EVALUATION OF HUMAN HEMOPOIETIC STEM CELL ASSAYS FOR TRANSPLANTATION AND GENE THERAPY

EVALUATIE VAN MENSELIJKE HEMOPOIETISCHE STAMCEL ASSAYS VOOR TRANSPLANTATIE EN GENTHERAPIE

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Een leven zonder dat men zoekt, is even ellendig als een leven nadat men gevonden heeft.

Jacob Israël de Haan, 1881-1924

Voor Merijn en mijn ouders

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

SCOPE OF THIS THESIS

Hemopoietic stem cell transplantation has become an important treatment modality for various hematological and non-hematological diseases, e.g. leukemia, lymphoma, congenital immunodeficiencies, autoimmune disease as well as solid tumors. In addition, the use of hemopoietic stem cell transplantation to induce donor-specific tolerance for organ transplantation is explored.

Due to increased use of graft manipulation prior to transplantation, including tumor cell purging, stem cell expansion or gene therapy, there is a strong need for in vitro assays able to assess the number and the quality of human in vivo repopulating stem cells. The stroma-supported cobblestone area forming cell (CAFC) assay allows for determination of the frequency of progenitor cell subsets in various hemopoietic materials. Additionally, the stroma-supported flask long-term culture colony-forming cell (LTC-CFC) assay provides means to assess the quality of the graft by determining the ability of the progenitor cells in the graft to produce progeny. Both assays have been extensively studied in the murine model and good correlations have been established with in vivo assays for transient and permanent engrafting stem cell subsets (1-5).

The work described in this thesis aims to clarify whether the CAFC and LTC-CFC assays provide a reliable measure for the human in vivo repopulating stem cells as well. In order to accomplish this, we have determined CAFC and LTC-CFC subsets in human mobilized peripheral blood (MPB) and bone marrow (BM) grafts, and correlated them with clinical parameters reflecting reconstitution of transplanted patients. In addition, data from in vitro assays were compared with those generated in a humanized immunodeficient mouse model.

GENERAL INTRODUCTION

1.1 The hemopoietic system

Except for lymphocytes, the mature peripheral blood cells have a limited lifespan and are incapable of self-renewal. The replacement of these cells depends on the function of less differentiated hemopoietic cells with proliferative capabilities. In combination with data suggesting that both the myeloid and the lymphoid cells originate from a single primitive precursor (6-12) this leads to a three compartment model for the hemopoietic system (Figure 1.1). The first, most primitive compartment consists of multipotential hemopoietic stem cells that possess extensive self-renewal capabilities and generate primitive progenitors that are programmed to differentiate (commitment). The second compartment consists of progenitors committed to differentiation to a single or a restricted number of lineages. Cells in this compartment respond to humoral regulators and are primarily responsible for maintaining the level of mature blood cells. The third compartment consists of mature cells such as erythrocytes and granulocytes, which have specialized functions and limited or no ability to proliferate (13).

The pluripotent hemopoietic stem cells (HSC) reside in the BM cavity during adult life (7) with only a few cell clones participating in the production of committed progenitors and eventually billions of mature blood cells (12, 14-17). The largest part of the stem cell pool remains in a quiescent state (18-22).

The process by which the progenitor and stem cell hierarchy of the hemopoietic system is established has been proposed to consist of stochastic mechanisms (23-27) in combination with signals provided by the microenvironment in the BM (12, 28).

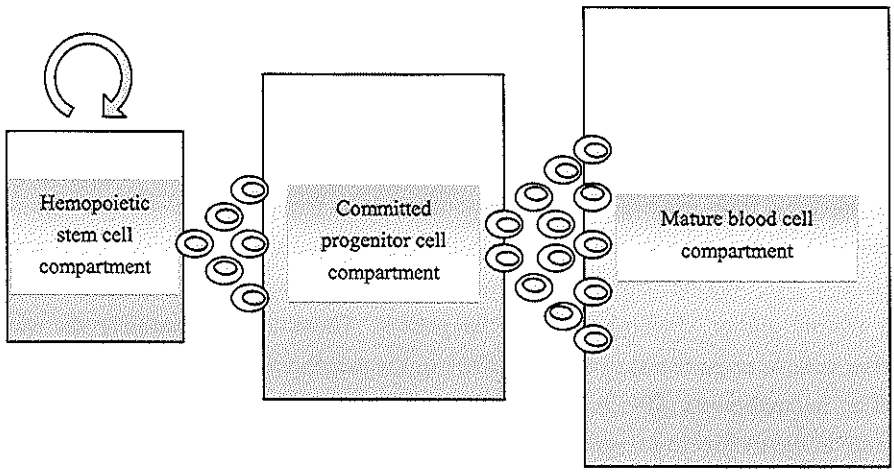

Figure 1.1 Schematic representation of the structure and organization of the hemopoietic system. The arrow indicates self-renewal.

1.2 Primitive human hemopoietic cells and transplantation

In infancy and early childhood all the BM cavities contain hemopoiesis while during adult life active hemopoiesis is confined to the central skeleton and the proximal ends of the femura and humeri.

BM is the classical source of hemopoietic cells in stem cell transplantation for various diseases, varying from leukemia to inborn errors of metabolism (reviewed in (29-32)). BM is used in the autologous as well as in the allogeneic transplantation setting. The autologous setting, where the donor is at the same time the recipient, is only feasible if the repopulating stem cells themselves are healthy and the graft is free of cells capable of inducing recurrence of the disease after transplantation. The latter can be pursued by extensive treatment of the patient previous to harvesting of the BM and/or purging of the marrow by using antibodies directed to the affected or the healthy cells (33-35). In case of allogeneic transplantation, where the graft is obtained from a healthy donor, the genetic differences between donor and recipient can initiate a cytokine mediated T-cell reaction directed to the recipient causing graft-versus-host disease (GvHD) (36-38). The incidence and severity of GvHD, which is fatal without proper

precautions (39), can be reduced by lymphocyte depletion of the graft (40) and administration of immunosuppressive agents (41-43). However, GvHD has also proven to be beneficial in case of malignant diseases of the BM as the reactive T-cells mediate a 'graft-versus-leukemia' effect as well (44, 45).

That human peripheral blood also contains stem cells with in vivo repopulating ability became apparent from case reports where patients were transplanted with autologous blood cells (46, 47). However, the frequency of stem and progenitor cells in the adult peripheral blood is low (48, 49) but can be dramatically increased by administration of hemopoietic growth factors and/or chemotherapy to the patient. These agents stimulate the stem and progenitor cells to migrate out of the BM cavity to the peripheral blood from which they can be isolated by leukapheresis (50-55). These MPB cells have shown to be a good alternative stem cell source in the treatment for numerous diseases allowing for a faster post-transplant recovery as compared to BM derived stem cells (56-60).

Blood harvested from the umbilical cord and the placental veins directly after birth of both term and pre-term pregnancies contains hemopoietic stem and progenitor cells as well (61). Umbilical cord blood (UCB) derived stem and progenitor cells have been shown to possess several distinct qualities: a) the number of committed progenitor cells are enriched in UCB as compared to adult peripheral blood (62, 63), b) in long-term hemopoietic in vitro cultures UCB progenitor cell production and culture life span are significantly increased compared to adult BM cultures (64) and c) UCB cells have an increased ability to repopulate the BM of an immunodeficient mouse (65-67) as compared to BM (68-70) and MPB (71, 72).

UCB as a stem cell source for transplantation has several advantages as compared to BM or MPB, including 1) immediate availability (minimum of 2 weeks for HLA-testing), 2) low donor risk, 3) a very low risk of transmissible diseases, such as cytomegalovirus or Epstein-Barr virus, 4) a potentially lower risk of acute GvHD (73) and 5) access to donor pools in targeted ethnic and racial minorities. However, disadvantages are 1) lower risk of acute GvHD might translate into a higher risk of relapse and 2) an insufficient number of stem and progenitor cells in UCB for larger recipients, limiting this stem cell source to predominantly pediatric patients (74). Still, UCB is used in the allogeneic stem cell transplantation setting for various diseases in children and young adults (74).

The speed of neutrophil recovery after UCB transplantation is comparable to that observed after allogeneic BMT but platelet recovery is delayed. An explanation for the latter

might be the finding that the number of megakaryocyte precursors in UCB graft, as assessed using phenotypical and functional assays, is reduced as compared to successful repopulating allogeneic MPB or BM transplants (73, 75).

In order to reduce the time needed for platelet recovery after UCB transplantation and to be able to transplant adult patients with initial small cord blood collections, extensive effort has been undertaken to increase the progenitor and stem cell content using ex vivo expansion procedures. Expansion protocols using serum-free or serum containing conditions, with or without stroma in combination with various cytokines have been evaluated using in vitro assays (64, 76-84) as well as immunodeficient mouse models (85-95). There have been case reports in which patients received an allogeneic cord blood transplant that contained ex vivo expanded cells (96, 97). However, clinical trials using grafts consisting of unmanipulated as well as genetically marked expanded cells should provide the definite answer about the effectiveness of ex vivo expansion procedures.

Another possible source of potent HSC is the fetal liver (FL) of the developing human (Löwenberg, thesis 1975). From studying the developing mouse and human it is known that the development of the HSC compartment in a fetus is a dynamic process dominated by migrating populations of hemopoietic stem and progenitor cells. The first signs of hemopoiesis can be detected in the yolk sac. This stage of hemopoiesis is called embryonic or primitive hemopoiesis and is characterized by the production of nucleated erythrocytes and a limited number of macrophages. The definitive hemopoiesis originates in the embryo itself, in the para-aortic splanchopleura, which later develops into the aorta-gonadal mesonephros region. The FL subsequently becomes the active site of hemopoiesis. Eventually the frequency of HSC in the FL decreases as hemopoiesis gradually shifts to the BM, the principal site of hemopoiesis in the adult. The FL remains the dominant hemopoietic site until the neonatal period (98).

Human FL cells have been extensively studied using in vitro and animal models, revealing that FL cells produce more (erythroid) colonies in long-term culture as compared to UCB or BM (99). Additionally, human FL cells generated multilineage hemopoiesis in immunodeficient mice (100, 101), humans (102) as well as pre-immune fetuses of sheep (103). Since 1988 a limited number of prenatal allogeneic FL transplantation in humans has been performed, resulting in functional donor-engraftment in absence of GvHD (104). However,

favorable results using in utero transplantation have thus far only been obtained in severe immunocompromized recipients (105).

Recently, the premise that stem cells capable of long-term hemopoiesis reside only in the BM cavity has been challenged in publications of various independent research groups. In 1996 Goodell et al (106) published the isolation of a cell population with extensive in vivo repopulating abilities from murine BM using a sorting procedure on the basis of Hoechst 33342 efflux, the so-called side population (SP). These cells lack the characteristic stem and progenitor cell marker CD34. Analysis of human, rhesus and miniature swine BM reveals a small, distinct population of cells that efflux the Hoechst 33342 dye in a manner identical to murine SP cells. Gussoni et al (107) and Jackson et al (108) isolated a functionally similar SP from murine striated muscle, though with a slightly different phenotype as compared to the SP cells isolated from BM. Additionally, Bjornson et al (109) published in 1999 that cultured brain cells were able to competitively repopulate the BM of sublethally irradiated hosts. Although extensive research is needed to characterize these apparently very primitive cells, these promising findings might eventually increase the variety of diseases for which cell transplantation is an important treatment option.

1.3 Human hemopoietic stem and progenitor cell assays

Because the possibilities to study hemopoiesis in humans are restricted, numerous surrogate in vitro and animal models have been developed to characterize and enumerate human stem and progenitor cells. However, the question remains whether each of these assays provides a reliable quantitative or qualitative indication of the in vivo repopulating stem cell subset. Therefore, it is important to list the advantages and limitations of the various in vitro and in vivo assays.

A. In vitro assays

a. Phenotypic analysis

Cells can be identified and sorted using flowcytometry on the basis of expression of proteins using fluorescently labeled antibodies or ligands.

Human HSC and progenitor cells are generally characterized by the expression of CD34. $CD34^+$ cells can be divided in immature stem cells and various committed progenitor cells on the basis of the absence or presence of additional cell surface markers. Immature pluripotent HSC are $CD34^+$ and lack the differentiation related marker CD38 (69, 110, 111), possess the myeloid marker CD33 (112) and express no or low levels of the HLA-DR antigen (113-115) and the Thy-1 antigen (116). Other phenotypic markers like KDR-1 (VEGF-R2) (117), AC133 (118, 119), c-kit (120) and the cytokine receptor CXCR-4 (121, 122) enable the discrimination of $CD34^+$ subsets with distinct functional characteristics in in vitro assays and immunodeficient mouse repopulation assays as well.

