.3%	29.5%

Mobilised autologous PBSC Patients						
PBSC Group	G-CSF+chemotherapy			G-CSF+AMD3100		
Time point	Whole Blood	Day 0 MNC	Day 2 MNC	Whole Blood	Day 0 MNC	Day 2 MNC
$CD66b^+$ Granulocytes	65.1%	27.7%	31.2%	78.8%	20.2%	9.0%
$CD14^+$ Monocytes	11.4%	11.0%	9.5%	8.9%	15.6%	32.1%
Lymphocytes	14.1%	19.2%	21.1%	10.4%	28.4%	26.0%
$CD235a^+$ Erythrocytes	—	34.6%	27.8%	—	29.5%	13.6%

Table 5.6 Comparison of leukocytes and erythrocytes between autologous patient groups. Samples obtained from non-mobilised and G-CSF or G-CSF+AMD3100 mobilised autologous PBSC patients. Figures are expressed as mean proportions of all events

5.2.6.3. 'Unclassified' events

There were relatively large populations of unclassified events ($CD45^-/235a^-$ or $CD45^+$ but not identified as $CD66b^+$ granulocytes, $CD14^+$ monocytes or lymphocytes) in day 2 MNC, particularly mobilised samples (11.9% in non-mobilised and 19.3% in mobilised day 2 MNC). There were substantial reductions in the proportions of $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes in day 2 MNC compared to day 0 MNC in mobilised samples and some of these cells lost from cell populations would now be included in the population of 'unclassified' events.

5.2.6.4. Conclusions

The changes in proportions of leukocyte subpopulations in whole blood and MNC samples following PBSC mobilisation with G-CSF+AMD3100 were comparable to the changes observed following PBSC mobilisation with $G\text{-}CSF^+$/-chemotherapy.

5.2.7. Use of SytoRed to define leukocyte subpopulations and CD235a$^+$ erythrocytes

SytoRed59 fluorescent nucleic acid staining was performed in order to determine whether a population of relevant (stem) cells was being overlooked during flow cytometry analysis when a gating strategies using CD45 were used. SytoRed59 is one of the members of the Syto dye family of permanent nucleic acid stains that will stain cytoplasmic, mitochondrial and nuclear elements of eukaryotic cells as well as most live and permeabilised bacteria. The Syto dyes differ from one another by characteristics including cell permeability, fluorescence enhancement upon binding nucleic acids, excitation and emission spectra, DNA/RNA selectivity and binding affinity. SytoRed59 absorption occurs at 622nm with maximal fluorescence emission at 645nm, detected in FL4 by the 633nm laser. A 0.05mM SytoRed working solution, prepared from the neat solution supplied, was used for all experiments in this section. For the remainder of this section, the term 'SytoRed' and abbreviation 'SR' refers exclusively to the SytoRed59 nucleic acid stain.

SytoRed was used to identify nucleated cells in mobilised and non-mobilised whole blood and MNC samples obtained from allogeneic or autologous PBSC patients referred for PBSC mobilisation with G-CSF. Results obtained using SytoRed gating were compared to those obtained when CD45 was used. MNC were examined at same two time points, day 0 MNC and day 2 MNC, as described in section 5.1. The 4 main cell populations were defined using the phenotypic definitions as stated in section 5.1.1, with expression of SR replacing CD45. CD66b$^+$ granulocytes were defined as events gating within the live cell gate co-expressing CD45 or SR and CD66b. CD14$^+$ monocytes were defined as events gating within the live cell gate co-expressing CD45 or SR and CD14. CD235a$^+$ erythrocytes were defined as low forward and side scatter CD45$^-$ or SR$^-$ (or SR$_{low}$) events expressing CD235a (Glycophorin A) but not CD14 or CD66b. Lymphocytes were small (low forward and side light scatter) CD45$^+$ or SR$^+$ events gating within the live cell gate and not expressing CD66b, CD14 or CD235a.

Patient samples

CD45 staining was directly compared with SR staining in 53 PBSC donor samples (28 non-mobilised samples (10 whole blood samples, 10 day 0 MNC, 8 day 2 MNC) and 25 mobilised samples (9 whole blood samples, 8 day 0 MNC, 8 day 2 MNC).

5.2.7.1. Proportions of $CD45^+$ versus SR^+ events

There were no significant differences in the proportions of whole blood or MNC events that were $CD45^+$ or SR^+. Overall, the proportions of $CD45^+$ and SR^+ events differed by <2%. Samples containing significant proportions of $CD235a^+$ erythrocytes (poorly-lysed whole blood samples and some MNC) recorded greater differences between the proportions of $CD45^+$ and SR^+ events identified in a sample.

Whole blood leukocytes

Flow cytometric analysis of lysed whole blood samples stained with SytoRed produced a narrow peak of SR^+ cells but when erythrocyte lysis was incomplete, a narrow peak of SR^+ events was present together with a population of erythrocytes that produced a broader, less intense SR^+ peak. Flow cytometric analysis of whole blood samples stained with CD45 resulted in either a single peak or dual $CD45^+$ peaks ($CD45^{BRIGHT}$ and $CD45^{REG}$ populations). $CD66b^+$ granulocytes were the main constituents of $CD45^{REG}$ cells with $CD14^+$ monocytes and lymphocytes contained mainly in the $CD45^{BRIGHT}$ fraction. A single $CD45^+$ peak occurred when a dominant population of $CD66b^+$ granulocytes swamped $CD45^{BRIGHT}$ cells (Figure 5.5).

Day 0 MNC

SR staining of day 0 MNC was more dispersed across a wider range of fluorescence intensities, producing a broader single peak of SR^+ cells, than whole blood samples. $CD235a^+$ erythrocytes stained with SR and produced broader and less intense peaks of activity. Similar to whole blood samples, the distribution of CD45 (and presence of a single or double (consisting of $CD45^{BRIGHT}$ and $CD45^{REG}$ cells) $CD45^+$ populations) in MNC depended on whether significant $CD66b^+$ granulocytic populations were present (Figure 5.5).

Day 2 MNC

SytoRed staining of day 2 MNC was less clean and spread across a wider range of fluorescence compared to day 0 MNC. A lack of distinction between SR^+ and SR^- populations was frequently observed, making it more difficult to isolate positive cells. CD45 staining was also seen to broaden and become less distinct during CFU-EPC culture but it remained easier to isolate $CD45^+$ from $CD45^-$ events (Figure 5.5).

5.2.7.2. Proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes

The proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes gated using either CD45 or SR to identify nucleated cells was determined in 28 patient samples (15 whole blood samples, 6 day 0 MNC, 7 day 2 MNC). Analysis of the proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes contained in whole blood and MNC samples from non-mobilised and mobilised patients showed no significant differences between SR gated and CD45 gated events (p=ns for all comparisons) (Table 5.7).

Non-mobilised samples displayed tighter agreements in the proportions leukocyte subpopulations identified using SR or CD45. On review, it was apparent this was the result of difficulties in setting a SR gate in the presence of significant numbers of $CD235a^+$ erythrocytes which blurred the distinction between SR^+ and SR^- gates (Table 5.7).

G-CSF	Sample	Whole Blood		Day 0 MNC		Day 2 MNC	
	Antibody	SR	CD45	SR	CD45	SR	CD45
Non-mobilised	SytoRed/CD45	96.2%	94.9%	59.1%	57.9%	68.6%	66.9%
	$CD66b^+$ Granulocytes	52.8%	52.3%	0.8%	0.9%	1.0%	0.8%
	$CD14^+$ Monocytes	4.9%	4.8%	6.6%	7.1%	6.4%	6.4%
	Lymphocytes	19.8%	19.4%	50.4%	48.0%	—	—
	$CD235a^+$ Erythrocytes	—	—	23.6%	24.9%	—	—
G-CSF Mobilised	SytoRed/CD45	95.4%	95.5%	65.1%	57.9%	89.8%	90.1%
	$CD66b^+$ Granulocytes	73.6%	71.5%	27.0%	22.6%	68.0%	68.7%
	$CD14^+$ Monocytes	11.5%	11.4%	18.1%	19.0%	4.0%	5.0%
	Lymphocytes	11.6%	13.3%	25.5%	26.4%	—	—
	$CD235a^+$ Erythrocytes	—	—	36.1%	36.5%	—	—

Table 5.7 SytoRed versus CD45 gating of leukocyte subpopulations and erythrocytes. Non-mobilised and mobilised whole blood and day 0 MNC samples gated using SytoRed (SR) and CD45. Figures are expressed as mean proportions of all events.

5.2.7.3. Conclusions

The use of SR did not alter the proportions of leukocyte subpopulations detected. Some of the minor differences in results between CD45 and SR gated cells could be attributed to difficulties in setting the SR gate in the presence of significant proportions of $CD235a^+$ erythrocytes. As the morphology of day 0 and day 2 MNC was not assessed, we do not know whether any SR^+ but $CD45^-$ $CD235a^+$ erythrocytes represented populations of nucleated erythrocytes or immature erythrocytes containing mitochondria and greater amounts of RNA. When SR was used in these situations it was difficult to cleanly differentiate between SR^+ and SR^- events and establish the SR gate. Technical issues overshadowed the use of SR. Difficulties were caused by deterioration of the SR working solution over time and the intermittent nature of PBSC donor samples. This resulted in variable intensity of fluorescence of SR^+ events. Intermittent difficulties with the FL4 laser on the flow cytometer, resulting in failure of detection of FL4 fluorescence (including SytoRed59) occurred. If this laser problem was not noted during flow cytometry invalid results were produced for the sample and data was lost. For the remainder of this work the use of CD45 in the antibody staining panels was therefore used in preference to SR staining.

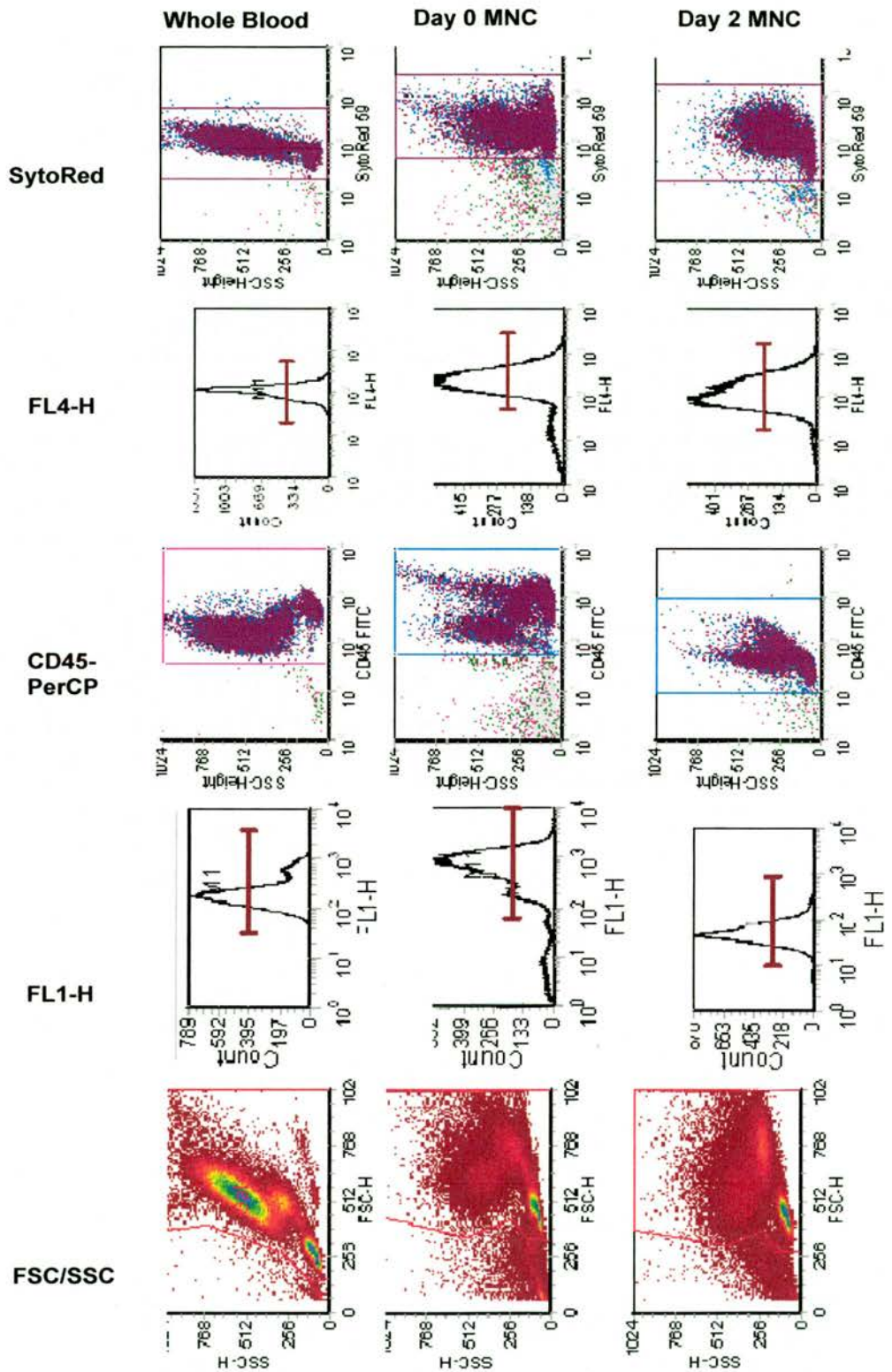


Figure 5.5 Changes in CD45 and SR gating during CFU-EPC culture.
Differences in whole blood, day 0 MNC and day 2 MNC gating of a G-CSF mobilised patient sample (patient 232).

5.2.8. Conclusions

A number of published studies reporting CFU-EPC suggested that in agreement with work within our group (136), CFU-EPC formation in normal (non-G-CSF treated) patients is $CD14^+$ monocyte dependent (129, 132, 137, 140). Additionally there is some evidence that T cells might have important roles in CFU-EPC (135, 137, 142) and that their interactions with $CD14^+$ monocytes could be important in initiation of CFU-EPC. CFU-EPC are heterogeneous in their cell composition and it would therefore be reasonable to suggest that changes in the proportions of these and other leukocyte subpopulations would affect CFU-EPC. The phenotypic analysis of the changes in cell proportions occurring in whole blood and MNC following G-CSF administration presented in section 5.2, and summarised in the table below (Table 5.8), indicated that that the reduction of CFU-EPC following the G-CSF administration cannot be attributed to reductions in the proportions of $CD14^+$ monocytes in day 0 or day 2 mobilised MNC (Figure 5.4).

Allogeneic PBSC donors			
	Whole Blood	Day 0 MNC	Day 2 MNC
$CD66b^+$ Granulocytes	↑*	↑*	↑*
$CD14^+$ Monocytes	↑*	↑*	↑
Lymphocytes	↓	↓*	↓*
$CD235a^+$ Erythrocytes	—	↔	↔

Autologous PBSC patients			
	Whole Blood	Day 0 MNC	Day 2 MNC
$CD66b^+$ Granulocytes	↔	↑*	↑*
$CD14^+$ Monocytes	↑*	↔	↔
Lymphocytes	↔	↓*	↓*
$CD235a^+$ Erythrocytes	—	↔	↔

Table 5.8 Changes in the proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes following G-CSF PBSC mobilisation.
Figures express the changes in the proportions of all events between non-mobilised and mobilised whole blood, day 0 MNC and day 2 MNC.
Allogeneic PBSC donors (upper diagram) and autologous PBSC patients (lower diagram).

↑ = increased, ↓ = decreased, ↔ = no change
* = statistically significant difference between non-mobilised and mobilised samples ($p<0.05$)

Whole blood samples from allogeneic and autologous PBSC donors contained significantly greater proportions of $CD14^+$ monocytes compared to non-mobilised samples. When $CD14^+$ monocytes were considered as proportions of all events, day 0 mobilised MNC from allogeneic PBSC donors contained significantly greater proportions of $CD14^+$ monocytes than non-mobilised MNC but this difference was not maintained on analysis of day 2 mobilised MNC. There were no significant differences in the proportions of $CD14^+$ monocytes in day 0 or day 2 MNC following G-CSF PBSC mobilisation of autologous PBSC patients. On account of the presence of considerable proportions of $CD235a^+$ erythrocytes in MNC from autologous patients, particularly in mobilised samples, $CD14^+$ monocytes as proportions of $CD45^+$ leukocytes were increased in mobilised autologous MNC compared to non-mobilised samples. The lack of dependence of CFU-EPC in mobilised MNC on $CD14^+$ monocytes was suggested by failure of plastic adherence to enhance CFU-EPC in mobilised MNC as it did for normal blood and UCB (136).

MNC samples from autologous PBSC patients contained significantly greater proportions of $CD235a^+$ erythrocytes than non-mobilised or G-CSF mobilised samples obtained from allogeneic PBSC donors (Figure 5.4). There were no changes in the proportions of $CD235a^+$ erythrocytes between non-mobilised and mobilised MNC for either allogeneic or autologous PBSC donors, the proposition that the $CD235a^+$ erythrocyte content alone was not responsible for the reduction in CFU-EPC following G-CSF administration.

The lymphocyte content of whole blood samples declined following G-CSF. The decline in whole blood lymphocyte count was minimal in autologous PBSC patients, largely due to the presence of higher proportions of $CD14^+$ monocytes and $CD66b^+$ granulocytes in non-mobilised samples from autologous PBSC patients, possibly the result of recent chemotherapy and recovery of cell counts from their nadir increasing the relative proportions of $CD66b^+$ granulocytes and $CD14^+$ monocytes. Alternatively, lymphocyte counts may have been reduced secondary to

chemotherapy or immunotherapy administrated to these haematology patients. There were clear reductions in MNC lymphocyte content following G-CSF administration to allogeneic PBSC donors but less marked reductions in autologous PBSC patient samples. Between day 0 and day 2 of CFU-EPC culture a moderate increase in MNC lymphocyte content of pre G-CSF samples (allogeneic and autologous PBSC donors) was observed but less change in lymphocytes occurred between day 0 and 2 in mobilised samples (Figure 5.4), perhaps due to lack of dilution on account of lower proportions of fibronectin adherent leukocytes.

The changes in the $CD66b^+$ granulocyte populations were the most significant (and consistent) change noted between pre G-CSF and mobilised samples. G-CSF mobilised samples from allogeneic and autologous PBSC donors contained elevated levels of $CD66b^+$ granulocytes compared to non-mobilised samples. The magnitude of the $CD66b^+$ granulocyte population was not altered by replating non-adherent MNC after 2 days of culture (Figure 5.4). Hence day 2 MNC from mobilised samples also contained high proportions of $CD66b^+$ granulocytes. The actions of G-CSF to increase myeloid proliferation and stimulate the release of enzymes and other factors from neutrophils means that it is likely that G-CSF-stimulated granulocytes within MNC continue to be metabolically active. Through the continued release of enzymes from granulocytes, these cells could affect cell behaviour and cell interactions leading to a reduction in CFU-EPC.

No link between the monocyte content of MNC preparations (expressed as absolute monocytes or expressed proportionally to lymphocytes and granulocytes) and the potential for CFU-EPC formation in mobilised samples was identified. This means that, although apparently essential for CFU-EPC development, monocytes are affected by the presence of other cells or soluble factors in cell culture. The wide variation in CFU-EPC formation observed in normal donors and patients made correlating CFU-EPC with cell numbers difficult, especially when some non-mobilised samples failed to generate CFU-EPC. No correlation was found

between changes in proportions of day 0 or day 2 MNC lymphocytes or $CD66b^+$ granulocytes, for although lymphocytes decreased and $CD66b^+$ granulocytes increased in mobilised samples, the inter-individual variations in CFU-EPC and lack of CFU-EPC development in significant proportions of non mobilised samples made comparisons difficult.

The most marked change in cell content of MNC post G-CSF was the increase in granulocytes in MNC preparations, which led to speculation that these cells were significant in producing the 'G-CSF effect' on CFU-EPC. It was proposed that the increased granulocytic content of mobilised samples, rather than altered $CD14^+$ monocyte levels, influenced CFU-EPC. This might be a result of the granulocytic content alone, direct or indirect effects of granulocytes on other cells (monocytes) through cellular factors or secreted factors (paracrine activity). Subsequent experiments, selectively enriching and depleting MNC for $CD66b^+$ granulocytes, explored the role of granulocytes in the reduction of CFU-EPC in mobilised samples.

5.3. Changes in T cells and putative angiogenic T cells following PBSC mobilisation with G-CSF or G-CSF+AMD3100

5.3.1. Introduction

A number of publications have commented on the heterogeneity of the cells identified within CFU-EPC and have highlighted the presence of monocytes and T cells in colonies (135, 137) with one group (142) reporting on the importance of a subpopulation of lymphocytes, so-called 'angiogenic' T cells, to CFU-EPC. The changes in proportions of $CD3^+$ T cells and angiogenic T cells, defined by the phenotype $CD3^+/31^+/CXCR4^+$ (142), following PBSC mobilisation with G-CSF are presented in section 5.4, with the decline in proportions of PB and MNC lymphocytes following PBSC mobilisation with G-CSF^+/-chemotherapy or G-CSF+AMD3100 presented earlier (section 5.2). Whole blood samples were stained with fluorochrome conjugated monoclonal antibodies and treated with FACS lyse prior to flow cytometric analysis. Lymphocytes were identified as low forward and side scatter $CD45^+$ cells within the live cell gate not expressing CD66b, CD14 or CD235a. Angiogenic T cells were low forward and side scatter events within the live cell gate (i.e. displaying light scatter characteristics of lymphocytes) that expressed CD45 together with CD3, CD31 and CXCR4. Data was presented as proportions of all events and proportions of $CD3^+$ T cells. For the data presented in this section, events identified as $CD66b^+$ granulocytes, $CD14^+$ monocytes and lymphocytes together accounted for greater than 98% of $CD45^+$ events. The proportions of angiogenic T cells were determined in whole blood samples. Based on the relative lymphocyte content of peripheral blood and MNC samples, these results were extrapolated to day 0 MNC.

5.3.2. Changes occurring following G-CSF^+/-chemotherapy

Whole blood angiogenic T cells were evaluated in 27 samples obtained from 17 PBSC patients (2 allogeneic donors and 15 autologous patients) undergoing PBSC

mobilisation with G-CSF, either given alone or sequentially with chemotherapy. The results of five samples were excluded from review (22 evaluable samples); due to spill of fluorescence from FL2 into FL1 and FL3 (an error in CXCR4 staining due the use of an incorrect fluorochrome-conjugated antibody) (n=4) and too few cells being present for analysis (n=1).

5.3.2.1. Non-mobilised (pre G-CSF or donor follow-up) PBSC samples

Analysis of the proportions of T cells and angiogenic T cells in non-mobilised PBSC samples (n=12, 10 pre G-CSF and 2 donor follow-up samples) showed that 14.4% of events (65.9% of lymphocytes) were $CD3^+$ T cells and that 23.0% of these were angiogenic T cells (3.2% of all events).

5.3.2.2. G-CSF mobilised PBSC samples

Analysis of the proportions of T cells and angiogenic T cells in $G\text{-}CSF^+$/-chemotherapy mobilised PBSC samples (n=10) showed that 8.6% of events (62.4% of lymphocytes) $CD3^+$ T cells and that 21.5% of these were angiogenic T cells (1.8% of all events).

5.3.2.3. Comparison of non-mobilised and G-CSF mobilised samples

Angiogenic T cell numbers (as proportions of $CD45^+$ events) declined significantly following PBSC mobilisation with $G\text{-}CSF^+$/-chemotherapy (3.2% and 1.8% in non-mobilised and mobilised samples respectively, $p<0.01$) (Figure 5.6). This can be attributed to the changes in the relative proportions of leukocyte subpopulations following G-CSF with significant decreases in lymphocytes (20.7% and 13.1% of whole blood leukocytes in non-mobilised and mobilised PB respectively, $p<0.05$) and $CD3^+$ T cells (14.4% and 8.6% respectively, $p<0.01$) observed (Table 5.9). When angiogenic T cells were expressed as proportions of $CD3^+$ T cells, no difference existed between non-mobilised and G-CSF mobilised patient samples (23.0% and 21.5% respectively, p=ns) (Figure 5.7). The expression of CD45, together with the proportions of $CD3^+$ T cells, angiogenic T cells and the expression of CD31 and CXC4 by $CD3^+$ T cells are displayed in Table 5.9.

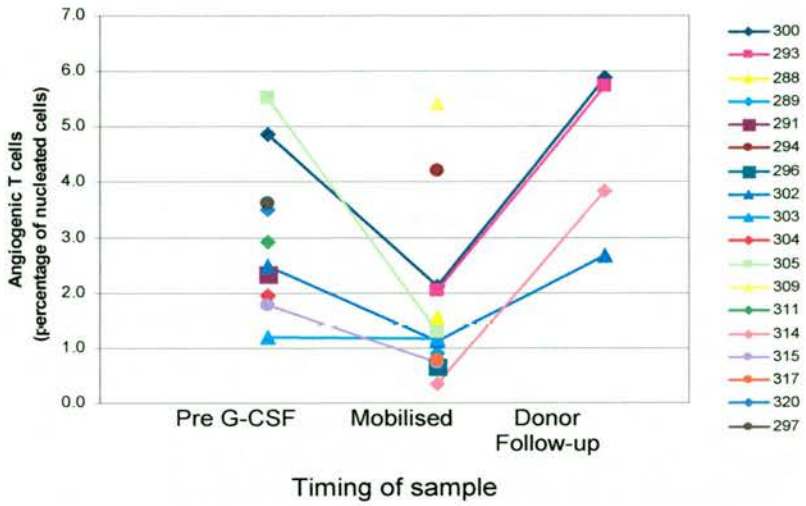


Figure 5.6 Changes in angiogenic T cells (as % of leukocytes) following G-CSF.
Samples obtained from PBSC patients pre G-CSF, after mobilisation and at donor follow-up.

G-CSF	$CD3^+$ T cells (% all events)	% $CD3^+$ T cells		Angiogenic T cells	
		$\%CD31^+$	$\%CXCR4^+$	% all events	% T cells
Non-mobilised	14.4	28.9	71.2	3.2	23.0
Mobilised	8.6	30.8	59.1	1.8	21.5
p value	<0.01	ns	ns	<0.01	ns

Table 5.9 Changes in angiogenic T cells and $CD3^+$ T cells post $G\text{-}CSF^+$/-chemotherapy.
Angiogenic T cells, $CD3^+$ T cells and T cell expression of CD31 and CXCR4 in non-mobilised and mobilised whole blood samples obtained from patients receiving $G\text{-}CSF^+$/-chemotherapy for PBSC mobilisation.

p value = comparison between non-mobilised and mobilised samples, Mann-Whitney test.
ns = non significant difference between the analysed populations.

5.3.2.4. Extrapolation to day 0 MNC

The proportions of $CD45^+$ events and lymphocytes in whole blood and day 0 MNC were used to extrapolate the proportions of PB $CD3^+$ T cells and PB angiogenic T cells to day 0 MNC in a subset of patients (non-mobilised n=10, mobilised n=8). Using these figures, non-mobilised day 0 MNC contained 31.8% $CD3^+$ T cells and 5.9% angiogenic T cells (9.7% of $CD45^+$ leukocytes). The proportions of day 0 MNC $CD3^+$ T cells and angiogenic T cells both declined in mobilised MNC (25.9% and 3.6% respectively, 5.1% of $CD45^+$ leukocytes).

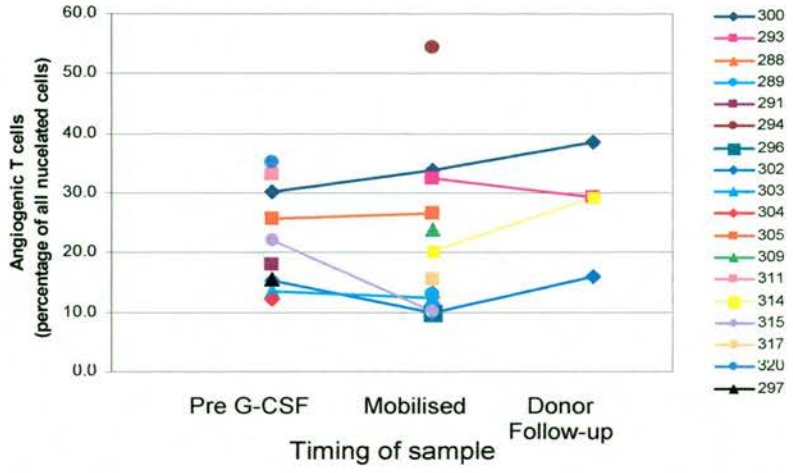


Figure 5.7 Changes in angiogenic T cells (as % of $CD3^+$ T cells) following G-CSF. Lack of difference between samples obtained from PBSC patients pre G-CSF, after G-CSF mobilisation and at donor follow-up.

5.3.3. Changes occurring following G-CSF+AMD3100

Whole blood angiogenic T cells were measured in 12 samples obtained from 6 patients undergoing PBSC mobilisation with G-CSF+AMD3100 (5 pre G-CSF, 5 G-CSF+AMD3100 mobilised and 2 donor follow-up samples) using identical methods and phenotypic definitions as presented in section 5.4.1.

5.3.3.1. Non-mobilised samples

Analysis of the proportions of angiogenic T cells in non-mobilised samples showed that 21.2% of $CD3^+$ T cells were angiogenic T cells (1.8% of all events) (Table 5.9). The proportion of $CD3^+$ T cells that were identified as angiogenic T cells was comparable to the results obtained from the non-mobilised samples from patients undergoing PBSC mobilisation with G-CSF+/-chemotherapy (section 5.4.2) (23.0%, p=ns) but non-mobilised samples from G-CSF+AMD3100 patients contained fewer $CD3^+$ T cells than non-mobilised samples from G-CSF patients (3.2%, $p<0.05$).

5.3.3.2. G-CSF+AMD3100 mobilised samples

Analysis of the proportions of angiogenic T cells in G-CSF+AMD3100 mobilised PBSC samples showed that 2.7% of $CD3^+$ T cells were angiogenic T cells (0.1% of all events) (Table 5.10). This was significantly different from angiogenic T cells as

proportions of all events (1.8%, p<0.005) and CD3+ T cells (21.2%, p<0.01) in non-mobilised G-CSF+AMD3100 PBSC patients. The changes in proportions of angiogenic T cells following G-CSF+AMD3100 PBSC mobilisation (expressed as proportions of leukocytes and proportions of CD3+ T cells) are illustrated in Figures 5.8 and 5.9 respectively. The decline in angiogenic T cells was disproportionately greater than the decline in lymphocytes and T cells in G-CSF+AMD3100 mobilised samples.

G-CSF+ AMD3100	$CD3^+$ T cells (% all events)	% $CD3^+$ T cells		Angiogenic T cells	
		$CD31^+$	$CXCR4^+$	% all events	% T cells
Non-mobilised	9.3	32.5	66.9	1.8	21.2
Mobilised	4.8	37.9	7.0	0.1	2.7
p value	<0.02	ns	<0.005	<0.005	<0.01

Table 5.10 Changes in angiogenic T cells and $CD3^+$ T cells following G-CSF+AMD3100. Angiogenic T cells, $CD3^+$ T cells and T cell expression of CD31 and CXCR4 in non-mobilised and mobilised whole blood samples obtained from patients receiving G-CSF+AMD3100 for PBSC mobilisation.

p value= comparison between non-mobilised and mobilised samples, Mann-Whitney test.
ns = non significant difference between the analysed populations.

5.3.3.3. Expression of CD31 and CXCR4 on $CD3^+$ T cells

CD31 expression

The CD3+ T cell-expression of CD31 in non-mobilised G-CSF+AMD3100 patients was equivalent to that of non-mobilised G-CSF patient samples (p=ns). Similarly, the expression of CD31 by CD3+ T cells in G-CSF+AMD3100 mobilised samples (37.9%) did not differ from that of non-mobilised G-CSF+AMD3100 patient samples (32.5%, p=ns) or non-mobilised G-CSF patient samples (28.9%) (p=ns) (Table 5.9).

CXCR4 expression

The CD3+ T cell-expression of CXC4 in non-mobilised G-CSF+AMD3100 patients was equivalent to that of non-mobilised G-CSF patient samples (p=ns). The expression of CXCR4 by CD3+ T cells was reduced in G-CSF+AMD3100 mobilised PBSC samples (7.0%) compared to G-CSF mobilised PBSC samples (59.1%, p<0.001) and to both non-mobilised G-CSF+AMD3100 (66.9%, p<0.05) and non-mobilised

G-CSF (71.2%, $p<0.005$ patient samples (Table 5.9 and Table 5.10). Expression of CXCR4 was reduced on $CD3^+$ T cells/lymphocytes from G-CSF+AMD3100 mobilised samples and was also reduced on granulocytes, consistent with the known mechanism of action of AMD3100 as a reversible inhibitor of CXCR4, the SDF-1α receptor.

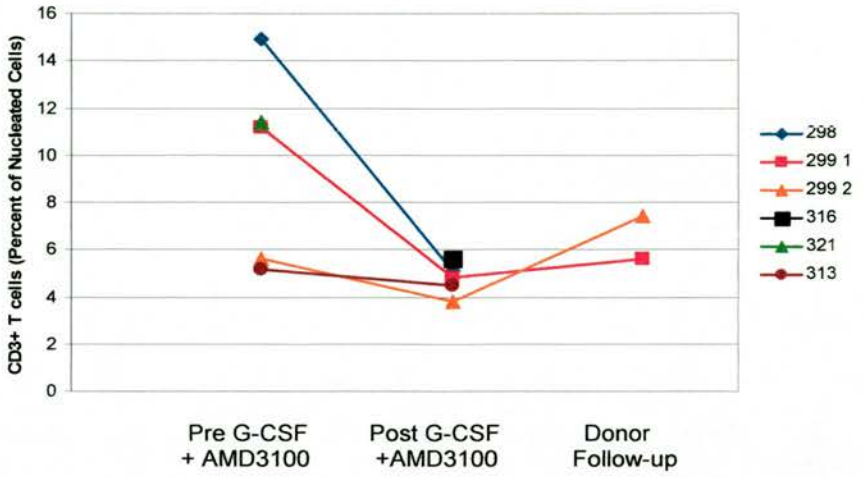


Figure 5.8 Changes in angiogenic T cells (as % leukocytes) following G-CSF+AMD3100.