Recently, also human $CD34^-$ cells that do not express lineage markers ($CD34^-/Lin^-$), have shown to posses the ability to repopulate the BM of the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse (123) as well as pre-immune sheep fetuses (124). In both in vivo repopulation models primary and secondary recipients engrafted following $CD34^-/Lin^-$ cells transplantation, indicative of the long-term repopulating ability of these cells.

At this moment it is still unclear how the $CD34^+$ and the $CD34^-$ cells within the Lin^- population are hierarchically related. As Sato et al (125) have shown in the murine system, it might be possible that the two phenotypes are interconvertible with CD34 as a marker for activation of the cell. On the other hand, culturing lineage-depleted $CD34^-$ cells resulted in an increase of the number of colony-forming cells and an increased ability to repopulate the NOD/SCID mouse BM (126). Additionally, unmanipulated $CD34^-$ cells have shown not to be exhaustible in pre-immune fetal sheep serial transplants while $CD34^+$ cells were (124). These data suggest that $CD34^-$ cells are a reservoir for the $CD34^+$ population. Additional studies need to be performed to elucidate this topic, as 'to select or not to select for $CD34^+$ cells' might be relevant for clinical transplantation.

It seems that functional capacity and phenotype of stem and progenitor cell populations correlate only to a certain extent. This means that a population of cells with a primitive

phenotype can contain the long-term repopulating stem cells, e.g. $CD34^+/CD38^-$ cells, but the phenotype itself is not representative for a stem cell. Additionally, as has become clear recently, the correlation between functionality and phenotype decreases even further as cells are manipulated in vitro (91, 95). Therefore, the relation between phenotype and functional capacities of tentative progenitor and stem cell populations need to be monitored during development of new graft manipulation procedures.

b. Colony-forming unit in culture (CFU-C) assay

Hemopoietic progenitor cells can be recognized and classified by the ability to form colonies in vitro and in vivo. The cell that forms a colony in vitro in a semi-solid medium in the presence of hemopoietic growth factors is called a CFU-C. The classification of the colony-forming cell depends on the cell types that are contained in the colony, e.g. colonies that contain granulocytes en macrophages are derived from a CFU-GM. Similarly, erythroid progenitors form under appropriate in vitro conditions the burst forming unit-erythroid (BFU-E). The types of colonies that can be found are directly related to the growth conditions in the assay.

The majority of cells that form colonies in a semi-solid medium lack in vivo repopulating ability. Primitive stem cells may form larger colonies in the CFU-C assay and have secondary plating abilities as compared to progenitor cells. It remains however difficult to distinguish colonies derived from more primitive stem cells or committed progenitor cells. In conclusion, the CFU-C assay allows assessment of the functional progenitor content of a graft.

c. High proliferative potential colony-forming unit in culture (HPP-CFU-C)

The HPP-CFU-C was initially described as a murine BM progenitor with a high proliferative potential in the CFU-C assay producing colonies containing more than 50 $x10^3$ cells and consisting mainly of macrophages (127). A major feature of the HPP-CFU-C is the resistance to the S-phase specific drug hydroxyurea (127). HPP-CFU-Cs can also be found in human BM (128). Several studies described a low correlation between HPP-CFU-C numbers and in vivo repopulating cells (129, 130). This may indicate that the majority of HPP-CFU-Cs is derived from primitive committed progenitor cells and not from HSC.

d. Stroma-supported long-term culture assays

In order to quantify and characterize stem cells with long-term in vivo repopulating ability in vitro, several stroma-supported assays have been developed. These assays are based on the observation that primitive hemopoietic progenitors can proliferate, produce committed progenitors and form visible clones beneath a stromal layer (131-133) due to the local production and presentation of cytokines and extracellular matrix components.

The long-term culture-initiating cell (LTC-IC) assay

Committed progenitors present in the graft disappear from stroma-based cultures during the initial 3 to 5 weeks of culture, whereas primitive progenitors can persist for more than 5 weeks and generate secondary CFU-Cs (113, 115). These cells are termed LTC-IC. In order to determine the absolute numbers of LTC-IC a limiting dilution setup needs to be applied (134). Using limiting dilution techniques the frequency of the LTC-IC in various hemopoietic materials has been established (135-137). Although the LTC-IC represent primitive progenitors, it is unknown whether they represent the pluripotent, self-renewing HSC.

The cobblestone area forming cell (CAFC) assay

To avoid replating large numbers of wells, a limiting dilution type LTC-IC assay was developed with a directly visible endpoint, namely the cobblestone area (CA). This resulted initially in the murine CAFC assay (1) but the assay was subsequently adapted for human hemopoiesis (138). The percentage of wells with at least one phase-dark hemopoietic clone, i.e. CA, consisting of at least five cells, is determined at several time points. In the mouse, this assay allows differential frequency analysis of transiently and permanently repopulating HSC as the moment at which a hemopoietic cell is active is directly related to the primitiveness of the stem cell clone (2). As with the LTC-IC assay it is unclear how the human primitive CAFC subsets (week 6 of culture and more) relate to the *true* in vivo repopulating stem cell.

Long-term culture colony-forming cell (LTC-CFC) assay

The progenitor cell production by stem cell subsets can be assessed by replating the adherent stromal layer containing hemopoietic cells and the non-adherent supernatant of long-term cultures to the CFU-C assay at various time points. The number of progenitors produced at a certain time point after initiation of the culture is a measure for the quality of the graft. This

assay allows for studying the heterogeneity of progenitor cell production of the various stem and progenitor cell subsets in various hemopoietic materials of different species, i.e. human, mouse, rat and rhesus monkey.

The frequency of the primitive progenitors (CAFC subsets and LTC-IC) or the ability of the graft to produce committed progenitors (LTC-CFC) is influenced by numerous variables of culture, for instance the kind of serum used, presence of additional cytokines (138-140), the type of stromal cell line used (141-143) and the presence and concentration of hydrocortisone (144, 145). Therefore, the results of these assays need to be interpreted with caution.

The potentials of the stroma-supported assays have been enhanced by extending the culture time of the long-term culture to at least 60 days (extended LTC-IC) (146, 147). By prolonging the culture time, more primitive progenitors, that may be functionally closer to the human long-term repopulating stem cell in vivo, will be activated to produce progenitors. As with the LTC-IC and the CAFC assessment, culture conditions influence the extended LTC-IC enumeration, e.g. the stromal layer (148). A disadvantage of the extended cultures is the increased labor intensity and chance for contamination.

e. Myeloid lymphoid-initiating cell (ML-IC) assay

This assay allows the identification of a single human BM progenitor, tentatively closely related to HSC, because of its capability of generating multiple secondary progenitors that can reinitiate long-term myeloid and lymphoid hemopoiesis in vitro (149). The ML-IC might prove to be useful to enumerate the number of very primitive human progenitors with multilineage potential and to evaluate new graft manipulation procedures. However, as this assay takes at least 10 weeks, it is very laborious and the practical applicability is limited.

A different way to assess the myeloid-lymphoid potential of cells is the use of 'switch cultures'. In these assays myeloid culture conditions are succeeded by conditions that facilitate lymphopoiesis (150, 151).

B. In vivo assays

Although some hemopoietic cells characterized in vitro have the multipotential and proliferative properties of pluripotent HSC it is necessary to provide evidence that these cell populations have also long-term repopulating ability in vivo. To study human HSC with in vivo repopulating ability, immunodeficient animal models allowing xenografting have been developed.

a. SCID hu model

In this model, fetal bone (152-155), thymus or liver (156) fragments are implanted under the skin or the renal capsule in the SCID mouse. The implants are subsequently injected with human cells. At various time points after grafting the implants are evaluated for CFU-C content or (multilineage) engraftment of human cells, using flowcytometry or DNA analysis. This assay allows for the assessment of the proliferative potential of the graft but does not provide for any quantitative information about the stem and progenitor cells present in the graft.

b. (NOD/)SCID mouse model

This model is used to study the repopulating ability of human hemopoietic cells in sublethally irradiated (NOD/)SCID mice. Five to 12 weeks after intravenous transplantation of the human cells the mice are sacrificed and the BM and other organs are evaluated for human cells using flowcytometry or DNA analysis. If this model is applied in a limiting dilution setup, i.e. transplanting limited numbers of cells in mice, it allows for a frequency analysis of the SCID repopulating cell (SRC) in various human hemopoietic materials (70). The SRC has been proposed to be a more immature stem cell than assessed by the CAFC week (wk.) 6 on the basis of differences in frequency, phenotype, transducability and multilineage outgrowth potential in immunodeficient recipients. Recently, Kollet et al (157) reported that the newly developed NOD/SCID ß2^{null} mice are better recipients for human hemopoietic cells than NOD/SCID mice as the SRC frequency in UCB is more than 11-fold higher in the NOD/SCID ß2^{null} mice.

A disadvantage of the immunodeficient mouse models is that the mice are prone to develop tumors and infections at an early time point in life. Therefore, the period in which the repopulating ability of the human cells in the mice can be studied is limited.

Instead of using immunodeficient animals to overcome the xenogeneic transplantation barrier, making use of a natural and passing period of immunodeficiency in the pre-immune fetus provides an alternative.

c. Fetal sheep assay

In 1986, Flake et al (158) published the fetal sheep transplantation model. In this model, pre-immune fetuses are intra-peritoneally transplanted with hemopoietic cells. If the pregnancy comes to term the newborn sheep can be evaluated for donor-recipient chimerism in the peripheral blood (PB) and BM. Several years later, Zanjani et al (103) transplanted human FL cells to sheep fetuses. Human cells from various lineages could be determined in the BM and the PB of the recipient sheep for several years.

Because of the 7 to 8 year life span of a sheep, the size of the recipients and the fact that secondary recipients are repopulated successfully as well (159), this model is suitable to assess the long-term in vivo repopulating ability of human hemopoietic cells. However, the number of unsuccessful transplantations, abortions and low level of human hemopoiesis in the recipient sheep limit the possibilities of this animal model.

d. Nonhuman primates

For several decades nonhuman primates have been used as a comparative in vivo model for human hemopoiesis. Especially the common marmoset, rhesus monkey and baboon are used in experiments to evaluate gene transfer protocols (160-168) or the effect of growth factors on steady state hemopoiesis (169, 170) and on hematological recovery after conditioning by chemotherapy or irradiation (171-181). A major advantage of using these nonhuman primates is that the experiments resemble the human setting to a high extent. However, the high costs involved in purchasing, maintaining and treatment of the animals prohibit large studies.

C. Overview of various progenitor and stem cell assays, as currently employed, and their mutual relation (Figure 1.2).

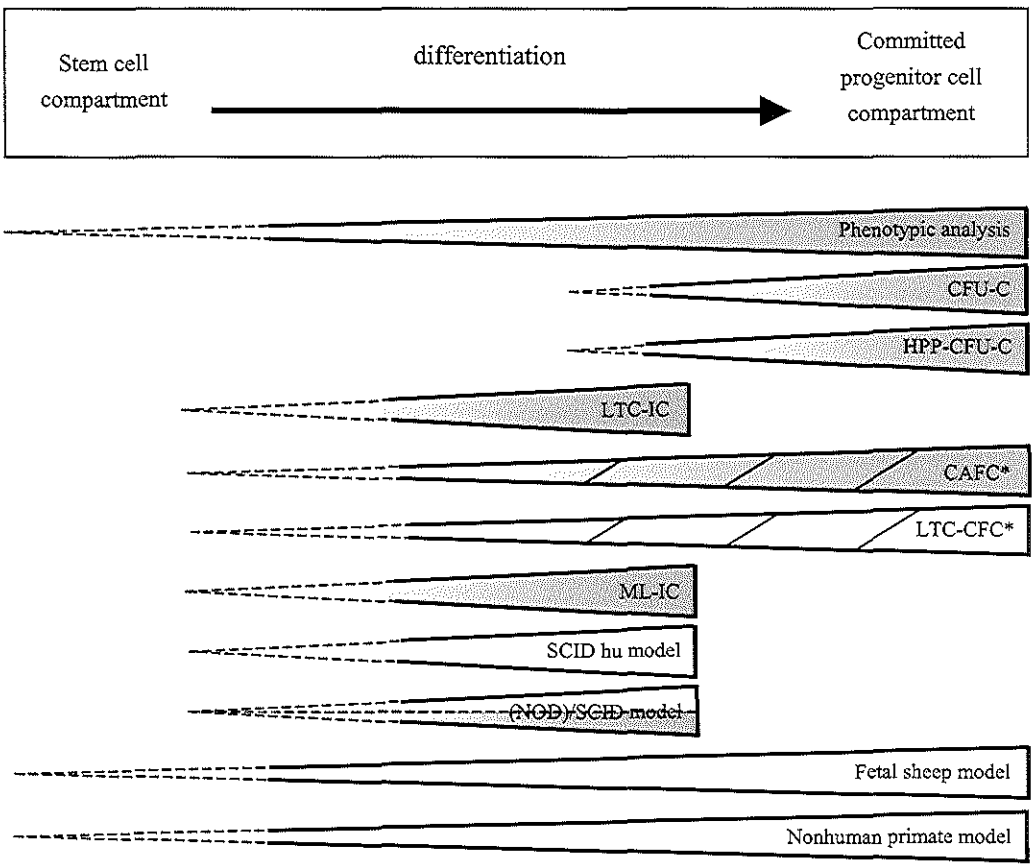

Figure 1.2 The assays indicated with grey shaded boxes allow for frequency analysis of stem and progenitor cells, while the assays indicated with white boxes assess the ability of the graft. Abbreviations: CFU-C, colony-forming unit in culture; HPP-CFU-C, high proliferative potential colony-forming unit in culture; LTC-IC, long-term culture-initiating cell; CAFC, cobblestone area forming cell; LTC-CFC, long-term culture colony-forming cell; ML-IC, myeloid lymphoid-initiating cell. *subsets are determined at various time points defining progenitor cell populations differing in primitivity.

1.4 Gene transfer into human HSC

Correcting genetically crippled cells by introducing the intact gene into the target cells may improve clinical outcome of various genetic diseases. As HSC or their progeny are affected in numerous diseases, e.g. Gaucher, ADA-SCID and thalassemia, and easily accessible, they have been subject in the development of efficient gene transfer methods for many years.

The most frequently used method to genetically modify HSC is by using retroviral vectors. They have the potential for stable virus integration and expression as long as cells go through a cell cycle. However, as expected, the efficiency with which the most primitive and thus quiescent hemopoietic cells can be genetically modified appears to be low. This has limited the clinical applicability of gene transfer protocols (182-184). Recently, several modifications have led to improved gene transfer into hemopoietic cells by the use of particular cytokine combinations for stimulation of the cells (185), modified vectors (186), lenti-virus based vectors (187-189), virus receptor targeting (168) and co-localization of the hemopoietic cells and virus by using the recombinant fibronectin fragment (CH-296) (190-192).

Clinical prove of the progress that has been made in improving the efficiency with which HSC can be genetically modified has recently been presented by Cavazzana-Calvo et al (193). This group demonstrated that 10 months after transplantation of genetically modified $CD34^+$ cells from X-linked SCID patients, NK and T-cells expressed the introduced gene and that T-, B- and NK cell counts and function were comparable with those of age-matched controls.

Gene transfer into human stem and progenitor cells in the experimental setting also provide means to assess the contribution of the marked cells to in vitro and in vivo repopulation and thereby determination of the relation between various in vitro parameters and in vivo repopulating ability.

1.5 Introduction to the experimental work

In the work described in this thesis, CAFC/LTC-CFC measurements in human hemopoietic material have been correlated with clinical parameters as well as with other in vitro assays and

data generated in a humanized immunodeficient mouse model. This was done to determine whether these assays provide a reliable indication for the human in vivo repopulating stem cell.

In chapter two the effect of chemotherapy on the number and the quality of progenitor cells that can be mobilized from BM is described. In addition, the relation between various phenotypic and functional in vitro parameters and the speed of post-transplant hematological recovery after autologous MPB transplantation is specified.

In order to explore the relation between phenotypic and functional in vitro parameters and in vivo recovery more closely, it has been investigated whether (partial) absence of hematological recovery after autologous stem cell transplantation (BM or MPB) can be caused a quantitative or qualitative stem cell defect in the transplant. The results are described in chapter three.

In chapter four is shown that counterflow centrifugal elutriation allows processing of BM and MPB transplants with sufficient yield of mature and immature progenitor cells, while CAFC and in vivo repopulating ability co-enrich in the same CCE fractions.

In chapter five is described that various subpopulations in UCB are capable of repopulating the (NOD/)SCID BM as an indicator for human in vivo repopulating ability.

In chapter six is shown, that a) genetically modified UCB cells still possess (NOD/)SCID BM repopulating ability and b) the CAFC wk.6 and the cells retrieved from the NOD/SCID BM show equal transduction efficiency.

As the NOD/SCID mouse model is used for frequency analysis of cells with SCID repopulating ability in human UCB, BM and MPB, it is important to determine the percentage of injected cells that can actually participate in repopulation of the BM. For that purpose, the percentage of the injected human $CD34^+$, CFC and CAFC subsets derived from various human hemopoietic materials homing to the BM and the spleen of the NOD/SCID mouse has been studied. The results, which are described in chapter seven, show that estimated frequencies of the CAFC wk.6 and the NOD/SCID repopulating cell are likely to be far more similar than previously assumed.

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CHAPTER 2

INDIVIDUAL STEM CELL QUALITY IN LEUKAPHERESIS PRODUCTS IS RELATED TO THE NUMBER OF MOBILIZED STEM CELLS

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ABSTRACT

Peripheral blood stem cells (PBSC) are used for stem cell support in patients following intensive chemotherapy and generally permit faster hematological recovery than bone marrow (BM). The development of different protocols for chemotherapy conditioning, mobilization and ex vivo manipulation of PBSC may potentially lead to loss of primitive hemopoietic stem cells (HSC) or reduction of their quality. In order to characterize the frequency of different stem cell subsets and their quality per mobilized PBSC, we have studied 47 leukapheresis products (LPs) of 21 cancer patients using stroma-dependent long-term culture (LTC) and limiting dilution type cobblestone area forming cell (CAFC) assays.

A large variation in CAFC week-type frequencies between the LPs was observed. The frequencies of CAFC week (wk.) 2 as a tentative indicator of mature progenitor cells and transiently repopulating HSC ranged from 0.89 to 205 per 10^5 mobilized nucleated cells (NC) and the frequencies of more primitive CAFC wk.6 varied between 0.37 and 48. The average total colony-forming cell (CFC) production per CAFC at week 6 varied between 1.2 and 730 as determined in parallel LTC. In contrast to LPs, BM samples generated 4.2 to 48 CFC per CAFC at week 6. Notably, a poor stem cell quality was consistently found in LPs that contained less than 5,000 CAFC wk.6 per kg bodyweight (BW).

Frequency analyses of CFC, CAFC-subtypes and immunophenotypic subsets showed a good level of mutual correlation, suggesting identical mobilization kinetics of different stem cell subsets. The pre-mobilization chemotherapy intensity was directly correlated with both decreasing frequency and quality of the CAFC wk.6 in LPs. The frequency of CFC, immunophenotypic subsets and CAFC subsets transplanted and the transplant quality as determined in LTC assays was related to the neutrophil and platelet recovery time after PBSC transplantation. Although the number of progenitor cells transplanted and the in vitro transplant quality showed the best correlation with early hematological recovery, the data did not permit determination of which stem cell subsets are predominantly responsible for early post-transplant recovery. As a result, frequency and quality analysis of stem cell subsets may be a useful tool to monitor and calibrate the efficacy of novel mobilization regimens and ex vivo manipulation of PBSC.

INTRODUCTION

PBSC mobilized after chemotherapy as an alternative source of HSC have been successfully used to rescue cancer patients after intensive chemotherapy requiring stem cell support and to treat hematological malignancies refractory to standard chemotherapeutic regimens (1-4). The finding that HSC are mobilized by hemopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) has greatly expanded the application of PBSC transplants (5-10). Moreover, PBSC mobilized by chemotherapy alone or in combination with hemopoietic growth factors have an advantage over BM transplants because they are associated with a more rapid hematological and immunological recovery after the reinfusion following myeloablative chemotherapy (4, 10-12), although the reason for this rapid hematological recovery is still ill-defined. A further optimization of PBSC mobilization for transplantation purposes and the development of ex vivo stem cell manipulation protocols require a study of the quality of mobilized PBSC. As a measure of graft potency, the ability of primitive HSC to produce primitive daughter cells and clonable progenitors over an extended period is determined in stroma-supported LTC assays (13-15). Limiting dilution LTC assays allow frequency analysis of the LTC-initiating cell (LTC-IC) by scoring for wells containing replatable CFC after 5 or 8 weeks LTC (16, 17). Such an assay requires large culture efforts, especially when assessment of frequencies at multiple time points is desired. The development of a human CAFC assay fit for routine use has made it possible to analyze a large series of clinical samples and to determine the frequency of both the 5-fluorouracil sensitive short-term culture initiating cell (CAFC wk.1 to 3) and the 5-fluorouracil resistant LTC-IC (CAFC wk.5 to 8) in the same assay (18).

Our first goal was to assess the quantity and quality of stem cells in LPs by determining the frequency of CAFC week-types and the CFC production in parallel LTC and to compare these data with immunophenotypic analyses and data from unmanipulated BM cell samples. Secondly, the effect of pre-mobilization treatment on the CAFC subset content was studied. Finally, by comparing our in vitro data with the short-term in vivo post-transplant recovery, we attempted to collect data that contribute to validation of the human CAFC assay.

MATERIALS AND METHODS

Patients

Fifteen patients with Non-Hodgkin lymphoma, four multiple myeloma patients, one patient with testicular cancer and one Hodgkin disease patient were included in this study. Before leukapheresis the patients were treated with several courses of chemotherapy summarized in table 2.1. Two days after the last course of chemotherapy, G-CSF (Filgrastim, recombinant-methionyl human G-CSF; 5 mg/kg/day; Roche, Mijdrecht, The Netherlands) was subcutaneously administered to induce HSC mobilization until the completion of the leukapheresis harvests. PBSC were harvested by leukapheresis once the leukocyte count was above 2.0 $x10^9$/l and the percentage CD34-positive ($CD34^+$) cells in the peripheral blood was more than 0.1%. One to three leukaphereses per patient were performed using either a Baxter CS3000+ (Baxter, Utrecht, The Netherlands) or a Cell Separator AS 104 (Fresenius, 's Hertogenbosch, The Netherlands). In one patient (patient 9) a suboptimal number of $CD34^+$ cells were harvested (0.15 $x10^7$ per kg) after three leukaphereses. Therefore, an additional chemotherapy course was performed and after G-CSF mobilization two additional LPs were harvested. After cell collection, any excess of erythrocytes was removed using buffy coat centrifugation. The cells were frozen and stored in liquid nitrogen. The LPs were thawed and reinfused into the patients after myeloablative radio/chemotherapy. All 21 patients were transplanted with PBSC without addition of BM cells and during the hematological recovery no relapse of disease was observed. In 20 patients a complete hematological recovery was observed, but one patient (patient 19) did not show any platelet recovery and was transfusion dependent for more than one year after transplantation. Therefore, this patient was not used for the platelet recovery correlation study. The patient characteristics are summarized in table 2.1. Thawed control ampoules of each LP were used for the experiments in this study. In total 47 LPs were studied. From eight patients the leukaphereses were pooled and subsequently analyzed in the same proportions as transplanted. For the comparison with BM cells the historic data from eight normal BM samples (18) together with four additional unmanipulated BM samples from one normal donor and from one Hodgkin disease and two Non-Hodgkin lymphoma patients in remission were used.

Table 2.1 Patient and transplantation characteristics.

Patient No.	Sex/ Age (years)/ Diagnosis	Prior Treatment (No. of Courses)	No. of Phereses	Transplant Total NC $x10^8$/kg	Transplant $CD34^+$ cells $X10^7$/kg
1	M/40/NHL	CHOP(3), CEMP(1)	3	1.69	0.52
2	M/51/MM	VAD(2), IDM(1)	3	2.87	2.97
3	F/37/MM	VAD(2), IDM(1)	2	5.29	2.28
4	M/50/MM	VAD(2), IDM(1)	3	3.20	0.51
5	M/56/MM	VAD(2), IDM(1)	2	1.97	1.84
6	M/23/TES	BOP/VIP(6), Ca/Et(1)	2	0.83	0.53
7	F/32/NHL	CVP(8), Ad/Te(2)	2	2.43	1.01
8	M/34/NHL	CVP(6), Cy(1)	2	2.27	0.34
9	M/26/HD	MOPP/ABV(8), DHAP(2)	5	4.21	0.20
10	M/31/NHL	CHOP(5)	1	2.41	0.44
11	M/24/NHL	HOVON27(2)	1	2.34	2.52
12	M/41/NHL	CHOP(6), DHAP(3)	2*	3.12	1.53
13	F/47/NHL	HOVON27(2)	1	1.71	2.39
14	M/38/NHL	HOVON27(2)	1	1.29	1.35
15	M/54/NHL	CHOP(8), CEMP(4)	2*	1.15	0.83
16	F/47/NHL	CHOP(8), CEMP(2)	2*	2.98	3.50
17	M/52/NHL	CHOP(8), CEMP(3)	3*	4.11	0.48
18	M/38/NHL	CHOP(8), EMP(3)	3*	1.86	0.71
19	M/50/NHL	CHOP(8), DHAP(6), CEMP(2)	3*	2.61	0.26
20	M/64/NHL	CHOP(8), CEMP(6)	2*	1.13	0.26
21	F/43/NHL	CVP(11), CEMP(4)	2*	2.82	2.42

Abbreviations: NC, nucleated cells; M, male; F, female; NHL, Non-Hodgkin Lymphoma; MM, Multiple Myeloma; TES, Testicular Cancer; HD, Hodgkin Disease; CHOP, Cyclophosphamide/ Adriamycin/Vincristine/Prednisone; CEMP, Lomustine/Etoposide/Mitozantrone/Prednisone; VAD, Vincristine/Adriamycin/Dexamethasone; IDM, Intermediate Dose Melphalan, BOP/VIP, Bleomycin/Vincristine/Prednisone/Vinblastine/Ifosfamide/Cisplatin; Ca, Carboplatin; Et, Etoposide; CVP, Cyclophosphamide/Vincristine/Prednisone; Ad, Adriamycin; Te, Teniposide; Cy, Cyclophosphamide; MOPP/ABV, Mustine/Vincristine/Prednisone/Procarbazine/Adriamycin/Bleomycin/ Vinblastine; DHAP, Cisplatin/Cytarabine/Dexamethasone; HOVON27, 2 Courses (Course 1: Prednisone/Adriamycin/Cyclophosphamide, Course 2: Prednisone/Mitozantrone/Etoposide); EMP, Etoposide/Mitozantrone/ Prednisone; *, pooled leukaphereses were analyzed.