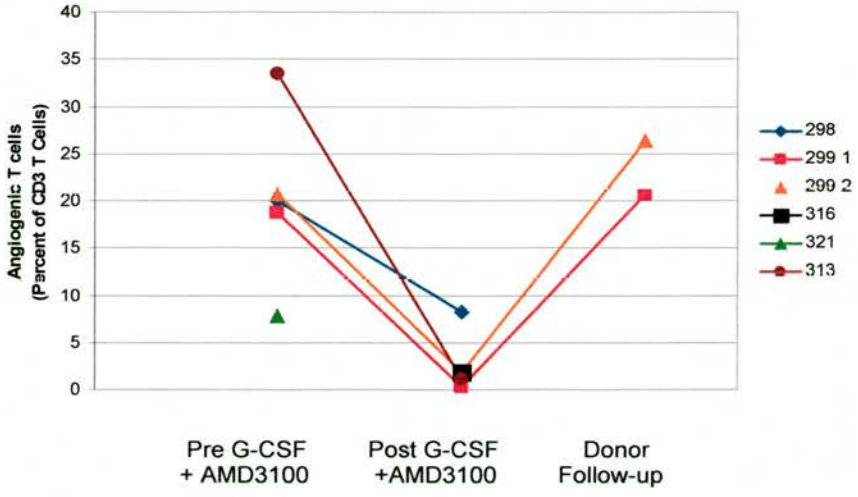


Figure 5.9 Changes angiogenic T cells (as % $CD3^+$ T cells) following G-CSF+AMD3100.

5.3.3.4. Angiogenic T cells in day 0 MNC

The proportions of $CD45^+$ events and lymphocytes in whole blood and day 0 MNC were used to extrapolate the proportions of PB $CD3^+$ T cells and angiogenic T cells

to day 0 MNC in a subset of patients (non-mobilised n=4, mobilised n=4). Using these figures, non-mobilised MNC contained 27.0% $CD3^+$ T cells and 4.7% angiogenic T cells (7.5% of $CD45^+$ leukocytes) with $CD3^+$ T cells (16.8%) and angiogenic T cells (0.6%, 1.0% of $CD45^+$ leukocytes) declining in mobilised MNC.

5.3.4. Conclusions

PBSC mobilisation with G-CSF^+/-chemotherapy was accompanied by a reduction in the proportions of lymphocytes, $CD3^+$ T cells and PB angiogenic T cells. However, when angiogenic T cells were considered as proportions of $CD3^+$ T cells, there was no difference in the proportions of angiogenic T cells between G-CSF mobilised and non-mobilised samples. The data presented earlier (sections 5.2.1-5.2.4) showed that the rise in the granulocytic content of blood samples following PBSC mobilisation with G-CSF was consistently accompanied by a reduction in the proportions of lymphocytes in these samples. It would seem that the decrease in angiogenic T cells in G-CSF mobilised PBSC samples paralleled these changes in leukocyte subpopulations. Extrapolation of data to day 0 MNC showed that T cells and angiogenic T cells both declined in mobilised samples. Analysis of PB angiogenic T cells in G-CSF+AMD3100 mobilised PBSC patient samples showed a profound reduction in angiogenic T cells following G-CSF+AMD3100. Extrapolation of data to day 0 MNC showed similar reductions in $CD3^+$ T cells and angiogenic T cells in mobilised samples. In contrast to the reduction in angiogenic T cells following PBSC mobilisation with G-CSF^+/-chemotherapy, the fall in the proportions of angiogenic T cells was not secondary to a fall in the proportions of $CD3^+$ T cells alone. The expression of CD31 by $CD3^+$ T cells was unaltered following G-CSF+AMD3100 but the expression of CXCR4 by $CD3^+$ T cells was markedly reduced.

Reductions in the proportions of T cells following G-CSF may contribute to falls in CFU-EPC. It is difficult to attribute the reduction in CFU-EPC formation following G-CSF+/-chemotherapy or G-CSF+AMD3100 to the decline in angiogenic T cells alone as changes also occur in lymphocytes and $CD3^+$ T cells.

5.4. Modification of CFU-EPC by manipulation of the cell content of cultured cells; Polymorphprep density gradient centrifugation

MNC recovered following Histopaque density gradient centrifugation of G-CSF mobilised PB contained significant numbers of $CD66b^+$ granulocytes. In the next sections, the affects of manipulating the proportions of $CD66b^+$ granulocytes in MNC on CFU-EPC are presented. The affects of $CD66b^+$ granulocytes on CFU-EPC was investigated using 3 main techniques to alter the proportions of these cells plated into CFU-EPC culture;

1. alteration of the cell content of interface layers (granulocyte depletion and collection of separated granulocytes) by use of alternate density gradient cell centrifugation techniques (Polymorphprep) (section 5.4);
2. enrichment or depletion for $CD66b^+$ or $CD14^+$ day 0 MNC using immunomagnetic separation (section 5.5); and
3. depletion of MNC $CD66b^+$ granulocytes by cryopreservation (section 5.6).

5.4.1. Introduction

Whole blood samples underwent buoyant density gradient centrifugation using Polymorphprep (PMP) (Axis Shield, UK) using the method described in section 2.2.2. PMP separation was performed *in lieu* of or in addition to standard Histopaque (1.077g/mL; Sigma Diagnostics, UK) MNC separation. Two interface layers were obtained with PMP; an upper MNC (MNC-p) layer and a lower granulocyte-rich (PMN-p) layer. The MNC interface layer produced following Histopaque density gradient centrifugation of whole blood is referred to as MNC-s in this and subsequent sections.

Cell concentrations (cell numbers) of recovered cells were determined using a Coulter cell counter. Coulter cell counter settings were set to exclude erythrocytes (and smaller events) from the cell count. The cell content of each interface layer

collected (MNC-s, MNC-p, PMN-p) was determined by flow cytometry. Recovered cells were plated into a CFU-EPC assay (CFU-Hill or CFU-Hill(direct) assays). Where cell numbers permitted, mixing studies using different interface layers were performed and cells were then plated into CFU-Hill(direct) assays.

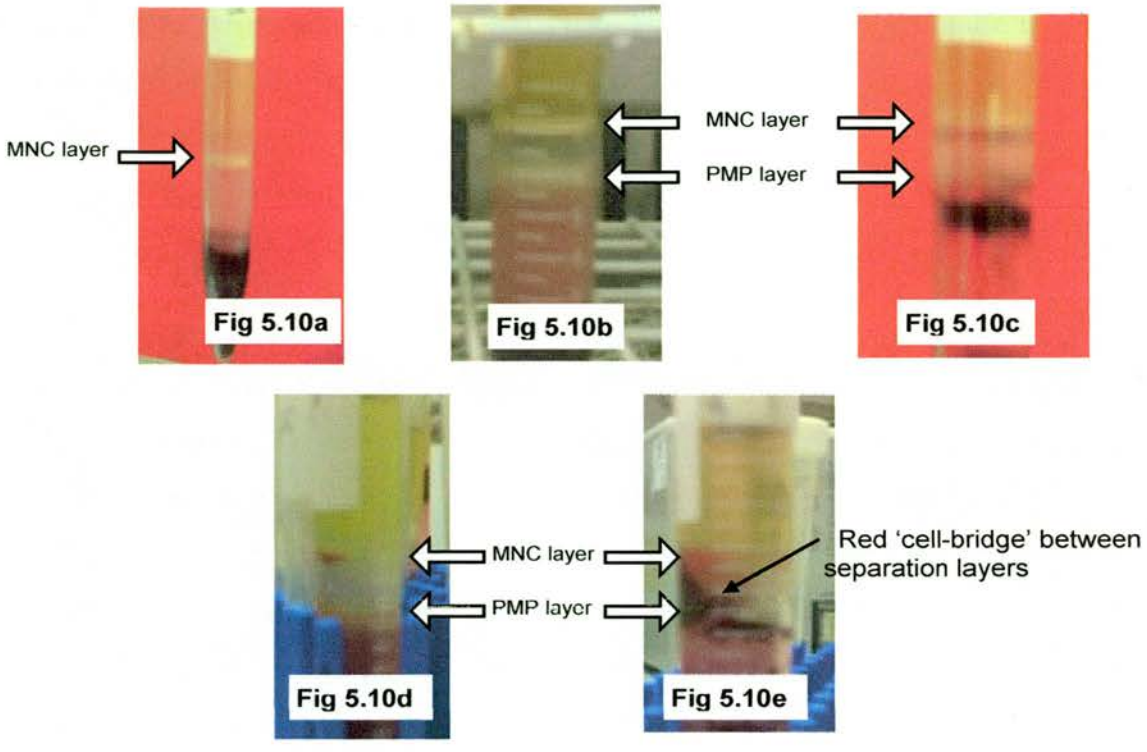


Figure 5.10 Cell interface layers after Histopaque or PMP density gradient centrifugation. MNC (and granulocyte) interface layers following density gradient centrifugation of non-mobilised and mobilised whole blood samples shown.

Fig 5.10a Histopaque MNC separation of a non-mobilised sample.
Fig 5.10b and 5.10c PMP separation of non-mobilised PBSC patient samples.
(allogeneic Fig 5.10b, autologous Fig 5.10c)
Fig 5.10d and 5.10e PMP separation of G-CSF mobilised samples.

5.4.1.1. Patient samples

Whole blood samples were obtained from 11 subjects (8 allogeneic PBSC donors, 3 autologous PBSC patients) on 3 occasions; pre G-CSF (n=10), after G-CSF mobilisation (n=3) and at donor follow-up (n=3). Two buffy coat samples from blood donors were obtained from the SNBTS were also used in these experiments.

5.4.2. Macroscopic findings

5.4.2.1. Histopaque density gradient centrifugation

MNC separations of whole blood samples from normal subjects in the absence of G-CSF (healthy volunteers and non-mobilised allogeneic PBSC donors) usually produced 'clean' cell separations with a white interface layer situated between plasma and Histopaque (Figure 5.10a, arrow). Macroscopically apparent erythrocyte contamination of the interface layer was frequently observed in MNC prepared from mobilised whole blood samples (and was also sometimes observed following recent chemotherapy without G-CSF (pre G-CSF samples from autologous PBSC patients)).

5.4.2.2. PMP density gradient centrifugation

Considerable erythrocyte contamination was observed when whole blood samples from normal subjects (non-mobilised allogeneic PBSC donors) underwent PMP separation. The degree of erythrocyte contamination of MNC-p was macroscopically much greater than the erythrocyte content of Histopaque-separated MNC-s (Figures 5.10b and 5.10c, arrows). Even when MNC-p appeared relatively erythrocyte-free, flow cytometric analysis confirmed the presence of considerably greater proportions of erythrocytes in MNC-p compared to MNC-s. Following G-CSF administration, the erythrocyte content of MNC-p and PMN-p increased. A red 'cell bridge', presumably with high red cell content, was frequently observed between the layers, making collection of two separate layers of interface cells more difficult (Figures 5.10d and 5.10e, arrows).

5.4.2.3. Buffy coat preparations from normal donors

PMP separations of buffy coat preparations were unsuccessful. PMP separations failed to produce distinct MNC-p or PMN-p layers. The bulk of erythrocytes remained suspended between the PMP and plasma layers following centrifugation and only a small proportion of erythrocytes passed through the gradient (Figure 5.11).

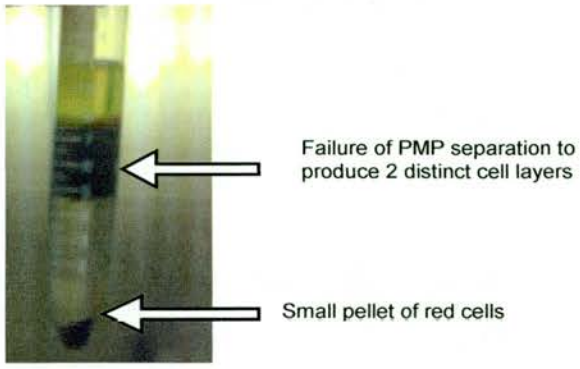


Figure 5.11 Failed Polymorphprep separation of buffy coat specimen.

Failure of PMP separation may have been a result of the high density of the original buffy coat preparations. Diluting the buffy coat (in PBS) prior to layering it on PMP might have overcome this. The manufacturers of PMP recommend that density gradient centrifugation is performed on samples less than four hours old. Buffy coat preparations were available for use as 'fresh' products. However, these were separated from whole blood donations received on the previous day and there was no possibility of obtaining a 'fresher' product. This is probably the main reason for the failure of PMP separation of buffy coat samples.

5.4.3. Flow cytometry analysis of MNC-s, MNC-p and PMN-p cells

Cell populations were defined phenotypically using identical definitions as used in section 5.1.2. A live cell gate was set using forward and side light scatter. Leukocytes were defined using a combination of their light scatter characteristics, expression of CD45 and expression of lineage-specific antigens ($CD66b^+$ granulocytes and $CD14^+$ monocytes). Erythrocyte lysis was not performed during antibody staining of MNC as the content of the cell suspension actually being plated into cell culture was being assessed. $CD235a^+$ erythrocytes were defined as low forward and side scatter $CD45^-$ events expressing CD235a but not CD66b or CD14. Lymphocytes were small (low FSC and SSC) $CD45^+$ events gating within the live cell

gate not expressing CD66b, CD14 or CD235a. Events that did not fall into one of these 4 defined populations (unclassified events) were excluded from the analysis.

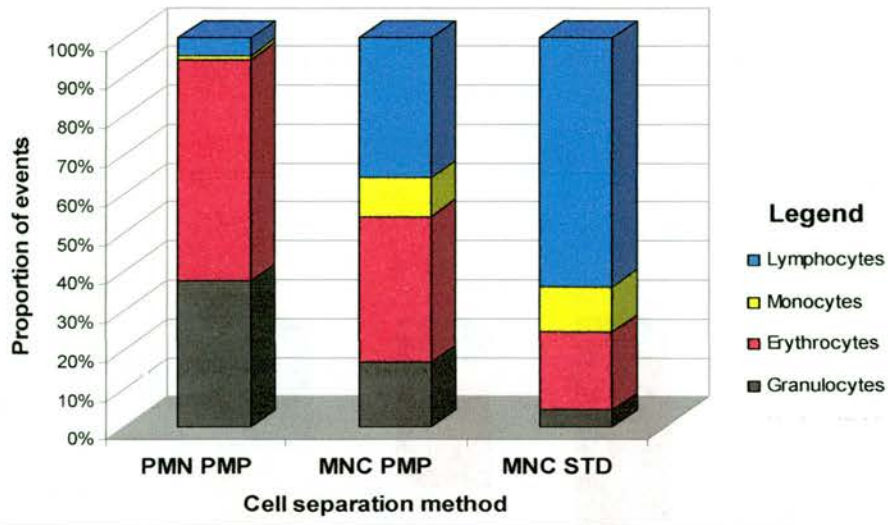
The cell content of MNC-s, MNC-p and PMN-p are presented as mean proportions of defined events (proportions of the sum of $CD66b^+$ granulocytes, $CD14^+$ monocytes, lymphocytes and $CD235a^+$ erythrocytes). Pre G-CSF and donor follow-up samples contained similar proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes and results from these samples are frequently combined into a single group of non-mobilised samples.

5.4.3.1. MNC (MNC-s and MNC-p) in non-mobilised samples

PMP cell separation of non-mobilised peripheral blood was inferior to Histopaque density gradient centrifugation. MNC-p contained substantially greater proportions of $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes and lower levels of lymphocytes than MNC-s. There was little difference in the $CD14^+$ monocyte content between the MNC preparations (Figure 5.12). The flow cytometry results of MNC-s (pre G-CSF samples from 3 allogeneic and 4 autologous PBSC patients) were comparable to the flow cytometry results presented in section 5.2.

5.4.3.2. PMN-p cells in non-mobilised samples

PMN-p cells were composed principally of $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes (>90% of all events) with very low levels of $CD14^+$ monocytes and lymphocytes present. PMP cell separation achieved a good separation between the interface layers in terms of $CD14^+$ monocyte and lymphocyte content but performed poorly with regard to $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes. The $CD235a^+$ erythrocyte content of MNC-p and PMN-p were considerably greater than the degree of $CD235a^+$ erythrocyte contamination expected with PMP according to the product information (Figure 5.12).



Cell Type	PMN-p	MNC-p	MNC-s
Number	10 (7 allo/3 auto)	5 (4 allo/1 auto)	7 (4 allo/3 auto)
$CD66b^+$ granulocytes	37.4%	16.5%	4.3%
$CD235a^+$ erythrocytes	57.0%	37.6%	20.1%
$CD14^+$ monocytes	1.2%	9.9%	11.8%
Lymphocytes	4.4%	36.0%	63.8%

Figure 5.12 Day 0 MNC content of non-mobilised MNC-s, MNC-p, PMN-p cells.
Pre or donor follow-up samples obtained from non-mobilised PBSC donor samples.

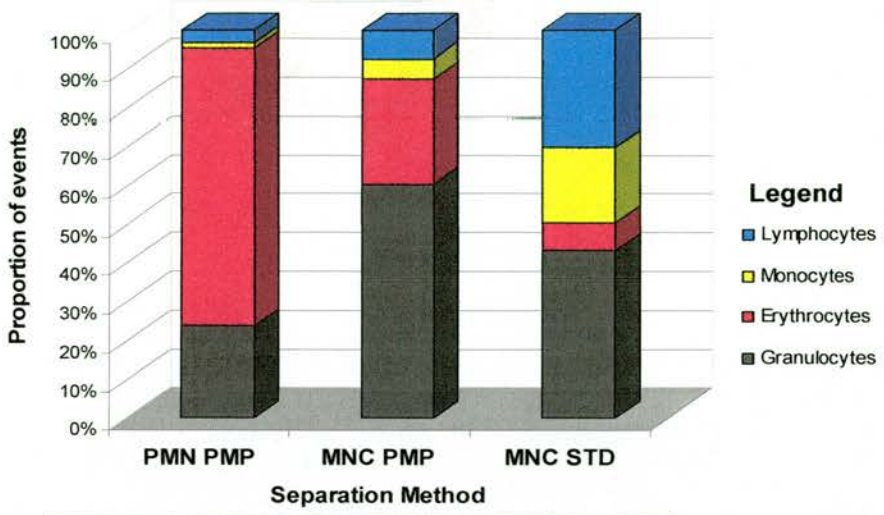
5.4.3.3. MNC (MNC-s and MNC-p) in mobilised samples

PMP separation of mobilised peripheral blood was inferior to Histopaque density gradient centrifugation. Similar to the results of non-mobilised samples (section 5.4.3.1) mobilised MNC-p contained greater proportions of $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes but contained substantially lower levels of $CD14^+$ monocytes and lymphocytes than mobilised MNC-s (Figure 5.13). Flow cytometry results of MNC-s (3 mobilised allogeneic PBSC donor samples) were comparable to the flow cytometry results presented in section 5.2.

5.4.3.4. PMN-p cells in mobilised samples

Similar to non-mobilised PMN-p, mobilised PMN-p were composed principally of $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes (>90% of all events) and very low levels of $CD14^+$ monocytes and lymphocytes were present. The increase in $CD235a^+$ erythrocytes in mobilised samples occurred at the expense of $CD66b^+$ granulocytes

(Figure 5.13). As seen in non-mobilised samples (section 5.4.3.2), PMP cell separation of mobilised samples achieved good separation between the interface layers in terms of their $CD14^+$ monocyte and lymphocyte content but performed poorly with regard to $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes.



Cell Type	PMN-p	MNC-p	MNC-s
Number	3	3	3
$CD66b^+$ granulocytes	23.8%	60.3%	43.1%
$CD235a^+$ erythrocytes	71.5%	27.2%	7.2%
$CD14^+$ monocytes	1.4%	5.1%	19.5%
Lymphocytes	3.3%	7.4%	30.2%

Figure 5.13 Day 0 MNC content of G-CSF mobilised MNC-s, MNC-p, PMN-p cells. Samples obtained from 3 G-CSF mobilised allogeneic PBSC donor samples.

5.4.4. CFU-EPC in PMN-p and MNC-p cells compared to MNC-s cells

MNC-s, MNC-p and PMN-p cells were enumerated and plated into either CFU-Hill or CFU-Hill(direct) assays. Comparisons were made between CFU-EPC obtained from each sample type.

5.4.4.1. CFU-EPC in PMN-p cells

PMN-p cells were counted and plated into CFU-EPC assays in identical fashion to MNC. Eight non-mobilised PMN-p (7 pre G-CSF samples and 1 donor follow-up

sample) and 4 mobilised PMN-p (all from 4 allogeneic PBSC donors) were plated into CFU-EPC culture. No CFU-EPC activity (0 CFU-EPC/10^6 cells plated) was recorded in any well containing PMN-p (mobilised or non-mobilised PMP-p).

5.4.4.2. CFU-EPC in MNC-p cells

Pre G-CSF samples from 5 allogeneic donors underwent PMP cell separation and the resulting MNC-p were plated into CFU-EPC assays. Despite the relatively high $CD66b^+$ granulocyte and $CD235a^+$ erythrocyte content of MNC-p, non-mobilised MNC-p showed CFU-EPC activity (mean 12.8 CFU-EPC/10^6 MNC, n=5). Mobilised MNC-p samples obtained from 5 allogeneic PBSC donors were plated into CFU-EPC assays. CFU-EPC activity was virtually absent in these samples (mean 1.4 CFU-EPC/10^6 MNC, n=5).

5.4.4.3. Changes in CFU-EPC activity in MNC-p cells following G-CSF

When the CFU-EPC in non-mobilised and mobilised MNC-p were assessed, these displayed the same (transient) decline in CFU-EPC formation following G-CSF exposure as MNC-s (Figure 5.14). The CFU-EPC activity of MNC-s from 3 allogeneic PBSC donors that had MNC separated by Histopaque and PMP are shown also in the figure (dotted lines) to provide comparison data. However, the MNC-p were not depleted of granulocytes (certainly not when compared to MNC-s) as had been expected, so this did not give information on the possible effect of granulocytes on reduced CFU-EPC following G-CSF PBSC mobilisation.

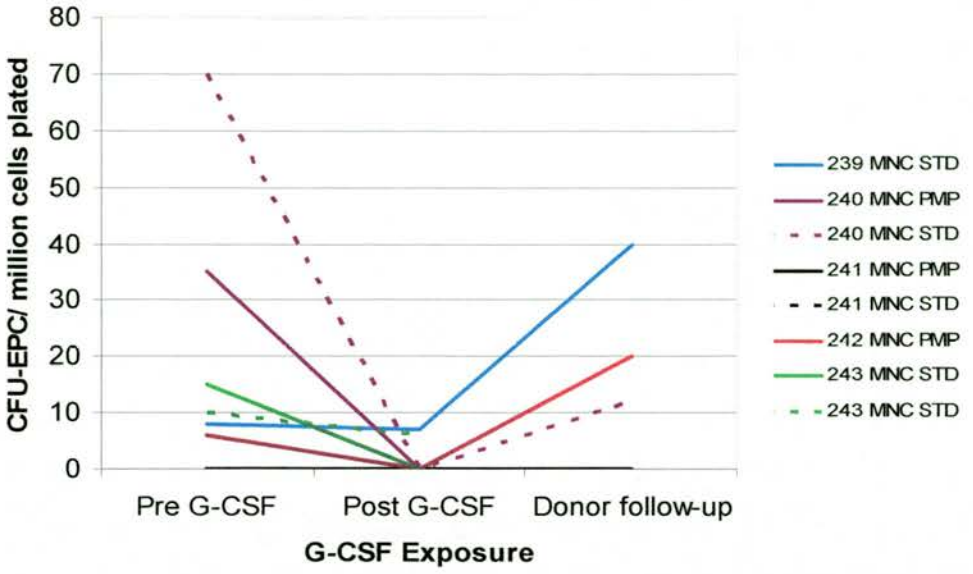


Figure 5.14 Changes in CFU-EPC frequency in MNC-p and MNC-s cells post G-CSF. Samples collected pre G-CSF, following G-CSF mobilisation and at donor follow-up.

5.4.5. Co-plating of cells in CFU-EPC assays; direct mixing or transwell experiments

5.4.5.1. Introduction

A series of co-plating experiments were carried out in order to determine whether changes in CFU-EPC activity following G-CSF could be attributed to altered proportions of granulocytes. The experiments were also to assess whether the 'G-CSF effect' on CFU-EPC formation was due to the presence of contaminating cells (granulocytes) or restricted to the presence of 'mobilised granulocytes (or other cell populations). Non-mobilised patient samples were used in most of these co-plating experiments as non-mobilised MNC had greater chances of generating CFU-EPC than mobilised MNC. One could therefore more readily see changes in CFU-EPC formation.

5.4.5.2. Cell separation

Whole blood samples underwent Histopaque and/or PMP density gradient centrifugation to obtain MNC and $CD66b^+$ granulocyte-rich cells. The flow cytometric analysis of PMP separated MNC-p and PMN-p was presented in section

5.4.3. The MNC-p and PMN-p used in these co-plating experiments contained very similar levels of $CD66b^+$ granulocytes, $CD14^+$ monocytes, lymphocytes and $CD235a^+$ erythrocytes to these cells.

MNC were depleted for $CD66b^+$ granulocytes by MACS microbead immunomagnetic separation using an indirect labelling technique. Aliquots of non-mobilised MNC were first stained with anti-CD66b-FITC monoclonal antibody and then labelled with anti-FITC microbeads, as outlined in section 2.2.4.2.

CD66b-depleted MNC were used in co-plating experiments. One of the issues concerning use of MACS microbead depleted MNC fractions is that although enriched MNC fractions consist of relatively pure cell populations, antigen depleted MNC contain mixtures of the remaining (antigen negative) cells as well as residual antigen positive cells. These antigen positive cells may not have stained with the antibody or with microbeads or were not removed by the magnetic columns.

5.4.5.3. Cell numbers used for CFU-EPC assays

When cell fractions were plated together into CFU-Hill assays, a total of $3x10^6$ cells were suspended in 1.5mL of CECM in a well of a 12 well fibronectin coated plate as day 0 MNC. 'Primary' cells were suspended in CECM and added to the well. Secondary cells were then added without delay and the well contents were mixed by gentle aspiration with a pipette. Following 2 days incubation, the non-adherent day 2 cells were collected and $1x10^6$ cells were transferred in 1mL CECM to 24 well fibronectin coated plates (day 2 MNC).

When cell fractions were plated together into CFU-Hill(direct) assays up to $1.5x10^6$ cells ($1.0\text{-}1.5x10^6$ cells) were suspended in 1mL of CECM and plated into a well of a 24 well fibronectin coated plate. Generally $1x10^6$ 'primary' cells were added to the well with CECM. The second cell fraction ($0.25x10^6$ or $0.5x10^6$ of secondary cells) was added without delay and the well contents were mixed by gentle aspiration with a pipette.

When secondary cells were added to the primary cells in a 24 well fibronectin coated plate in a ThinCert™ insert, $1x10^6$ primary cells suspended in 0.8mL CECM were first added to the well. The ThinCert™ insert was carefully placed over the well containing primary cells and then the chosen number ($0.25x10^6$ or $0.5x10^6$) of secondary cells suspended in 0.2mL of CECM were added to the insert.

Either $0.25x10^6$ or $0.5x10^6$ secondary cells were added to the primary cells. These ratios were chosen on the basis of the analysis of the proportions of $CD66b^+$ granulocytes in mobilised day 0 MNC, whereby mixing of $1x10^6$ MNC and $0.5x10^6$ PMN-p might result in $CD66b^+$ granulocyte content close to 30%.

5.4.5.4. Co-plating experiments using cell fractions obtained from the same donor

5.4.5.4.1. Co-plating MNC-p cells with PMN-p cells

Non-mobilised whole blood samples from 3 allogeneic PBSC donors underwent PMP separation to yield MNC-p and PMN-p fractions. In these experiments MNC-p were co-plated with PMN-p (Table 5.11).

CFU-EPC formation in MNC-p and PMN-p cells

When plated alone, 3 of 3 PMN-p failed to generate CFU-EPC (0 CFU-EPC/10^6 cells). Two of the 3 MNC-p generated CFU-EPC (Table 5.11).

MNC-p cells plus PMN-p cells

Addition of PMN-p to MNC-p resulted in clear reductions in CFU-EPC formation. This occurred when PMN-p were added directly to the MNC-p in the well or were separated from them using ThinCert™ inserts. No evidence of a dosing effect of PMN-p could be seen in these experiments (Table 5.11).

The sample that generated no CFU-EPC from MNC-p also failed to generate any colonies (0 CFU-EPC/10^6 cells) on the addition of PMN-p to the culture wells. Subsequent samples from this patient (mobilised and donor follow-up samples) also

failed to generate CFU-EPC from MNC-p or MNC-s. This suggested that patient factors rather than experimental or technical factors resulted in the lack of CFU-EPC formation in the sample obtained pre G-CSF mobilisation (Table 5.11).

5.4.5.4.2. Co-plating MNC-s cells with PMN-p cells

Non-mobilised whole blood samples from 4 PBSC donors (1 autologous and 3 allogeneic) underwent PMP separation to yield MNC-p and PMN-p fractions as well as Histopaque separation to yield MNC-s, which were co-plated with PMN-p (Table 5.12 and Table 5.13).

MNC-s were chosen for co-plating rather than MNC-p as MNC-s fractions contained lower proportions of $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes. This meant that when co-plating MNC with PMN-p the differences in the cell content between primary and secondary cells was maximised by using MNC-s (Table 5.12 and Table 5.13).

CFU-EPC formation in MNC-s cells and PMN-p cells

When plated alone, all (3 of 3) PMN-p failed to generate CFU-EPC (0 CFU-EPC/10^6 cells). Three of the 4 MNC-s samples generated CFU-EPC (Table 5.12 and Table 5.13).

MNC-s cells plus PMN-p cells

PMN-p were added directly to the culture wells containing MNC-s and were also added in ThinCert™ inserts so to remain separated from MNC-s. Addition of PMN-p to MNC-s resulted in clear reductions (or abolition) of CFU-EPC formation (assessed by either CFU-Hill or CFU-Hill(direct) assays). This change in CFU-EPC formation occurred when PMN-p were added to wells in a ThinCert™ insert or when PMN-p were mixed directly with MNC-s in the culture well (Table 5.12 and Table 5.13). The patient sample that failed to generate CFU-EPC from MNC-s also failed to generate CFU-EPC when MNC-s were co-plating with PMN-p.

MNC-s cells plus MNC-s cells

In 3 of the 4 experiments, additional MNC-s were introduced to the culture well in ThinCert™ inserts. The level of CFU-EPC activity generated in these wells was virtually identical to that observed when MNC-s were plated alone (Table 5.12 and Table 5.13). MNC-s from one patient failed to generate CFU-EPC. None of the co-plating experiments performed using this patient sample generated CFU-EPC.

5.4.5.4.3. Co-plating of mobilised MNC-s cells with CD66b-depleted MNC

Mobilised whole blood samples from 5 PBSC donors (4 autologous and 1 allogeneic) underwent Histopaque separation to yield MNC-s. An aliquot of each mobilised MNC-s then underwent CD66b depletion using MACS microbead separation (CD66b-depleted MNC) (Table 5.14) (section 2.4.2.2).

These experiments co-plated MNC-s together with CD66b-depleted MNC. Cell fractions were plated into wells using the same ratios of MNC to granulocyte-enriched fractions as described in section 5.5.5.3. In one case MNC-s were also plated together with CD66b-enriched MNC.

$CD66b^+$ granulocyte content of MNC-s and CD66b-depleted MNC

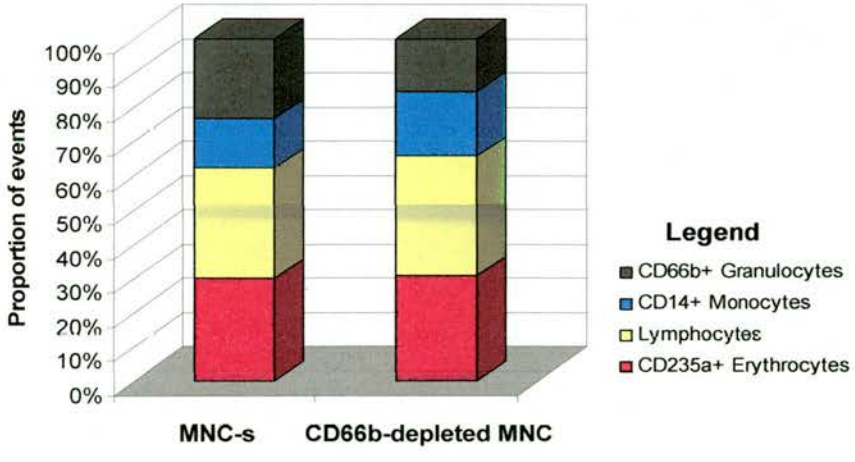
The change in $CD66b^+$ granulocyte content of CD66b-depleted MNC from the levels contained in MNC-s was variable and often surprisingly marginal. MNC-s contained 22.8% (8.2-33.1% of all events). Following CD66b depletion by MACS, the CD66b-deplete MNC contained an average 14.0% $CD66b^+$ granulocytes (range 7.63%-25.0%), a mean reduction of 8.8% (range 0.6%-24.5% of all events) and mean relative reduction of 38.6%. When proportions were corrected to take into account any populations of unclassified events, slight differences are noted in the values. The mean reduction in the proportions of $CD66b^+$ granulocytes was then 32.6% (Figure 5.15).

CFU-EPC activity of mobilised MNC-s cells

Four of 5 mobilised MNC-s failed to generate any CFU-EPC (0 CFU-EPC/10^6 MNC). One mobilised MNC-s showed 12 CFU-EPC/10^6 MNC. No non-mobilised samples were available for that patient (Table 5.14).