Immunofluorescence analysis

CD34 staining was performed by incubating 10^6 NC after erythrocyte lysis for 30 minutes with anti-CD34-FITC (anti-HPCA-2 monoclonal antibody; Becton Dickinson, San Jose, CA, USA). For combined CD34 and HLA-DR or CD38 staining, the LPs were incubated in one step for 30 minutes on ice with anti-CD34-FITC and anti-HLA-DR-PE (Becton Dickinson) or anti-CD38-PE (Becton Dickinson). The incubations were performed in phosphate-buffered saline (PBS; Gibco, Breda, The Netherlands) containing 5% fetal calf serum (FCS; Hyclone, Logan, UT, USA). After the incubation the cells were washed in PBS with 5% FCS and resuspended in 0.5 ml PBS. Analysis was performed using a FACScan (Becton Dickinson). Twenty to thirty thousand events per sample were analyzed. Figure 2.1 shows the dot plot analysis of one of the LPs with the windows that were used. CAFC analyses with combined CD34 and CD38 sorted LPs have shown that the highest CAFC wk.6 to 8 frequencies are found in the $CD34^+$/CD38-negative fraction. However, the majority of the late CAFC wk.6 to 8 was found in the $CD34^+$/CD38-dim fraction (R.E. Ploemacher et al, unpublished observations). Therefore, the percentage of $CD34^+$/CD38-negative and dim ($CD34^+/CD38^{neg/dim}$) cells was determined using dot plot 1A, window 1. Several authors have shown that the highest frequency of LTC-IC and late CAFC wk.5 and 6 are contained in the $CD34^+$ and HLA-DR-low ($CD34^+/HLA\text{-}DR^{low}$) fraction (16, 18). As a result, in dot plot 1B, window 2 the percentage $CD34^+/HLA\text{-}DR^{low}$ cells was determined.

Hemopoietic growth factors

For the in vitro studies the following purified recombinant growth factors were kindly provided: human stem cell factor (SCF) and human G-CSF (Amgen, Thousand Oaks, CA, USA), human GM-CSF and murine SCF (Genetics Institute, Cambridge, MA, USA) and human interleukin-3 (IL-3; Gist Brocades, Delft, The Netherlands).

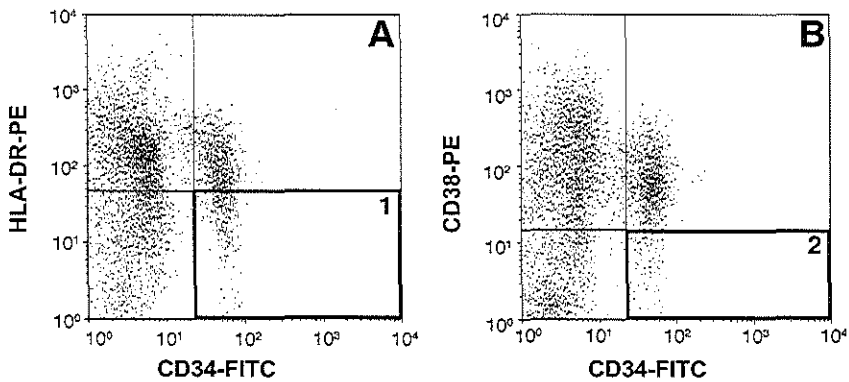

Figure 2.1 Immunophenotyping of leukapheresis products. Dot plot image showing the windows from which the percentages of $CD34^{+}/CD38^{neg/dim}$ cells (Dot plot 1A, window 1) and $CD34^{+}/HLA\text{-}DR^{low}$ cells (Dot plot 1B, window 2) were determined.

Colony-forming cell (CFC) assay

Quantification of the number of colony-forming units-granulocyte macrophage (CFU-GM) and burst-forming units-erythroid (BFU-E) was performed using a semisolid (1.2% methylcellulose; Methocel, Stade, Germany) culture medium (Iscove's modified Dulbecco's medium (IMDM); Gibco) at 37°C and 5% CO_2. The cultures contained 30% FCS supplemented with 0.75% bovine serum albumin (Sigma, St Louis, MO, USA), penicillin (100 U/ml; Gibco), streptomycin (100 mg/ml; Gibco), β-mercapto-ethanol (βme; 5 $x10^{-5}$ M; Merck, Darmstadt, Germany), erythropoietin (1 U/ml; Boehringer, Mannheim, Germany), IL-3 (15 ng/ml), G-CSF (50 ng/ml), GM-CSF (5 ng/ml) and human SCF (50 ng/ml) or murine SCF (100 ng/ml) all at final concentrations. CFU-GM and BFU-E were counted on day 14 of culture in the same dish.

Stromal feeders

The FBMD-1 murine stromal cell line was used as described before (18). Briefly, stromal feeders were prepared by seeding 10^5 FBMD-1 cells from log-phase cultures into 25 cm^2 culture flasks (Costar, Cambridge, MA, USA) or 10^3 cells per well into flat-bottom 96-well

plates (Falcon, Lincoln Park, NJ, USA). Culture plastics destined for establishment of FBMD-1 stromal feeders were incubated overnight at 4°C with 0.2% gelatin (Sigma) in demineralized water to improve adherence of the stromal layer. The FBMD-1 cells were cultured in IMDM with Glutamax-1 (Gibco) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), βme (10^{-4} M), 10% FCS, 5% horse serum (HS; Gibco) and hydrocortisone 21-hemisuccinate (HC; 10^{-5} M; Sigma). After seven to ten days of culture at 33°C and 10% CO_2 the stromal layers had reached confluence and were overlaid with LP cells within the subsequent two weeks.

Long-term cultures (LTC) in flasks

Confluent stromal layers of FBMD-1 cells in 25 cm^2 flasks were overlaid with 0.50 or 1.0 x10^6 LP NC. The cells were cultured in IMDM with Glutamax-1 supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), βme (10^{-4} M), 20% HS and HC (10^{-6} M). IL-3 (12 ng/ml) and G-CSF (20 ng/ml) were added weekly to the cultures. Flask cultures of each LP were setup in duplicate and maintained at 33°C and 10% CO_2 for six weeks with weekly half-medium changes and therefore removal of only half of the non-adherent (NA) cells. The NA-CFC output of individual flask cultures was determined on week 2, 4 and 6 and was not corrected for the weekly demi-depopulations. At the end of six weeks the number of CFC in the adherent layer was also determined. To this purpose, the NA cells were collected from the flasks and after two rinses with PBS replaced by 3 ml of 0.1% trypsin-EDTA (Gibco) at 37°C for 5 minutes. The digestion was stopped by adding 1 ml of ice-cold FCS or HS and the flasks were scraped with a cell scraper (Greiner, Alphen a/d Rijn, The Netherlands) to include strongly adherent cells. A single cell suspension was made by sieving the cell suspension through a 100 μm nylon filter. The cell suspension was taken up in IMDM and several concentrations of the suspension were plated in a semisolid CFC assay.

Cobblestone area forming cell (CAFC) assay

Confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates were overlaid with LP in a limiting dilution setup. Input values ranged between 24 and 50,000 NC per well. Twelve dilutions two-fold apart were used for each sample with 15 replicate wells per dilution. The cells were cultured in the same medium and under the same conditions as the LTC in flasks. To diminish the excessive superficial cell production of LPs and consequently increase the

visibility of the cobblestone areas a HC concentration of 10^{-5} M instead of 10^{-6} M was used in the CAFC culture medium. The percentage of wells with at least one phase-dark hemopoietic clone of at least five cells (i.e. cobblestone area) beneath the stromal layer was determined weekly for six weeks and CAFC frequencies were calculated using Poisson statistics as described previously (19, 20).

Data analysis

Data were analyzed using Slide write Plus for DOS - Version 6.0 (Advanced Graphics Software, Carlsbad, CA, USA). To characterize the data, curve fits were performed using a least squares regression fit. Correlation coefficients for the curve fit (R) were calculated. Statistical analysis was performed using GraphPad Instat (GraphPad Software, San Diego, CA, USA). The Spearman's rank correlation coefficient (r_s) was determined to quantitate the degree of linear association between two variables. A two-sided p-value was calculated testing the null hypothesis that the population correlation coefficient equals zero. The means of two populations were compared using the Student's *t* test. The two-sided p-value was determined testing the null hypothesis that the two population means are equal.

RESULTS

CFC production in flask LTC

Thirty-six samples containing 47 LPs of 21 patients were assayed for their content of CFC and ability to produce NA-CFC on stromal FBMD-1 feeders in LTC flasks at week 2, 4 and 6 following a known input of CFC. At week 6 the CFC number in the adherent stromal layer was also determined following trypsinization and replating. In figure 2.2, the mean CFU-GM, BFU-E and total CFC production in flask LTC of all LPs are shown. As compared to input values (week 0), an increasing number of CFU-GM and a decreasing number of BFU-E were harvested from the supernatant during six weeks of LTC. At week 6 the stromal layer still contained CFC on average two-fold less than were detected in the supernatant of the same flasks at that time.

The dashed lines in figure 2.2B showed the maximum and minimum CFC production of the 36 LP samples, which varied greatly for different LPs, showing an up to four-log difference

on the basis of the NC input. The ongoing high CFC production that we observed in LPs at four weeks and later was at variance with our observation of a declining CFC production by BM samples in LTC as previously reported (18).

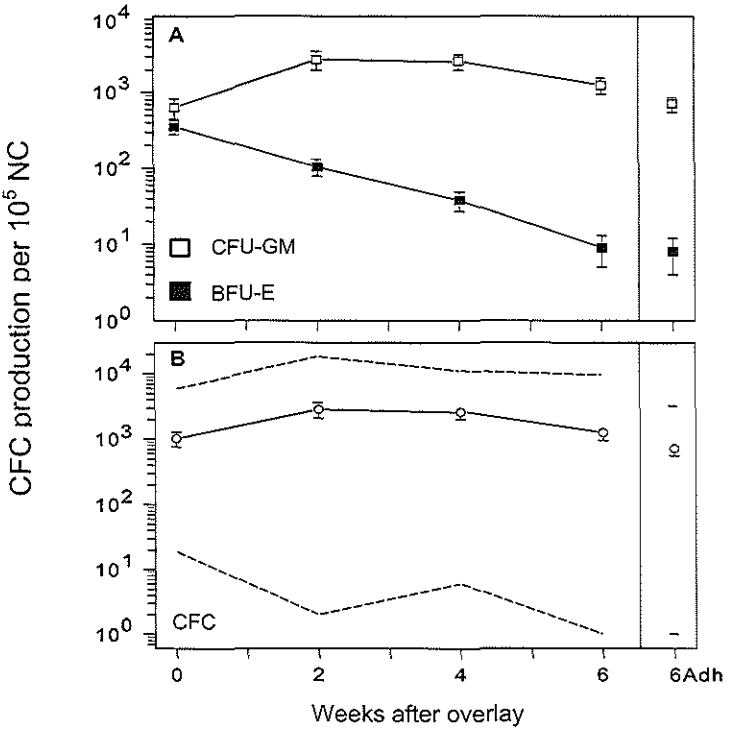

Figure 2.2 Mean (±1SEM) primary CFU-GM (Figure 2.2A, □), BFU-E (Figure 2.2A, ■) and total colony-forming cell (CFC; Figure 2.2B, O) content (week 0), non-adherent CFC production at week 2, 4 and 6 and CFC numbers in the adherent stromal layer at week 6 (6Adh) in flask LTC per 10^5 nucleated cells (NC) of 36 samples containing 47 leukapheresis products from 21 patients. Dashed lines in figure 2.2B show the maximum and minimum total CFC production of the 36 samples.