CFU-EPC activity of CD66b-depleted MNC

Two of 5 CD66b-depleted MNC formed CFU-EPC when cells were plated into CFU-Hill(direct) assays. Of note, CD66b-depleted cells that showed the greatest reduction in $CD66b^+$ granulocyte content from the levels contained in the mobilised MNC-s were the cells to show some CFU-EPC activity. Matched non-mobilised (all pre G-CSF) samples were available for 4 patients. All (4 of 4) non-mobilised MNC-s generated CFU-EPC. There appeared to be no correlation between the numbers of CFU-EPC from non-mobilised samples and CFU-EPC from mobilised CD66b-depleted MNC for these patients (Table 5.14).



Leukocyte subpopulation and erythrocyte content	Mean proportions of all events	
	MNC-s	CD66b depleted MNC
$CD66b^+$ Granulocytes	22.8% (23.3)	14.0% (15.7)
$CD14^+$ Monocytes	14.7% (15.0)	14.5% (16.3)
Lymphocytes	31.2% (31.8)	32.1% (36.1)
$CD235a^+$ Erythrocytes	29.3% (29.9)	28.3% (31.8)

Figure 5.15 Leukocyte subpopulations and erythrocytes in MNC-s and CD66b-depleted mobilised MNC.
Expressed as mean proportions of all events; proportions of $CD45^+$ cells in parentheses.

Mobilised MNC-s cells plus CD66b-depleted MNC

Co-plating MNC-s with CD66b-depleted MNC produced CFU-EPC activity in 2 cases. This included one sample that generated CFU-EPC when CD66b-depleted MNC were plated alone in CFU-EPC assay and one sample that did not (Table 5.14).

Mobilised MNC-s cells plus CD66b-enriched MNC

On one occasion MNC-s were also co-plated with the CD66b-enriched MNC. No CFU-EPC formation was recorded, as was seen when the fractions were plated alone into CFU-Hill(direct) assays (Table 5.14).

Details	CFU-EPC activity MNC-p or PMN-p			CFU-EPC activity MNC-p plus PMN-p			
Sample	240	240	240	240	240	240	240
Primary cells ($x10^6$)	1.0 MNC-p	0.25 PMN-p	0.5 PMN-p	1.0 MNC-p	1.0 MNC-p	1.0 MNC-p	1.0 MNC-p
Secondary cells ($x10^6$)	—	—	—	0.25 PMN-p	0.5 PMN-p	0.25 PMN-p	0.5 PMN-p
Well set-up	Standard	Standard	Standard	Standard	Standard	Transwell	Transwell
CFU-EPC per 10^6 cells	70	0	0	45	55	45	42

Details	CFU-EPC activity MNC-p or PMN-p			CFU-EPC activity MNC-p plus PMN-p			
Sample	241	241	241	241	241	241	241
Primary cells ($x10^6$)	1.0 MNC-p	0.25 PMN-p	0.5 PMN-p	1.0 MNC-p	1.0 MNC-p	1.0 MNC-p	1.0 MNC-p
Secondary cells ($x10^6$)	—	—	—	0.25 PMN-p	0.5 PMN-p	0.25 PMN-p	0.5 PMN-p
Well set-up	Standard	Standard	Standard	Standard	Standard	Transwell	Transwell
CFU-EPC per 10^6 cells	0	0	0	0	0	0	0

Details	CFU-EPC activity MNC-p			CFU-EPC activity MNC-p plus PMN-p			
Sample	242	242	242	242	242	242	242
Primary cells ($x10^6$)	3.0 MNC-p	3.0 MNC-p	1.0 MNC-p	1.0 MNC-p	1.0 MNC-p	1.0 MNC-p	1.0 MNC-p
Secondary cells ($x10^6$)	—	—	—	0.25 PMN-p	0.5 PMN-p	0.25 PMN-p	0.5 PMN-p
D0/D2 MNC	Day 2 NA 1.0 MNC-p	Day 2 Adherent	Day 0	Day 0	Day 0	Day 0	Day 0
Well set-up	Standard	Standard	Standard	Standard	Standard	Transwell	Transwell
CFU-EPC per 10^6 cells	6	0	0	0	0	0	0

Table 5.11 Results of co-plating non-mobilised MNC-p with PMN-p cells (section 5.4.5.4.1).

Details	CFU-EPC activity MNC-s			CFU-EPC activity MNC-s plus PMN-p or MNC-s		
Sample	288	288	288	288	288	288
Primary cells ($x10^6$)	3.0 MNC-s	3.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s
Secondary cells ($x10^6$)	—	—	—	0.5 MNC-s	0.5 PMN-p	0.5 PMN-p
D0/D2 MNC	Day 2 NA 1.0 MNC-s	Day 2 Adherent	Day 0	Day 0	Day 0	Day 0
Well set-up	Standard	Standard	Standard	Transwell	Transwell	Standard
CFU-EPC per 10^6 cells	5	0	3	4	0	0

Details	CFU-EPC activity MNC-s			CFU-EPC activity MNC-s plus PMN-p or MNC-s		
Sample	293	293	293	293	293	293
Primary cells ($x10^6$)	3.0 MNC-s	3.0 MNC-s	0.8 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s
Secondary cells ($x10^6$)	—	—	—	0.2 MNC-s	0.2 PMN-p	0.2 PMN-p
D0/D2 MNC	Day 2 NA 1.0 MNC-s	Day 2 Adherent	Day 0	Day 0	Day 0	Day 0
Well set-up	Standard	Standard	Standard	Transwell	Transwell	Standard
CFU-EPC per 10^6 cells	0	0	0	0	0	0

Details	CFU-EPC activity MNC-s			CFU-EPC activity MNC-s plus PMN-p or MNC-s				
Sample	297	297	297	297	297	297	297	297
Primary cells ($x10^6$)	3.0 MNC-s	3.0 MNC-s	1.0 MNC-s	2.0 MNC-s	2.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s
Secondary cells ($x10^6$)	—	—	—	1.0 MNC-p	1.0 MNC-p	0.5 PMN-p	0.5 PMN-p	0.5 MNC-s
D0/D2 MNC	Day 2 NA 1.0 MNC-s	Day 2 Adherent	Day 0	Day 2 NA 1.0 MNC-s	Day 2 Adherent	Day 0	Day 0	Day 0
Well set-up	Standard	Standard	Standard	Standard	Standard	Transwell	Transwell	Transwell
CFU-EPC per 10^6 cells	2	0	5	0	0	1	0	4

Table 5.12 Results of co-plating of non-mobilised MNC-s with PMN-p cells (section 5.6.5.4.2).

Details	CFU-EPC activity MNC-s		CFU-EPC activity MNC-s plus PMN-p or MNC-s			
Sample	294	294	294	294	294	294
Primary cells ($x10^9$)	3.0 MNC-s	3.0 MNC-s	2.0 MNC-s	2.0 MNC-s	1.0 MNC-s	1.0 MNC-s
Secondary cells ($x10^9$)			1.0 PMN-p	1.0 MNC-s	2.0 PMN-p	2.0 PMN-p
D0/D2 MNC	Day 2 NA 1.0 MNC-s	Day 2 Adherent	Day 2 NA 1.0 MNC Mixed	Day 2 Adherent	Day 2 NA 1 .0 Mixed	Day 2 Adherent
Well set-up	Standard	Standard	Standard	Standard	Standard	Standard
CFU-EPC per 10^6 cells	2	0	0	0	3	0

Table 5.13 Results of co-plating of non-mobilised MNC-s with PMN-p cells (section 5.4.5.4.2).

ID	Non-G-CSF MNC-s	Mobilised MNC-s				Mobilised CD66b-deplete MNC				Pre G-CSF	Mobilised Sample		
	$CD66b^+$ PMN	$CD66b^+$ PMN	$CD14^+$ Mono	Lymphs	$CD235a^+$ RBC	$CD66b^+$ PMN	$CD14^+$ Mono	Lymphs	$CD235a^+$ RBC	MNC-s	MNC-s	CD66b deplete MNC	MNC-s+ CD66b deplete MNC
287	3.4 (4.3)	8.2 (24.8)	2.8 (8.3)	22.1 (66.7)	63.9	7.6 (21.2)	4.3 (11.9)	22.5 (62.4)	61.9	3	0	0	0
291	3.6 (5.5)	30.0 (47.4)	13.5 (21.4)	22.9 (36.1)	33.2	25.0 (38.4)	17.5 (26.8)	22.7 (34.8)	30.9	3	0	0	0
274	0.2 (0.3)	33.1 (38.7)	28.0 (32.8)	31.0 (36.3)	11.0	8.6 (9.9)	29.0 (33.5)	50.6 (58.4)	11.6	24	0	3.8	0
316	ND	20.9 (23.7)	15.7 (17.8)	47.2 (53.6)	10.2	18.8 (20.7)	25.2 (27.7)	40.3 (44.4)	8.0	ND	12	30	15
315	0.6 (0.8)	21.9 (33.4)	10.4 (15.9)	32.8 (50.0)	28.3	10.2 (22.5)	11.5 (25.3)	24.4 (53.9)	29.4	1	0	0	2

Table 5.14 Results of co-plating mobilised MNC-s with mobilised CD66b-depleted MNC (section 5.4.5.4.3).

5.4.5.5. Co-plating experiments - cell fractions obtained from different patients (PBSC donor and non PBSC sources)

5.4.5.5.1. Co-plating non-mobilised donor MNC-p or PMN-p cells with buffy coat MNC-s cells

Non-mobilised (all pre G-CSF) samples from 3 allogeneic PBSC donors underwent PMP separation of whole blood to obtain donor MNC-p and donor PMN-p. Buffy coat cells underwent Histopaque density gradient centrifugation to yield MNC-s (BC MNC-s). Donor MNC-p or donor PMN-p were plated together with BC MNC-s into CFU-EPC assays. BC MNC-s were always plated into the culture wells as the primary cells. Due to concerns about the immune compatibility of samples, MNC-p were not mixed directly with BC MNC-s and were added to BC MNC-s in ThinCert™ inserts. Instead, due to the low lymphocyte count of PMN-p, these cells were either mixed directly with BC MNC-s or added in ThinCert™ inserts (Table 5.15).

CFU-EPC development in BC MNC-s cells

Of the 2 buffy coat samples used in these experiments, one failed to generate CFU-EPC when BC MNC-s were plated alone into a CFU-Hill(direct) assay. This was considered an aberrant result and was judged as a technical failure due to the use of an aged fibronectin coated plate in the CFU-EPC assay. BC MNC-s from this sample plated into other CFU-Hill(direct) assays and day 2 MNC from the second stage of CFU-Hill assay were plated into a fresh 24 well fibronectin coated plate (the same plate for all) and CFU-EPC formation was observed in these wells. The results of BC MNC-s plated into the CFU-Hill have been used as the figure for BC MNC-s CFU-EPC in experiments using this buffy coat sample (Table 5.15).

BC MNC-s from the other buffy coat sample generated CFU-EPC in the CFU-Hill and CFU-Hill(direct) assays.

Donor PMN-p cells plus BC MNC-s cells

The addition of donor PMN-p directly to BC MNC-s produced different effects on CFU-EPC formation than the addition of donor PMP-p to BC MNC-s in ThinCert™ well inserts.

The addition of donor PMN-p directly to BC MNC-s appeared to negatively affect the rate of CFU-EPC formation overall. Two of 3 experiments showed reduced CFU-EPC formation on the addition of donor PMN-p directly to a well containing BC MNC-s. CFU-EPC formation was reduced from BC MNC-s levels but it was not abolished. The remaining experiment showed no negative effects of the direct introduction of donor PMN-p on CFU-EPC formation (Table 5.15).

The rates of CFU-EPC formation increased from that observed when BC MNC-s were plated alone into culture wells (CFU-Hill or CFU-Hill(direct) assays) when donor PMN-p were added to BC MNC-s in ThinCert™ well inserts (3 of 3 cases) (Table 5.15).

Donor MNC-p cells plus BC MNC-s cells

The addition of donor MNC-p to BC MNC-s in ThinCert™ well inserts produced no demonstrable negative effect on CFU-EPC formation. In one case CFU-EPC formation substantially exceeded (doubled) that of BC MNC-s CFU-EPC. In the other 2 cases, the density of CFU-EPC in the wells containing donor MNC-p plus BC MNC-s was marginally higher than seen in wells containing BC MNC-s plated alone but lower than the CFU-EPC activity of donor PMN-p plus BC MNC-s (using ThinCert™ inserts) wells (Table 5.15).

5.4.5.5.2. Co-plating non-mobilised patient MNC-s or PMN-p cells with volunteer donor MNC-s cells

Two freshly collected whole blood samples were used in this co-plating experiment. This was to overcome any effect on CFU-EPC formation produced from using buffy coat MNC-s (which are 24 hours old when they are released for use) in experiments.

A non-mobilised (pre G-CSF) whole blood sample from an autologous PBSC patient underwent PMP separation to yield patient MNC-p and patient PMN-p as well as Histopaque separation to yield patient MNC-s. The same cell separations were performed on a whole blood sample from a volunteer (non PBSC donor) resulting in volunteer MNC-s, volunteer MNC-p and volunteer PMN-p. These co-plating experiments plated various combinations of patient MNC-s, volunteer MNC-s and patient PMN-p. Patient cells were added to volunteer MNC-s using ThinCert™ inserts. Cells were not mixed together in the culture wells (Table 5.16).

CFU-EPC activity of MNC-s or PMN-p cells

Both patient MNC-s and volunteer MNC-s generated CFU-EPC when MNC-s were plated alone into CFU-Hill(direct) assays. Volunteer MNC-s developed more colonies than patient cells (8 CFU-EPC/10^6 cells versus 2 CFU-EPC/10^6 cells respectively) perhaps a reflection of effects of chemotherapy or disease related factors in CFU-EPC in this patient (Table 5.16). Only patient (and not volunteer) PMN-p were plated into CFU-EPC assays. No CFU-EPC formation was observed in patient PMN-p.

Patient MNC-s cells with volunteer MNC-s cells

The addition of patient MNC-s to volunteer MNC-s increased CFU-EPC formation from that of volunteer MNC-s alone. Greater numbers of CFU-EPC were generated in the well that contained the higher concentration (and number) MNC. The level of CFU-EPC formation in these wells fell below that observed in a control well, where additional volunteer MNC-s (also using ThinCert™ inserts) were added to a well containing volunteer MNC-s (Table 5.16).

When considering the CFU-EPC formation in MNC-s, the level of CFU-EPC formation in the co-plated wells (patient MNC-s plus BC MNC-s) was in excess of the expected colony formation.

Patient PMN-p cells plus volunteer MNC-s cells

Similar to the previous co-plating experiments using BC MNC-s and donor PMP-p, CFU-EPC formation increased when patient PMN-p were added to volunteer MNC-s. The rate of CFU-EPC formation was above that observed in the wells containing patient MNC-s and volunteer MNC-s. No control wells, where volunteer PMN-p were plated with volunteer MNC-s, were performed (Table 5.16).

5.4.5.5.3. Co-plating of mobilised PBSC donor MNC-s with buffy coat MNC-s cells

A mobilised whole blood sample from an allogeneic PBSC donor underwent Histopaque density gradient centrifugation to yield donor MNC-s. Buffy coat cells from two different blood donors underwent Histopaque density gradient centrifugation to yield MNC-s in addition to undergoing PMP separation. PMP separation failed to work (section 5.4.2.3).

BC MNC-s were plated into the culture wells as the primary cells. Due to concerns about the immune compatibility of samples, donor or buffy coat cells were not directly mixed with BC MNC-s but were added to wells in ThinCert™ inserts (Table 5.17).

CFU-EPC formation in post G-CSF donor and BC MNC-s cells

BC MNC-s from both buffy coat specimens generated CFU-EPC from CFU-Hill(direct) assays. Both recorded 6 CFU-EPC/10^6 MNC. Mobilised donor MNC-s did not generate colonies (0 CFU-EPC/10^6 MNC) (Table 5.17).

Post G-CSF donor MNC-s cells plus BC MNC-s cells

Three of the four culture wells containing BC MNC-s and mobilised donor MNC-s recorded reductions in CFU-EPC formation from that of BC MNC-s plated alone. Mixed results were obtained for one buffy coat specimen that showed reduced CFU-EPC formation (compared to BC MNC-s plated alone) when low dose mobilised donor MNC-s were co-plated with BC MNC-s at and increased CFU-EPC

when greater numbers of donor MNC-s were co-plated with BC MNC-s. This result is not readily explainable (Table 5.17).

No control wells, where additional BC MNC-s were plated together with BC MNC-s or additional donor MNC-s were plated together with donor MNC-s were set up in this experiment. The use of mobilised MNC-s cells as primary cells, adding BC MNC-s in ThinCert™ inserts as secondary cells, may have provided some interesting additional information on the influence of non-mobilised cells on mobilised MNC.

Details	CFU-EPC activity MNC-s		BC MNC-s plus MNC-p or PMN-p					
Buffy Coat 1 cells ($x10^6$)	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s
Donor 240 cells ($x10^6$)	-	-	0.25 PMN-p	0.5 PMN-p	0.25 MNC-p	0.5 PMN-p	0.25 MNC-p	0.5 MNC-p
D0/D2 MNC	Day 2 NA	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0
Well set-up	Standard	Standard	Standard	Standard	Transwell	Transwell	Transwell	Transwell
CFU-EPC per 10^6 cells	35	25	48	42	45	36+	55	49

Details	CFU-EPC activity MNC-s		BC MNC-s plus MNC-p or PMN-p					
Buffy Coat 1 cells ($x10^6$)	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s
Donor 241 cells ($x10^6$)	-	-	0.25 PMN-p	0.5 PMN-p	0.25 MNC-p	0.5 PMN-p	0.25 MNC-p	0.5 MNC-p
D0/D2 MNC	Day 2 NA	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0
Well set-up	Standard	Standard	Standard	Standard	Transwell	Transwell	Transwell	Transwell
CFU-EPC per 10^6 cells	16	0*	6	3	23	20	10	15

Details	CFU-EPC activity MNC-s		BC MNC-s plus MNC-p or PMN-p					
Buffy Coat 2 cells ($x10^6$)	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s
Donor 242 cells ($x10^6$)	-	-	0.25 PMN-p	0.5 PMN-p	0.25 MNC-p	0.5 PMN-p	0.25 MNC-p	0.5 MNC-p
D0/D2 MNC	Day 2 NA	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0	Day 0
Well set-up	Standard	Standard	Standard	Standard	Transwell	Transwell	Transwell	Transwell
CFU-EPC per 10^6 cells	16	0*	10	3	42	35	25	25

Table 5.15 Results of co-plating non-mobilised donor MNC-s or PMN-p with buffy coat MNC-s (section 5.4.5.5.1)

Details	CFU-EPC activity MNC-s or PMN-p			CFU-EPC activity BC MNC-s plus MNC-s or PMN-p				
Volunteer cells ($x10^6$)	-	-	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s 0.25 MNC-s
Patient 297 cells ($x10^6$)	1.0 PMN-p	1.0 MNC-s		0.25 PMN-p	0.5 PMN-p	0.25 MNC-s	0.5 MNC-s	
Well set-up	Standard	Standard	Standard	Transwell	Transwell	Transwell	Transwell	Transwell
CFU-EPC per 10^6 cells	0	2	8	18	17	12	15	28
CFU-EPC Comment	RBC	RBC	Typical	Typical	Shorter spindle	Clear colonies	Clear colonies	Stunted spindle

Details	CFU-EPC activity MNC-s or PMN-p			CFU-EPC activity BC MNC-s plus MNC-s or PMN-p				
Volunteer cells ($x10^6$)	-	-	1.0 MNC-s			1.0 MNC-s	1.0 MNC-s	1.0 MNC-s 0.25 MNC-s
Patient 254 cells ($x10^6$)	1.0 PMN-p	1.0 MNC-s		1.0 MNC-s 0.25 PMN-p	1.0 MNC-s 0.5 PMN-p	0.25 MNC-s	0.5 MNC-s	
Well set-up	Standard	Standard	Standard	Standard	Transwell	Transwell	Transwell	Transwell
CFU-EPC per 10^6 cells	0	2	8	0	3	12	15	28
CFU-EPC Comment	RBC	RBC	Typical			Clear colonies	Clear colonies	Stunted spindle

Table 5.16 Results of co-plating non-mobilised patient MNC-s with volunteer MNC-s (section 5.4.5.5.2).

Details	CFU-EPC activity MNC-s			BC MNC-s plus MNC-s			
Buffy Coat		BC 571Z	BC 5532	BC 571Z	BC 571Z	BC 5532	BC 5532
Buffy coat cell ($x10^6$)		1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s	1.0 MNC-s
Donor 251 cells ($x10^6$)	1.0 MNC-s			0.25 MNC-s	0.5 MNC-s	0.25 MNC-s	0.5 MNC-s
Well set-up	Standard	Standard	Standard	Transwell	Transwell	Transwell	Transwell
CFU-EPC per 10^6 cells	0	6	6	3	9	1	0
CFU-EPC Comment	No CFU-EPC	Sparse spindle	Early dispersal	Sparse spindle	Increased spindle		

Table 5.17 Results of co-plating mobilised donor MNC-s with buffy coat MNC-s (section 5.4.5.5.3)

5.4.6. Conclusions

PMN-p obtained from PMP separation of whole blood samples from non-mobilised or G-CSF mobilised PBSC patients consistently failed to generate CFU-EPC (0 CFU-EPC/10^6 cells), suggesting that $CD66b^+$ granulocytes do not participate in CFU-EPC. However it remains unclear as to whether (non-mobilised or G-CSF mobilised) PMN-p could influence CFU-EPC formation by other cells. Summarised results of co-plating experiments performed using MNC and PNN-p are presented below (Table 5.18).

Cell sources	G-CSF	Primary cells	Secondary cells				
			—	PMN-p	PMN-p	MNC-s	Additional primary cells
		Well	Std	Std	T/well	T/well	Std
Cells from same subject	Non-mobilised	MNC-p	++ 0 +	↓ 0 0	↓ 0 0	—	—
		MNC-s	+ + 0 +	0 0 0 0	0 0/↓ 0 0	↔ ↔ 0 0	—
		MNC	**+**	**↓**	**↓**	**↔**	—
Cells from different subjects	Non-mobilised	MNC-BC	++ + +	↑ ↓ ↓	↑ ↑ ↑	↑ ↔ ↑	—
		MNC-Vol	+	—	↑	↑	↑↑
		MNC	**+**	**↓**	**↑**	**↑**	**↑**
	G-CSF	**MNC**	**+**	—	**↓**	**↓ ↓**	—

Cells from same subject	G-CSF mobilised	CD66b MACS	—	66b-deplete	66b-enriched	MNC-s + 66b-dep	Additional MNC-s
		MNC	**0**	**↑**	**0**	**↑**	—

Table 5.18 Summarised results from co-plating experiments (sections 5.4.5.4, 5.4.5.5).

Legend: 0 = no CFU-EPC, ↑ = increased, ↓ = decreased, ↔ = no change
Std = cells mixed together, T/well = secondary cells added in a Thinsert™ insert

When primary and secondary cells were obtained from the same donor, the addition of non-mobilised MNC to non-mobilised MNC in a ThinCert™ insert did not affect CFU-EPC. In contrast, when primary and secondary cells were obtained from different donors, the addition of non-mobilised MNC to non-mobilised MNC in a ThinCert™ insert increased CFU-EPC whilst the addition of mobilised MNC to non-mobilised MNC had the opposite effect on CFU-EPC.

The addition of PMN to culture wells affected CFU-EPC differently to MNC. When both primary and secondary cells came from the same donor, addition of non-mobilised PMN-p to non-mobilised MNC reduced CFU-EPC, regardless of whether cells were directly mixed or added to MNC in ThinCert™ inserts. In contrast, when primary and secondary cells were sourced from different donors, the addition of non-mobilised PMN-p to non-mobilised MNC reduced CFU-EPC when cells were directly mixed but increased CFU-EPC were observed when PMN-p were added in ThinCert™ inserts. However, when mobilised PMN-p were added to non-mobilised MNC from another patient in ThinCert™ inserts CFU-EPC were reduced. Co-plating experiments that plated mobilised MNC and mobilised CD66b-depleted mobilised MNC from the same patient had the opposite effect and increased CFU-EPC were observed.

These results showed that CFU-EPC can be manipulated by altering the proportions of $CD66b^+$ granulocytes in cells plated into the CFU-EPC assay. The results suggested that even in samples from non-mobilised patients, the degree of 'contamination' of the interface layer by $CD66b^+$ granulocytes affected CFU-EPC; whether this was due to the physical presence of granulocytes in the well or due to secreted granulocytic factors that affected CFU-EPC. Further work to examine the influence of leukocyte subpopulations on CFU-EPC formation in mobilised and non-mobilised PBSC patient samples was performed using MACS microbead separation methods.

5.5. Modification of CFU-EPC by manipulation of the cell content of cultured cells; immunomagnetic separations

5.5.1. Introduction

Co-plating experiments using MNC (MNC-s or MNC-p) and PMN-p provided some evidence that $CD66b^+$ granulocytes (pre G-CSF or G-CSF mobilised) can influence CFU-EPC. However, MNC-p were not sufficiently depleted in granulocytes/myeloid content for any firm conclusions to be drawn on their affect on CFU-EPC formation when PMP cells were plated in well together with MNC. This series of experiments was performed in order to assess further the contribution of $CD66b^+$ granulocytes to CFU-EPC formation and in particular to establish whether modification (reduction) of $CD66b^+$ granulocytes in mobilised MNC samples could reverse the reduction of CFU-EPC activity observed in mobilised PBSC donor MNC.

5.5.1.1. Patient samples

Non-mobilised whole blood samples were obtained from 9 subjects; (4 pre G-CSF autologous PBSC patients, 3 PBSC donor follow–up samples (1 allogeneic and 3 autologous) and 2 healthy volunteers). Mobilised samples were obtained from 19 PBSC donors (5 allogeneic and 14 autologous PBSC donors (G-CSF+ chemotherapy mobilised (n=11) and G-CSF+ AMD3100 mobilised (n=3)).

MNC-s were obtained from whole blood samples by Histopaque density gradient centrifugation. Aliquots of the resulting MNC-s were selectively enriched or depleted for $CD66b^+$ granulocytes, $CD14^+$ monocytes or $CD3^+$ lymphocytes using MACS microbead separation techniques (section 2.4.2).

5.5.2. Cell content of MNC prior to MACS microbead separations

The phenotype of MNC-s prior to MACS microbead separation was assessed by flow cytometry. The proportions of $CD66b^+$ granulocytes, $CD14^+$ monocytes, lymphocytes and $CD235a^+$ erythrocytes in MNC-s (starting cell population) were

comparable to the phenotypic analyses of non-mobilised and mobilised samples presented in previous sections (Table 5.19).

G CSF	Sample Source	n	CD45	$CD66b^+$ Granulocytes	$CD14^+$ Monocytes	Lymphocytes	$CD235^+$ Erythrocytes
Non-mobilised	PBSC donors (1 allo, 5 auto)	6	52.7	3.8	9.6	33.8	37.6
	Healthy volunteer	2	85.3	0.12	13.4	70.0	10.7
Mobilised	All PBSC donors	19	69.4	28.7	12.7	26.1	24.6
	Allogeneic PBSC donors	5	85.7	33.2	16.9	37.1	10.5
	Autologous PBSC patients	14	63.6	27.2	11.2	22.2	29.7

Table 5.19 Leukocyte subpopulations and erythrocytes present in MNC-s.
Mean proportions of all events presented.
(allo = allogeneic PBSC donor, auto = autologous PBSC patient).

5.5.3. MACS microbead separations for CD66b

Samples from 16 PBSC donors underwent MACS microbead separation for CD66b. These consisted of 15 mobilised PBSC patient samples (5 allogeneic and 10 autologous PBSC patients) and 1 non-mobilised sample (allogeneic PBSC donor follow-up sample).

CD66b MACS separation of the non-mobilised patient sample was unsuccessful. The starting MNC contained 0.4% $CD66b^+$ granulocytes and CD66b-enrichment or depletion was not achieved. CD66b separation of non-mobilised samples was not repeated.

The CD66b-enriched MNC and CD66b-depleted MNC from mobilised PBSC donors were fairly consistent in their cell contents. The compositions of starting MNC were comparable to previous results with the same differences between samples from allogeneic and autologous PBSC donors being noted (section 5.2).

CD66b-enriched MNC contained high levels of $CD66b^+$ granulocytes with very low numbers of $CD14^+$ monocytes, lymphocytes and $235a^+$ erythrocytes present. CD66b-depleted MNC contained reduced proportions of $CD66b^+$ granulocytes and

higher proportions of $CD14^+$ monocytes and lymphocytes than starting MNC (Table 5.20). Overall, CD66b-depleted MNC showed a 31.0% reduction in $CD66b^+$ granulocyte content from starting MNC; identical in allogeneic and autologous donor samples. In MACS separated cells an increase in the proportions of events that were 'unclassified' using our definitions for leukocytes and erythrocytes were noted; increasing from 3.9% to 8.1% (CD66b-depleted MNC) and 14.1% (CD66b-enriched MNC). This might be attributed the MACS separation process itself causing disruption and damage to these large, granular and stimulated mobilised myeloid cells.

PBSC	MNC fraction	CD45	$CD66b^+$ granulocytes	$CD14^+$ monocytes	Lymphocytes	$CD235^+$ erythrocytes
Mobilised patient samples	Starting MNC (n=15)	69.3	30.0 (43.3)	13.4	27.3	25.4
	CD66b-depleted	71.2	21.3 (29.9)	18.1	27.9	24.6
	CD66b-enriched	91.6	78.6 (85.8)	1.7	1.8	3.4
Allogeneic PBSC donors	Starting MNC (n=5)	85.7	33.2 (38.7)	16.9	37.1	10.5
	CD66b-depleted	87.3	23.2 (26.6)	21.8	40.8	10.4
	CD66b enriched	97.1	80 6 (83.0)	2.2	2.6	1.2
Autologous PBSC patients	Starting MNC (n=10)	61.0	28.4 (46.6)	11.5	21.9	33.7
	CD66b-depleted	63.2	20.2 (32.0)	16.1	23.9	32.5
	CD66b-enriched	88.2	77.3 (87.6)	1.7	5.2	1.6

Table 5.20 Leukocyte subpopulations and erythrocytes present in CD66b-enriched and CD66 depleted MNC.
Figures expressed as mean proportions of all events; proportions of $CD45^+$ events in parentheses.

5.5.3.1. Influence of CD66b depletion on CFU-EPC (allogeneic PPSC donors)

We have evidence from CFU-EPC of PMN-p that granulocytes do not form CFU-EPC. This was the case for both non-mobilised and mobilised PMN-p. It is unclear whether or not the physical presence of $CD66^+$ granulocytes alone is sufficient to suppress CFU-EPC formation or whether cellular or secreted factors are important influences of CFU-EPC formation.

5.5.3.1.1. CFU-EPC formation in non-mobilised samples

In 4 of 5 cases pre G-CSF MNC-s from allogeneic PBSC donors generated CFU-EPC (mean 9 CFU-EPC/10^6 cells). Two of 5 donor follow-up samples generated CFU-EPC (mean 3.8 CFU-EPC/10^6 cells) whilst 3 of 5 did not. In 1 of 3 samples that failed to generate CFU-EPC from donor follow-up MNC-s, CFU-EPC failed to form due to technical reasons (Table 5.21).

MNC-s obtained from one further allogeneic PBSC donor at follow-up failed to show CFU-EPC activity. No CFU-EPC formation was noted in CD66b-depleted MNC either. No pre G-CSF sample was available from this patient to provide additional non-mobilised CFU-EPC data.

5.5.3.1.2. CFU-EPC formation in mobilised samples

Mobilised MNC-s cells

CFU-EPC formation was severely curtailed but not completely absent in MNC-s following G-CSF administration. 1 of 5 mobilised MNC-s generated CFU-EPC, albeit at reduced rate compared to the matched pre-CSF sample (mean 0.6 CFU-EPC/10^6 cells, n=5) (Table 5.21).

Pt ID	CFU-EPC/x10^6 cells plated				
	Pre G-CSF	Mobilised			Follow-up
	MNC-s	MNC-s	CD66b-depleted MNC	CD66b-enriched MNC	MNC-s
274	24	0	3.75	0	0*
276	15	3	5	0	14
293	0	0	0	0	0
294	2	0	0	0	0
300	4	0	15	0	1

Table 5.21 CFU-EPC in MNC-s, CD66b-enriched and CD66b-depleted MNC (1).
Samples were obtained from 5 allogeneic PBSC donors; pre G-CSF, day 1 PBSCH and at donor follow-up.

Mobilised CD66b-enriched MNC

No CFU-EPC activity was noted (0 CFU-EPC/10^6 cells) in CD66b-enriched MNC (Table 5.21).

Mobilised CD66b-depleted MNC

Plating mobilised CD66b-depleted MNC into CFU-EPC culture demonstrated partial recovery of CFU-EPC activity (mean 4.75 CFU-EPC/10^6 cells). CFU-EPC formation was restricted to those samples from allogeneic PBSC donors that developed CFU-EPC from non-mobilised samples. Subjects with higher CFU-EPC activity in non-mobilised samples (24/15/4 CFU/10^6 cells versus 2/0 CFU-EPC/10^6cells) demonstrated CFU-EPC recovery in mobilised CD66b-depleted MNC (Table 5.21).

Changes in CFU-EPC colony morphology were also noted. CFU-EPC colonies from mobilised CD66b-depleted MNC were similar in appearance to non-mobilised CFU-EPC. Longer and more slender spindle cells were associated with tight cores of rounded cells in wells containing CD66b-depleted MNC. This was not observed in any CFU-EPC culture wells containing mobilised MNC-s (see Appendix).

5.5.3.2. Influence of CD66b depletion on CFU-EPC (autologous PBSC patients)

Ten samples were obtained from 9 autologous PBSC patients (diagnoses of MM (n=7) or NHL (n=2)) following PBSC mobilisation with G-CSF+chemotherapy (n=7) or G-CSF+ AMD3100 (n=3). Information on the CFU-EPC generation of pre G-CSF samples was available in 7 cases and the CFU-EPC activity of donor follow-up samples was available in 6 cases.