Frequency analysis of CAFC subsets

Weekly CAFC frequencies of the LPs were determined for six weeks. The mean, maximum and minimum CAFC week-type frequencies of the 36 samples containing 47 LPs of 21 patients

were calculated and presented in figure 2.3A. Similar to the CFC output in flask LTC, the CAFC frequencies differed among the various patients.

The frequencies of CAFC wk.2 as a tentative indicator of progenitor cells and transiently repopulating HSC ranged from 0.89 to 205 per 10^5 NC, and the frequencies of the primitive CAFC wk.6 ranged from 0.37 and 48. However, following a correction of the data for the number of $CD34^+$ cells these ranges of values were condensed to less than a 40-fold difference between the observed extremes (Figure 2.3B). On average, lower CAFC wk.1 to 6 frequencies were found in LPs as compared to BM samples (Table 2.2). This difference was larger at early weeks (week 2: 3.7-fold) than at later weeks (week 6: 2.3-fold).

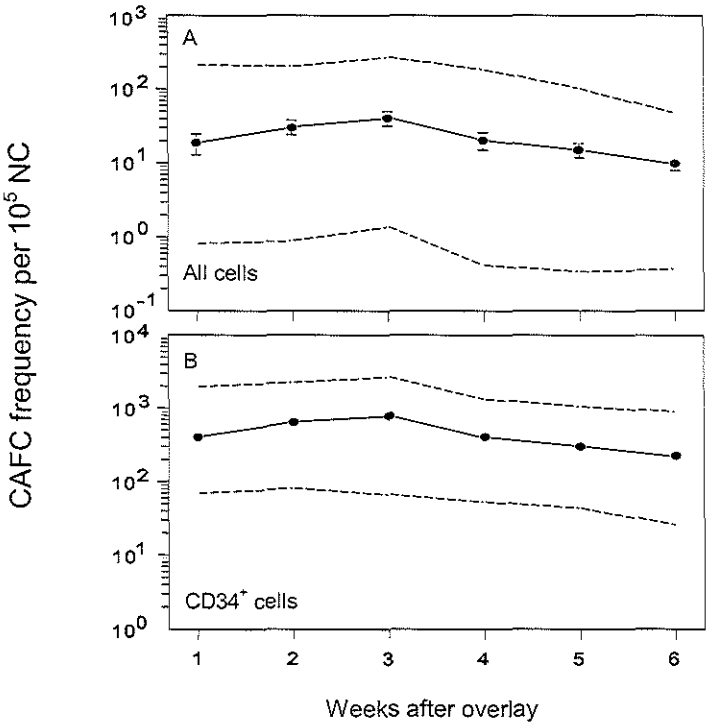

Figure 2.3 Mean (±1SEM) frequency distribution of CAFC week-types per 10^5 all nucleated cells (NC) (Figure 2.3A) and per 10^5 $CD34^-$ cells (Figure 2.3B) in 36 samples containing 47 leukapheresis products from 21 patients. Dashed lines show the maximum and minimum CAFC frequency of the 36 samples.

Table 2.2 Mean CAFC week-type frequencies of bone marrow and leukapheresis products (±1SEM).

	CAFC week-type frequencies per 10^5 nucleated cells					
	1	2	3	4	5	6
Bone Marrow	47	114	110	69	36	23
(n=12)	(±9)	(±28)	(±34)	(±25)	(±10)	(±7)
Leukapheresis Products	19	31	41	21	15	10
(n=36)	(±6)	(±7)	(±9)	(±5)	(±3)	(±2)

Quality analysis of LP and BM as determined using parallel LTC and CAFC assays

In addition to the quantitative estimations of different CAFC week-types, we set out to assess the ability of CAFC to generate CFC in parallel LTC in the same week.

Table 2.3 shows the average number of NA-CFC produced per CAFC wk.2, 4 and 6 and the number NA- and stroma-adherent (SA) CFC per CAFC at week 6 in the 36 LP samples. As compared to BM, an eight to 13-fold higher CFC production per CAFC week-type was observed. In order to compare the quality of primitive HSC in LPs and BM, the total NA- and SA-CFC production in LTC at week 6 was related with the CAFC wk.6 frequency (Figure 2.4). In BM any increase in CAFC wk.6 frequency was linearly related to a similar increase in CFC production (R=0.74). The average total NA- and SA-CFC production per CAFC at week 6 of BM ranged from 4.2 to 48. In contrast, in LPs the best curve fit had a logarithmic shape (R=0.85). The average total NA- and SA-CFC production per CAFC at week 6 of LPs ranged from 1.2 to 730. In addition, the total NA- and SA-CFC output per CAFC at week 6 as a quality index was related to the total number of CAFC wk.6 harvested (Figure 2.5). LPs with an absolute yield of less than 5,000 CAFC wk.6 harvested per kg body weight (BW) expressed an average poor stem cell quality (mean±1SEM: 74±31), while harvests containing a total of 5,000 or more CAFC wk.6 per kg BW were able to generate a statistically significant (Student's *t* test: p=0.0071) higher number of CFC per CAFC at week 6 (mean±1SEM: 252±46).

Table 2.3 Mean long-term culture non-adherent and stroma-adherent colony-forming cell production per cobblestone area forming cell of bone marrow and leukapheresis products (±1SEM).

	NA-CFC per CAFC week-type						NA+SA CFC/ CAFC wk.6
	1	2	3	4	5	6	
Bone Marrow	16	8	12	11	13	9	23
(n=12)	(±3)	(±2)	(±5)	(±2)	(±2)	(±2)	(±5)
Leukapheresis Products	N.D.	73	N.D.	115	N.D.	114	188
(n=36)		(±11)		(±19)		(±23)	(±33)

Abbreviations: CAFC, cobblestone area forming cell; CFC, colony-forming cell; NA, non-adherent; SA, stroma-adherent; N.D., not determined.

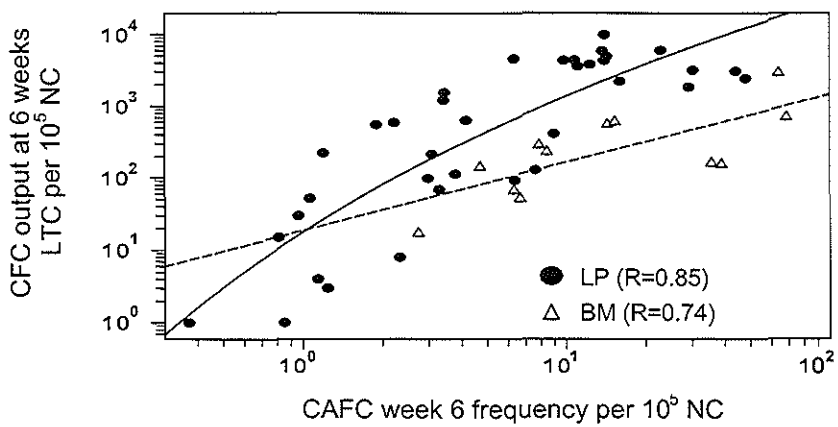

Figure 2.4 Qualitative comparison of leukapheresis products (LP) and bone marrow (BM). The non-adherent and stroma-adherent colony-forming cell (CFC) output at 6 weeks long-term culture (LTC) is related to the proportion of CAFC wk.6. The figure represents the data sets from 36 LP samples and from 12 BM samples. In BM this relation is linear (dashed line; R=0.74). In LPs the best curve fit has a logarithmic shape (curved line; R=0.85). NC, nucleated cells.

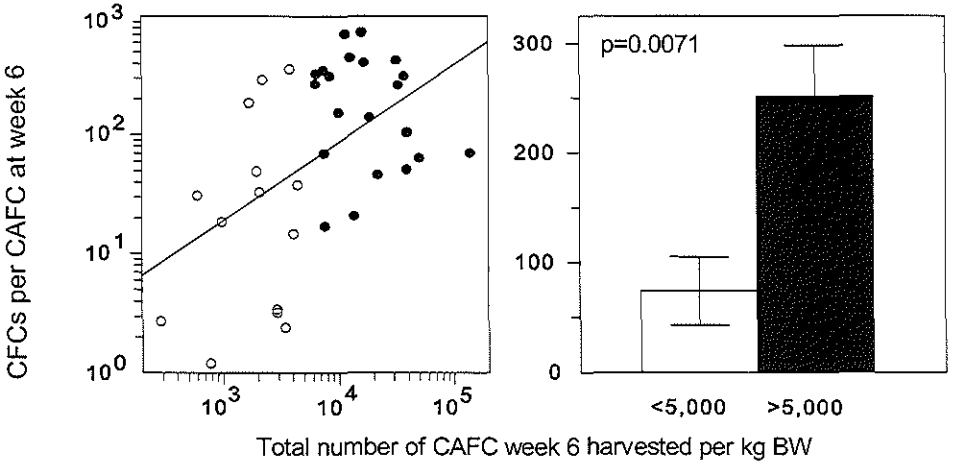

Figure 2.5 The non-adherent and stroma-adherent colony-forming cells (CFC) per CAFC at week 6 stem cell quality in leukapheresis products is a function of the total number of CAFC wk.6 mobilized. Leukapheresis products with a total CAFC wk.6 number per kg BW below 5,000 (O) had a statistically significant (Student's *t* test: two-sided p-value=0.0071) lower total CFC production per CAFC at week 6 (Mean±1SEM: 74±31, range 1.2 to 357) than had CAFC wk.6 numbers exceeding 5,000 (● ; Mean±1SEM: 252±46, range 17 to 730).

Relation between frequencies of immunophenotypic subsets, CFC and CAFC week-types

The percentage immunophenotypic subsets in the LPs were determined as described in materials and methods (Figure 2.1). $CD34^+$ cell percentages ranged from 0.31% to 18%. The percentage of $CD34^+/CD38^{neg/dim}$ cells varied between 0.01% and 3.6% and $CD34^+/HLA\text{-}DR^{low}$ cells ranged from 0.005% to 2.3%. The degree of linear association between the immunophenotypic subsets, CFC and CAFC week-type frequencies in the LPs was quantitated by determining the Spearman's rank correlation coefficient between the variables (Table 2.4). CFC frequencies showed a good correlation with $CD34^+$ cells (r_s=0.82, $p<0.0001$), $CD34^+/CD38^{neg/dim}$ cells (r_s=0.86, $p<0.0001$) and all CAFC week-types (r_s between 0.79 and 0.87, $p<0.0001$). The correlation coefficients between the immunophenotypic subsets and all CAFC week-types were lower but still statistically highly significant (r_s between 0.59 and 0.79, $p\leq0.0002$).

Table 2.4 Spearman's rank correlation coefficients between immunophenotypic subset percentages, colony-forming cell frequency and CAFC week-type frequencies in 36 leukapheresis samples ($p<0.0001$).

	$CD34^+$	$CD34^+/CD38^{neg/dim}$	$CD34^+/HLA\text{-}DR^{low}$	CFC
CFC	0.82	0.86	0.74	X
CAFC wk.1	0.73	0.68	0.71	0.79
CAFC wk.2	0.72	0.74	0.59*	0.84
CAFC wk.3	0.79	0.72	0.68	0.86
CAFC wk.4	0.77	0.72	0.68	0.87
CAFC wk.5	0.77	0.73	0.71	0.83
CAFC wk.6	0.69	0.73	0.62	0.82

Abbreviations: CFC, colony-forming cells; *, p=0.0002.

Effect of pre-mobilization treatment on the CAFC subset content of LPs

Twenty-one chemotherapy and G-CSF induced mobilizations were analyzed. Before leukapheresis the patients received two to 16 courses of chemotherapy (Table 2.1). The degree of linear association between the number of pre-mobilization chemotherapy cycles and the mean number of CAFC week-types harvested per kg BW was quantitated by determining the Spearman's rank correlation coefficient. Figure 2.6A shows a negative correlation (r_s=-0.74, p=0.0001) between the number of chemotherapy cycles and the number of CAFC wk.6 harvested. Moreover, all leukaphereses with a low quality of less than 50 NA- and SA-CFC per CAFC at week 6 were harvested from patients who had received at least seven chemotherapy cycles. These observations indicate that the pre-mobilization chemotherapy significantly limits both the number of CAFC that can be mobilized as well as their quality. Figure 2.6B shows that the negative correlation increased with later CAFC week-types.

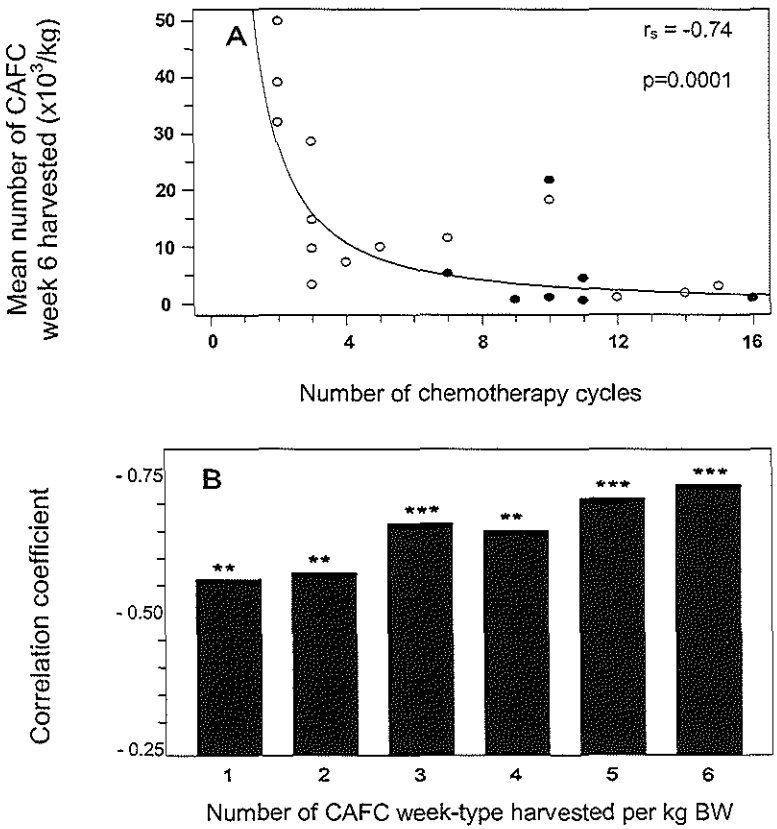

Figure 2.6 Relation between CAFC analysis and pre-mobilization treatment. Figure 2.6A shows the Spearman's rank correlation (r_s=-0.74, p=0.0001) between the number of chemotherapy cycles and the number of CAFC wk.6 harvested. Leukaphereses with a low quality of less than 50 colony-forming cells per CAFC at week 6 (●) were harvested from patients who had received at least seven chemotherapy cycles. Figure 2.6B shows the Spearman's rank correlation coefficients to quantitate the degree of linear association between the number of pre-mobilization chemotherapy cycles and the mean number of CAFC week-types harvested per kg BW. Two-sided p-value: **, p<0.01; ***, p<0.001.

Predictive value of in vitro LP analyses for in vivo post-transplant hematological recovery

The total number of NC, $CD34^+$ cells, $CD34^+/CD38^{neg/dim}$ cells, $CD34^+/HLA\text{-}DR^{low}$ cells, CAFC week-types and CFC transplanted and total NA-CFC production at week 2, 4, 6 and total combined NA- and SA-CFC production at week 6 in flask LTC of the transplants were related to the post-transplant time to recover to values of neutrophils >0.5 $x10^9/l$ (Mean±1SEM: 15±1 days, range 11 to 21 days; n=21) or platelets >50 $x10^9/l$ (Mean±1SEM: 19±2 days, range 10 to 52 days; n=20) of the patients. The Spearman's rank correlation coefficients of the analyses between the different cell subsets are presented in figure 2.7. No correlation was found between the number of NC transplanted and the hematological recovery. The number of $CD34^+$ cells, $CD34^+/CD38^{neg/dim}$ cells, CFC and CAFC wk.3 and 4 showed the best correlation with the recovery time to 0.5 $x10^9/l$ neutrophils after PBSC transplantation (r_s=-0.58 to -0.66, $p<0.007$). However, the number of CAFC wk.1 and 2 transplanted showed the best relation with the recovery time to 50 $x10^9/l$ platelets (r_s=-0.63, p=0.0031 and r_s=-0.59, p=0.0065). The more primitive CAFC wk.6 stem cell subset showed a lower correlation with the neutrophil recovery (r_s=-0.54, p=0.012) and no correlation with the platelet recovery (r_s=-0.39, p=0.092). This suggests that the CAFC wk.6 contributes less to early post-transplant recovery than the less primitive CAFC wk.1 to 4. Interestingly, the highest correlation was found between the neutrophil recovery and the total transplant quality as determined in flask LTC (r_s=-0.67 to -0.71, $p\leq0.0008$).

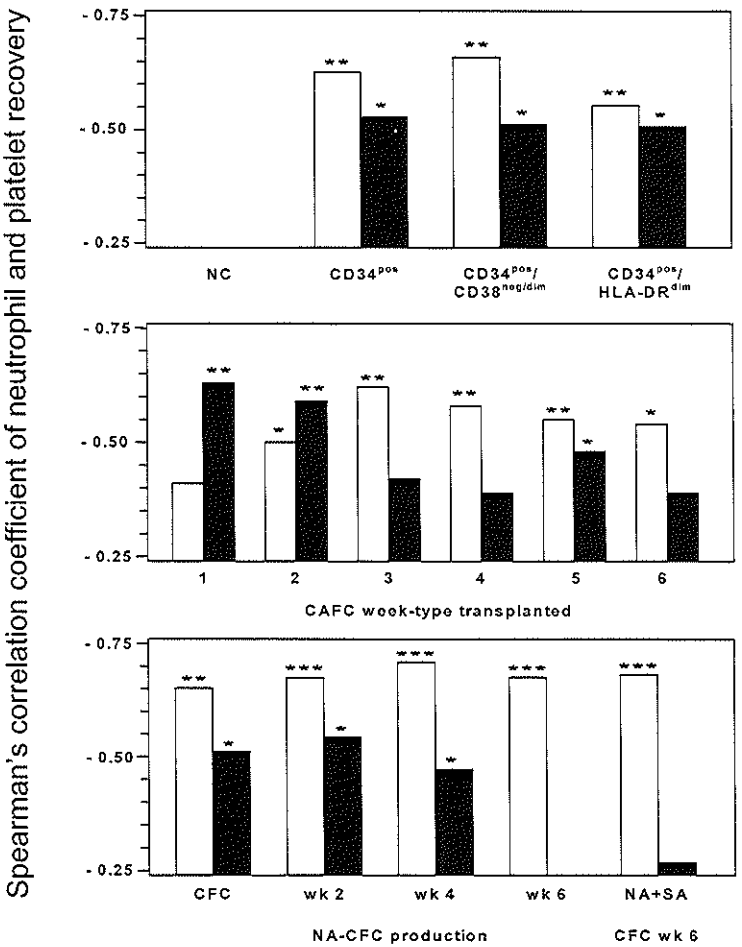

Figure 2.7 Relation of in vitro leukapheresis analyses with in vivo hematological recovery. The linear associations between the number of nucleated cells (NC), $CD34^+$ cells, $CD34^+/CD38^{neg/dim}$ cells, $CD34^+/HLA\text{-}DR^{low}$ cells, CAFC week-types and colony-forming cells (CFC) transplanted and total non-adherent (NA) CFC production at week 2, 4, 6 and total combined NA- and stroma-adherent (SA) CFC production at week 6 in flask LTC of the transplants and the post-transplant time to recover to values of neutrophils >0.5 $x10^9/l$ (□; Mean±1SEM: 15±1 days, range 11 to 21 days; n=21) or platelets >50 $x10^9/l$ (■; Mean±1SEM: 19±2 days, range 10 to 52 days; n=20) of the patients were quantitated with the Spearman's rank correlation coefficient. Two-sided p-value: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

DISCUSSION

The human CAFC assay on the stromal cell line FBMD-1 enables frequency analysis of different stem cell subsets by determining frequencies at multiple time points (18). The extensively in vivo validated murine CAFC assay strongly supports the concept that the human CAFC assay can be used as an in vitro frequency analysis of both transiently and long-term repopulating stem cells in vivo (19-24). However, frequency analysis of stem cell subsets may not suffice to estimate their proliferative ability e.g. in active disease or post-therapy. In order to allow quality analysis, parallel flask LTC can be included to measure the clonogenic potency per CAFC. In unmanipulated low density BM samples the clonogenic potency per HSC shows variation with means of 4 CFC per LTC-IC wk.5 (range 1 to 30) (16) and 23 CFC per CAFC at week 6 (range 4.2 to 48) (18).

Even more so than in normal BM, the present data show large quantitative and qualitative differences in primitive HSC contained in the analyzed LPs. We observed a mean CAFC wk.6 frequency of 10 (range 0.37 to 48) per 10^5 NC and a quality range of 1.2 to 730 NA- and SA-CFC generated per CAFC at week 6. Interestingly, the lowest primitive HSC quality was found in LPs with less than 5,000 CAFC wk.6 harvested per kg BW. The CAFC frequencies observed in our study was within the ranges reported earlier for LP stem cells, i.e. 1.8 to 166 LTC-IC per 10^5 NC (25) and 1.9 to 44 CAFC wk.5 per 10^5 NC (26). However, the extreme variability of progenitor cell generation per CAFC at week 6 contrasted with the low quality of LTC-IC in LPs as reported by Sutherland et al in a study of seven patients (i.e. 1.1 to 2.7) (25). These differences may have been caused by the previous treatment and mobilization protocols. Our observations demonstrate that stem cell quantification by means of immunophenotyping or CAFC/LTC-IC assays does not present information on the proliferative potential of HSC. In addition, the extreme variability in the stem cell quality and quantity between different patients does not permit calculation of HSC frequency by dividing the total CFC output per LTC flask at week 5 by an average CFC output per stem cell (i.e. 4) as proposed by the Vancouver group (25).

Because all frequency determinations have been done with unsorted cell fractions, this can partly explain the high correlation between the various immunophenotypic subsets, CFC and CAFC week-types. It also suggests that different stem cell subsets were mobilized with comparable kinetics and extend in these patients. A statistically highly significant, negative

correlation was found between the number of chemotherapy cycles and the number of CAFC wk.6 harvested. Haas et al recently reported a similar relation between chemotherapy cycles and the number of $CD34^+$ cells harvested (27). Furthermore, all leukaphereses with a low quality of less than 50 NA- and SA-CFC per CAFC at week 6 were harvested from patients who had received at least seven chemotherapy cycles. This observation shows that not only quantity but also quality of HSC is affected by consecutive courses of chemotherapy. Interestingly, the negative correlation increased with later CAFC week-types, suggesting that primitive HSC compartment is most affected by chemotherapy.

As reported by other investigators (4, 11, 27), the hematological recovery showed a good correlation with the number of CFC or $CD34^+$ cells transplanted. The present data also suggest that a correlation exists between the neutrophil recovery and the number of $CD34^+/CD38^{neg/dim}$ cells and CAFC wk.3 and 4. The number of CAFC wk.1 and 2 transplanted showed the best relation with the platelet recovery. These data may suggest a role of these stem cell subsets in early neutrophil and platelet recovery. However, other CAFC subsets correlate with early hematological recovery as well. Therefore, it will be very difficult to prove which stem cell subset is predominantly involved in early neutrophil and platelet recovery, unless marked or sorted stem cell subsets are used in the transplants, as has been done in animal studies (23, 24, 28). Furthermore, the high correlation between the neutrophil recovery and the total transplant quality as determined in flask LTC indicates that not only the number of HSC transplanted but also the quality of the transplant contributes to the hematological recovery.

For unexplained reasons post-transplant platelet and neutrophil recoveries of patients transplanted with mobilized PBSC are often more rapid than following BM transplantation (4, 10-12). In our study the more mature CAFC wk.1 to 4 subsets show the highest correlation with the speed of early hematological recovery. Although BM contains a higher frequency of CAFC wk.1 to 4 than LPs (2.5- to 3.7-fold), the average quality of the CAFC wk.2 and 4 in LPs is a nine-fold and ten-fold, respectively, higher. Therefore, the total progenitor stem cell potency of leukapheresis harvests is approximately three-fold higher than that of normal BM. This may explain the rapid post-transplant hematological recovery. This difference in stem cell quality may be caused by a priming effect of the mobilization protocol leading to increased progenitor cell generation from HSC or by a possible preferential mobilization of more potent stem cells from the marrow by G-CSF.

In conclusion, our data show that LP may contain extremely variable numbers of mobilized stem cells. In addition, their quality appeared to vary greatly and was especially poor in LPs containing few CAFC. These observations suggest an increased risk for transplantation of patients with LPs containing low HSC numbers or quality. Therefore, combined frequency and quality analyses of stem cell subsets may be used as a tool to qualify novel mobilization regimens and to monitor ex vivo manipulation of PBSC.