5.5.3.2.1. CFU-EPC formation in non-mobilised MNC

Five of 7 pre G-CSF patient samples showed CFU-EPC activity. In these samples, mean colony formation was 1.9 CFU-EPC/10^6 MNC; a lower rate of CFU-EPC formation compared to other pre G-CSF autologous PBSC patients samples. All 6 donor follow-up samples showed CFU-EPC formation (mean 13.3 CFU-EPC/10^6 MNC). Matched pre G-CSF CFU-EPC data was available for comparison in 4 of

these 6 cases. In 2 cases, CFU-EPC formation in donor follow-up samples matched that of the pre G-CSF sample. However, in the other 2 cases, CFU-EPC formation in donor follow-up samples exceeded that of the pre G-CSF samples. This might suggest a possible negative effect of chemotherapy, patient treatment or disease related factors on CFU-EPC formation, which is independent of any G-CSF effects (Table 5.22).

Pt ID	G+C or G+A	CFU-EPC/x10^6 cells plated				
		Pre G-CSF	Mobilised			Follow-up
		MNC-s	MNC-s	CD66b-depleted MNC	CD66b-enriched MNC	MNC-s
277	G+C	—	2	16	0	3
278	G+C	0	1.4	11.3	0	20
279	G+C	—	1	1	0	48
283	G+C	1	0	0	0	4
287	G+C	1	0	0	0	—
291	G+C	5	1	0	0	—
315	G+C	0	0	0	—	—
299	G+A	3	0	0	0	2
299	G+A	3	0	8	0	3
316	G+A	—	12	30	0	—

Table 5.22 CFU-EPC in MNC-s, CD66b-enriched MNC and CD66b-depleted MNC (2). Samples obtained from 10 autologous PBSC patients; 7 mobilised with G-CSF+chemotherapy (G+C) and 3 with G-CSF+AMD3100 (G+A); samples collected pre G-CSF, day 1 PBSCH and at donor follow-up.

5.5.3.2.2. CFU-EPC formation in mobilised cells

Mobilised MNC-s cells

Five of 10 mobilised autologous PBSC patient MNC-s generated CFU-EPC whilst 5 samples had no CFU-EPC formation (0 CFU-EPC/10^6 MNC). Apart from one sample that recorded 12 CFU-EPC/10^6 MNC, CFU-EPC formation was at a low rate; 1.1 CFU-EPC/10^6 MNC in those forming colonies (overall 1.7 CFU-EPC/10^6 cells (n=10)) (Table 5.22).

Mobilised CD66b-enriched MNC

No CFU-EPC formation was observed in any mobilised CD66b-enriched MNC fraction (0 CFU-EPC/10^6 cells) (Table 5.22).

Mobilised CD66b-depleted MNC

Five of 10 samples showed CFU-EPC formation in mobilised CD66b-depleted MNC (mean 6.6 CFU-EPC/10^6 cells). This included a sample from 1 patient that failed to generate CFU-EPC from a pre G-CSF sample. A follow-up sample from this particular patient subsequently generated CFU-EPC in high numbers (20 CFU-EPC/10^6 cells) (Table 5.22, see also section 4.7). Similar changes in colony morphology in mobilised CD66b-depleted MNC were noted as observed in mobilised CD66b-depleted MNC from allogeneic PBSC donors (see Appendix).

5.5.3.3. Increased CFU-EPC activity in mobilised CD66b-depleted MNC

Similar changes in CFU-EPC formation were present in MNC-s and CD66b-depleted MNC obtained from allogeneic and autologous PBSC donors. The changes in CFU-EPC formation using CD66b-depleted MNC in mobilised samples (in addition to use of mobilised MNC-s) are illustrated in Figure 5.16.

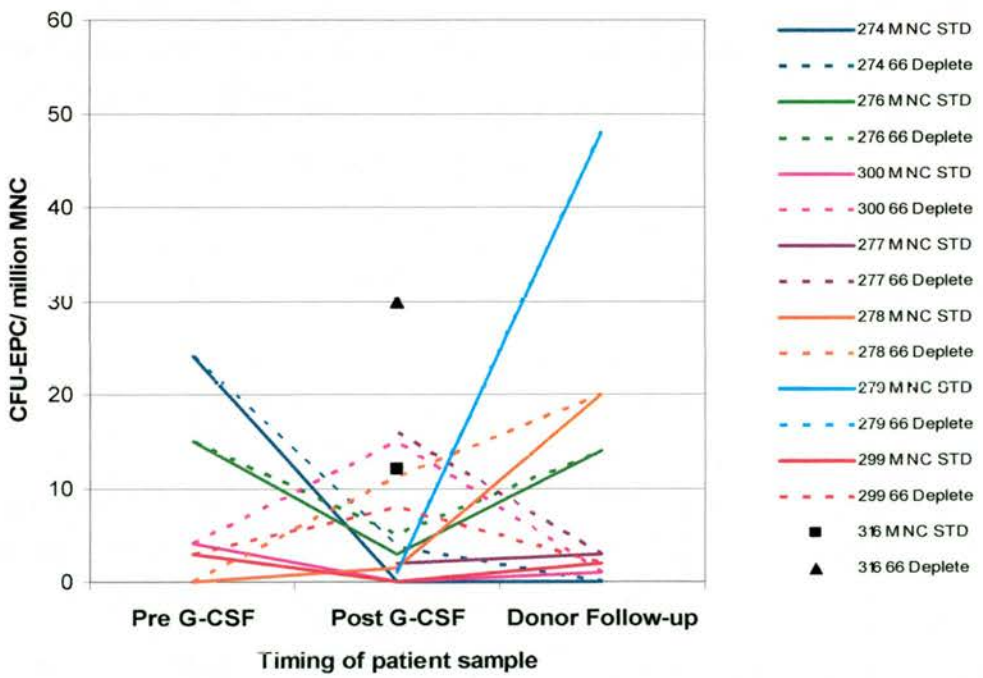


Figure 5.16 CFU-EPC in MNC-s (MNC STD) and CD66b-depleted MNC (66 Deplete). Cells obtained from PBSC donors on 3 occasions; pre G-CSF, day 1 PBSCH and at donor follow-up. CFU-EPC frequency is expressed per 10^6 cells plated.

5.5.3.4. Correlations between $CD66b^+$ granulocyte content, $CD14^+$ monocyte content, $CD14^+$ monocyte: $CD66b^+$ granulocyte ratio and CFU-EPC formation

There appeared to be a correlation between the magnitude of reduction of $CD66b^+$ granulocytes in mobilised CD66b-depleted MNC from the level contained in mobilised MNC-s and CFU-EPC formation but this failed to achieve statistical significance (p=ns). Overall, there was a mean reduction of $CD66b^+$ granulocyte content from MNC-s of 41.9% in CD66b-depleted MNC that generated CFU-EPC compared to a mean reduction of 15.7% in those CD66b-depleted MNC that did not generate CFU-EPC.

Greater reductions in $CD66b^+$ granulocyte content (as seen in samples generating CFU-EPC in mobilised CD66b-deplete MNC) were accompanied by greater increases in $CD14^+$ monocytes and lymphocytes in the CD66b-depleted MNC samples.

It appears that the ratios of $CD14^+$ monocytes and $CD66b^+$ granulocytes in a sample might affect CFU-EPC generation in post G-CSF samples. The patient samples that generated CFU-EPC from CD66b-depleted fractions had higher ratios of $CD14^+$ monocytes: $CD66b^+$ granulocytes than CD66b-depleted MNC that failed to generate CFU-EPC ($p<0.02$) and the magnitude of change in the $CD14^+$ monocyte: $CD66b^+$granulocyte ratios from mobilised MNC-s to CD66b-depleted MNC was significant ($p<0.02$).

5.5.4. MACS microbead separations for CD14

MACS microbead separations for CD14 were performed on 20 samples; 2 allogeneic PBSC donors, 16 autologous PBSC patient and 2 healthy volunteer samples. Mobilised samples (n=11) were obtained from 2 allogeneic PBSC donors and 9 autologous PBSC patients (mobilised with G-CSF+chemotherapy (n=6) or G-CSF+AMD3100 (n=3)). Non-mobilised samples (n=9) consisted of 2 healthy volunteer, 4 pre mobilisation and 3 follow-up samples. Many of these samples undergoing MACS separation for CD14 had additional MACS separations

performed (for CD66b or CD3). CD14-enriched MNC and CD14-depleted MNC were fairly consistent in their cell contents. Starting MNC (MNC-s) were comparable to previous results and the same differences between samples from allogeneic and autologous PBSC donors were noted (section 5.2).

Compared to CD66b-enriched MNC, CD14-enriched MNC were less 'pure'. Similar to CD66b MACS cell separations, there were populations of 'unclassified events'. These were most prevalent in CD14-enriched MNC. There was significant $CD66b^+$ granulocytic content of CD14-enriched MNC, principally due to the low expression of CD14 on a proportion of granulocytes. Non-mobilised samples, including the samples from 2 healthy volunteers, also showed significant CD66b expression on CD14-enriched MNC, illustrating that this problem was not limited to mobilised samples (Table 5.23). Weak CD14 expression was displayed by a proportion of $CD66b^+$ granulocytes. In standard flow cytometric analysis CD14 expression on granulocytes doesn't interfere with gating of cell populations but in MACS cell separations there would have been sufficient CD14 expression on these cells for some $CD66b^+$ granulocytes to be retained in the magnetic column with $CD14^+$ monocytes.

Cell separation by MACS will therefore not distinguish between $CD14^+$ and CD66b+/14 $^{low+}$ events unless a second stage of bead labelling is performed, first depleting for $CD66b^+$ events and then enriching for $CD14^+$ events. Due to the presence of a significant proportion of 'unclassified' events after one labelling stage, performing a second one was thought to be undesirable. Flow cytometry cell sorting might overcome this issue as low and high CD14 expression could be distinguished during the sort.

PBSC	MNC fraction	CD45	CD66b$^+$ granulocytes	CD14$^+$ monocytes	Lymphocytes	CD235$^+$ erythrocytes
Mobilised samples	Starting MNC (n=11)	76.7	28.8	12.7	29.8	17.8
	CD14-depleted	70.4	29.1	5.1	33.6	24.1
	CD14-enriched	93.4	19.3	64.1	3.8	0.7
Mobilised Allogeneic PBSC donors	Starting MNC (n=2)	85.7	33.1	16.9	37.1	10.4
	CD14-depleted	81.9	37.2	1.4	41.4	16.7
	CD14-enriched	98.7	32.2	53.4	7.8	0.1
Mobilised Autologous PBSC patient	Starting MNC (n=9)	63.6	27.2	11.2	22.2	29.7
	CD14-depleted	65.7	26.5	6.4	30.6	28.0
	CD14-enriched	91.5	14.4	68.1	2.3	0.9
Non mobilised PBSC patient	Starting MNC (n=7)	53.9	4.5	10.2	32.9	44.3
	CD14-depleted	49.5	4.2	3.1	46.2	43.6
	CD14-enriched	93.4	15.6	73.9	4.4	1.6
Non G-CSF Healthy Volunteers	Starting MNC (n=2)	85.3	0.1	13.4	70.0	10.7
	CD14-depleted	85.7	0.1	0.8	—	—
	CD14-enriched	97.2	0.6	91.5	—	—

Table 5.23 Leukocyte subpopulations and erythrocytes present in MNC-s, CD14-enriched MNC and CD14-depleted MNC.
Samples obtained from non-mobilised PBSC donors, mobilised PBSC donors and healthy volunteers. Figures are expressed as proportions of all events.

5.5.4.1. Influence of CD14-enrichment on CFU-EPC formation

5.5.4.1.1. CFU-EPC formation in non-mobilised samples

Four of 4 pre G-CSF samples (1 allogeneic and 3 autologous PBSC patient samples) generated CFU-EPC (mean 2.5 CFU-EPC/10^6 cells, n=4) and 2 of 3 donor follow-up samples (1 allogeneic and 2 autologous PBSC patient samples) generated CFU-EPC (mean 3.7 CFU-EPC/10^6 cells, n=3). Only 1 of 7 non-mobilised samples that underwent MACS separation for CD14 showed any CFU-EPC within CD14-enriched MNC. This was a donor follow-up sample from an autologous PBSC patient. Neither MNC-s nor CD14-depleted MNC generated CFU-EPC but 1 CFU-EPC/10^6 cells developed in CD14-enriched MNC (n=1). No CFU-EPC were observed in CD14-depleted MNC from any of the 7 samples (see Appendix).

5.5.4.1.2. CFU-EPC formation in mobilised samples

Mobilised MNC-s cells

CFU-EPC activity was observed in 7 of 11 mobilised MNC. Except for one patient sample that generated 12 CFU-EPC/10^6 cells (no pre G-CSF or donor follow-up sample was available on this patient) most samples had low level CFU-EPC formation (overall mean 2.4 CFU-EPC/10^6 cells, n=11, mean 1.5 CFU-EPC/10^6 cells if sample that generated 12 CFU-EPC/10^6 is excluded, n=10) (see Appendix).

Mobilised CD 14 enriched MNC

CD14-enriched MNC from 3 patient samples (1 allogeneic PBSC donor and 2 autologous PBSC patients) showed some CFU-EPC activity (mean 2 CFU-EPC/10^6 cells). Although the level of CFU-EPC activity in CD14-enriched MNC was modest, the comparative non G-CSF samples also had low CFU-EPC activity (mean 3 CFU-EPC/10^6 cells) (see Appendix).

There were no apparent links between $CD14^+$ monocyte content, $CD66b^+$ granulocyte content, ratios of $CD14^+$ monocytes to $CD66b^+$ granulocytes or change in cell proportions from MNC-s to CD14-enriched MNC and the CFU-EPC development observed in these samples.

Mobilised CD14-depleted MNC

A single sample, from a patient mobilised with G-CSF+AMD3100 generated CFU-EPC from the CD14-depleted MNC but not the CD14-enriched MNC. MNC-s generated CFU-EPC at 12 CFU-EPC/10^6 cells whilst CD14-enriched MNC had 0 CFU-EPC/10^6 cells and CD14 deleted MNC showed 4 CFU-EPC/10^6 cells. This was not be readily explained by the cell contents of MNC. CD14-enriched MNC contained 18.8% $CD66b^+$ granulocytes and 86.8% $CD14^+$ monocytes and CD14-depleted MNC contained 17.6% $CD66b^+$ granulocytes and 5.4% $CD14^+$ monocytes, which were comparable to other patient samples (see Appendix).

5.5.4.1.3. CFU-EPC formation in samples from healthy volunteers

MNC-s from 2 healthy volunteers generated CFU-EPC (20 and 8 CFU-EPC/10^6 cells). Following MACS separation, CD14-depleted MNC failed to form CFU-EPC (0 CFU-EPC/10^6 MNC).

There were insufficient cells in the CD14-enriched MNC fraction for these cells to be plated alone into CFU-Hill assays. Instead, CD14-enriched MNC were mixed with MNC-s (ratio 1x10^6 CD14-enriched MNC + 2x10^6 MNC-s) and then plated into CFU-EPC assays. On day 2, 1x10^6 (mixed) cells were transferred into a 24 well fibronectin coated plate. Both volunteer samples showed prolific CFU-EPC formation from the MNC-s + CD14-enriched MNC mix (35 and 50 CFU-EPC/10^6 cells). These colonies showed typical CFU-EPC morphology.

5.5.5. MACS microbead separations for CD3

MACS microbead separations for CD3 were performed on samples from 5 patients. These were 2 healthy volunteer samples, 2 autologous G-CSF+chemotherapy mobilised patient samples and 1 autologous patient donor follow-up sample. Samples undergoing MACS separation for CD3 had additional MACS separations performed (for CD66b or CD14) (Table 5.24).

5.5.5.1. Influence of CD3-enrichment on CFU-EPC formation

5.5.5.1.1. CFU-EPC formation in non-mobilised samples

The two samples from healthy volunteers generated CFU-EPC from MNC-s (8 and 20 CFU-EPC/10^6 cells) but the other non G-CSF sample (autologous PBSC patient follow-up sample) showed no CFU-EPC activity in MNC-s. CFU-EPC activity was not observed in any of the CD3-enriched MNC or any of the CD3-depleted MNC (see Appendix).

PBSC	MNC fraction	CD45	$CD66b^+$ granulocytes	$CD14^+$ monocytes	Lymphocytes ($CD3^+/45^+$)	$CD235^+$ erythrocytes
Mobilised autologous PBSC	Starting MNC (n=2)	69.6	27.6	18.5	23.3 (Lymph) (CD3 NA)	24.8
	CD3-depleted	47.4	22.9	13.6	2.11 ($CD3^+$)	—
	CD3-enriched	52.3	3.8	4.0	42.4 ($CD3^+$)	—
Non-mobilised autologous PBSC	Starting MNC (n=2)	43.2	10.0	9.0	23.2 (Lymph) (CD3 NA)	54.9
	CD3-depleted	34.9	8.0	10.4	4.8 ($CD3^+$)	—
	CD3-enriched	95.6	1.1	1.1	77.1 ($CD3^+$)	—
Healthy volunteer	Starting MNC (n=2)	85.3	0.12	13.4	70.0 (Lymph) (CD3 NA)	10.7
	CD3-depleted	85.1	0.2	22.2	7.9 ($CD3^+$)	—
	CD3 Enriched	98.2	0.2	0.7	81.7 ($CD3^+$)	—

Table 5.24 Leukocyte subpopulations and erythrocytes in CD3 enriched and depleted MNC. Samples obtained from non-mobilised PBSC donors, mobilised PBSC donors and healthy volunteers. Figures are expressed as proportions of all events.
(Lymph - lymphocytes, NA - not available)

5.5.5.1.2. CFU-EPC formation in mobilised samples

Neither of the MNC-s from the 2 mobilised samples formed CFU-EPC. CFU-EPC activity was not observed in any of the CD3 enriched MNC nor in any of the CD3-depleted MNC (see Appendix).

5.5.5.1.3. CFU-EPC in mixing experiments; mixing of CD3 enriched or CD3-depleted MNC with other cells

CD3 enriched MNC were mixed with CD14-enriched MNC and plated into CFU-Hill assay (1 healthy volunteer sample and 1 autologous PBSC patient follow-up sample). Neither sample generated CFU-EPC. When CD3-depleted MNC were mixed with CD14-enriched MNC and plated into CFU-EPC assay (other healthy volunteer sample), prolific CFU-EPC activity (120 CFU-EPC/10^6 cells) in excess of that recorded for either the MNC-s or the mix of MNC-s and CD14-enriched MNC (see Appendix).

5.5.6. Conclusions

The generation of colonies from non-mobilised or G-CSF (and G-CSF+AMD3100 and G-CSF+chemotherapy) mobilised samples does not simply reflect the cell content of the MNC suspension. Use of MACS to manipulate the cell content of MNC preparations in order to selectively enrich or deplete a cell population was successful in 'recovering' some CFU-EPC activity following G-CSF administration. However, increases in CFU-EPC were not consistent. Overall, patients who had higher frequencies of CFU-EPC in their non-mobilised samples were more likely to recover CFU-EPC activity on manipulation of a G-CSF mobilised sample.

It appears that neither the CD66b/granulocytic enriched population nor the CD14/monocyte depleted population generate CFU-EPC, either in the non-mobilised or G-CSF mobilised setting. The CD66b MACS separation results were consistent with those results obtained using PMP for cell separations, demonstrating a lack of CFU-EPC forming activity within the polymorphonuclear/lower interface layer.

On occasion CFU-EPC have been generated from a 'failed' MACS separation (when there has been no depletion/enrichment of the desired cell fraction). However, overall, mobilised samples that showed some recovery of CFU-EPC activity following MACS CD66b depletion of MNC had lower CD66b/granulocyte levels than the samples that failed to generate CFU-EPC post MACS separation. MACS separations for CD14, to date, produced inconsistent results and it appears to be inferior to CD66b depletion in enhancing CFU-EPC in mobilised samples. MACS separation for CD14 failed to produce pure positively selected fractions and it was found that the weak CD14 expression on a proportion of $CD66b^+$ granulocytes led to the presence of some granulocytes in CD14-enriched MNC.

5.6. Investigation of the use of thawed MNC as a source of CFU-EPC

5.6.1. Introduction

MNC obtained from G-CSF mobilised PBSC donors contained significantly greater proportions of $CD66b^+$ granulocytes (31.3% in allogeneic and 27.7% in autologous PBSC MNC) and showed marked reductions in CFU-EPC activity compared to non-mobilised MNC ($CD66b^+$ granulocytes 2.9% and 5.0% in allogeneic and autologous non-mobilised PBSC samples respectively). It is proposed that G-CSF, possibly by altering the proportions of leukocyte subpopulations, contributed to the reduction in CFU-EPC in mobilised blood samples. If the proportions of the leukocyte subpopulations in mobilised blood MNC could be altered to be similar to that of non-mobilised MNC then the frequency of CFU-EPC might increase towards that of non-mobilised MNC. Myeloid cells do not survive cryogenic preservation well. It was proposed that freezing and thawing MNC would destroy $CD66b^+$ granulocytes and that thawed MNC would therefore contain lower proportions of $CD66b^+$ granulocytes which would reduce the impact of 'contaminating' $CD66b^+$ granulocytes on CFU-EPC.

A source of cryopreserved samples from patients known to generate endothelial colonies (CFU-EPC) would also be an extremely useful research tool. Use of thawed MNC would enable a number of experiments to be completed on a single sample, providing comparative data for review. Mixing studies, using samples collected at different time points and MNC/cells obtained from patients receiving different treatments, would also be possible. Experiments could also be performed on a number of samples at once, allowing streamlining of experimental activity.

MNC were isolated from diluted whole blood samples by Histopaque density gradient centrifugation. After enumeration (Coulter cell counter), MNC (fresh MNC) were plated into CFU-Hill assays with stem cell and leukocyte phenotypes

determined by flow cytometry. Aliquots of fresh MNC suspended in culture medium were cryopreserved after the addition of foetal calf serum and DMSO using rate controlled freezing (section 2.1.2). MNC were frozen at minus 80C until thawed (thawed MNC) (sections 2.1.4). In these experiments using thawed MNC we sought to establish;

1. whether thawed MNC (from non-mobilised or mobilised PBSC donors) were capable of CFU-EPC formation;
2. the degree of attenuation of CFU-EPC activity in thawed MNC, by comparing CFU-EPC development in fresh and thawed MNC;
3. the differences in the proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes between fresh and thawed MNC; and
4. whether there were any differences in stem cell phenotypes between fresh MNC and thawed MNC.

5.6.1.1. Patient Samples

Eleven non-mobilised samples were obtained from 8 subjects (5 non-mobilised autologous PBSC patients and 3 healthy volunteers). G-CSF mobilised samples were obtained from 4 patients (3 autologous PBSC patients and 1 allogeneic PBSC donor). Flow cytometry was performed to assess the major cell populations (leukocyte subpopulations and $CD235a^+$ erythrocytes) and putative EPC phenotypes contained in 10 of 11 non-mobilised and all 4 mobilised samples using the same methods and phenotypic definitions as described sections 5.1.2 and 6.1. MNC were plated into CFU-EPC assays.

5.6.2. CFU-EPC development in thawed MNC

We have previously determined that plating MNC directly into wells of 24-well fibronectin coated plates (CFU-Hill(direct)) resulted in the reliable appearance of CFU-EPC in samples that formed CFU-EPC when plated into a CFU-Hill assay. In order to have sufficient MNC available for comparative studies using fresh and thawed MNC, CFU-EPC were assessed in fresh MNC using CFU-Hill(direct) assays,

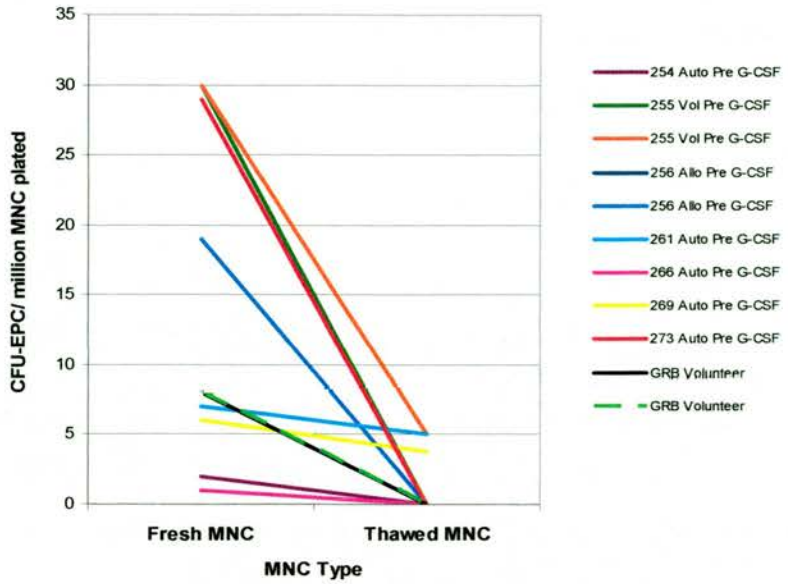
using 0.8-1.0x10^6 MNC suspended in 1.0mL of CECM. When MNC numbers permitted, fresh MNC were also plated into CFU-Hill assays (3x10^6 fresh MNC plated into a 12 well fibronectin coated plate). Results were considered in two groups; non-mobilised (pre G-CSF, donor follow-up samples and volunteer samples) and G-CSF mobilised patients.

5.6.2.1. CFU-EPC activity in thawed MNC compared to fresh MNC

5.6.2.1.1. Non-mobilised samples

CFU-EPC colonies were generated by all 11 non-mobilised fresh MNC in CFU-Hill(direct) assays. Colonies displayed typical CFU-EPC morphology. Only 3 of 11 thawed MNC (27.3%) generated CFU-EPC in CFU-Hill(direct) assays.

CFU-EPC frequency in thawed MNC was greatly reduced from that of the comparable fresh MNC sample. Overall, samples that displayed the greatest CFU-EPC activity in fresh MNC were more likely to generate CFU-EPC from thawed MNC. One sample that generated CFU-EPC from an aliquot of thawed MNC failed to show consistent results as another aliquot of thawed MNC from the same patient did not generate CFU-EPC (Figure 5.17).



Patient ID		254	255 1	255 2	256 1	256 2	261	266	269	273	GRB 1	GRB 2
Subject Type		Auto	Vol	Vol	Allo	Allo	Auto	Auto	Auto	Auto	Vol	Vol
G-CSF		Pre	Pre	Pre	Pre	Pre	Pre	Pre	Pre	Pre	No	No
CFU-EPC/ 10^6 MNC	Fresh MNC	2	30	30	19	19	7	1	6	29	8	8
	Thawed MNC	0	0	5	0	0	5	0	3.8	0	0	0

Figure 5.17 Comparison of CFU-EPC in fresh and thawed non-mobilised MNC. (Vol=volunteer, Auto=autologous PBSC patient, Allo=allogeneic PBSC donor).

5.6.2.1.2. G-CSF Mobilised Samples

No (0 of 4) fresh mobilised MNC generated CFU-EPC when cultured in either CFU-Hill(direct) or CFU-Hill assays. On thawing, aggregates of non re-suspended cells/debris were frequently observed. No thawed mobilised MNC generated CFU-EPC when plated into CFU-EPC assays (CFU-Hill(direct) assay (n=3) or CFU-Hill assay (n=1)) (Table 5.25).

Cell Culture	CFU-EPC (per 10^6 MNC plated)			
G-CSF	Non-mobilised	Non-mobilised	Mobilised	Mobilised
Sample	Fresh	Thawed	Fresh	Thawed
253	—	—	0	0
256	19	0	9	0
259	40	—	2	0
266	1	0	0	0

Table 5.25 CFU-EPC activity of fresh and thawed G-CSF mobilised MNC.

We failed to observe any increase in CFU-EPC in G-CSF mobilised thawed MNC compared to fresh mobilised MNC, suggesting that the degree of reduction in $CD66b^+$ granulocytic and $CD235a^+$ erythrocyte content of MNC by freeze-thawing was possibly insufficient to restore CFU-EPC activity. Unlike selection of cells by MACS, unwanted cells were not removed from the cell culture. Dead cells or fragmented cells remained as debris within sample and could still affect cell behaviour (including CFU-EPC activity) of viable cells remaining in culture.

5.6.2.1.3. CFU-EPC morphology non-mobilised samples

Two of three samples that generated CFU-EPC from thawed MNC had sparse, short spindle cells associated with the CFU-EPC core in the fresh sample. Following freezing and thawing, CFU-EPC morphology did not change appreciably. One sample (255) had prominent spindle formation observed in the CFU-EPC assay of fresh MNC. The thawed MNC sample generated CFU-EPC with reduced spindle cell number and size compared to fresh MNC. Representative photographs of CFU-EPC are presented (Figure 5.18).

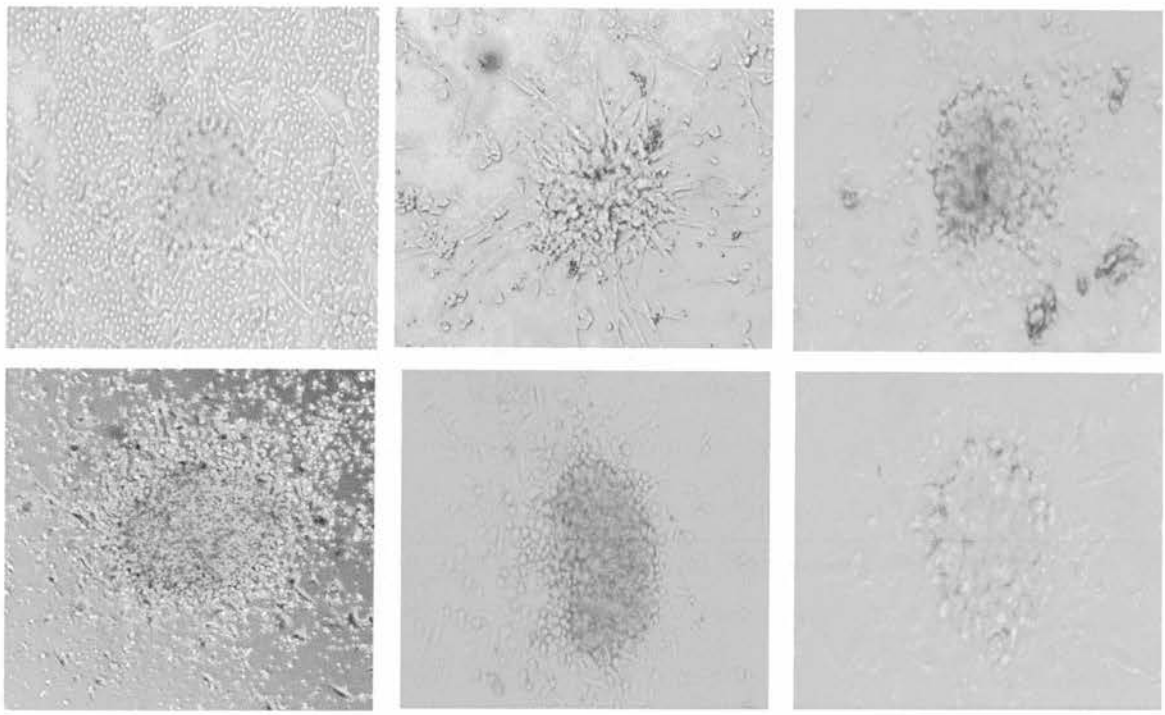


Figure 5.18 Images of CFU-EPC generated from fresh and thawed non-mobilised MNC. Three non-mobilised samples; autologous PBSC patient 261 (left), volunteer 255 (centre images) and autologous PBSC patient 269 (right); paired data with fresh MNC shown in upper images and thawed MNC in lower images.

5.6.3. Flow cytometric analysis of leukocyte subpopulations and CD235a$^+$ erythrocytes

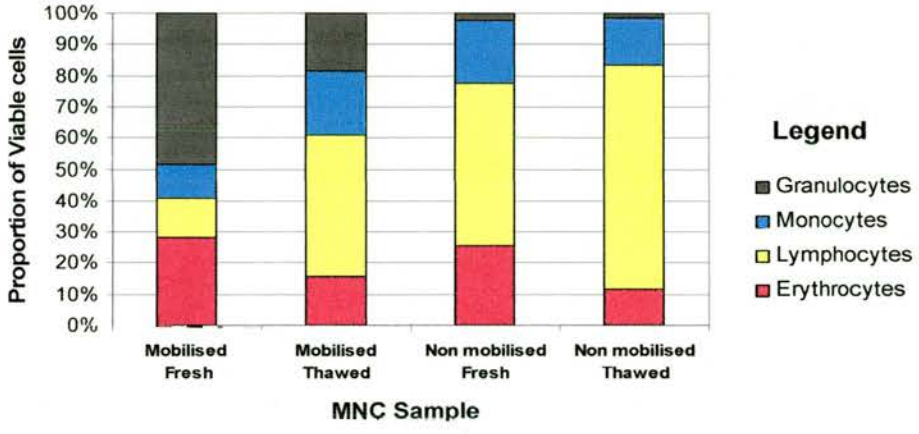
The proportions of leukocytes subpopulations and CD235a$^+$ erythrocytes were determined in freshly MNC and in thawed MNC. The same definitions for cell populations were used as were employed in other analyses (see section 5.1.1). CD66b$^+$ granulocytes were defined as events gating within the live cell gate co-expressing CD45 and CD66b. CD14$^+$ monocytes were defined as events gating within the live cell gate co-expressing CD45 and CD14. CD235a$^+$ erythrocytes (red blood cells) were defined as CD45$^-$ events expressing CD235a$^+$ (Glycophorin A) and falling outside of the live cell gate. Lymphocytes were small (low forward and side light scatter), CD45$^+$ events gating within the live cell gate and not expressing CD66b, CD14 or CD235a.

5.6.3.1. Fresh MNC compared to thawed MNC

Analysis of the proportions of leukocyte subpopulations and CD235a$^+$ erythrocytes in fresh MNC and thawed MNC from non-mobilised and mobilised autologous PBSC patients showed significant differences between MNC (Figure 5.19).