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CHAPTER 3

STROMA-SUPPORTED PROGENITOR PRODUCTION AS PROGNOSTIC TOOL FOR GRAFT FAILURE FOLLOWING AUTOLOGOUS STEM CELL TRANSPLANTATION

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ABSTRACT

In order to analyze the involvement of a possible numerical or qualitative stem cell defect in the development of sustained graft failure after autologous transplantation, we have determined the graft's content of $CD34^+$ nucleated cells (NC), colony-forming cells (CFC) and cobblestone area forming cell (CAFC) subsets, as well as the transplant's ability to produce progenitors using the long-term culture colony-forming cell (LTC-CFC) assay. We evaluated material from graft reference ampoules of 13 graft failure patients after bone marrow transplantation (BMT) and 4 graft failure patients and 4 isolated thrombocytopenia patients after peripheral blood stem cell transplantation (PBSCT). Next, we compared these data with those from 6 successfully engrafted BMT patients and 20 engrafted PBSCT patients, respectively.

In the BMT setting, the LTC-CFC week (wk.) 6 assay represented a highly significant graft failure predictor. In the PBSCT setting the total number of LTC-CFC wk.2 and wk.6 transplanted per kg bodyweight (BW) showed the highest significant difference between the engrafted and the graft failure patients, as well as between the engrafted patients and the patients suffering from isolated thrombocytopenia after transplantation. These data show that the ability of a graft to generate progenitors in vitro rather than the number of primitive progenitors transplanted can have prognostic value for post-transplant hematological reconstitution.

INTRODUCTION

For several hematological and solid malignancies autologous hemopoietic stem cell (HSC) transplantation is a well-established treatment option. Both bone marrow (BM) and peripheral blood stem cells (PBSC) can be used as a source of HSC. Failure of engraftment results in delayed or absent hematological recovery and therefore increased morbidity and mortality. In the autologous setting, graft failure may be caused by an insufficient number of HSC in the transplant, a decreased ability of the graft to produce progenitors, or undefined patient characteristics (e.g. stroma-related).

The transplant quality and the number of HSC and progenitors present in the graft can be determined by in vitro stem and progenitor cell assays, i.e. the stroma-supported flask LTC-

CFC, the limiting dilution type CAFC assay (1) and the CFC assay, respectively. For obvious reasons, stem cell assays cannot be performed on grafts on a routine basis, therefore, in clinical practice the decision to transplant is based on the total graft content of living NC, progenitor cells i.e. CFC and/or $CD34^+$ cells. However, the balance between the various parameters used for graft evaluation and the characteristics of the HSC compartment might be affected by the increasing manipulation of grafts when aiming at tumor cell purging, stem cell expansion or gene therapy, prior to transplantation. Therefore, stem cells assays might prove to be essential for initial evaluation of new graft manipulation procedures.

In order to determine to which extent numerical and qualitative progenitor and stem cell deficiencies contribute to the development of sustained graft failure, we evaluated samples of the grafts given to patients who showed no (complete) hematological recovery after autologous BMT or PBSCT transplantation. The data were compared with graft sample data from patients who showed successful recovery after BMT or PBSCT.

MATERIALS AND METHODS

Patients

Patient characteristics of the BMT and the PBSCT patients are summarized in tables 3.1A and 3.1B, respectively. The patients gave informed consent for the use of their hemopoietic material for scientific purposes. The following transplantation centers in the Netherlands contributed to this study: a) University Hospital Dijkzigt, Rotterdam, b) Dr Daniel den Hoed Cancer Center, Rotterdam, c) Emma Children's Hospital/University Medical Center, Amsterdam, d) Leiden University Medical Center, Leiden.

The BM cells were collected in Hanks' Balanced Salt Solution (HBSS; Gibco, Breda, The Netherlands) with heparin from patients in complete remission by posterior iliac crest puncture. Mononucleated cells were isolated using a ficoll-gradient (1.077 g/cm^2; Nycomed, Oslo, Norway). The cells were cryopreserved in 10% dimethyl sulfoxide (DMSO; BDH, Poole, United Kingdom) and 20% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, USA or Summit Biotechnology, Fort Collins, CO, USA) or 20% Human Serum Albumin (CLB, Amsterdam, The Netherlands) until use.

To mobilize hemopoietic progenitor cells the patients received granulocyte-colony stimulating factor (G-CSF; Filgrastim or Lenograstim, recombinant methionyl human G-CSF; 5 μg/kg/day; Roche, Mijdrecht, The Netherlands) subcutaneously, in most cases subsequent to the last chemotherapy cycle until the completion of the leukapheresis harvests. PBSC were harvested by leukapheresis once the leukocyte count was more than 2.0 $x10^9$/L and the percentage of $CD34^+$ cells in the peripheral blood was more than 0.2%. One to five cycles per patient were performed using either a Baxter CS3000+ (Baxter, Utrecht, The Netherlands), a MCS+ (Haemonetics, Zaventem, Belgium) or a Cobe Spectra (Cobe, Zaventem, Belgium). Excess erythrocytes was removed from the material using buffy coat centrifugation. The cells were frozen and stored in liquid nitrogen as described above.

Successful engrafted patients were defined as having more than 50 $x10^9$/L platelets and more than 1.0 $x10^9$/L neutrophils within the first month after transplantation. Patients who did not reach 100 $x10^9$/L platelets in the first 6 months and 1.0 $x10^9$/L neutrophils during the first 3 months after transplantation were considered as having graft failure. Patients who had a successful neutrophil recovery but were not able to produce more than 100 $x10^9$/L platelets in the first 6 months were defined as having an isolated thrombocytopenia. None of the latter patients showed signs of an autoimmune process as a cause of lasting low platelet levels.

In this study reference ampoule material has been used to determine the graft content of the NC, $CD34^+$ NC, CFC, CAFC week-types and LTC-CFC week-types. If the transplant consisted of more than one BM or PBSC harvest, reference ampoule material was pooled in the same proportion as the graft to fully represent the transplant in the analysis.

Table 3.2 shows the time (days) to more than 50 and 100 $x10^9$/L platelets and to more than 1.0 $x10^9$/L neutrophils after autologous BMT (A) or PBSCT (B) for the various patient populations.

Table 3.1A Patient and transplantation characteristics.

A. Autologous BM transplantation patients with successful hematological recovery.				
Diagnosis	No. Patients	Median no. of chemotherapy cycles (range)		Transplant
		total	cont. alkylating agents	Median no. of NC x10^6/kg (range)
NHL	5	3 (3-4)	3 (3-4)	79 (21-297)
HD	1	12	12	21
B. Autologous BM transplantation patients with graft failure.				
AML-M0	1	4	0	144
AML-M2	5	3 (2-6)	0 (0-1)	162 (15-452)
AML-M4	1	9	0	57
AML (MDS-RAEB-t)	1	3	0	238
NHL	3	2 (2-4)	1 (0-2)	138 (13-141)
ALL	2	5.5 (4-7)	1.5 (1-2)	48 (6-89)
p-value[◊]		N.S.	0.00	N.S.

[◊] Engrafted patients as compared to graft failure patients. NC: nucleated cells cont.: containing N.S.: not significant

NHL Non-Hodgkin lymphoma
HD Hodgkin disease
AML-M0 Acute undifferentiated myeloblastic leukemia
AML-M2 Acute myeloblastic leukemia with maturation
AML-M4 Acute myelo/monocytic leukemia
AML (MDS-RAEB-t) Acute myeloid leukemia due to progression of MDS-RAEB-t (myelodysplastic syndrome-refractory anemia with excess of blasts in transformation)
ALL Acute lymphocytic leukemia
alkylating agents: cyclophosphamide, etoposide, vincristine, vinblastine
Mann Whitney-U-test has been used for statistical analysis. P-value <0.05 is considered significant.

Table 3.1B Patient and transplantation characteristics.

C. Autologous PBSC transplantation patients with successful hematological recovery.

Diagnosis	No. of Patients	Median no. of chemotherapy cycles (range)		Transplant median no. of NC $x10^6$/kg (range)	Transplant median no. of $CD34^+$ NC $x10^6$/kg (range)
		total	cont. alkylating agents		
NHL	14	9.5 (4-15)	7.5 (2-15)	231 (113-411)	9 (3-35)
MM	4	3 (3-3)	1 (1-1)	304 (197-529)	21 (5-30)
TES	1	7	6	83	5
HD	1	10	8	421	2

D. Autologous PBSC transplantation patients with graft failure.

NHL	2	9 (2-16)	5.5 (1-10)	167 (73-261)	1 (0.2-3)
HD	1	13	4	200	1
MM	1	4	2	143	0.3
p-value$^{\phi}$		N.S.	N.S.	N.S.	0.004

Table 3.1B Patient and transplantation characteristics (continued).

E. Autologous PBSC transplantation patients with isolated thrombocytopenia.

Diagnosis	No. of Patients	Median no. of chemotherapy cycles (range) total	cont. alkylating agents	Transplant median no. of NC $x10^6$/kg (range)	Transplant median no. of $CD34^+$ NC $x10^6$/kg (range)
NHL	2	12 (12-12)	7.5 (7-8)	381 (200-561)	2.8 (2.7-2.8)
AML-M5	1	3	0	337	N.D.
MM	1	3	2	*	2.3
p-value$^{\square}$		N.S.	N.S.	N.S.	N.S.
p-value$^{\varphi}$		N.S.	N.S.	N.S.	N.S.

ϕ Engrafted patients as compared to graft failure patients.
$\square$ Engrafted patients as compared to isolated thrombocytopenia patients.
φ Graft failure patients as compared to isolated thrombocytopenia patients.
NC: nucleated cells cont.: containing N.D.: not determined N.S.: not significant
NHL Non-Hodgkin lymphoma
HD Hodgkin disease
MM Multiple myeloma
TES Tesicular cancer
AML-M5 Acute monoblastic leukaemia
* Transplant consisted of $CD34^+$ selected cells.
alkylating agents: cyclophosphamide, etoposide, vincristine, vinblastine
Mann Whitney-U-test has been used for statistical analysis. P-value <0.05 is considered significant.

$CD34^+$ NC enumeration

The percentage of $CD34^+$ NC in the peripheral blood of the patients was determined using the SIHON method as initially described by Gratama et al (2). Briefly, the SIHON method is a three-color flowcytometric analysis based on laser dye solution (LDS) -751 as nuclear acid dye, CD14 (stains monocytes) and CD66e (stains granulocytes) monoclonal antibodies (MAb) and a class III CD34 MAb. During data acquisition, a live gate was set on nucleated cells ($LDS\text{-}751^+$) and 50,000 events were acquired. During analysis, mature myelomonocytic cells, i.e. $CD14^+$ and/or $CD66e^+$, were excluded in order to eliminate the interference of CD34 MAb binding by the Fc receptor on these cells. A window on $SSC^{low}/CD34^+$ cells was then set on the $CD14^-/CD66e^-$ events. The percentage of $CD34^+$ cells was expressed as fraction of nucleated cells, i.e. $LDS\text{-}751^+$ cells.

Table 3.2A Time (days) to 50 or 100 x10^9/L platelets and 1.0 x10^9/L neutrophils after autologous BMT.

Patient no.	Time (days) to 50 x10^9/L platelets	Time (days) to 100 x10^9/L platelets	Time (days) to 1.0 x10^9/L neutrophils
Engrafted			
1	20	25	20
2	13	15	12
3	17	20	17
4	26	35	22
5	22	30	25
6	10	12	16
Median (days)	18.5	22.5	18.5
Min (days)	10	12	12
Max (days)	26	35	25
Graft failure			
1	>180	>180	>90
2	>180	>180	100
3	95	>180	>90
4	>180	>180	>90
5	>360	>360	>90
6	>180	>180	>90
7	178	>180	>90
8	>180	>180	>90
9	>180	>180	>90
10	>180	>180	>90
11	>180	>180	90
12	180	>180	>90
13	>180	>180	>90

Table 3.2B Time (days) to 50 and 100 x10^9/L platelets and 1.0 x10^9/L neutrophils after autologous PBSCT.

Patient no.	Time (days) to 50 x10^9/L platelets	Time (days) to 100 x10^9/L platelets	Time (days) to 1.0 x10^9/L neutrophils
Engrafted			
1	15	21	23
2	12	13	16
3	22	29	24
4	16	23	17
5	18	26	22
6	13	14	18
7	16	44	16
8	10	13	16
9	16	22	14
10	16	150	19
11	10	11	21
12	26	50	15
13	28	39	18
14	30	150	17
15	29	150	30
16	24	60	14
17	22	26	22
18	16	28	12
19	11	13	13
20	14	16	14
Median (days)	16	26	17
Min (days)	10	11	12
Max (days)	30	150	30

Table 3.2B Time (days) to 50 and 100 x10^9/L platelets and 1.0 x10^9/L neutrophils after autologous PBSCT (continued).