5.6.3.1.1. Non-mobilised MNC

In non-mobilised samples, there were no statistically significant changes in the proportions of leukocyte subpopulations between fresh and thawed MNC (paired analysis, n=10). There was a significant reduction in the proportion of CD235a$^+$ erythrocytes (18.4% and 14.0% in fresh MNC and thawed MNC respectively, $p<0.02$) with a relative increase in the proportion of lymphocytes (37.9% and 50.6% respectively, p=ns) between fresh and thawed MNC. Less marked changes were noted in the proportions of CD14$^+$ monocytes (14.4% and 11.1%, p=ns) and CD66b$^+$ granulocytes (1.6% and 1.0%, p=ns) (Figure 5.19).



Cell Type	Mobilised MNC		Non-mobilised MNC	
	Fresh	Thawed	Fresh	Thawed
CD66b$^+$ Granulocytes	43.2%	9.9%	1.6%	1.0%
CD14$^+$ Monocytes	9.7%	10.9%	14.4%	11.1%
Lymphocytes	11.1%	24.2%	37.9%	50.6%
CD235a$^+$ Erythrocytes	25.3%	8.3%	18.4%	14.0%

Figure 5.19 Leukocyte subpopulations and CD235a$^+$ erythrocytes in fresh or thawed MNC. Samples obtained from non-mobilised and mobilised PBSC samples. Figures are expressed as proportions of all events.

5.6.3.1.2. Mobilised MNC

Overall, comparing the distribution of leukocytes between fresh and thawed G-CSF mobilised MNC in paired analysis (n=7) showed that there was little change in the proportion of $CD14^+$ monocytes (9.7% in fresh MNC and 10.9% in thawed MNC, p=ns). However, freezing resulted in a reductions in the proportions of $CD66b^+$ granulocytes (43.2% and 9.9% respectively, $p<0.02$) and $CD235a^+$ erythrocytes (25.3% and 8.3% respectively, p=ns) with a concomitant increase in lymphocytes (11.1% and 24.2%, respectively, $p<0.02$) (Figure 5.19).

5.6.3.1.3. Non-viable cell populations

When MNC were thawed, reductions in the proportions of viable cells in the samples were noted. Non-viable cells displayed altered light scatter properties (low FSC/low SSC) and cells were not contained within the live cell gate. They generally expressed CD45 together with lineage specific markers, but the intensity of fluorescence was reduced compared to intact cells. These non-viable cells appeared to be mainly granulocytic or monocytic in origin, as evidenced by CD66b or CD14 expression on these events, but they were excluded from cell counts.

5.6.3.2. Conclusions

Thawed mobilised MNC contained lower proportions of $CD66b^+$ granulocytes than fresh MNC; approximately a third of the $CD66b^+$ granulocytes of the comparative fresh mobilised MNC. There was little change in the proportion of $CD14^+$ monocytes between fresh and thawed MNC. The lymphocyte populations appeared to be quite robust. Thawed MNC contained relatively higher proportions of lymphocytes than fresh MNC largely due to the loss of viable $CD235a^+$ erythrocytes and $CD66b^+$ granulocytes after freezing. Thawed MNC (non-mobilised and mobilised) contained lower proportions of $CD235a^+$ erythrocytes than fresh MNC.

5.6.4. Flow cytometric analysis of proportions of $CD133^+$ and $CD34^+$ stem cells

Stem cells were defined as events gating within the live cell gate displaying light scatter characteristics similar to that of lymphocytes (low FCS and low SSC) and

expressing CD133 or CD34 (section 6.1). By definition, $CD34^+$ events, using ISHAGE guidelines, are all $CD45^+$ (191). The analysis of the stem cell phenotypes of autologous and allogeneic PBSC donors undergoing PBSC mobilisation with G-CSF^+/-chemotherapy presented in chapter 6 showed that there were no differences between stem cell populations when analysed as proportions of $CD45^+$ stem cells gating within the live cell gate (Stems CD45+R1) or as proportions of stem cells gating within the live cell gate (and not considering CD45 expression) (Stems R1). To include any $CD45^-/34^+$ or $CD45^-$ stem cells present within mobilised samples, stem cells were analysed and the data presented in this section either as proportions of all events or proportions of Stems R1 (Figure 6.1).

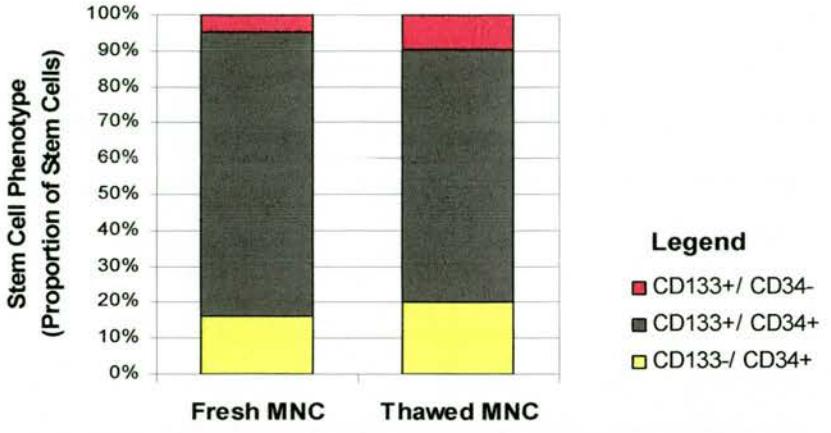
5.6.4.1. Proportions of stem cells

Analysis of the proportions of stem cells in mobilised samples showed that thawed MNC contained significantly greater stem cells (as proportions of all events) than fresh MNC (3.2% and 0.8% respectively, $p<0.01$). Thawed MNC contained greater proportions of $CD34^+$ (2.3%) and $CD133^+$ cells (3.0%) than fresh MNC (CD34 0.7%, $p<0.01$ and CD133 0.7%, $p<0.02$). Populations of KDR^+ stem cells were not detected in either patient group. Non-mobilised thawed MNC contained greater proportions of $CD34^+$ cells (0.31% and 0.15% in thawed and fresh MNC respectively), $CD133^+$ cells (0.28% and 0.21% respectively) and stem cells (0.48% and 0.23% respectively) than fresh MNC but these differences were not statistically significant (p=ns).

5.6.4.2. Distribution of stem cell phenotypes

Analysis of stem cell phenotypes in mobilised fresh MNC and mobilised thawed MNC confirmed that there were no significant differences in the proportions of $CD133^+/34^-$, $CD133^+/34^+$ and $CD133^-/34^+$ stem cells between fresh and thawed mobilised MNC (p=ns for all comparisons). Fresh MNC contained on average 4.8% $CD133^+/34^-$, 78.8% $CD133^+/34^+$ and 16.4% $CD133^-/34^+$ stem cells whilst thawed MNC contained 9.6% $CD133^+/34^-$. 70.6% $CD133^+/34^+$ and 20.2% $CD133^-/34^+$ stem cells (Figure 5.20). The stem cell phenotypes in this experiment were very similar to mean values presented for all G-CSF mobilised autologous PBSC samples (section

6.1.8.2.2 and Table 6.6). There were no significant differences in stem cell phenotypes between fresh and thawed non-mobilised MNC. Fresh MNC contained 38.4% $CD133^+/34^-$, 44.1% $CD133^+/34^+$ and 18.5% $CD133^-/34^+$ stem cells whilst thawed MNC contained 25.9% $CD133^+/34^-$. 45.8% $CD133^+/34^+$ and 29.0% $CD133^-/34^+$ stem cells (p=ns for all comparisons).



Day 0 MNC	Stem cell phenotype (mean proportions of stem cells)		
MNC	$CD133^+/34^-$	$CD133^+/34^+$	$CD133^-/34^+$
Fresh MNC	4.9%	78.8%	16.4%
Thawed MNC	9.5%	70.5%	20.0%
Autologous mobilised MNC*	6.1%	72.4%	21.8%

Figure 5.20 Stem cell phenotypes in fresh and thawed mobilised MNC. Distribution of $CD133^-/34^+$, $CD133^+/34^+$ and $CD133^-/34^+$ stem cells shown. Figures are expressed as mean proportions of stem cells.

* Comparative data from all G-CSF moblised autologous PBSC patients (table 6.6)

5.6.5. Conclusions

It was found that CFU-EPC could not be consistently generated from thawed MNC even when the comparative fresh MNC sample displayed moderate CFU-EPC activity. In samples that generated CFU-EPC from both fresh and thawed MNC, marked reductions in CFU-EPC frequency occurred in thawed MNC. On this basis the use of thawed MNC for CFU-EPC assessment was not pursued further.

The proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes in thawed MNC differed substantially from fresh MNC. Significant reductions in the

proportions of $CD66b^+$ granulocytes and increases in lymphocyte proportions were noted after thawing mobilised patient samples. Substantial reductions in the proportions of $CD235a^+$ erythrocytes were observed, statistically significant in non-mobilised samples, with no changes in the proportions of $CD14^+$ monocytes occurring. There were no differences between fresh MNC and thawed MNC in the distribution of CD34 and CD133 on stem cells. Thawed MNC contained significantly greater proportions of stem cells, $CD34^+$ cells and $CD133^+$ cells than fresh MNC, likely a reflection of losses of other less robust cell populations during freezing.

5.7. Addition of autologous plasma to CFU-EPC colony assays can influence colony forming activity

Results of co-plating experiments using Thinsert inserts™ suggested that soluble factors and cellular factors could influence CFU-EPC. The contribution of secreted/soluble factors released into the plasma by cells (mainly neutrophils) stimulated by G-CSF was assessed by the addition of plasma, obtained from non-mobilised and mobilised patients to CFU-EPC cell cultures. Non-mobilised plasma was added to CFU-EPC culture to explore whether any effects on CFU-EPC were specifically related to G-CSF stimulation.

5.7.1. Method

Venous blood samples were collected into lithium heparin anticoagulated blood collection tubes. Following centrifugation of the sample, the plasma was collected and reserved. Autologous patient plasma (autologous referring to plasma and MNC being obtained from the donor) was added in various concentrations (2-10%) to wells containing MNC, plated into either the CFU-Hill or the CFU-Hill(direct) assay. When MNC numbers were limited, CFU-EPC was assessed using CFU-Hill(direct) assays only. In CFU-Hill assays plasma was added to CECM in the specified concentrations on day 0 when freshly isolated MNC were plated into 6 or 12 well fibronectin-coated plates and also when re-plating non-adherent MNC on day 2.

5.7.2. CFU-EPC formation

5.7.2.1. CFU-EPC in non-mobilised samples

Autologous non-mobilised patient plasma was added to 3 non-mobilised MNC samples (patients 276, 278, 280). Patients who formed CFU-EPC using standard culture methods (2 of 3 patients) displayed enhanced CFU-EPC formation following the addition of 2% autologous patient plasma to the CECM in the culture wells.

Patient 280- MNC were plated into CFU-Hill and CFU-Hill(direct) assays. In the direct plated assay peak CFU-EPC was recorded with the addition of 2% plasma whilst in the CFU-Hill assay peak CFU-EPC occurred with the addition of 5% autologous plasma. However, some CFU-EPC activity was retained within the day 2 adherent MNC suggesting that the CFU-EPC activity with 2% plasma might have been curtailed on account of this (Table 5.26).

Proportions of autologous plasma		0%	2%	5%	10%
CFU-EPC (per 10^6 MNC)	CFU-Hill(direct)	1	15	5	5
	CFU-Hill day 2 non-adherent MNC	0	10	15	—
	CFU-Hill day 2 adherent MNC	0	2	1	—

Table 5.26 CFU-EPC on addition of non-mobilised plasma to non-mobilised MNC. (Patient 280).

Patient 278- CFU-EPC were not formed from baseline (0% plasma) culture or on the addition of autologous plasma.

Patient 276- CFU-EPC (CFU-Hill(direct) assay) increased following the addition of 1% or 2% autologous plasma; 4 CFU-EPC/10^6 MNC with 0% plasma, increasing to 19 CFU-EPC/10^6 MNC with 2% plasma. Escalation of plasma concentrations above 2% (5% and 10%) did not result in CFU-EPC. In this case, CFU-EPC activity in the CFU-Hill(direct) assay containing 2% autologous pre G-CSF plasma exceeded the CFU-EPC activity of MNC plated into the CFU-Hill assay (15 CFU-EPC/10^6 MNC, 0% plasma) (Table 5.27).

CFU-EPC assay	CFU-Hill(direct)					CFU- Hill
% Autologous plasma	0%	1%	2%	5%	10%	0%
CFU-EPC (per 10^6 MNC)	4	14	19	0	0	15

Table 5.27 CFU-EPC on addition of non-mobilised plasma to non-mobilised MNC. (Patient 276).

5.7.2.2. CFU-EPC in G-CSF mobilised samples

Inconsistent changes in CFU-EPC activity were found on the addition of autologous (mobilised) plasma (n=2). CFU-EPC numbers were marginally increased following the addition of autologous mobilised plasma to mobilised MNC in 1 case (patient 273) whilst in the other addition of autologous mobilised plasma to mobilised MNC failed to change (increase) CFU-EPC activity (patient 279).

Patient 273 – Mobilised MNC plated into CFU-Hill(direct) assays recorded no CFU-EPC with 0% autologous mobilised plasma (0 CFU-EPC/10^6 MNC) but 1 CFU-EPC/10^6 MNC with 2% autologous mobilised plasma. In this case co-plating of mobilised MNC with autologous non-mobilised plasma (at a concentration of 2% plasma) enhanced CFU-EPC activity to 6 CFU-EPC/10^6 MNC but CFU-EPC remained well below that of the paired non-mobilised MNC sample (29 CFU-EPC/10^6 MNC). Other levels of plasma content were not assessed. Of interest, increased spindle formation, whereby increasing proportions of individual cells adopted a spindle-shaped morphology, was noted in the co-plated well containing autologous non-mobilised plasma and mobilised MNC. This is in contrast to the appearance of wells containing autologous mobilised plasma and mobilised MNC which did not display significant spindle formation.

Patient 279- Addition of autologous mobilised plasma to mobilised MNC failed to increase CFU-EPC. Mobilised MNC plated into the CFU-Hill assay produced 2 CFU-EPC/10^6 MNC but cells in the CFU-Hill(direct) assay failed to generate any colonies Addition of autologous mobilised plasma (2% and 5% concentrations)

failed to increase CFU-EPC. Non-mobilised autologous plasma was not added to mobilised MNC in this case.

5.7.3. Conclusions

These experiments indicate that autologous non-mobilised plasma has some effect on CFU-EPC formation. Autologous plasma produced enhancement of CFU-EPC activity in non-mobilised samples. Interestingly, the addition of non-mobilised plasma to mobilised MNC had greater effect on CFU-EPC activity than the addition of mobilised plasma to mobilised MNC, though the level of colony formation was still below that observed in the paired non-mobilised sample.

These findings could be interpreted as providing supportive evidence for the importance of soluble/secreted factors to CFU-EPC development. It is speculative as to whether non-mobilised patient plasma could enhance CFU-EPC formation by neutralising or antagonising some factor present in mobilised MNC cell culture.

5.8. Discussion

The proportions of leukocyte subpopulations in whole blood and MNC (day 0 and day 2) differ significantly between non-mobilised and mobilised PBSC donor samples. Significant differences were also present in the proportions of $CD235a^+$ erythrocytes present in both MNC obtained from non-mobilised and mobilised PBSC donors and samples obtained from autologous PBSC patients which also contained greater proportions of $CD235a^+$ erythrocytes than samples from allogeneic PBSC donors. Although a number of groups (including our own) have explored and commented on the importance of monocytes to the development of CFU-EPC (93, 130-132, 136, 139, 141) our data showed that the reduction in CFU-EPC following G-CSF administration was not attributable to reductions in MNC $CD14^+$ monocyte content. Day 0 and day 2 MNC obtained from mobilised patient samples contained greater proportions of $CD14^+$ monocytes than comparative non-mobilised day 0 and day 2 MNC. Mobilised peripheral blood and MNC contained lower proportions of lymphocytes, T cells and $CD3^+/31^+/CXCR4^+$ 'angiogenic' T cells than non-mobilised samples. A number of studies have described interactions between T cells and monocytes in initiation of colony formation (135, 137, 142) which might be relevant to the decline in CFU-EPC following G-CSF.

The lack of CFU-EPC formation in non-mobilised MNC following MACS immunomagnetic bead separation for CD14 could possibly be a reflection of the relatively high purity of $CD14^+$ cells in these cultures, has been suggested by van Been et al (135) to result in loss of CFU-EPC formation. This is not supported by earlier work using non-mobilised blood in our laboratory (136) or by enhanced CFU-EPC when non mobilised MNC from healthy volunteers were co-cultured with CD14-enriched MNC.

It appeared that the most significant difference between non-mobilised and mobilised samples was the CD66b$^+$ granulocyte content of mobilised samples. Using PMP density gradient centrifugation, MACS immunomagnetic MNC separations and cryopreservation of MNC the affects of alterations to the CD66b$^+$ content of non-mobilised and mobilised MNC on CFU-EPC formation were assessed. CD66b$^+$ granulocyte enriched MNC fractions failed to show any CFU-EPC development. The series of co-plating experiments (using CD66b$^+$ granulocyte enriched cells obtained from PMP separation of whole blood or CD66b MACS immunomagnetic separations of MNC) showed that the addition of non-mobilised or mobilised CD66b$^+$ granulocytes to CFU-Hill or CFU-Hill(direct) assays affected colony formation. Interestingly, mixing CD66b$^+$ granulocytes in direct contact with MNC reduced CFU-EPC whilst addition of these cells in a ThinCert™ insert either enhanced CFU-EPC (non-mobilised CD66b$^+$ granulocytes) or reduced CFU-EPC (mobilised CD66b$^+$ granulocytes). Similarly, MACS immunomagnetic separations plating CD66b$^+$ enriched or depleted MNC into CFU-EPC assays in isolation or mixed with MNC showed that CD66b$^+$ granulocyte depletion can increase CFU-EPC in samples obtained from mobilised patients. Cryopreservation of mobilised MNC reduced the CD66b$^+$ granulocyte and CD235a$^+$ erythrocyte content of MNC. Thawed mobilised MNC failed to form CFU-EPC but CFU-EPC were seen in some thawed non-mobilised MNC. It might be that the initiator of CFU-EPC is sensitive to freeze/thaw or that the responder cells (or both) are affected by this process.

There is evidence that the effects of CD66b$^+$ granulocytes of CFU-EPC extend beyond their physical presence in the culture well and that factors released from these cells influences CFU-EPC. Preliminary studies investigating the effects of autologous plasma from non-mobilised and G-CSF patients showed effects of 2% non-mobilised plasma in enhancing CFU-EPC of non-mobilised and mobilised MNC.

The stimulation of granulocytes by G-CSF results in the release of enzymes which have important roles in the mobilisation of HSC and HPC from the BM into the circulation. G-CSF stimulated granulocytes will continue to be metabolically active in culture and secreted factors will affect the behaviour of MNC. G-CSF induces alterations in cell adhesion molecules including L-selectin, VCAM-1 expressed on $CD34^+$ cells which contribute to their mobilisation from the BM. G-CSF might also alter the monocyte expression of these cells and through this affect the ability of MNC (including monocytes) to adhere to fibronectin, a key factor in the development of CFU-EPC. The influence of G-CSF on the expression of adhesion molecules by leukocytes is now being investigated. It is suggested that G-CSF could either affect CFU-EPC responder or initiator cells directly (e.g. the down regulated expression of receptors on $CD14^+$ monocytes) or indirectly, through its effects on myeloid cells that secondarily influence other leukocytes including $CD14^+$ monocytes. Evidently there is an interaction between the plated cells and the fibronectin coating the culture plates required in order for the CFU-EPC assay to generate colonies. If this stimulus cannot take place colonies will then not form. Fibronectin receptor down-regulation might therefore affect the ability to make CFU-EPC and investigation of G-CSF-induced changes in molecules involved in fibronectin adhesion is now being undertaken within the laboratory.

6. Flow cytometric analysis of putative EPC phenotypes in patients undergoing G-CSF PBSC mobilisation

6.1. Analysis of non-mobilised and mobilised PBSC samples

6.1.1. Introduction

The flow cytometric characterisation of samples obtained from autologous and allogeneic donors undergoing PBSC mobilisation for the presence of cells exhibiting putative endothelial cell phenotypes is presented in section 6.1. Similar to previous chapters, samples were collected from patients on three occasions; at donor review prior to PBSC mobilisation (non-mobilised or pre G-CSF sample), at day 1 PBSCH (mobilised or post G-CSF sample) and at donor follow-up 4-6 weeks following PBSCH (donor follow-up sample). In section 6.1 at least 100,000 events gating in the live cell gate were acquired from each tube. Whole blood samples and MNC were examined for the presence of cells exhibiting putative endothelial cell phenotypes with day 0 and day 2 MNC assessed as described in section 5.1. In this chapter, day 2 MNC refers specifically to MNC that remained non-adherent to fibronectin after 2 days of incubation.

6.1.1.1. Defining haematopoietic $CD34^+$ cells and stem cells

Haematopoietic $CD34^+$ cells

The International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines were established for enumerating $CD34^+$ events to determine the number of HSC and HPC within patient samples. A combination of side light scatter (SSC), forward light scatter (FSC), CD45 antigen expression and CD34 antigen expression is used to identify all low SSC $CD45^+/34^+$ events and to exclude other nucleated cells, erythrocytes, platelets and debris from the calculation of $CD34^+$ events (191). CD34 enumeration can be carried out as a single or dual platform analysis (193), with the dual platform method using the patient's peripheral blood leukocyte count to convert a proportional CD34 count into an absolute value. The immunophenotypes

of HPC and other precursor cells are well documented and this knowledge is used clinically to identify normal and abnormal cell populations.

Stem cells

'Stem cells' were defined as events gating within the live cell gate displaying light scatter characteristics similar to that of lymphocytes and expressing CD133 and/or CD34, and therefore not restricted to $CD45^+$ events as identified using ISHAGE guidelines. The term 'stem cells' does not specifically relate to haematopoietic stem cells though the vast majority of cells identified by flow cytometric analysis were haematopoietic according to their expression of CD45.

6.1.1.2. Stem cell populations

There were no difficulties in identifying discrete $CD34^+$ and/or $CD133^+$ 'stem' cell populations in samples from mobilised patients (Figure 6.1). Stem cells were rare events in normal (non-mobilised) samples. In some cases, even with acquisition of >150,000 events during flow cytometry, insufficient cells were identified as stem cells in non-mobilised samples for stem cell immunophenotyping to be performed (Table 6.1).

Cells	PBSC Patient Sample	Timing of Sample		
		Pre G-CSF	Mobilised	Follow-up
Whole blood	Allogeneic donor	10 (9)	9 (9)	8 (7)
	Autologous donor	35 (29)	29 (29)	7 (7)
	All PBSC patients	45 (38)	38 (38)	15 (14)
Day 0 MNC	Allogeneic donor	15 (15)	12 (12)	12 (12)
	Autologous donor	38 (30)	30 (30)	7 (7)
	All PBSC patients	53 (45)	42 (42)	19 (19)
Day 2 MNC	Allogeneic donor	9 (5)	7 (7)	6 (3)
	Autologous donor	28 (16)	24 (24)	4 (2)
	All PBSC patients	37 (21)	31 (31)	10 (5)

Table 6.1 Allogeneic and autologous PBSC donor samples analysed in section 6.1. Numbers of whole blood, day 0 MNC and day 2 MNC samples, with numbers of evaluable samples in parentheses.

6.1.2. Stem cell gating strategies

6.1.2.1. Stems CD45+R1 versus Stems R1 gates

When only haematopoietic progenitor cells (HPC) are being assessed, CD45 expression should be included in an antibody panel used to define HPC by flow cytometry. However, the data presented in this section is not restricted to examination of HPC and considers any non-haematopoietic stem cells. If gating strategies that included CD45 expression as part of the obligatory set of antigens for the assignment of $CD34^+$ or stem cell status to identify the stem cell populations, $CD45^-$ stem cells expressing CD133 and/or CD34 would be missed.

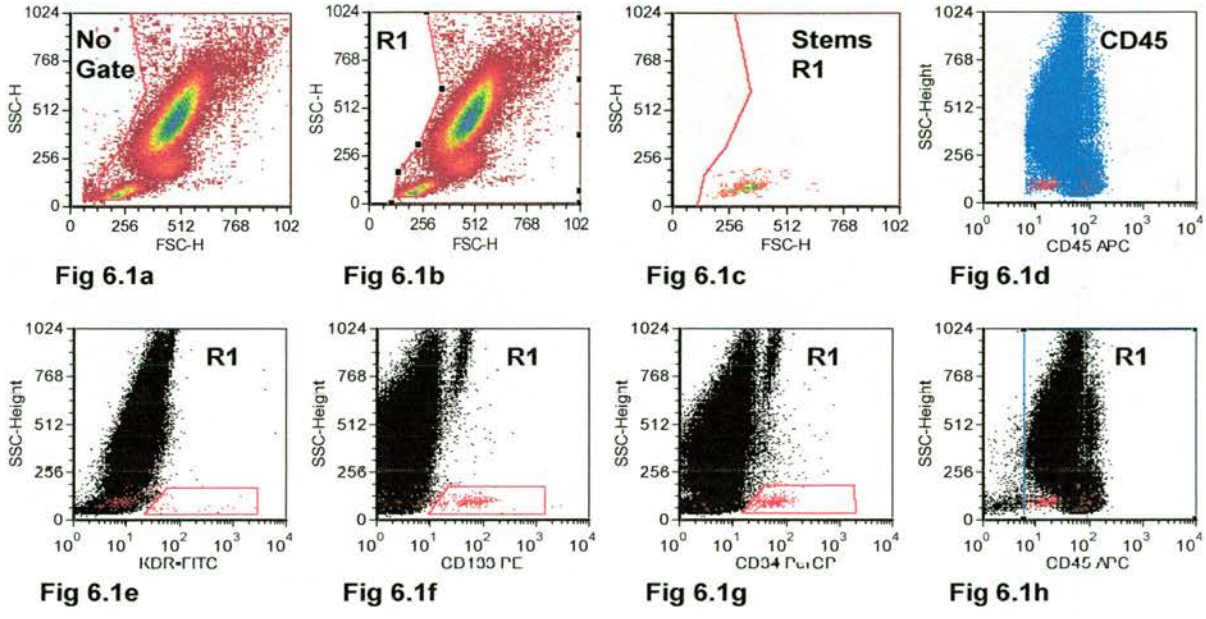


Figure 6.1Representative flow cytometry dot plots from a G-CSF mobilised PB sample.

Figure 6.1a FSC v SSC of all events (Fig 6.1a) with live cell gate indicated (in red outline).
Figure 6.1b FSC v SSC of R1 gated events in figure 6.1a.
Figure 6.1c FSC v SSC of Stems R1 cells identified in figures 6.1f and 6.1g (Stems R1 gate).
Figure 6.1d CD45-APC expression of stem cells (red), figure gated on CD45.
Figures 6.1e, 6.1f, 6.1g and 6.1h - KDR-FITC, CD133-PE, CD34-PerCP and CD45-APC expression of events gating in R1 (figure 6.1b).

Polygonal stem cell gates were used to restrict the analysis/area of interest to events of similar size to lymphocytes (which includes stem cells) within the live cell gate (Stems R1).
Stem cells are $CD34^+$ and/or $CD133^+$ events gating in the Stems R1 region.
Stem cells (shown in red) are $CD45^{l}$ but KDR^-.

6.1.2.1.1. Data presentation

To ensure that any $CD45^{-}/34^{+}$ cells, $CD45^{-}/133^{+}$ cells or $CD45^{-}$ stem cells present within a sample were not overlooked, these cells were defined using light scatter characteristics and CD34 and/or CD133 antigen expression, to define low SSC events within the live cell gate (Stems R1 gate) expressing CD34 and/or CD133 (Figure 6.1 and Figure 6.2). $CD34^{+}$ events and stem cells were also identified using the parameters established in the ISHAGE guidelines.

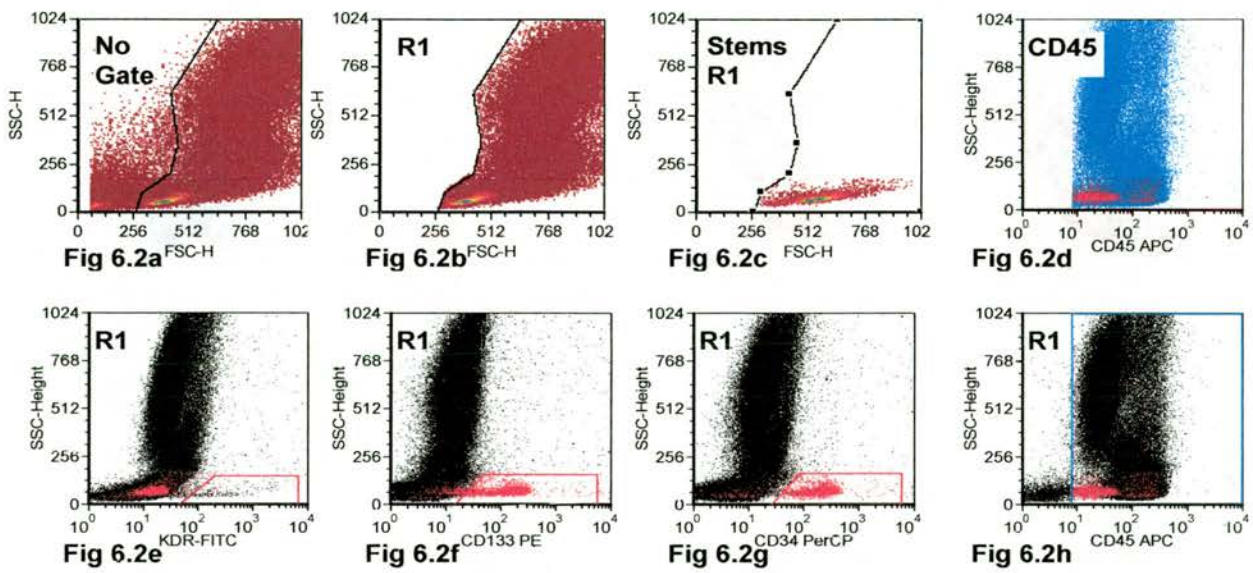


Figure 6.2 Representative flow cytometry dot plots from day 0 G-CSF mobilised MNC. Mobilised MNC sample contains proportions of $CD66b^{+}$ granulocytes and $CD45^{-}$ erythrocytes.

Figure 6.2a FSC v SSC of all events (Fig 6.1a) with live cell gate indicated (in black outline).
Figure 6.2b FSC v SSC of R1 gated events in figure 6.2a.
Figure 6.2c FSC v SSC of Stems R1 identified in figures 6.2f and 6.2g (Stems R1 gate).
Figure 6.2d CD45-APC expression of stem cells (red), figure gated on CD45.
Figures 6.2e, 6.2f, 6.2g and 6.2h - KDR-FITC, CD133-PE, CD34-PerCP and CD45-APC expression of events gating in R1 region (figure 6.2b).

Polygonal stem cell gates were used to restrict the analysis/ area of interest to events of similar size to lymphocytes (which includes stem cells) within the live cell gate (Stems R1 gate). Stem cells, identified by their expression of CD133 and/or CD34 (shown in red) are $CD45^{+}$ but KDR^{-}. KDR^{+} events (figure 6.2e) were neither $CD133^{+}$ nor $CD34^{+}$.

6.1.3. Proportions of $CD34^+$ stem cells

Analysis of the proportions of $CD34^+$ cells in non-mobilised and mobilised PBSC patient samples showed that the proportions of $CD34^+$ cells increased in whole blood (0.07% versus 0.60%), day 0 MNC (0.12% versus 0.92%) and day 2 MNC (0.05% versus 0.20%) following the administration of G-CSF+/-chemotherapy ($p<0.0001$, $p<0.0001$ and $p<0.01$ for CD34 for whole blood day 0 and day 2 MNC respectively) (Stems R1 gate). The changes in the proportions of $CD34^+$ cells following G-CSF remained significant at the 3 time points when allogeneic and autologous PBSC patients were considered separately. The populations of $CD34^+$ cells mobilised by the administration of G-CSF were clearly evident (Figure 6.1g and Figure 6.2g). There were no differences in the proportions of $CD34^+$ cells identified using Stems R1 or a Stems CD45+R1 gates (Table 6.2 and Table 6.3). There were no significant differences between the numbers of $CD34^+$ cells identified in pre G-CSF and donor follow-up samples (p=ns). There were no differences in the proportions of $CD34^+$ cells in non-mobilised samples from allogeneic and autologous PBSC patients in whole blood (0.06% and 0.08%), day 0 MNC (0.12% and 0.13%) and day 2 MNC (0.02% and 0.06%) (p=ns for the 3 time points).

Mobilised whole blood samples from autologous PBSC patients contained significantly greater proportions of $CD34^+$ cells (0.74%) than samples from allogeneic PBSC donors (0.09%, $p<0.005$). Although greater in MNC from mobilised autologous PBSC patients, there were no differences between patient groups in the proportions of $CD34^+$ cells in day 0 MNC (0.57% and 1.12% for allogeneic and autologous PBSC donors respectively, p=ns) or day 2 MNC (0.30% and 0.17% respectively, p=ns).

6.1.4. Proportions of $CD133^+$ stem cells

Analysis of the proportions of $CD133^+$ cells in non-mobilised and mobilised PBSC patient samples showed that the proportions of $CD133^+$ cells increased in whole blood (0.05% versus 0.50%), day 0 MNC (0.13% versus 0.74%) and day 2 MNC

(0.08% versus 0.48%) following the administration of G-CSF+/-chemotherapy ($p<0.0001$, $p<0.0001$, $p<0.0001$ for CD133 for whole blood day 0 and day 2 MNC respectively) (Stems R1 gate). The changes in the proportions of CD133+ following G-CSF remained significant at the 3 time points when allogeneic and autologous PBSC patients were considered separately. The populations of CD133+ cells mobilised by G-CSF administration were clearly evident (Figure 6.1f and Figure 6.2f). There were no differences in the proportions of CD133+ cells identified using Stems R1 gates (Table 6.2) or Stems CD45+R1 gates (Table 6.3). There were no significant differences between the numbers of CD133+ cells identified in pre G-CSF and donor follow-up samples.

Timing of samples	Time point	All PBSC patients		Allogeneic PBSC donors		Autologous PBSC patients	
		CD34	CD133	CD34	CD133	CD34	CD133
Pre G-CSF	Whole blood	0.07%	0.05%	0.06%	0.04%	0.08%	0.06%
	Day 0 MNC	0.12%	0.13%	0.12%	0.12%	0.13%	0.13%
	Day 2 MNC	0.05%	0.08%	0.02%	0.04%	0.06%	0.10%
G-CSF mobilised	Whole blood	0.60%	0.50%	0.09%	0.09%	0.74%	0.61%
	Day 0 MNC	0.92%	0.74%	0.57%	0.44%	1.12%	0.92%
	Day 2 MNC	0.20%	0.48%	0.30%	0.43%	0.17%	0.49%
Donor Follow-up	Whole blood	0.04%	0.03%	0.04%	0.03%	0.04%	0.03%
	Day 0 MNC	0.08%	0.08%	0.10%	0.10%	0.05%	0.04%
	Day 2 MNC	0.06%	0.06%	0.03%	0.03%	0.10%	0.09%

Table 6.2 Proportions of $CD34^+$ and $CD133^+$ cells in PBSC donor samples. Figures are expressed as proportions of all events (Stems R1 gate).

Timing of samples	Time point	All PBSC patients		Allogeneic PBSC donors		Autologous PBSC patients	
		CD34	CD133	CD34	CD133	CD34	CD133
Pre G-CSF	Whole blood	0.07%	0.05%	0.05%	0.04%	0.07%	0.03%
	Day 0 MNC	0.12%	0.12%	0.09%	0.11%	0.13%	0.13%
	Day 2 MNC	0.03%	0.08%	0.02%	0.03%	0.04%	0.07%
G-CSF mobilised	Whole blood	0.60%	0.5%	0.10%	0.09%	0.72%	0.60%
	Day 0 MNC	0.90%	0.71%	0.52%	0.43%	1.06%	0.91%
	Day 2 MNC	0.17%	0.44%	0.24%	0.40%	0.15%	0.44%
Donor Follow-up	Whole blood	0.04%	0.03%	0.04%	0.03%	0.01%	0.02%
	Day 0 MNC	0.05%	0.07%	0.06%	0.08%	0.04%	0.04%
	Day 2 MNC	0.02%	0.03%	0.06%	0.07%	0.06%	0.07%

Table 6.3 Proportions of $CD34^+$ and $CD133^+$ cells in PBSC donor samples. Figures are expressed as proportions of all events (Stems CD45+R1 gate).

There were no differences in the proportions of $CD133^+$ cells in non-mobilised samples from allogeneic and autologous PBSC patients in whole blood (0.04% and 0.06%), day 0 MNC (0.12% and 0.13%) and day 2 MNC (0.04% and 0.10%) (p=ns for the 3 time points). Mobilised whole blood samples from autologous PBSC patients contained significantly greater proportions of $CD133^+$ cells (0.61%) than samples from allogeneic PBSC donors (0.09%, $p<0.05$). However, there were no differences between patient groups in the proportions of $CD133^+$ cells in day 0 or 2 MNC (day 0 MNC 0.44% and 0.92% for allogeneic and autologous PBSC donors respectively, p=ns, and day 2 MNC 0.43% and 0.49%, p=ns).

6.1.5. Proportions of KDR^+ stem cells

A population of stem cells expressing KDR was not identified by flow cytometric analysis in this cohort of non-mobilised and mobilised PBSC patient samples. Using 'fluorescence minus 1' gating we failed to detect KDR expression above the levels contained in control tubes. Given the scarcity of stem cells in non-mobilised samples we did not anticipate being able to identify KDR^+ stem cells in these samples. The majority of mobilised samples had 100,000-200,000 events acquired for FACS analysis which were insufficient to detect KDR^+ stem cells.

6.1.5.1. KDR gating strategies

The expression of CD133 and CD34 antigens were restricted to stem cells, producing discrete cell populations that displayed similar FSC and SSC characteristics as lymphocytes (Figure 6.1). The expression of KDR, however, was not restricted to stem cells and was frequently detected on leukocytes, particularly $CD66b^+$ granulocytes and $CD14^+$ monocytes. This was particularly apparent in mobilised samples and was observed to be more pronounced on day 0 MNC than on whole blood samples, suggesting possible activation of KDR expression on leukocytes during cell handling. To ensure that a population of KDR^+ stem cells was not being overlooked, the proportions of stem cells expressing KDR were determined using

two gating strategies. In addition to detection of KDR expression using a 'stem cell' gate, set using negative controls and a 'fluorescence minus one' staining strategy (Figure 2.3) to define the positive region with light scatter characteristics similar to lymphocytes (KDR), a wider gate, which was set using unstained cells and CD45 expression (KDR_{WIDE}) was employed (Figure 6.3).

Large populations of leukocytes expressing KDR were detected when the KDR_{WIDE} gate was used. However, the expression of KDR by stem cells, defined by the expression of CD133 and/or CD34, was not altered by using this alternate KDR_{WIDE} gating and no increase in cells co-expressing CD133, CD34 and KDR was detected using KDR_{WIDE} gates (Figure 6.3). Unless otherwise stated, KDR expression was calculated (section 6.2) using polygonal stem cell gates, though for most samples KDR_{WIDE} gates were also examined. On account of the lack of a definable KDR^+ stem cell population (section 6.1.5) the latter half of section 6.1 examines the distribution of CD133 and CD34 (but not KDR) antigen expression by stem cells.

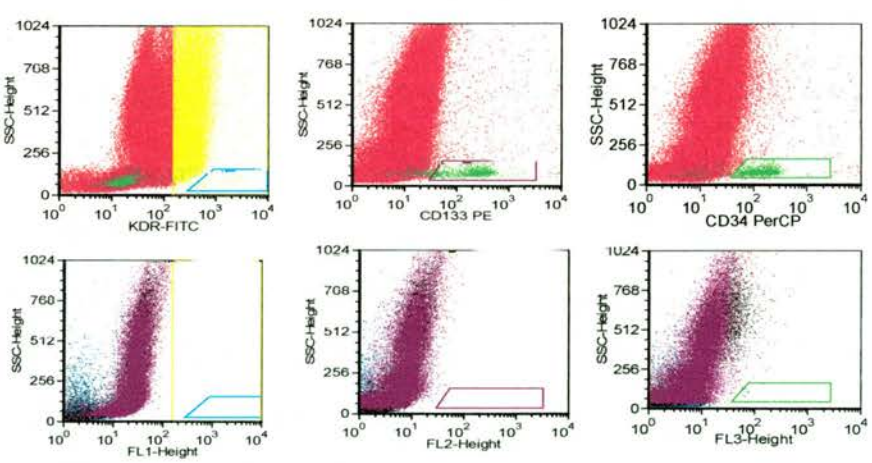


Figure 6.3 Flow cytometry dot plots of KDR expression in a G-CSF mobilised PB sample.

Upper figures show the expression of KDR-FITC (KDR_{WIDE} gates in yellow and KDR stem cell gates in blue), CD133-PE and CD34-PerCP by events gating in the live cell gate (red). The stem cell population, indicated in green, is clearly KDR^-.
Lower figures display FL1, FL2 and FL3 dot plots for unstained cells.

expression of KDR by UCB $CD34^+$ cells compared to mobilised PB $CD34^+$ cells. Differences in the expression of some myeloid antigens by $CD34^+$ cells were also noted with significantly lower expression of CD13 and CD33 identified on UCB $CD34^+$ cells.

Section 6.3 focussed on the identification of a novel population of $CD34^+$ cells ($CD34^{BRIGHT}$ cells) in UCB samples. In contrast to G-CSF mobilised samples, flow cytometry of UCB samples demonstrated $CD34^+$ cells with a differential of fluorescence intensity and $CD34^+$ cells were divided into $CD34^{REG}$ and $CD34^{BRIGHT}$ populations. When UCB samples and G-CSF mobilised peripheral blood samples were compared, it was found that $CD34^+$ cells in G-CSF mobilised blood formed a tight cell population with relatively low intensity CD34 expression, comparable to the $CD34^{REG}$ population seen in UCB samples. UCB $CD34^{REG}$ and UCB $CD34^{BRIGHT}$ cells differed significantly in the co-expression of stem cell related and other antigens. UCB $CD34^{BRIGHT}$ cell populations contained higher proportions of $CD45^-$ and $CD133^-$ cells, were more likely to express KDR but less likely to express CD38 than UCB $CD34^{REG}$ cells.

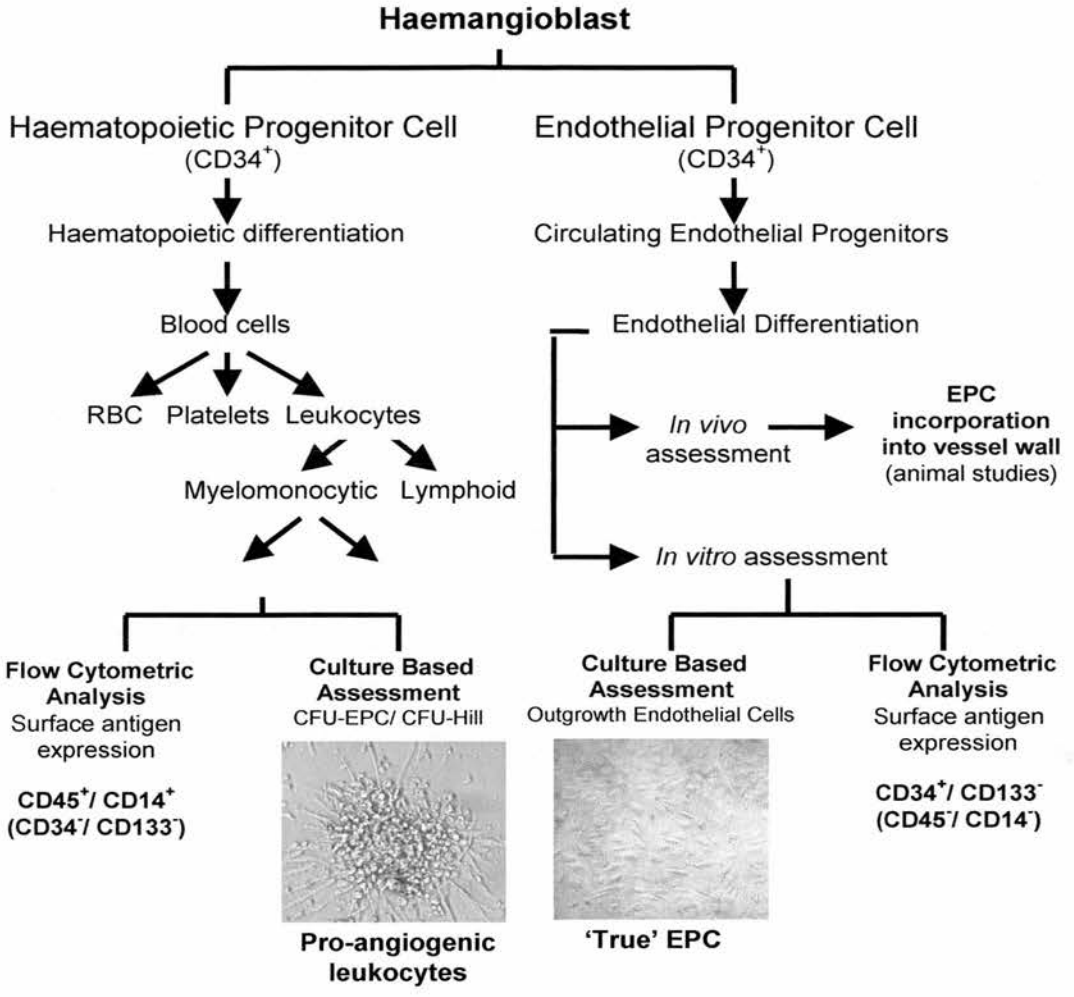
On the basis of higher proportions of $CD45^-$ and $CD133^-$ cells in UCB $CD34^{BRIGHT}$ cell populations it was investigated whether these cells could be circulating endothelial cells. Small numbers, but significantly greater proportions of cells were found with phenotypes consistent with CEC in UCB $CD34^{BRIGHT}$ cells compared to UCB $CD34^{REG}$ cells, suggesting consideration should be given as to whether EOC formation from $CD34^+/133^-$ cells could be attributed to the presence of CEC.

7. Discussion

The material presented in this thesis relate to the study of the effects of G-CSF on EPC, assessed using flow cytometric analysis and culture based colony assays, mainly CFU-Hill but also EOC. Recent opinion is that two broad types of EPC exist, as defined by cell culture and although these share some features (including lectin binding, ac-LDL uptake, CD31 expression) the cell types differ in their proliferative capacity, morphology and functional characteristics (93, 108, 131, 133, 134); summarised in Figure 7.1

Cells identified as EOC derived from the adherent fraction of MNC plated onto collagen are now believed to represent true EPC (115). Cells identified in short term culture on fibronectin coated plates are believed to be myelomonocytic cells of haematopoietic origin that may facilitate angiogenesis and vascularisation through their secretion of pro-angiogenic factors and paracrine activity (96, 131, 132, 142). A role for other inflammatory cells in vascular repair, particularly T cells and their interactions with monocytes has also been raised (135, 137, 142). Consequently, it is difficult to relate the results of earlier publications to our current framework of understanding of EPC and to compare the phenotypic and culture-based detection of EPC which do not appear to correlate with each other (169, 199).

At present, the term 'EPC' is still used to describe the haematopoietic cells identified in short term culture of MNC on fibronectin that contribute to neovessel formation through secretion of pro-angiogenic factors and paracrine activity. The use of 'progenitor' implies that these cells can differentiate into endothelial cells whereas it is becoming clear that some EPC (e.g. $CD14^+$ monocytes) cannot, but they may be pro-angiogenic. Perhaps 'pro-angiogenic cells' rather than EPC is a better term for such cells.



Characteristic	Early EPC (CFU-Hill)	Endothelial Outgrowth Cell
Cell of Origin	Haematopoietic (myelomonocytic)	Non-Haematopoietic
Essential Phenotype	$CD45^+/ CD14^+$	$CD45^-/ CD34^+/ CD133^-$
Formation CFU-Hill	Yes	No
Outgrowth Colony Formation	No	Yes
Incorporation into vessels	No	Yes
Paracrine effects	Yes	No

Figure 7.1 Suggested relationship between Endothelial Outgrowth Cells and early 'EPC'.

This body of work follows on from observations made by O Tura that CFU-EPC numbers declined following administration of G-CSF to patients mobilised with G-CSF+/-chemotherapy for PBSC collection (136). After first reviewing the characteristics of patients referred to our stem cell collection facility for PBSC mobilisation (Chapter 3) peripheral blood samples obtained from allogeneic and

autologous PBSC patients were studied and the initial observations were confirmed (Chapter 4). CFU-EPC frequency, assessed using a commercially available CFU-EPC assay (CFU-EPC, Stem Cell Technologies) according to manufacturer instructions, varied considerably in normal healthy subjects (allogeneic PBSC donors) and in haematology patients referred for consideration of autologous PBSC collection. A proportion of healthy donor and non-mobilised patient samples failed to generate CFU-EPC in the absence of G-CSF or chemotherapy exposure. The administration of G-CSF for PBSC mobilisation (given alone, sequentially with chemotherapy or with AMD3100) profoundly reduced CFU-EPC. The effect of G-CSF was consistently demonstrated for both allogeneic and autologous PBSC donors, regardless of PBSC mobilisation regimen used. The decline in CFU-EPC following G-CSF was reversible, as follow-up samples obtained 4-6 weeks after PBSC mobilisation showed recovery of CFU-EPC frequency to pre mobilisation levels. Whilst G-CSF produced a significant leukocytosis in allogeneic PBSC donors, peripheral blood leukocyte counts remained within the normal range following PBSC mobilisation in autologous PBSC patients, indicating that the reduction of CFU-EPC in mobilised MNC was not merely due to the dilution of colony-forming cells within an increasingly cellular peripheral blood.

These findings were consistent with and extend the previous results presented by our group(136) but are contrary to a number of studies, published in the setting of G-CSF mobilisation of EPC in patients with cardiovascular disease (163, 165, 167), patients undergoing PBSC mobilisation (162, 170) or healthy volunteers (167, 189). These studies assessed early EPC in fibronectin adherent (165, 189) or non-adherent MNC fractions (162, 163, 167, 170). All introduced modifications from the methods published by Hill et al(110) and the commercially available assay kit (CFU-EPC, Stem Cell Technologies) or identified EPC in fibronectin adherent MNC, so it is difficult to make direct comparisons between these studies and to compare directly with the work presented here. No other study has described the reduction in

CFU-EPC following G-CSF administration for HPC mobilisation that we have consistently observed.

The assay published by Hill *et al*, designed to assess spontaneous rather than stimulated colony formation, plated freshly isolated MNC onto fibronectin coated plates in Medium 199 supplemented with 20% foetal calf serum (FCS) and streptomycin without additional growth factors, and day 2 fibronectin non-adherent MNC were transferred to fresh fibronectin plates(110). The availability of a commercial CFU-EPC assay (Stem Cell Technologies, UK) that, when followed according to manufacturer instructions, would produce reproducible and comparable results between different laboratories, and overcome variations in results due to differences in FCS, produced a large number of reports from different laboratories in changes in CFU-EPC in a variety of clinical conditions (110, 111, 135, 137, 199, 200). This assay was chosen for this study on this basis.

Variations in CFU-EPC assays, particularly in time (approaching EOC timescale) and with the addition of cytokines that possibly counteract the G-CSF effect but certainly abrogate the spontaneous colony formation that Hill stresses is the basis for the assay, mean that results cannot be compared to other reports or interpreted readily. Three studies (162, 163, 167) used Medium 199 supplemented with 20% FCS but differed in the timing of CFU-EPC assessment, MNC replating and changing of the media. Three other studies of the effects of G-CSF on EPC failed to assess spontaneous colony formation as growth factors, particularly VEGF, were added to cultures (165, 170, 189). The study by Allen *et al* supplemented RPMI not only with 20% FCS and heparin but with endothelial cell growth factor (170). Honold *et al* assessed EPC in fibronectin adherent MNC using Medium 199 supplemented with FCS and atorvastatin, an agent reported to increase CFU-EPC (201) for the initial 3 days of primary culture with adherent day 3 MNC then cultured in methylcellulose in the presence of VEGF for a further 2 weeks (165).

A single healthy volunteer study (Shepherd *et al*) that reported increased PB EPC numbers following either AMD3100 or G-CSF failed to assess spontaneous colony formation as the authors used VEGF supplemented cultured medium to assess EPC in fibronectin adherent MNC (189). In this study EPC frequency was presented per mL blood rather than per 10^6 cells plated, making it difficult to establish the role of G-CSF or AMD3100 in increasing the proportions of CFU-EPC forming cells independently of any cytokine induced leukocytosis (which would occur in healthy volunteers) altering CFU-EPC per unit volume of peripheral blood. A murine study (190), found that pre-treatment with VEGF increased AMD3100 mobilisation of EPC and suggested that HPC and EPC were differentially mobilised. However, like the previous study, the CFU-EPC assay used by Pitchford *et al* plated MNC onto fibronectin in supplemented EGM2 media to which additional VEGF was added and, although cultured on fibronectin, the cells cultured represented late outgrowth colonies (190). An earlier study, again using a murine model, assessed clinical outcomes without phenotypic or culture based EPC assessments (202). However, the G-CSF and AMD3100 doses administered (190, 202) were not comparable those used in human studies (189). Also, since 'EPC' remain poorly and inconsistently defined, it is not certain that mouse and human studies are equivalent.

The CFU-EPC assay as described by Hill *et al* (110) relies on colony morphology rather than phenotype for measurement of colony frequency. Although the results for CFU-EPC presented in this thesis reflected the observations of an individual, the change in CFU-EPC following G-CSF administration has been observed independently by a number of group members. Photographs of CFU-EPC cultures have been reviewed by other group members to reach similar conclusions as how to 'score' cell aggregates. The recommendations by Hill and accompanying the commercial reagents were used and these criteria have been stringently applied in our assessments. Concerns remain that representative published images of CFU-EPC from G-CSF mobilised MNC (163) are not consistent with the colony morphology reported for CFU-EPC (110). In scoring cells as colonies the primary

decision to make is whether cell morphology is consistent with CFU-EPC and secondarily to demonstrate 'endothelial features'. It may be that other research groups are relying too heavily on immunostaining (CD31, CD105, CD144 etc.), cell uptake of ac-LDL or Ulex Lectin binding, all non specific characteristics that do not identify a unique population of endothelial or haematopoietic cells (93, 131-134), to score a cell cluster as CFU-EPC rather than to first examine 'colony' morphology to ascertain whether it meets CFU-EPC definitions. Uptake of ac-LDL and Ulex Lectin binding and the expression of endothelial genes and proteins are properties shared with peripheral blood monocytes (139).

The lack of CFU-EPC in G-CSF mobilised samples could not be attributed to a number of technical issues that were explored in chapter 4 and the results show that the effect of G-CSF in reducing or abolishing CFU-EPC is real and not an artefact. Delays in MNC separation, which might have led to underestimation of the differences in CFU-EPC between non-mobilised and mobilised samples, were shown not to be causative. The lack of CFU-EPC in mobilised samples was shown not to be due to loss of 'colony forming' cells from the CFU-Hill assay as colony forming cells were not retained within the day 2 fibronectin adherent MNC at the expense of CFU-EPC formation in the day 2 non-adherent MNC. When the sum of CFU-Hill and colonies within day 2 adherent MNC was considered as 'total CFU-EPC' or CFU-Hill(direct) colonies were examined, similar substantial reductions in CFU-EPC following G-CSF were found. The frequency of CFU-EPC in G-CSF mobilised samples was not altered by modifying the concentrations of cells plated into the cells or by changing the culture medium during the culture period.

Previous work by our group (136) and others (129, 132, 140) have explored the importance of $CD14^+$ monocytes for early EPC formation (generated from fibronectin non-adherent or adherent MNC). Based on the increase in PB monocyte counts following G-CSF (42, 43) it would be reasonable to suppose that CD14-dependent CFU-EPC might also increase in mobilised samples. However, results of

previous studies demonstrating increased CFU-EPC following $CD14^+$ monocyte enrichment of normal blood MNC were not replicated when G-CSF mobilised MNC were used. Two-hour plastic adherence of G-CSF mobilised MNC failed to increase CFU-EPC (136).

The proportions of leukocyte subpopulations and $CD235a^+$ erythrocytes in peripheral blood and MNC plated into CFU-EPC culture were investigated and the changes that occurred following G-CSF administration to allogeneic and autologous PBSC patients noted. The effect of alterations in the proportions of $CD14^+$ monocytes and other leukocyte subpopulations on CFU-EPC in mobilised MNC was assessed by phenotypic analysis of whole blood, day 0 MNC and day 2 MNC. Significant changes in the cells that floated on the Histopaque medium following G-CSF compared to non mobilised samples were observed. MNC recovered from buoyant Histopaque density gradient centrifugation of mobilised peripheral blood contained significantly greater proportions of $CD66b^+$ granulocytes and $CD235a^+$ erythrocytes than non-mobilised samples and whilst the proportions of $CD14^+$ monocytes were preserved, the proportions of lymphocytes were reduced. Similar to non-mobilised samples, there were some losses of $CD14^+$ monocytes from the non-adherent cells between day 0 MNC and day 2 MNC as a result of retention of 2 day fibronectin adherent MNC within the original well, leading to reductions in the proportion of $CD14^+$ monocytes in day 2 MNC. However, there was no significant difference between the proportions of $CD14^+$ monocytes in day 2 mobilised MNC and day 2 non-mobilised MNC for any patient group. The reduction in CFU-EPC in mobilised samples could not be attributed to reductions in the proportions of (day 0 or day 2) MNC monocytes following the administration of G-CSF (alone or sequentially with either chemotherapy or AMD3100). The effect, if any, of $CD66b^+$ granulocytes on CFU-EPC, the cell population most changed in MNC following G-CSF was therefore investigated.

G-CSF mobilised day 0 and day 2 MNC from both allogeneic and autologous PBSC donors contained significantly greater proportions of $CD66b^+$ granulocytes than non-mobilised samples (greater than 5-fold increase), a cell population that is not isolated in buoyant density separation of normal blood. G-CSF induced stimulation of proliferation and differentiation of HPC is mediated via myeloid G-CSF receptors (35, 36). G-CSF also stimulates the proliferation of myeloid cells and activation of mature neutrophils, inducing the release of their cytoplasmic granules and the contents of secretory vesicles (27, 37, 38). Elastases, proteases and metalloproteinases (MMP) released from G-CSF stimulated neutrophils facilitate the release of HPC from the BM (37). The reduction in CFU-EPC frequency observed in G-CSF mobilised MNC could simply have been due to dilution of the cell populations (other than $CD14^+$ monocytes) involved in the generation of CFU-EPC or it might be that the enzymes and factors released by stimulated neutrophils present in mobilised MNC and therefore plated into CFU-Hill assays affect CFU-EPC forming cells.

We presented novel data that illustrated the effects of $CD66b^+$ granulocytic populations on CFU-EPC (Chapter 5). Assessing the relative proportions of $CD66b^+$ granulocytes in G-CSF mobilised samples showed no differences between day 0 MNC and day 2 fibronectin non-adherent MNC. 'Contaminating' myeloid cell proportions were equivalent at both time points. Co-plating studies, where purified $CD66b^+$ granulocyte populations from non-mobilised and mobilised MNC were cultured in direct contact and separated in transwell inserts, showed that both sources of $CD66b^+$ granulocytes modulated CFU-EPC. CFU-EPC frequency of G-CSF mobilised MNC could be modified by immunomagnetic separation to reduce MNC $CD66b^+$ granulocytic content. Depletion of $CD66b^+$ granulocytes from G-CSF mobilised MNC was partially successful in increasing CFU-EPC towards that of non-mobilised MNC. $CD66b^+$ granulocyte enriched cell fractions (PMN-p cells or CD66b-depleted MNC) did not generate CFU-EPC when plated alone into cell culture. Contaminating myeloid cells exert a negative influence on CFU-EPC to

inhibit colony formation but the mechanism of their actions is yet to be elucidated. The effects of manipulating the CD66b+ granulocytic content on CFU-EPC were not consistent but there was enough evidence to suggest a strong contributory effect of myeloid elements on CFU-EPC within mobilised blood samples but it is clearly not the whole story. It is likely that additional factors are involved in the reduction of CFU-EPC in mobilised samples, whether this is mediated through granulocytes and other myeloid cells or a direct action of G-CSF to affect cells involved in CFU-EPC (e.g. $CD14^+$ monocytes or lymphocytes).

The proportions of PB $CD3^+$ T cells were found to decline in direct proportion to the reduction in total lymphocytes following G-CSF^+/-chemotherapy or G-CSF+AMD3100 PBSC mobilisation. $CD3^+/31^+/CXCR4^+$ 'angiogenic' T cells, a T cell subpopulation reported by Hur *et al* to be of significance to CFU-EPC (generated in fibronectin adherent MNC) was assessed in a subset of non-mobilised and mobilised PBSC patients (142). In G-CSF^+/-chemotherapy mobilised peripheral blood, the proportions of $CD3^+/31^+/CXCR4^+$ angiogenic T cells decreased as a consequence of reduced proportions of total lymphocytes in mobilised samples rather than due to specific alterations in T cell subpopulations. The reduction in angiogenic T cells following G-CSF+AMD3100 PBSC mobilisation differed from that observed in G-CSF+chemotherapy mobilised PBSC patients. T cell numbers were reduced in proportion to the decline in lymphocytes in G-CSF+AMD3100 mobilised peripheral blood but angiogenic T cells were disproportionately lowered, attributable to the reduction in the co-expression of CXCR4 by $CD3^+$ T cells, either a direct result of AMD3100 or its combination with G-CSF. The potential contributions of G-CSF mobilised $CD66b^+$ granulocytes to CFU-EPC have been highlighted but it is possible that the decline in lymphocytes following G-CSF had some effect on the initiation of CFU-EPC, whether independently or through interactions with $CD14^+$ monocytes, an area that should be further explored. Previous work by our group, using plastic adherence and immunomagnetic separation to enrich normal blood and UCB for $CD14^+$ monocytes, demonstrated

that CFU-EPC were contained within CD14-enriched fractions and whilst $CD4^+$, $CD8^+$ and $CD19^+$ cells could be identified in CFU-EPC from unselected MNC, these cells were not demonstrated in CFU-EPC arising from CD14-enriched fractions (136). Based on these results it is unlikely that alterations in T cell frequency would affect CFU-EPC frequency. It may be that a very small numbers of T cells are required for CFU-EPC but so far our data has not shown this.

There is a lack of information on the expected proportions of leukocyte subpopulations and erythrocytes in MNC recovered from mobilised peripheral blood samples after buoyant density gradient centrifugation which makes it difficult to critically appraise our results compared to published studies. Although there was evidence published on the haematopoietic ($CD14^+$ monocyte) origin of CFU-EPC generated by short term culture on fibronectin plates at the time, other studies of the effects of G-CSF on CFU-EPC (early EPC) formation did not present phenotypic data on constitution of MNC actually plated into cell culture (162, 163, 165, 167, 170, 189). Despite evidence that short term culture of MNC on fibronectin results in colonies arising from myelomonocytic cells, it is the $CD34^+$ peripheral blood cells that the authors' identify and correlate with CFU-EPC. Work within our group (136) using subtractive analysis demonstrated that CFU-EPC were independent of $CD34^+$ or $CD133^+$ cells. The initial publication by Korbling *et al* that described collection of HPC from the circulation reported 10% granulocytes in the leukapheresis product (15). One murine study (202) using G-CSF+AMD3100 to enhance blood flow in a hindlimb ischaemia model (202), is notable for its examination of G-CSF+AMD3100 mobilised MNC prepared from density gradient centrifugation of whole blood and the contributions of different cell fractions to recovery of limb perfusion. MACS immunomagnetic bead separation was used to separate the $CD11b^+$ cells from MNC, which comprised 36% of isolated 'MNC' and consisted mainly of neutrophils. Following further separation of $CD11b^+$ cells (FACS sort) into neutrophils, monocytes, and NK cells the authors demonstrated clinical benefits of $CD11b^+$ MNC

and $CD11b^+$ monocytes but not of $CD11b^+$ neutrophils but cell fractions were not plated into CFU-EPC assays (202).

Expression of adhesion molecules including VLA-4 (CD49d) and L-selectin by $CD34^+$ cells is reduced (55, 56) and soluble factors including sL-selectin, sE-selectin and sCD44 are increased following G-CSF administration (43, 54). It is reasonable to suppose that G-CSF stimulated myeloid cells contained in CFU-EPC culture might affect cell expression of adhesion markers by other cell populations apart from $CD34^+$ cells including $CD14^+$ monocytes. The effects of G-CSF administration in vivo on the expression of adhesion molecules by leukocytes is currently being assessed. Differences in the expression of adhesion molecules between non-mobilised and mobilised patient samples and differences between UCB and samples from adult PBSC donors have been observed. Preliminary studies have shown alterations in the monocytic expression of CD29, CD49d, CD49e and CD49c with differences noted between UCB, non-mobilised peripheral blood and mobilised peripheral blood. Alterations in adhesion markers, through the effects of this on the adherence of potential of colony forming cells to fibronectin, might prove significant in colony based EPC assessments. Additionally, interruption of signalling through fibronectin receptors as a result of G-CSF might alter cell activation and cell function.

EPC, variously defined according to their expression of CD34, KDR and CD133 antigens, with expression of these antigens alone or in combination being used to define them, have been correlated with disease states (110, 111). There is little debate about the existence of endothelial and haematopoietic progenitors within the $CD34^+$ fraction of BM, UCB and PB MNC. KDR (VEGFR2) and CD133, both used to define EPC, are also expressed by HSC/HPC (93, 112, 113). $CD34^+/133^+/VEGFR2^+$ cells identified by Piechev and others (112, 122, 125, 128) and present in extremely low numbers (0.0084%+/-0.0052% in whole blood and 2.07%+/-0.15% in mobilised PB $CD34^+$ cells according to Case *et al* are haematopoietic (112). The direct contribution of $CD34^+/133^+/KDR^+$ cells to vasculogenesis has not been reported (113) so the earlier

phenotypic definitions of EPC, based on the hypothesis by Asahara *et al*, may be incorrect.

The phenotypic identification of EPC using CD34 in combination with CD133 and/or KDR has been often presented together with results of short term culture of MNC on fibronectin to identify EPC, including those studies examining effects of G-CSF on EPC (162, 163, 165, 167, 170). In these studies EPC were identified using a number of different phenotypes; $CD34^+$ (162, 170), $CD34^+/133^+/KDR^+$ (162, 163), $CD34^+/133^+$ (167), $CD133^+/KDR^+$ (167) with some comparing their flow cytometry results to CFU-EPC and finding no correlation between frequency of putative EPC phenotypes and culture based EPC assessments (162, 169, 170). However, phenotypically identified $CD34^+$ EPC, are now believed to have key roles in EOC formation rather than in early EPC formation (129, 132, 139) leading us to question the relevance of these putative EPC phenotypes to EPC identified in short term culture on fibronectin.

Our data demonstrated that in the non-mobilised state, progenitor cells (haematopoietic or endothelial) are present at very low levels in peripheral blood and MNC. Similar to others, when defined phenotypically as $CD34^+/KDR^+$ or $CD34^+/133^+/KDR^+$ cells, EPC were not identifiable by flow cytometry in the non-mobilised state (128). A stem cell gate (Stems R1), defined on the basis of light scatter characteristics and the expression of CD133 and/or CD34 antigens was used to capture all events expressing CD133 or CD34 and all haematopoietic and non-haematopoietic progenitor cells (Chapter 6). In non-mobilised samples there was a relatively even distribution of stem cell phenotypes between $CD133^+/34^-$ (36.8%), $CD133^+/34^+$ (32.3%) and $CD133^-/34^+$ cells (30.8%) in freshly isolated MNC , with no differences noted between normal donors (allogeneic PBSC donors) and non-mobilised autologous PBSC patients, confirming earlier reports (123, 136). Virtually all $CD34^+$ and/or $CD133^+$ cells were $CD45^+$ and no populations of $CD45^-$ cells could reliably be identified. Analysis of the proportions of stem cells contained in G-CSF

mobilised peripheral blood and MNC showed significant differences in stem cell numbers and phenotypes compared to non-mobilised samples. G-CSF mobilised $CD133^+$ stem cells and $CD34^+$ stem cells to similar extents, though the proportions of $CD34^+$ stem cells always exceeded those of $CD133^+$ stem cells.

Consistent with other studies reporting on the effects of G-CSF on EPC, G-CSF increased the numbers of $CD34^+$ (162-164), $CD34^+/133^+$ (164, 167, 168) and $CD34^+/133^-/KDR^+$ cells (162, 163, 166). Increases in $CD133^+/34^-$ and $CD34^+/133^-$ stem cells were also noted following G-CSF. G-CSF mobilised stem cell populations contained large proportions of $CD133^+/34^+$ dual positive stem cells (3.9% $CD133^+/34^-$, 75.9% $CD133^+/34^+$ and 20.1% $CD34^+/133^-$ in mobilised whole blood and 6.1% $CD133^+/34^-$, 72.2% $CD133^+/34^+$ and 22.0% $CD34^+/133^-$ in mobilised day 0 MNC), figures comparable to that reported by O Tura and de Wynter *et al* (123, 136). No differences were seen in stem cell phenotypes between allogeneic and autologous PBSC donors. Increases in the proportions of $CD133^+/34^+$ cells occurred principally at the expense of $CD133^-/34^+$ cells which were present in reduced proportions in mobilised compared to non-mobilised samples. Similar to non-mobilised samples, almost all stem cells were $CD45^+$ with only very small populations of $CD45^-$ stem cells ($CD34^+$ and/or $CD133^+$ cells, Stems R1 gate) identified in a proportion of samples. When present, $CD45^-$ stem cells appeared to contain greater proportions of $CD133^+/34^-$ cells compared to $CD45^+$ stem cells. Preliminary data from the analysis of a small group of patients undergoing PBSC mobilisation with G-CSF and AMD3100 (without chemotherapy) showed similar changes in stem cell phenotypes of mobilised samples with a predominance of $CD133^+/34^+$ stem cells in mobilised samples.

Examination of putative EPC phenotypes found in G-CSF mobilised peripheral blood and UCB samples confirmed that virtually all $CD34^+$ progenitor cells mobilised by G-CSF were haematopoietic and expressed CD45 whilst $CD34^+/45^-$ cells were identified in UCB. Although UCB stem cells were enriched in $CD133^+/34^+$

cells (62% of stem cells), figures comparable to that reported by other studies (123, 169), this differed significantly from G-CSF mobilised peripheral blood. Despite being rich in $CD34^+$ cells, preliminary studies failed to identify EOC within unselected G-CSF mobilised MNC. However, UCB MNC, another CD34-rich source, reliably generated EOC. There are a lack of reports relating to EOC formation in G-CSF mobilised MNC. A single human study (Shepherd *et al*) reported increased EOC (per mL blood) following the administration of G-CSF and also following AMD3100 when MNC (collected by leukapheresis) were cultured in EGM2 supplemented with FCS together with VEGF (189). A murine study, similarly used supplemented EGM2 with additional VEGF to assess late outgrowth cells appearing on fibronectin (190). In our laboratory, selection of MNC according to their expression of CD34 and CD133 has demonstrated enhanced EOC within $CD34^+/133^-$ UCB MNC (O Tura, personal communication) which is in agreement with reports by other groups (112, 134, 150). A unique population of $CD34^+$ cells ($CD34^{BRIGHT}$), defined by their bright CD34 expression, was identified in UCB $CD34^+$ but not in G-CSF mobilised peripheral blood $CD34^+$ or non-mobilised PB $CD34^+$ cells. UCB $CD34^{BRIGHT}$ cells were more likely to be $CD133^-$ and $CD38^-$ and to express KDR compared to UCB $CD34^{REG}$, mobilised PB $CD34^+$ cells (all $CD34^{REG}$) and non-mobilised PB $CD34^+$ cells (all $CD34^{REG}$). EOC were enriched in UCB $CD34^+/133^-$ MNC fractions. However, G-CSF mobilised MNC, which is also enriched in $CD34^+$ cells, and have $CD34^+/133^-$ cells accounting for just over 20% of ($CD34^+$ and/or $CD133^+$) stem cells, are yet to be shown to produce EOC. We propose that these UCB $CD34^{BRIGHT}$ cells might be critical for EOC formation and could contain the EOC progenitors.

The source of EOC within a subset of $CD34^+/133^-$ cells, possibly from this $CD34^{BRIGHT}$ fraction, would be consistent with conclusions published by three groups investigating the phenotype of cells contributing to EOC, that EOC arose from $CD45^-/34^+$ cells that did not express CD133. (112, 134, 150). The belief that EPC could be defined phenotypically by cell expression of three antigens, CD34, CD133

and KDR, has been refuted. Untergasser *et al* reported that EOC arose in peripheral blood and UCB $CD34^+/133^-$ but not $CD34^+/133^+$ cells (150). Case *et al* reported that BM and UCB $CD34^+/45^-$ cells formed EOC but failed to generate CFU-EPC or haematopoietic activity whilst $CD34^+/45^+$ cells were enriched for haematopoietic colony forming activity (112). Timmermans *et al* reported that UCB and BM MNC derived EOC arise from $CD34^+/45^-$ cells and identified $CD34^+/133^-/KDR^+$ cells as the source of EOC (134). $CD34^+/133^+/KDR^+$ cells from UCB and mobilised PB $CD34^+$ cells were $CD45^+$ (>99%) and failed to generate CFU-EPC or EOC but produced low and high proliferative potential HPC in haematopoietic assays (112). Our own work showing increased EOC in $CD34^+/133^-$ UCB cells is consistent with their conclusions (O Tura).

UCB $CD34^{REG}$ and $CD34^{BRIGHT}$ cells were examined for CEC phenotypes using the antibody combinations $CD34^+/31^+/45^-$, $CD34^+/45^-/146^+$ and $CD34^+/45^-/146^+/31^+$ reported by others (116, 194-196) to identify CEC. Although there was no difference in CD31 expression between UCB $CD34^{REG}$ and $CD34^{BRIGHT}$ cells, UCB $CD34^{BRIGHT}$ cells contained greater proportions of $CD34^+/31^+/45^-$ (36.1%), $CD34^+/45^-/146^+$ (5.4%) and $CD34^+/45^-/146^+/31^+$ (5.0%) than UCB $CD34^{REG}$ cells (8.0% ($p<0.0.01$), 1.0% and 0.8% respectively). We should therefore question whether the $CD34^+/133^-$ cells (112, 134, 150) or $CD34^+/133^-/KDR^+$ cells (112), thought to be critical to EOC formation, do in fact arise from circulating cells that are endothelial-committed and may be quite mature, and not a primitive progenitor as implied in some interpretations (96, 153).

$CD34^+$ cells expressing CD31 and CD146 have been reported by another group as identifying EOC (189). In this study of EPC phenotype by Shepherd *et al*, EPC were identified in G-CSF mobilised peripheral blood and in day 14-28 cultured EOC, as $CD34^+/31^+/144^+/146^+/105^+$ cells that did not express CD45 or CD14 with cell numbers increasing (per mL peripheral blood) following G-CSF. Insufficient EOC ($CD34^+/31^+/144^+/146^+/105^+/45^-/14^-$ cells) were obtained for transplantation into a murine hindlimb ischaemia model and cells did not survive cryopreservation. Cells

were only passaged three times and no reference to continued cell proliferation was made by the authors (189). A study examining KDR^+ cell populations (Vroling *et al*) identified increased numbers of $CD45^-/34^+/KDR^+$ cells in the peripheral blood of patients with cancer compared to healthy controls(203). These cells expressed CD31, CD146 and CD105 but were $CD133^-$ and $CD13^-$(203). Vroling *et al* considered these cells to be distinct from phenotypically defined $CD146^+$ circulating endothelial cells, largely on the basis of their light scatter characteristics. Further phenotypic and culture based assessments of $CD34^+$ subsets within UCB and mobilised peripheral blood are now being performed within our group.

It would appear that in most areas, the co-administration of sequential G-CSF and AMD3100 prevented any AMD3100-specific effects being noted. It was found that G-CSF+AMD3100 produced similar changes in CFU-EPC formation, leukocyte subpopulations, and stem cell phenotypes as observed following $G\text{-}CSF^+$/-chemotherapy PBSC mobilisation. The only clear difference between G-CSF and G-CSF+AMD3100 mobilised patient samples was reductions of 'angiogenic' T cells observed, as a result of the reduction in co-expression of CXCR4 by $CD3^+$ T cells in the latter group. AMD3100, a reversible CXCR4 antagonist, does not cleave the CXCR4 receptor. It is possible that the epitope targeted by the particular anti-CXCR4 antibody used in flow cytometry analysis was still affected by AMD3100 at the time of sampling. Sequential administration of AMD3100 with G-CSF might have had additive effects on the CXCR4 receptor or perhaps more likely the timing of the 5^{th} dose of G-CSF, administered 1-2 hours prior to collection of mobilised samples led to the profound reduction in CXCR4 expression by $CD3^+$ T cells. Recently published microarray data using an animal (macaque) model, suggests the differential mobilisation of progenitors by G-CSF and AMD3100 (204). In this study, 3 gene expression clusters were identified, with sequential G-CSF +AMD3100 up-regulating genes distinct from mobilisation with either G-CSF or AMD3100 alone. There is no immediate prospect of using AMD3100 alone for PBSC mobilisation in the local clinical setting, restricting our ability to investigate this

agent separately but animal studies suggest that this agent does mobilise different progenitors to G-CSF and could therefore be useful clinically in the setting of vascular repair (190, 204).

The clinical outcomes of a number of studies using G-CSF to mobilised cells for vascular repair, mostly in patients following myocardial infarction has been reported. The use of G-CSF mobilised progenitor cells for cardiovascular regeneration stalled once adverse findings of increased incidence of instent stenosis in following intracoronary implantation of autologous G-CSF mobilised MNC were reported (171). Subsequent to this, a small uncontrolled trial reported improved cardiac function following intracoronary infusion of autologous G-CSF mobilised MNC (172). A number of studies demonstrated no clinical benefit of using G-CSF alone, without the collection and local implantation of autologous mobilised cells, as an adjunct to standard medical care following myocardial infarction (166, 168, 173-175). Two studies reporting clinical improvements following G-CSF (176, 177) have been criticised and a meta-analysis on the use of G-CSF in patients with acute MI concluded that G-CSF administration failed to demonstrate any overall benefits compared with conventional treatment (178).

Whilst a handful of studies have reported enhanced CFU-EPC following G-CSF, this work confirmed our previous findings of reductions in CFU-EPC following G-CSF administration. There are difficulties in interpreting phenotypic and culture based assessments of EPC, due to differences in methodologies used and also because our understanding of the cells encompassed by the term 'endothelial progenitor cells' has changed. Whilst some studies identified increased proportions of phenotypically defined EPC following G-CSF, and some studies identify increased numbers of CFU-EPC, it is difficult to relate these results to studies that have failed to demonstrate clinical benefits (on revascularisation and cardiac function for example) of G-CSF. In such studies the clinical effects of G-CSF were assessed by a range of standard tests of cardiac function. The role of G-CSF in mobilising EOC is yet to be explored to any great extent. Using phenotypic analysis to compare UCB

and mobilised MNC differences between these sample groups were identified which when related to results of recent publications investigating EOC (112, 134, 150) and to results of our EOC cultures led to further investigation of $CD34^+$ subpopulations.

The cells contributing to the repair of damaged tissue are not well defined. It may be that BM derived endothelial cells (91, 107, 205) and vascular wall derived endothelial precursors (113) are important but there is likely a large , possibly indirect, contribution made by BM-derived cells of haematopoietic origin. A number of recent studies suggest that transplanted G-CSF mobilised $CD34^+$ or $CD133^+$ cells may provide a microenvironment supportive of tissue regeneration and vascular repair through autocrine and paracrine actions (183, 202). A phase I/IIa trial of locally implanted autologous G-CSF mobilised $CD34^+$ cells in patients with critical limb ischaemia reported clinical benefits in the ischaemic limb (188). Two recent studies also reported clinical benefits of locally implanted human G-CSF mobilised $CD34^+$ or $CD133^+$ cells in animal models of injury (185, 187) and suggested that implanted cells enhanced angiogenesis of recipient cells by creating a favourable microenvironment for tissue regeneration and by producing pro-angiogenic cytokines.

With recognition that pro-angiogenic effects of cell therapy extend beyond incorporation of transplanted cells into the vessel wall, the affect of cytokines (VEGF, IL-8) and proteases, including matrix metalloproteinases (MMP) released by EPC as well as other functions of EPC, identified in short term culture on fibronectin, on vessel formation has being investigated (130-132). The role G-CSF in stimulating the release of enzymes including MMP from neutrophils (53) may also be relevant to vascular repair. The Moldovan group has reported on the influence of monocytes and macrophages, through formation of cord-like structures and release of factors including macrophage specific metalloelastase 12 (MMP-12) which degrade the extracellular matrix, in facilitating the co-localisation and spatial

arrangement cells involved in neovessel formation (143, 144, 206). If G-CSF reduces the ability of $CD14^+$ monocytes to participate in CFU-EPC, it may also down-regulate other angiogenesis-related 'monocyte' activities. With the recognition of the contributions of mature haematopoietic cells and haematopoietic progenitors to vascular repair and the roles of the microenvironment, cytokines and other cellular elements to this process, current *in vitro* models and assays may be inadequate. The contribution of different processes and cells to a global measure of vascular repair will not easily be resolved.

Whilst it would be of great benefit to be able to prospectively identify and quantify EPC it may not be possible to assess 'vasculogenic potential' in a single test, whether this be culture based, phenotypic or another form of assessment. The ability to study bone marrow, which was not explored during the course of this work, would be beneficial in providing comparative data for mobilised samples, something that is now being actively pursued as it may be that the changes that occur in progenitor cells within the bone marrow and the bone marrow stroma leading to progenitor cell mobilisation have significant effects on our assessments of putative EPC. Recent publications reporting clinical benefits of mobilised $CD34^+$ and $CD133^+$ cells leads us to speculate that the cells delivering the therapeutic effect may be true haematopoietic progenitor cells rather than major contributions by EPC.

The rationale behind use of GCSF mobilised blood in vascular repair is that it contains a high level of putative EPC to address the reduced level of EPC found in patients with vascular disease and cardiovascular disease. This study has confirmed earlier findings in our research group that contradict what has previously been understood. GCSF administered for the mobilisation of HPC does not lead to a high level of EPC, as assessed by CFU-EPC (or EOC) assays. If our findings of reduced CFU-EPC in G-CSF mobilised blood are translated from the laboratory and into the clinic, any clinical benefits reported from the administration of G-CSF or the implantation of G-CSF-mobilised cells in the setting of myocardial or vascular

damage may not have been delivered by EPC but by HPC and other haematopoietic cells. This would suggest that the clinical basis for use of GCSF mobilised blood may need to be reconsidered. Once the different components of angio/vasculogenesis are better defined and their functions understood it may then be possible to design therapeutic regimens that can answer these questions and move cell-based therapy from an experimental to a mainstream therapeutic option.

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Appendix

CFU-EPC formation in CD66b-depleted mobilised MNC.

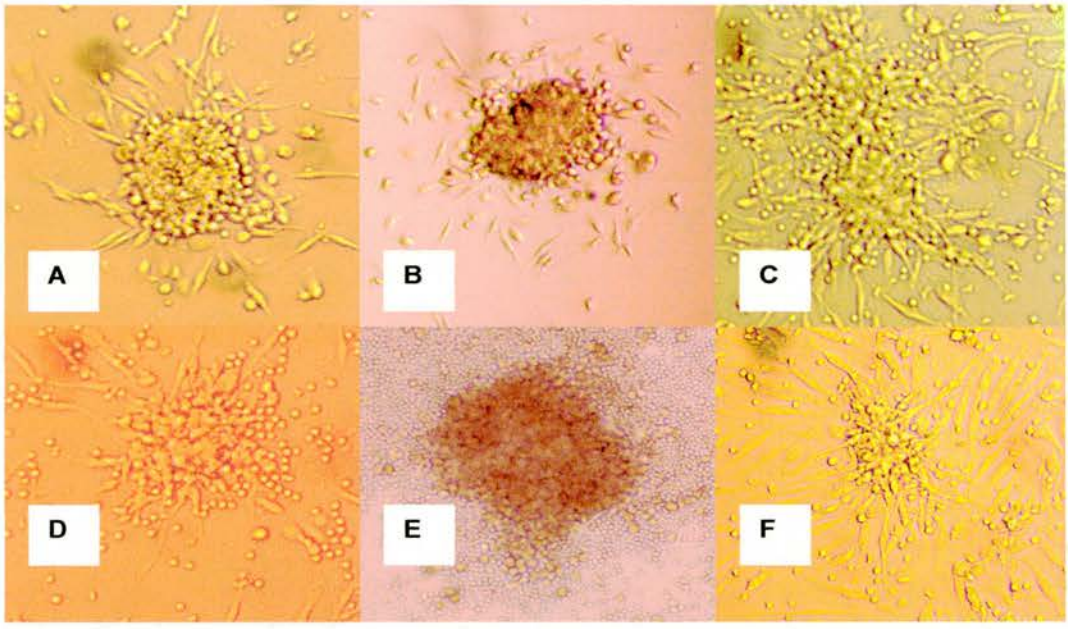

Figure 1 CFU-EPC in non mobilised MNC-s cells (A and D), CFU-EPC and cell clusters in G-CSF mobilised MNC-s cells (B and E) and CFU-EPC in G-CSF mobilised CD66b-depleted MNC (C and F); allogeneic PBSC patient (patient 276).

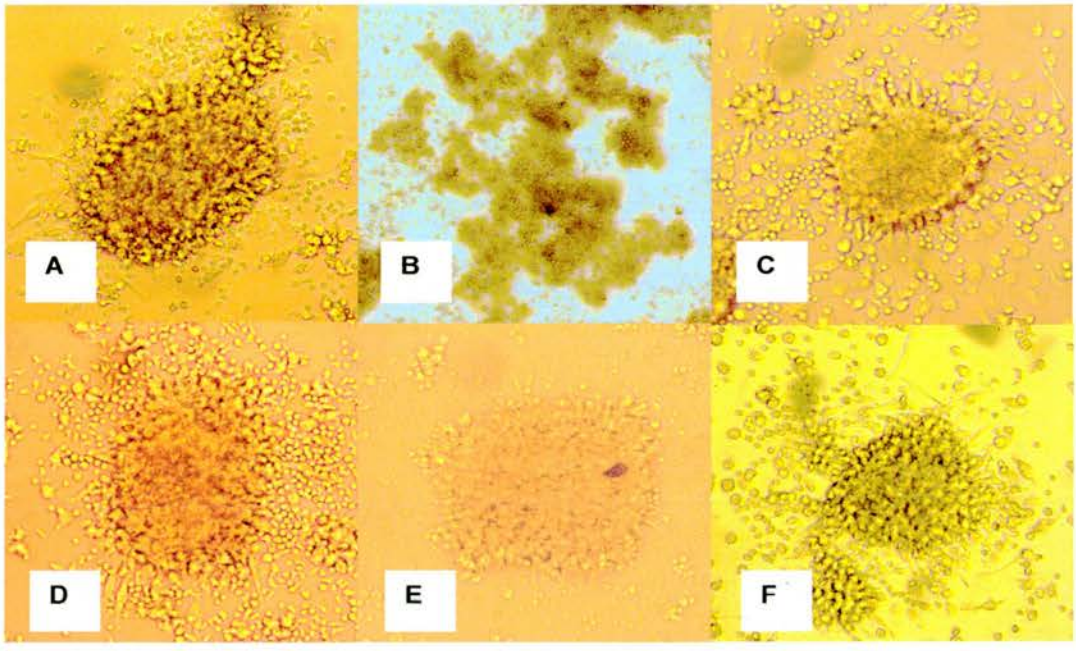

Figure 2 CFU-EPC in non mobilised MNC-s cells (A and D), well appearance of CD66b-enriched MNC (B), CFU-EPC in G-CSF+AMD3100 mobilised MNC-s cells (E) and CFU-EPC in G-CSF+AMD3100 mobilised CD66b-depleted MNC (C and F); autologous PBSC patient mobilised with G-CSF+AMD3100 (patient 316).

Appendix

Pt ID	G+C, G or G+A	G-C SF	Non-mobilised			
			MNC-s	CD14-depleted MNC	CD14-enriched MNC	Mixed culture
299	G+A	No	4	0	0	ND
299	G+A	No	0	0	0	ND
314	G+C	No	0	0	0	ND
300	G	No	1	0	0	ND
304	G+C	No	2	0	0	ND
305	G+C	No	2	0	0	ND
311	G+C	No	2	0	0	ND
HV01	Nil	No	20	0	ND	30 (14+MNC-s)
HV02	Nil	No	8	0	ND	50 (14+MNC-s)
299	G+A	Yes	1	0	0	ND
293	G	Yes	0	0	0	ND
294	G	Yes	1	0	0	ND
314	G+C	Yes	0	0	1	ND
309	G+C	Yes	0	0	4	ND
300	G	Yes	1	0	0	ND
302	G+C	Yes	3	0	0	ND
305	G+C	Yes	1	0	0	ND
315	G+C	Yes	0	0	ND	0 (14+MNC-s)
316	G+A	Yes	12	4	0	2 (14+MNC-s)

Table CFU-EPC results for CD14 MACS immunomagnetic separation experiments.

Pt ID	G+C or nil	MNC-s	CD3-depleted MNC	CD3 enriched MNC	CD14-depleted MNC	CD14-enriched MNC	Mixed culture	Mixed culture
314	G+C	0	2	ND	ND	1	0 (MNC-s+14^+)	0 (3^-+14^+)
315	G+C	0	0	0	0	0	0 (MNC-s+14^+)	0 (MNC-s+3^+)
HV02	nil	8	10	0	0	ND	50 (MNC-s+14^+)	100 (3^-+14^+)
HV01	nil	20	0	ND	0	ND	30 (MNC-s+14^+)	0 (3^-+14^+)
314	nil	0	0		0		0 (MNC-s+14^+)	0 (3^-+14^+)

Table CFU-EPC results for CD3 MACS immunomagnetic separation experiments.

Appendix

Tube	FL-1	FL-2	Fl-3	Fl-4
1	—	—	—	—
2	—	—	—	Anti 45-APC
3	—	Anti 133-PE	Anti 34-PerCP	Anti 45-APC
4	Anti KDR-FITC	Anti 133-PE	Anti 34-PerCP	Anti 45-APC
5	Anti 38-FITC	Anti 133-PE	Anti 34-PerCP	Anti 45-APC
6	Anti KDR-FITC	Anti CXCR4-PE	Anti 34-PerCP	Anti 45-APC
7	Anti 31-FITC	Anti CXCR4-PE	Anti 3-PerCP	Anti 45-APC
8	Anti 31-FITC	Anti CXCR4-PE	Anti 19-PerCP	Anti 45-APC
9	—	—	—	—
10	Anti 38-FITC	Anti 15-PE	Anti 34-PerCP	Anti 13-APC
11	Anti 33-FITC	Anti 14-PE	Anti 34-PerCP	Anti 13-APC
12	Anti 64-FITC	Anti 14-PE	Anti 34-PerCP	Anti 45-APC
13	Anti 66B-FITC	Anti 15-PE	Anti 34-PerCP	Anti 45-APC
14	Anti 66B-FITC	Anti 235a-PE	Anti 14-PerCP	Anti 45-APC

Stem cell subset analysis - antibody panel

Tube	FL-1	FL-2	Fl-3	Fl-4
1	—	—	—	—
2	—	—	—	Anti 45-APC
3	—	Anti 133-PE	Anti 34-PerCP	Anti 45-APC
4	Anti KDR-FITC	Anti 133-PE	Anti 34-PerCP	Anti 45-APC
5	Anti 38-FITC	Anti 133-PE	Anti 34-PerCP	Anti 45-APC
6	Anti KDR-FITC	Anti CXCR4-PE	Anti 34-PerCP	Anti 45-APC
7	Anti 31-FITC	Anti 146PE	Anti 34-PerCP	Anti 45-APC
8	Anti 31-FITC	Anti CXCR4-PE	Anti 3-PerCP	Anti 45-APC
9	Anti 66B-FITC	Anti 235a-PE	Anti 14-PerCP	Anti 45-APC

Stem cell subset analysis – abbreviated antibody panel

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ORIGINAL ARTICLE

Granulocyte colony-stimulating factor (G-CSF) depresses angiogenesis *in vivo* and *in vitro*: implications for sourcing cells for vascular regeneration therapy

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Summary. *Background:* The most common source of hematopoietic progenitor cells (HPCs) for hematopoietic reconstitution comprises granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cells (PBSCs). It has been proposed that endothelial progenitor cells (EPCs) share precursors with HPCs, and that EPC release may accompany HPC mobilization to the circulation following G-CSF administration. *Objective:* To investigate EPC activity following HPC mobilization, and the direct effects of exogenous G-CSF administration on human umbilical vein endothelial cells (HUVECs) and endothelial outgrowth cells (EOCs), using *in vitro* and *in vivo* correlates of angiogenesis. *Patients/Methods:* Heparinized venous blood samples were collected from healthy volunteers and from cord blood at parturition. G-CSF-mobilized samples were collected before administration, at apheresis harvest, and at follow-up. PBSCs were phenotyped by flow cytometry, and cultured in standard colony-forming unit (CFU)-EPC and EOC assays. The effect of exogenous G-CSF was investigated by addition of it to HUVECs and EOCs in standard tubule formation and aortic ring assays, and in an *in vivo* sponge implantation model. *Results:* Our data show that G-CSF mobilization of PBSCs produces a profound, reversible depression of circulating CFU-EPCs. Furthermore, G-CSF administration did not mobilize CD34+CD133− cells, which include precursors of EOCs. No EOCs were cultured from any mobilized PBSCs studied. Exogenous G-CSF inhibited CFU-EPC generation, HUVEC and EOC tubule formation, microvessel outgrowth, and implanted sponge vascularization in mice. *Conclusions:* G-CSF administration depresses both endothelial cell angiogenesis and monocyte proangiogenic activity, and we suggest that any angiogenic benefit observed following implantation of cells mobilized by G-CSF may come only from a paracrine effect from HPCs.

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Introduction

The recent discovery that endothelial progenitor cells (EPCs), which play a role in *de novo* vascularization (vasculogenesis), circulate in adult blood and reside in the bone marrow [1,2] prompted a range of studies based on localized implantation of autologous cells, aimed at vascularizing ischemic tissue, particularly in myocardial and critical limb ischemias. EPCs have not yet been definitively characterized, but have been linked with hematopoietic progenitor cells (HPCs). EPCs and HPCs share a common ancestor, the hemangioblast, in the developing fetus that may be retained in adult life [3]. HPCs derived from bone marrow [bone marrow stem cells (BMSCs)] or peripheral blood (PB) [peripheral blood stem cells (PBSCs)] following granulocyte colony-stimulating factor (G-CSF) administration have been utilized as sources of EPCs for regenerative vascularization. A recent meta-analysis showed that BMSC treatment generally improves short-term measurements of cardiac function after myocardial infarction. However, there is, as yet, little evidence with which to assess the long-term clinical effects of this treatment [4]. Although most studies so far reported have used BMSCs for therapeutic angiogenesis, those in which G-CSF-mobilized PBSCs were used gave comparable, mild improvements in cardiovascular lesions [5], and both sources are generally regarded as adequate for therapeutic angiogenesis, just as they are for hematopoiesis. To date, some studies have shown that EPCs, as well as HPCs, are demonstrably mobilized by G-CSF [6–8]. However, this

depends on how EPCs are defined and interpreted: we can measure increases or decreases in EPC numbers following G-CSF administration, depending on how EPCs are defined [9].

The current characterizations of EPCs have been based on phenotype and on colony assays. HPCs are routinely defined for clinical use by their expression of CD34 or CD133 [10]. A link between CD34/CD133 expression and the EPC phenotype was proposed almost from the initial discovery of circulating EPCs [11,12], but recent studies have indicated that cells expressing CD133 and their progeny remain hematopoietic, and only CD34+CD133− cells are true EPCs [13,14]. True EPCs are defined as cells that, in culture over 3–4 weeks on collagen, can give rise to endothelial outgrowth cells (EOCs) [14–16], whereas cells that give rise over 5–6 days to colonies on fibronectin (colony-forming unit endothelial progenitor cells) (CFU-EPCs), formerly proposed to be EPCs [17], are now recognized to be generated by monocytes [16,18,19]. CFU-EPCs stain for many endothelial markers [20,21] but also retain CD14 expression [22]. Monocytes can themselves mimic endothelial cells (ECs) by upregulating expression of many markers held to be endothelial, and have probably been mistaken for ECs in many investigations [22,23]. Although it is implicit in many studies that the observed clinical benefit is delivered by EPCs, which are ultimately incorporated as ECs into new vasculature, it is becoming apparent that neovascularization can also be promoted indirectly by cells that release paracrine factors that promote angiogenesis without being incorporated as ECs [24]. Although such cells may not be true EPCs, their proangiogenic effect may be important, and this may be why so many different cell phenotypes have been proposed to be EPCs [25,26].

To date, G-CSF PBSC mobilization has been generally regarded as a practical and feasible source of cells for therapeutic angiogenesis [27–29]. However, a recent meta-analysis reported that G-CSF infusion alone had no significant clinical benefit in myocardial infarction [30], and it was reported that G-CSF-mobilized PBSCs were less effective in inducing ulcer healing than were BMSCs [31]. The mechanism by which G-CSF may mobilize EPCs and/or enhance angiogenesis is still unknown. Similarly, the effects of G-CSF on ECs and the vasculature have not been extensively studied [32]. In this study, we have investigated circulating EPC activity following HPC mobilization by G-CSF administration, and the direct effects of G-CSF on *in vitro* and *in vivo* correlates of angiogenesis.

Materials and methods

Animals

Male C57B6J mice aged 10–12 weeks were purchased from Charles River Laboratories (Tranent, UK) or Harlan Olac Ltd (Loughborough, UK). Experimental procedures were approved by the University of Edinburgh ethics committee, and were authorized by the Home Office under the Animals (Scientific Procedures) Act 1986.

Cell sources and sampling

Peripheral venous blood samples from healthy adults (normal PB) were collected into heparin and from cord blood (CB) following elective caesarean section. For sequential studies, healthy PBSC donors (mobilized PB donors) and PBSC transplant patients (mobilized PB patients) donated 10 mL of venous PB before G-CSF mobilization (pre-G-CSF); at apheresis harvest (post-G-CSF), and 1–2 months after harvest (follow-up). The G-CSF protocol used in this work was the standard local clinical mobilization regimen. The G-CSF (lenograstim) dose for healthy donors was 10 $\mu g\ kg^{-1}\ d^{-1}$, given for four consecutive days before collection of post-G-CSF cells (at apheresis) on day 5. Patient samples were subjected to chemotherapy — salvage chemotherapy (lymphoma patients) or cyclophosphamide (multiple myeloma patients) — followed by G-CSF (lenograstim), starting at least 24 h following the last dose of chemotherapy — 5 $\mu g\ kg^{-1}\ d^{-1}$ (lymphoma patients) or 10 $\mu g\ kg^{-1}\ d^{-1}$ (multiple myeloma patients) — and given for 6–7 days before collection of post-G-CSF cells when CD34+ counts exceeded $10 \times 10^6\ L^{-1}$. Pre-G-CSF treatment cells were collected 10–30 days prior to G-CSF administration. Healthy adult donors (for allogeneic transplant) are the primary study subjects, and results for patients are included for comparison. Further clinical, hematologic and laboratory data are reviewed elsewhere (J. Crawford, MD thesis, University of Edinburgh, submitted). Appropriate ethical informed consent was obtained from subjects in all cases. Mononuclear cells (MNCs) were isolated by buoyant density centrifugation over Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden).

Isolation of short-term (2 h) plastic-adherent MNCs

MNCs (30×10^6) in 5 mL of IMDM (Invitrogen, Paisley, UK) containing 10% fetal bovine serum (Sigma, Dorset, UK) were plated in 25-cm^2 Corning tissue culture flasks (Fisher Scientific, Loughborough, UK) and incubated at 37 °C. After 2 h, adherent cells were detached using 1 mL of trypsin–EDTA in saline (Sigma). Harvested cells were resuspended in IMDM and characterized by flow cytometry for use in further experiments.

Flow cytometry analysis and sorting

Cells were directly stained and analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Oxford, UK), using CellQuest Pro software for phenotypic expression of surface markers, and analyzed using FCS Express (DeNovo Software, Los Angeles, CA, USA), as described previously [9]. Cells were sorted using a FACS Aria flow cytometer (Becton Dickinson), using Diva software. Sorted populations were recovered and characterized by further analysis. The anti-human monoclonal antibodies used for flow cytometry included anti-CD34–fluorescein isothiocyanate, anti-CD14–phycoerythrin, anti-CD45–PerCP (Becton Dickinson), and anti-CD133–allophycocyanin (Myltenyi Biotec, Bisley, UK).

CFU-EPCs

This assay, based on the method of Hill *et al.* [17], was performed using a commercial kit according to the manufacturer's recommendations (Stem Cell Technologies, Grenoble, France). As previously described [9,20,21], 5×10^6 unmodified MNCs were resuspended in EndoCult Liquid Medium (Stem Cell Technologies) and plated on fibronectin-coated six-well plates (Becton Dickinson) for 2 days. The non-adherent cells were recovered, resuspended in fresh medium, and transferred to a fibronectin-coated 24-well plate (Becton Dickinson) at 1×10^6 per well in the presence or absence of G-CSF (100 ng mL^{-1}) for a further 3 days; the colonies per well were then counted, and the EPC frequency was calculated. The concentration of G-CSF used in this and other *in vitro* assays described below was based on a previously determined optimal dose for CD34+ PBSC expansion/differentiation to neutrophils [33].

Culture of EOCs

EOC culture was performed as described by Ingram *et al.* [34]. Briefly, 30×10^6 MNCs from normal PB or 10×10^6 MNCs from CB were resuspended in endothelial growth medium (EBM-2; Lonza, Slough, UK) and plated onto type I rat tail collagen-coated six-well tissue culture plates (Becton Dickinson). The cells were incubated at 37 °C with 5% CO_2 for 3–4 weeks. The medium was changed every 2 days for 7 days, and then twice a week until first passage. Colonies were counted when they became evident but before they became confluent.

In vitro *vascular tubule formation assay*

Matrigel matrix (Becton Dickinson) solution was thawed overnight at 4 °C, and all plasticware was precooled. Human umbilical vein endothelial cells (HUVECs) (Lonza) and EOCs were resuspended at 1×10^5 mL^{-1} in EBM-2 in the presence or absence of G-CSF (100 ng $^{-1}$mL). Five hundred microliters of cells were added to duplicate wells precoated with 250 μL of Matrigel that had been allowed to solidify for 1 h at 37 °C. Capillary structures and EC networks were examined by phase contrast microscopy (× 40 lens), using an inverted microscope (Nikon Eclipse TS100-F, Nikon Instruments, Kingston Upon Thames, UK). Pictures were taken at 4 h and at 22 h. The EC network was quantified from the image fields by scoring the number of cell–cell connections.

In vitro *angiogenesis: aortic ring assay*

C57Bl6 mice were killed by asphyxiation in CO_2. The thoracic aorta was removed, washed in serum-free MCDB 131 medium (Invitrogen), cleaned of periadventitial tissue, and divided into 1-mm rings. Aortic rings were embedded in 200 μL of Matrigel (Becton Dickinson) and incubated at 37 °C in serum-free MCDB 131, with heparin, ascorbic acid and GA1000 (Cambrex Biosciences, Wokingham, UK) in the presence or absence of G-CSF (100 ng mL^{-1}). The medium was changed every 48 h. All assays were performed in triplicate. The growth of new vessels was counted at day 4 and day 8 by light microscopy.

Subcutaneous sponge implantation assay for in vivo vascularization

Mice were anesthetized with halothane, and a sterilized sponge cylinder (0.5 cm diameter, 1 cm long) (Caligen Foam, Accrington, UK) was implanted subcutaneously on each flank. Each animal had an intervention-impregnated sponge [growth-factor-reduced (GFR)-Matrigel + G-CSF] on one side and a control, vehicle-impregnated sponge (GFR-Matrigel alone) on the other side. Twenty days after implantation, mice were killed, and sponges were excised. Sponges were fixed in 4% formalin and embedded in paraffin wax. Sections (5 μm) were stained with hematoxylin–eosin for identification of blood vessels, as previously described [35]. Vessel density within sponges was determined using the mean of triplicate Chalkley counts on each of two sections per sponge [36].

Statistical analysis

Unless otherwise stated, continuous variables are reported as mean ± standard error of the mean. Statistical analyses were performed with GraphPad Prism 4 (Graph Pad Software, La Jolla, CA, USA), using two-tailed Student's *t*-test, Mann–Whitney *U*-test or Wilcoxon paired tests where appropriate. A *P*-value of < 0.05 was considered to indicate statistical significance.

Results

The effects of in vivo *administration of a PBSC-mobilizing G-CSF regimen on indicators of angiogenesis*

The induced depression of CFU-EPC capacity in PB MNCs following G-CSF administration is profound but transient When individual healthy subjects (PBSC donors) were followed sequentially, G-CSF administration caused a profound decline in CFU-EPC activity from normal levels, which recovered with time (Fig. 1A). The CFU-EPC activity was virtually abolished after G-CSF administration as compared with the pre-G-CSF sample ($P < 0.001$, paired *t*-test, $n = 21$). CFU-EPC activity returned to almost basal levels in follow-up samples after 1–2 months following completion of G-CSF treatment ($P < 0.01$, paired *t*-test, $n = 13$). Similar depression and recovery of CFU-EPC activity following G-CSF mobilization of PBSCs (for autologous transplantation) was seen in a large series of hematology patients (data not shown).

G-CSF-mobilized PB MNCs are unable to generate CFU-EPCs and these are not recovered by monocyte enrichment Confirming what we have previously reported [9], CFU-EPCs were most prevalent in normal PB MNCs and were virtually absent from G-CSF-mobilized PB MNCs,

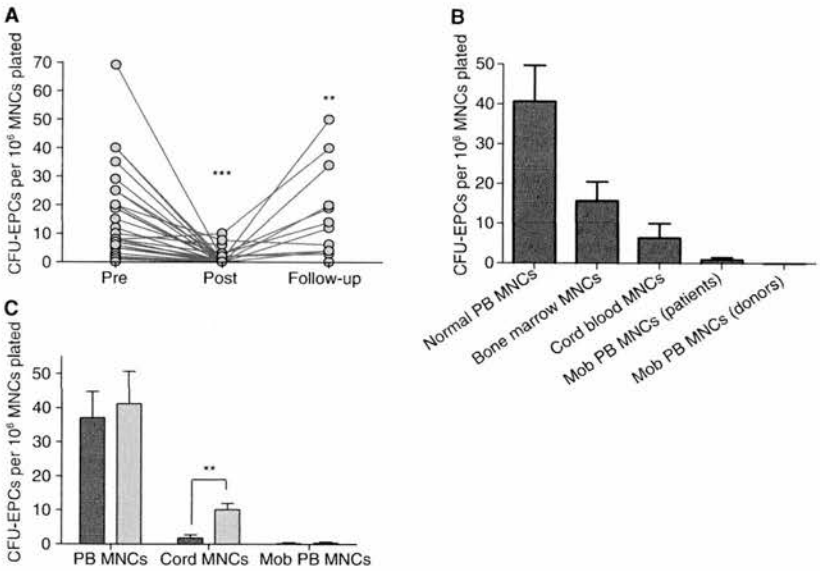


Fig. 1. Influence of administration of granulocyte colony-stimulating factor (G-CSF) for hematopoietic progenitor cell (HPC) mobilization (Mob) on colony-forming unit endothelial progenitor cells (CFU-EPCs) in peripheral blood (PB) mononuclear cells (MNCs). (A) There was a fall in CFU-EPCs following G-CSF administration to healthy adult HPC donors and a subsequent rise in CFU-EPCs at 1–2-month follow-up after mobilization. (B) CFU-EPCs in normal PB MNCs and in HPC-rich MNC sources. (C) CFU-EPCs in MNCs (black) and following monocyte (CD14) enrichment by plastic adherence (grey). A P-value of < 0.05 was considered to indicate statistical significance ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Pre, pre-G-CSF administration; Post, post-G-CSF administration.

whether from patient or healthy donor sources (Fig. 1B). We found that CFU-EPCs were slightly increased (e.g. from 21 to 25 per 10^6 MNCs, $n = 6$) when normalPB MNCs were enriched for monocytes (to around 80% CD14+) by 2 h of adherence on uncoated culture plates. Further enrichment of monocytes (to 98%) by CD14+ selection by FACS further increased CFU-EPCs (e.g. from 25 to 41 per 10^6 MNCs, $n = 6$) and completely removed CFU-EPCs from the CD14-depleted adherent cells. CFU-EPCs were not found in CD34-enriched (> 90%) or CD133-enriched (> 90%) MNCs from different HPC-rich sources (Table 1).

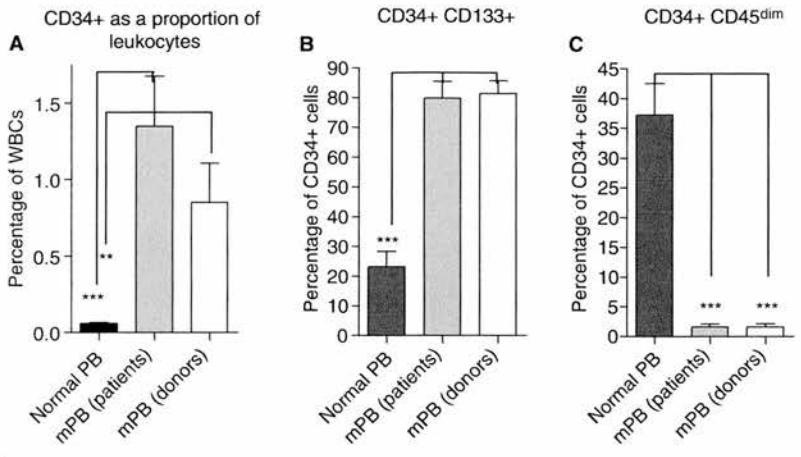
In a larger series studied by plastic adherence enrichment alone, normalPB MNCs generated a mean of 37 CFU-EPCs per 10^6 cells plated, CB MNCs generated fewer than five CFU-EPCs per 10^6 cells plated, and G-CSF-mobilized PB MNCs were virtually unable to form any CFU-EPCs (0.6 CFU-EPCs per 10^6 cells plated (Fig. 1C). Whereas enrichment of CD14+ cells by plastic adherence increased the number of CFU-EPCs slightly in normal PB MNCs and significally in CB MNCs, no CFU-EPCs were seen when CD14-enriched cells from G-CSF-mobilized MNCs were cultured.

Mobilization of PB HPCs with G-CSF alters the phenotype proportions of CD34+ cell subpopulations G-CSF-mobilized PB samples had a more than 10-fold higher proportion of CD34+ cells than normal PB samples (Fig. 2A). In agreement with our earlier studies [9], G-CSF-mobilized PB samples had markedly higher coexpression of CD133 by CD34+ cells (81.4% ± 10.5%) than normal PB samples (23.1% ± 18.2%). This was true for both healthy donors ($P < 0.001$, Mann–Whitney U-test, $n = 9$) and for autologous patients (Fig. 2B). Furthermore, in contrast to normal PB, in which a mean of 37% of CD34+ cells were $CD45^{low}$, G-CSF-mobilized PB samples contained very low proportions of CD34+$CD45^{low}$ cells (mean G-CSF-mobilized

Table 1 Effect of subpopulation enrichment/depletion on colony-forming unit endothelial progenitor cells (CFU-EPCs) in mononuclear cell (MNC) populations

Source	Number of CFU-EPCs per 10^6 MNCs plated
Unfractioned MNCs*	21
2-h adherent cells (> 80% CD14+)*	25
2-h non-adherent cells*	2
CD14-enriched (> 98%)*	41
CD14-depleted*	0
CD34-enriched (> 90%)†	0
CD34-depleted†	3
CD133-enriched (> 90%)†	0
CD133-depleted†	8

CFU-EPCs are slightly increased when normal peripheral blood MNCs are enriched for monocytes (to about 80% CD14+) by 2 h of adherence on uncoated culture plates. Further enrichment of monocytes (to 98%) by CD14+ selection by fluorescence-activated cell sorting further increased CFU-EPCs and completely removed CFU-EPCs from the CD14-depleted plastic-adherent cells. CFU-EPCs were not found in CD34-enriched (> 90%) or CD133-enriched (> 90%) MNCs from different HPC-rich cell sources. Their depleted column eluates tend to show reduced CFU-EPC activity as compared with the unfractioned starting MNCs, which may imply some loss of CFU-EPC activity by retention on columns by adhesion, implying these cells are adherent. *Paired normal peripheral blood samples ($n = 6$). †Unpaired HPC-rich samples (bone marrow, cord blood, mobilized blood).



D

Mean (SD)	Normal PB	Mob PB donors	Mob PB patients
Sample size (*n*)	11	9	11
CD34+ (10^6 L^{-1})	0.421 (0.110)	41.14 (37.27)	13.64 (10.91)
CD34+ CD133+ (10^6 L^{-1})	0.097 (0.020)	33.49 (03.91)	10.87 (01.60)
CD34+ $CD45^{low}$ (10^6 L^{-1})	0.156 (0.088)	0.671 (0.559)	0.224 (0.274)

Fig. 2. Influence of administration of granulocyte colony-stimulating factor (G-CSF) on hematopoietic progenitor cell (HPC) mobilization on CD34+ cells and CD34+ subpopulations in peripheral blood (PB) leukocytes. (A) CD34+ cells as a proportion of total leukocytes in normal PB and in G-CSF-mobilized PB from healthy donors and from hematologic malignancy patients in remission (mPB). (B) The proportion of CD34+ cells coexpressing CD133 in normal PB and in G-CSF-mobilized PB from healthy donors and from hematologic malignancy patients in remission. (C) The proportion of CD34+ cells with low to negligible CD45 expression in normal PB and in G-CSF-mobilized PB from healthy donors and from hematologic malignancy patients in remission. (D) Absolute numbers of circulating HPC subpopulations per liter of PB: all CD34+ cells; CD34+ cells coexpressing CD133; and CD34+ cells low in CD45 expression. The results are calculated from research laboratory determination of total leukocyte counts and proportional subpopulations, expressed as mean ± standard deviation (SD). A *P*-value of < 0.05 was considered to indicate statistical significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Mob, mobilized; WBC, white blood cell.

PB autologous, 1.64%; mean G-CSF-mobilized PB allogeneic, 1.63%) ($P < 0.001$, Mann–Whitney *U*-test, $n = 9$) (Fig. 2C), and their absolute numbers were little increased in G-CSF-mobilized PB as compared with normal PB, in contrast to total CD34+ numbers (Fig. 2D).

G-CSF-mobilized PB MNCs are unable to generate EOCs CB MNCs ($n = 15$) reliably generated EOC colonies when 10×10^6 MNCs were plated. For normal PB MNCs ($n = 12$), at least 30×10^6 MNCs had to be plated. G-CSF-mobilized blood MNCs were unable to form any EOC colonies for up to 30×10^6 MNCs plated ($n = 7$), either for healthy donors or for patients (Table 2).

EOC potential is associated with CD34+CD133− cells Enrichment of the CD34+ fraction of CB MNCs by magnetic bead cell sorting (> 90% purity) showed that this fraction was the source of all EOC colonies. No colonies were found in CD34-depleted MNCs (Table 2). Conversely, the CD133-enriched fraction from CB MNCs (> 90% purity) gave no EOC colonies, and all of the EOC colonies were generated from the CD133-depleted fraction. When the CD133− fraction was further sorted into CD34+ and CD34− fractions, only the CD34+ (CD133−) fraction gave EOC colonies. The number of cells required to produce EOC colonies fell dramatically with enrichment of CD34+CD133− cells. Enrichment for the CD34+CD133− cell population increased the frequency of EOC generation from CB MNCs, but not when G-CSF-mobilized blood MNCs were used.

Table 2 Attainment of endothelial outgrowth cell (EOC) colonies from different mononuclear cell (MNC) sources and subpopulations

Source (cord blood MNCs)	EOC colonies found	Number of MNCs plated for one EOC colony
Unfractioned MNCs	Yes	3×10^6
CD133-enriched (> 90%)	No	($> 1.3 \times 10^6$)
CD133-depleted	Yes	0.5×10^6
CD34-enriched (> 90%)	Yes	1.7×10^6
CD34-depleted	No	($> 3 \times 10^6$)
CD34+CD133+ (> 90%)	No	($> 1.65 \times 10^6$)
CD34+CD133-depleted	Yes	0.002×10^6

EOCs can be routinely cultured from cord blood MNCs plated at 5–10×10^6 per well on collagen. For normal adult peripheral blood MNCs, 30×10^6 MNCs per well are required to give approximately one EOC colony. From cord blood MNCs separated by magnetic beads, CD34-enriched cells form EOCs, but their CD34-depleted eluates do not. CD133-enriched cells do not form EOCs, but their CD133-depleted eluates do: if the CD133-depleted cells are further fractionated according to CD34 expression, the CD34-enriched (CD34+CD133−) cells form EOCs but the CD34− eluates do not.

The effect of direct addition of exogenous G-CSF on in vitro *indicators of angiogenesis*

The number of CFU-EPCs is reduced by addition of G-CSF to colony cultures The addition of G-CSF to normal PB MNCs *in vitro* significantly reduced the CFU-EPC frequency as compared with controls without G-CSF ($P < 0.01$, paired *t*-test, $n = 8$) (Fig. 3). The addition of 100 ng mL^{-1} vascular endothelial growth factor (VEGF) or stromal cell-derived factor-1 (SDF-1) to the wells to which G-CSF had been added did not rescue colony formation (data not shown).

Tubule formation by HUVECs and EOCs is reduced by G-CSF Vascular tubule formation by HUVECs in Matrigel showed a significant reduction at 22 h in the presence of G-CSF as compared with controls ($P < 0.01$, paired *t*-test, $n = 5$) (Fig. 4C); a paired example is shown in Fig. 4A,B. The addition of 100 ng mL^{-1} VEGF or SDF-1 to the wells with G-CSF did not rescue tube formation (data not shown). EOCs grown on collagen behave like HUVECs in many ways, and form tubules in Matrigel. As with HUVECs, tubule formation by EOCs in Matrigel was inhibited by G-CSF ($n = 5$) (Fig. 4D–F).

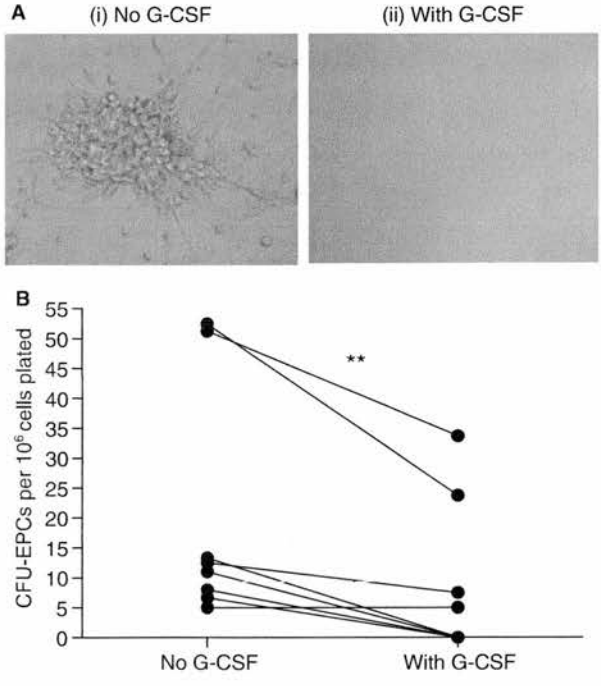


Fig. 3. Influence of exogenous granulocyte colony-stimulating factor (G-CSF) *in vitro* on colony-forming unit endothelial progenitor cells (CFU-EPCs). (A) Representative microscopy images of CFU-EPC formation (i) by normal peripheral blood mononuclear cells (MNCs) and (ii) in the presence of exogenous G-CSF (100 ng mL^{-1}). There was a significant reduction in MNC CFU-EPCs in the presence of G-CSF as compared with paired samples without G-CSF. A *P*-value of < 0.05 was considered to indicate statistical significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Angiogenesis from mouse aortic rings in vitro is reduced by G-CSF The numbers of vessels formed from murine aortic rings cultured *in vitro* and scored after 4 and 8 days were reduced in the presence of G-CSF as compared with controls at both time points ($P < 0.05$, paired *t*-test, $n = 4$, means of triplicates). Results scored at 8 days are shown in Fig. 5C. A paired example is shown in Fig. 5A,B.

In vivo *spontaneous angiogenesis in subcutaneous sponge implants in mice is inhibited by G-CSF* Control vehicle-impregnated sponges (GFR-Matrigel only) and G-CSF-impregnated sponges (G-CSF in GRF-Matrigel) excised after 20 days following implantation both appeared red on gross inspection, with lace-like coverings of blood vessels. They also both showed infiltration of organized matrix and an abundance of blood vessels. On histologic examination, all sponges exhibited vascularization, but G-CSF-impregnated sponges had significantly fewer blood vessels than controls when scored by Chalkley counts ($P < 0.001$, Mann–Whitney *U*-test, $n = 4$) (Fig. 6). A group of mice ($n = 4$) implanted with untreated sponges (no GFR-Matrigel vehicle) on both flanks exhibited a similar level of vasculogenesis as that seen in vehicle-impregnated sponges (GFR-Matrigel), demonstrating that GFR-Matrigel as vehicle has no intrinsic effect on vascularization (not shown).

Discussion

It is well established that G-CSF administration successfully mobilizes progenitor cells to PB, and these cells are able to reconstitute the hematopoietic system; therefore, it is thought that G-CSF mobilization might also increase the number of circulating EPCs. In this study, we used PBSCs from subjects receiving G-CSF for HPC mobilization, to examine the possibility that EPCs are mobilized into the circulation concurrently. We were unable to detect EPCs in G-CSF-mobilized PBSCs, using several of the published EPC phenotypes. G-CSF administration for HPC mobilization not only failed to mobilize EPCs, but also inhibited angiogenesis *in vitro* and *in vivo*.

Neither G-CSF-mobilized PB MNCs nor enriched monocytes are able to generate CFU-EPCs

Initial observations showed that, following administration of G-CSF to healthy allogeneic PBSC donors, there was a profound depression of CFU-EPC generation [9]. This reduction in endothelial colony potential has been consistently shown by independent observers in our group and in over 70 different samples studied. Longitudinal analysis of sequential PB samples from healthy donors undergoing PBSC mobilization showed that CFU-EPC numbers were severely reduced immediately after G-CSF administration, but returned to almost pretreatment levels within 2 months (Fig. 1A). Similar results have been obtained in hematologic malignancy patients undergoing PBSC mobilization for autologous transplantation, and may indicate that preceding

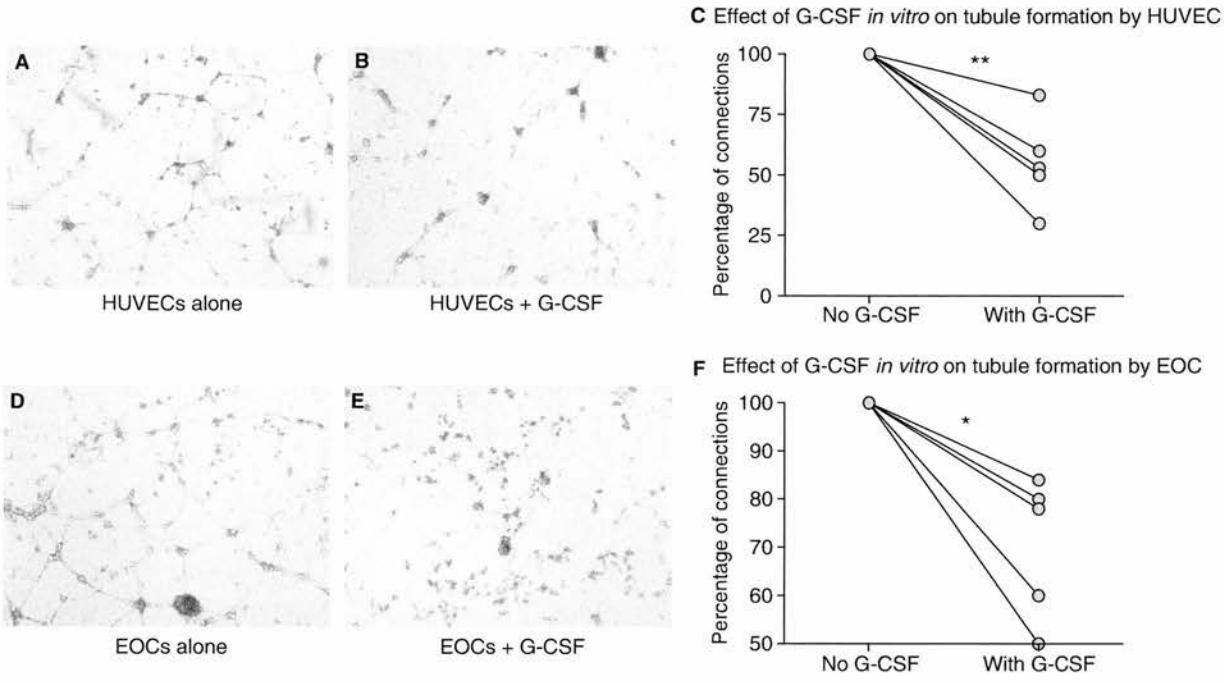


Fig. 4. Influence of exogenous granulocyte colony-stimulating factor (G-CSF) *in vitro* on tubule formation. (A, B) Representative microscopy images of (A) normal tubule formation by human umbilical vein endothelial cells (HUVECs) and (B) tubule formation by HUVECs in a paired culture with exogenous G-CSF added. (C) There was a significant reduction in HUVEC tubule connections in the presence of G-CSF as compared with paired samples without G-CSF. (D, E) Representative microscopy images of (D) normal tubule formation by endothelial outgrowth cells (EOCs) and (E) tubule formation by EOCs in a paired culture with exogenous G-CSF added. (F) There was a significant reduction in EOC tubule connections in the presence of G-CSF as compared to paired samples without G-CSF. A P-value of < 0.05 was considered to indicate statistical significance ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

chemotherapy does not markedly affect many putative laboratory measures of PBSC or endothelial function. Although CFU-EPCs were originally proposed by Hill *et al.* [17] as a correlate of EPC frequency, it is now established that they represent an expression of the activity of CD14+ monocytes [37], which possibly constitute a key proangiogenic monocyte subpopulation, not related to any HPC population. Monocyte enrichment by plastic adherence increased the frequency of CFU-EPC generation from umbilical cord MNCs but did not increase the frequency of CFU-EPC generation from G-CSF-mobilized blood MNCs (Fig. 1C), which, in contrast to CB monocytes, appear to be unresponsive in this assay.

A recognized, strong, inverse correlation between CFU-EPC frequency and cardiovascular risk has been reported extensively (reviewed in [20,21]). This accumulation of reports is not trivial, and indicates that CFU-EPC measurement can assess some aspects of angiogenic capacity. Thus, although CFU-EPC measurement seems not to be an indicator of EPC frequency, as originally proposed, depressed CFU-EPC activity in G-CSF-mobilized samples probably reflects reduced monocyte proangiogenic capacity.

G-CSF-mobilized PB MNCs are unable to generate EOCs

There are a number of claims that G-CSF mobilizes EPCs [6–8], but these depend on how EPCs are defined and interpreted. A very few studies are based on a reported increase in CFU-EPC frequency [7], and nearly all are based on putative phenotype characterization of EPCs. Although the proposed phenotypes for EPC have been dominated by variants based on coexpression of CD34 and CD133, the definitive phenotype of an EPC remains elusive. Recently, it has been reported that true circulating EPCs (EOCs) are CD34-positive but CD133-negative and CD45-negative, whereas cells expressing CD133 and CD45 remain hematopoietic and do not give rise to true ECs [13,14,38]. Previous observations showed that the HPCs in G-CSF-mobilized blood are predominantly CD34+CD133+ [9,39], so by phenotype alone there is no evidence of mobilization of EOCs (contained in the CD34+CD133− subpopulation) by G-CSF (Fig. 2B), and nor is there evidence that CD34+ cells with low or negligible expression of the panleukocyte marker CD45 are selectively mobilized by G-CSF (Fig. 2C). Indeed, no EOCs could be cultured from G-CSF-mobilized blood MNCs (30×10^6) from either autologous patients or allogeneic donors, whereas, in most cases, at least one EOC colony can be found in comparable normal PB MNCs, so there is no evidence that EOCs are mobilized by G-CSF. As there is little or no selective mobilization of CD34+CD133−CD45− cells by G-CSF, this may account for the failure to find EOCs in these samples. Furthermore, enrichment for the proposed EOC precursor population (CD34+CD133−) by magnetic beads increased the frequency of EOC generation from umbilical cord MNCs, but not from

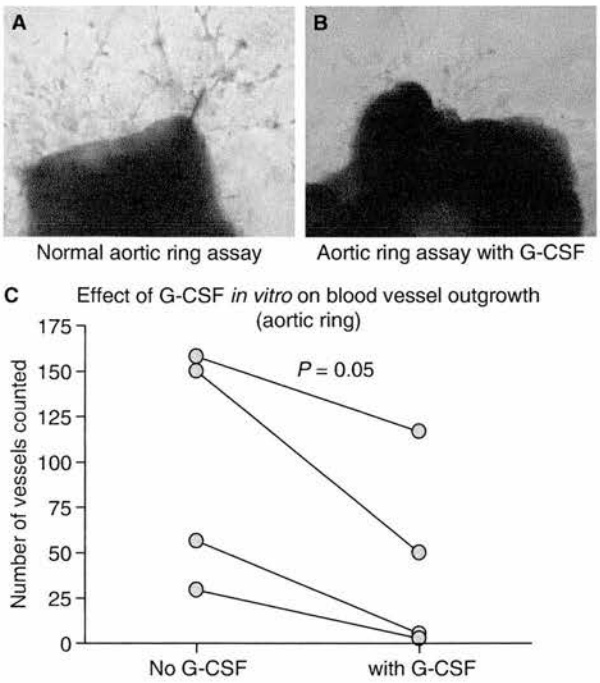


Fig. 5. Influence of exogenous granulocyte colony-stimulating factor (G-CSF) *in vitro* on microvessel outgrowth from mouse aortic rings. Representative microscopy images of (A) normal microvessel outgrowth from mouse aortic ring and (B) microvessel outgrowth from a paired aortic ring sample from the same mouse in the presence of added exogenous G-CSF. (C) There was a significant reduction in microvessel outgrowth from mouse aortic rings in the presence of G-CSF as compared with paired samples without G-CSF.

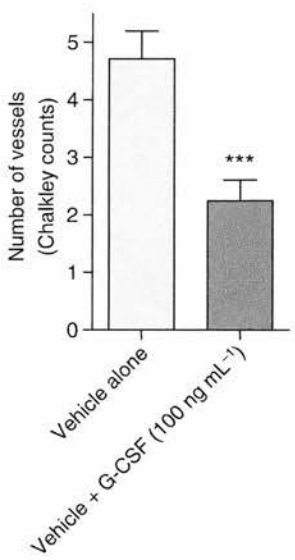


Fig. 6. Inhibition of angiogenesis *in vivo* by granulocyte colony-stimulating factor (G-CSF). There was a reduction of spontaneous vascularization of subcutaneously implanted sponges containing G-CSF in growth-factor-reduced Matrigel as compared with contralateral sponges in the same animal containing growth-factor-reduced Matrigel alone. A P-value of < 0.05 was considered to indicate statistical significance ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

G-CSF-mobilized blood MNCs. We have no evidence to judge whether EOCs are prsent at low frequency/absent or inactive in G-CSF-mobilized MNCs.

G-CSF has a direct inhibitory effect on angiogenesis

Although some of the effects of G-CSF on CFU-EPCs *in vivo* might result from alteration of the balance of different cell types in the circulation and dilution of some MNC subpopulations by others, it can be shown that G-CSF has a direct effect *in vitro* on CFU-EPCs. In paired MNC samples, the addition of G-CSF resulted in a decrease in CFU-EPC frequency (Fig. 3). Furthermore, the addition of known angiogenesis-promoting cytokines such as VEGF or SDF-1 to the G-CSF-treated MNCs did not rescue colony formation. Similarly, it can be shown that G-CSF depresses the expression *in vitro* of accepted endothelial cell functions, such as the formation of cell–cell links in human EC (HUVEC and EOC) tubule formation in Matrigel (Fig. 4) and in microvessel outgrowth from mouse aortic rings (Fig. 5).

The direct effect of G-CSF on angiogenesis was ultimately confirmed with the use of an *in vivo* mouse model of angiogenesis (subcutaneous sponge implantation). Localized G-CSF substantially inhibited spontaneous vascularization of sponges *in vivo* (Fig. 6), in direct contrast to what was seen in paired sponges lacking G-CSF in the same animals. Honold *et al.* [8] showed that EPCs in G-CSF-mobilized samples were transiently dysfunctional, owing to the cleavage of the chemokine receptor CXCR4, which is directly involved in stem cell homing. Thus, the observed reduction of endogenous blood vessel formation in the G-CSF-treated sponge may reflect a localized decline in the ability to recruit potential murine angiogenic cells. Preliminary evidence from our current work suggests that G-CSF may downregulate the expression of certain cell surface receptors and adherence molecules, which may impair the ability of cells to function in certain environments; this could explain the observed CFU-EPC depression and might be important in endothelial function and/or angiogenesis. This is currently under investigation.

In agreement with our findings, a recent meta-analysis reported that G-CSF infusion alone has no significant clinical benefit in myocardial infarction [30], and G-CSF-mobilized PBSCs were reported to be less effective in inducing ulcer healing than BMSCs [31]. However, a significant number of reports to date have shown that cellular therapies employing G-CSF-mobilized cells have some clinical benefit [27–29]. Thus, although G-CSF may not selectively mobilize true EPCs as defined by EOCs, and although it may inhibit monocyte proangiogenic activity and EC angiogenic activity, G-CSF does induce an increase in the number of circulating HPCs, which might home to ischemic lesions [27] and could therefore provide a paracrine effect without any incorporation into new vessels. These may be equivalent to the cells provided from bone marrow, and if that is the principal effect required in some aspects of therapeutic vascularization, then mobilized PBSCs may be as beneficial as BMSCs in clinical use.

In summary, this study has shown that there is a profound reduction in the number of CFU-EPCs following G-CSF administration, which recovers with time. To the best of our knowledge, we are the first to show that there is no evidence of circulating EOCs following G-CSF administration for mobilization of HPCs. G-CSF-mobilized PBSC were predominantly CD34+CD133+ cells, which are almost certainly hematopoietic cells. The presence *in vitro* of exogenous G-CSF had a direct antiangiogenic effect that was not abrogated by the addition of proangiogenic factors.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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