Patient no.	Time (days) to 50 x10^9/L platelets	Time (days) to 100 x10^9/L platelets	Time (days) to 1.0 x10^9/L neutrophils
Graft failure			
1	>480	>480	135
2	>180	>180	150
3	>180	>180	140
4	>180	>180	150
Median (days)	-	-	145
Min (days)	-	-	135
Max (days)	-	-	150
Isol. thr. cyt.			
1	>270	>270	28
2	55	>210	20
3	180	>210	29
4	>210	>210	19
Median (days)	-	-	24
Min (days)	-	-	19
Max (days)	-	-	29

Isol. thr. cyt.: isolated thrombocytopenia

Hemopoietic growth factors used in culture assays

Purified recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) and murine stem cell factor (SCF) were kindly provided by Genetics Institute, Cambridge, MA, USA. Human G-CSF and human interleukin-3 (IL-3) were gifts from Amgen, Thousand Oaks, CA, USA and Gist Brocades, Delft, The Netherlands, respectively.

Colony-forming cell (CFC) assay

Quantification of the number of colony-forming unit-granulocyte macrophage (CFU-GM) and burst forming unit-erythroid (BFU-E) was performed using a semi-solid (1.2% methylcellulose; Methocel, Stade, Germany) culture medium (Iscove's modified Dulbecco's

medium (IMDM); Gibco) at 37°C and 5% CO_2. The cultures contained 30% FCS supplemented with penicillin (100 U/ml; Gibco), streptomycin (100 µg/ml; Gibco), ß-mercapto-ethanol (ßme; 5 x10^{-5} M; Merck, Darmstadt, Germany), erythropoietin (1 U/ml; Boehringher, Mannheim, Germany), IL-3 (15 ng/ml), G-CSF (50 ng/ml), GM-CSF (5 ng/ml) and murine SCF (100 ng/ml) all at final concentrations. The number of cells plated per dish varied between 1,000 and 50,000 NC. CFU-GM and BFU-E consisting of more than 50 cells were counted on day 14 of culture in the same dish.

Stromal feeders

The FBMD-1 murine stromal cell line was used as described before (1). In short, culture plastics destined for establishment of FBMD-1 stromal feeders were incubated overnight at 4°C with 0.3% gelatin (Sigma, St Louis, MO, USA) in demineralized water to improve adherence of the stromal layer. After removal of the gelatin, stromal feeders were prepared by seeding 10^3 FBMD-1 cells per well into flat-bottom 96-wells plates (Falcon, Lincoln Park, NJ, USA) from log-phase cultures. The FBMD-1 cells were cultured in FBMD-1 medium consisting of IMDM with glutamax-1 (Gibco) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), ßme (10^{-4} M), 10% FCS, 5% horse serum (HS; Integro, Zaandam, The Netherlands) and hydrocortisone 21-hemisuccinate (HC; 10^{-5} M; Sigma). After seven to ten days of culture at 33°C and 10% CO_2 the stromal layers had reached confluence and were overlaid with BM NC or PBSC from reference ampoules within the subsequent week.

Long-term culture colony-forming cell (LTC-CFC) assay

Confluent stromal layers of FBMD-1 cells in 25 cm^2 flasks (Costar, Cambridge, MA, USA) were overlaid with 0.5 or 1.0 x10^6 BM NC or PBSC. The cells were cultured in 6 ml FBMD-1 medium with IL-3 and G-CSF at final concentrations of 10 ng/ml and 20 ng/ml, respectively. Flask culture of each BM or PBSC sample was setup in duplicate and maintained at 33°C and 10% CO_2 for six weeks with weekly half-medium changes and therefore removal of only half of the non-adherent (NA) cells. The NA-CFC output of the flask cultures pooled per sample was determined on week 2, 4 and 6 and was not corrected for the weekly demi-populations. At the end of six weeks the number of CFC in the stroma-adherent layer was also determined. For that purpose, the NA cells were collected from the flasks and after two rinses with PBS replaced by 3 ml 0.1% trypsin-EDTA (Gibco) at 37°C for 5 minutes. The digestion was

stopped by adding 1 ml FCS or HS and the flasks were scraped with a cell scraper (Greiner, Alphen a/d/ Rijn, The Netherlands) to include strongly adherent cells. A single cell suspension was made by sieving the cell suspension through a nylon filter. The cells were suspended in IMDM and several cell concentrations were plated in a semi-solid CFC assay. The stroma-adherent and the non-adherent CFC data generated at week 6 were pooled and referred to as LTC-CFC wk.6.

Cobblestone area forming cell (CAFC) assay

Confluent stromal layers of FBMD-1 cells in flat-bottom 96-wells plates were overlaid with BM NC or PBSC in a limiting dilution setup. The number of cells per well in the first dilution ranged from 27,000 to 100,000 per well. Twelve dilutions, two-fold apart with 15 replicate wells per dilution were used to analyze each sample. The cells were cultured at 33°C and 10% CO_2 for six weeks with weekly half-medium changes. The medium consisted of FBMD-1 medium supplemented with IL-3 and G-CSF at final concentrations of 10 ng/ml and 20 ng/ml, respectively. The percentage of wells with at least one phase-dark hemopoietic clone of at least five cells (i.e. cobblestone area) beneath the stromal layer was determined weekly or every two weeks. Frequencies of the CAFC were calculated using Poisson statistics as described previously (3).

Data analysis

Microsoft Excel 97 (Microsoft, Redmond, WA, USA) and SPSS for Windows Release 7.5.2. (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data are expressed as median (range). Statistical comparisons were performed using the Mann Whitney U-test. The two-sided p-value was determined testing the null hypothesis that the medians of two populations are equal. P-values of <0.05 were considered significant.

RESULTS

Number of chemotherapy cycles administered to engrafted and graft failure BMT patients

It has been shown that chemotherapy, especially alkylating agents, administered prior to autologous transplantation can damage the stem cell compartment. Therefore, we investigated whether BMT graft failure patients were administered a higher total number of chemotherapy cycles or a higher number of cycles containing alkylating agents as compared to the BMT engrafted patients.

The total number of cells transplanted per kg BW and the total number of chemotherapy cycles administered to engrafted and graft failure BMT patients did not differ significantly (Table 3.1A). However, the engrafted patients had received significantly more chemotherapy cycles that included alkylating agents than did the graft failure patients. These data indicate that the number of (alkylating) chemotherapy cycles administered to the patient is not likely to be responsible for the graft failure after autologous bone marrow transplantation in this patient population.

Number of chemotherapy cycles administered to graft failure, isolated thrombocytopenia and successfully transplanted PBSCT patients

The number of chemotherapy cycles previously administered to graft failure, isolated thrombocytopenia and engrafted PBSCT patients did not differ significantly (Table 3.1B). We observed also no significant difference between the various patient populations with regard to the number of alkylating agent containing chemotherapy cycles. Therefore, as in the BMT setting, the severity of the applied chemotherapy does not seem to explain the delayed or absent hematological recovery after PBSCT.

The total number of NC per kg BW returned to the engrafted and the (partial) graft failure patients did not differ significantly. The number of $CD34^+$ NC administered to the graft failure patients was significantly lower as compared to the engrafted patients. However, there was no significant difference in the dose of $CD34^+$ cells returned to the isolated thrombocytopenia as compared to the engrafted or graft failure patients.

Post-transplant hematological recovery after autologous BMT or PBSCT

Tables 3.2A and 3.2B show the time (days) to 50 and 100 $x10^9$/L platelets and 1.0 $x10^9$/L neutrophils for the various patient populations after BM or PBSC transplantation, respectively. In the BMT setting the engrafted patients had a median speed of recovery for 50 and 100 $x10^9$/L platelets of 18.5 (range, 10-26) and 22.5 (12-35), respectively, and for 1.0 $x10^9$/L neutrophils of 18.5 (12-25) days (Table 3.2A). In contrast, the BMT graft failure patients all needed at least 95 days for 50 $x10^9$/L, 180 days for 100 $x10^9$/L platelets and more than 90 days for 1.0 $x10^9$/L neutrophils. The PBSCT engrafted patients needed a median number of 16 (10-30), 26 (11-150) and 17 (12-30) days to reach 50 and 100 $x10^9$/L platelets and 1.0 $x10^9$/L neutrophil recovery, respectively (Table 3.2B). However, the PBSCT graft failure patients needed at least 180 days to obtain 50 or 100 $x10^9$/L platelets and 145 (135-150) days for 1.0 $x10^9$/L neutrophils. The isolated thrombocytopenia patients after PBSCT had a median speed of neutrophil recovery of 24 (19-29) days but all these patients needed more than 55 days to obtain 50 $x10^9$/L platelets and more than 210 days for 100 $x10^9$/L platelets.

Number of NC, CFC, CAFC week-types and LTC-CFC week-types transplanted per kg BW in autologous BMT

The engrafted patients were transplanted with a median (range; $x10^6$) number of 63 $x10^6$ (21-297) NC per kg BW, while the graft failure patients received median 138 $x10^6$ NC (6-452) per kg BW (Table 3.3). This difference was not statistically significant (Table 3.4). The median number of CFC transplanted per kg BW to the graft failure patients was median 2.6-fold lower ($p>0.05$) as compared to the engrafted patients.

Furthermore, the graft failure patients received median 1.6-, 3-, and 5.4-fold less CAFC wk.2, CAFC wk.6 and LTC-CFC wk.2 per kg BW, respectively, than the successfully transplanted patients. These differences were not statistically significant. The graft failure patients received median 11.6-fold less LTC-CFC wk.6 per kg BW than the engrafted patients did, which is a significant difference ($p=0.009$). In conclusion, of the parameters studied, the number of LTC-CFC wk.6 transplanted per kg BW seems to be the only prognostic parameter for graft failure in the BMT setting.

Number of NC, $CD34^+$ NC, CFC, CAFC week-types and LTC-CFC week-types transplanted per kg BW in autologous PBSCT

The median number of NC $x10^6$ transplanted per kg BW was 237 (range, 83-529), 202 (73-277) and 337 (200-561) in case of engrafted, graft failure and isolated thrombocytopenia patients, respectively (Table 3.3). These data were not significantly different (Table 3.4). The median number of $CD34^+$ grafted per kg BW was significantly lower in the graft failure than in the engrafted patients after PBSCT, but not significantly different between the isolated thrombocytopenia and the engrafted patients. The median number of CFC transplanted per kg BW to the graft failure patients was 16-fold lower ($p<0.05$) and to the isolated thrombocytopenia patients 4-fold lower ($p<0.05$), as compared to the engrafted patients. The graft failure patients received a median ± 6-fold less CAFC wk.2 ($p<0.05$) and wk.6 (not significantly different) per kg BW than the successfully transplanted patients. Additionally, the isolated thrombocytopenia patients received twice as few CAFC wk.2 per kg BW (not significantly different) but similar numbers of CAFC wk.6 per kg BW as compared to the engrafted patients. The graft failure and the isolated thrombocytopenia patients received a median 29- ($p<0.05$) and 111-fold ($p<0.05$) less LTC-CFC wk.2 per kg BW, respectively, than the engrafted patients. Additionally, the median number of LTC-CFC wk.6 transplanted per kg BW was 243-fold lower ($p<0.05$) in the graft failure patients and 174-fold lower ($p<0.05$) in the isolated thrombocytopenia patients, as compared to the successfully transplanted patients.

These data indicate that in the PBSCT setting limited numbers of primary CFC, $CD34^+$ NC, CAFC wk.2 as well as LTC-CFC wk.2 and wk.6 transplanted per kg BW predict graft failure. However, the total number of LTC-CFC wk.2 and wk.6 transplanted per kg BW shows the highest significant difference between the engrafted and the graft failure patients, as well as between the engrafted patients and the patients suffering from isolated thrombocytopenia after transplantation. None of the studied parameters (NC, $CD34^+$ NC, CFC, CAFC week-types and LTC-CFC week-types) transplanted per kg BW yielded a significant difference between the graft failure and the isolated thrombocytopenia patients.

Table 3.3 Number of NC, $CD34^+$ NC, CFC, CAFC week-types and LTC-CFC week-types transplanted per kg BW in the autologous BMT or PBSCT setting.

	# NC $x10^6$/kg BW	# $CD34^+$ NC $x10^6$/kg BW	# CFC $x10^3$/kg BW	# CAFC wk.2 $x10^3$/kg BW	# CAFC wk.6 $x10^3$/kg BW	# LTC-CFC wk.2 $x10^3$/kg BW	# LTC-CFC wk.6 $x10^3$/kg BW
BMT eg (N=6)	63 (21-297)	-	193 (50-4,777)	11 (5-279)	9 (1-43)	293 (54-2,436)	58 (39-709)
BMT gf (n=13)	138 (6-452)	-	73 (9-389)	7 (0.2-42)	3 (*-39)	54 (1-874)	5 (**-103)
PBSCT eg (n=20)	237 (83-529)	9.2 (2-35)	925 (126-13,673)	53 (7-350)	17 (2-138)	2,876 (206-46,573)	2,431 (97-13,717)
PBSCT gf (n=4)	202 (73-277)	0.7 (0.2-2.6)	58 (12-366)	9 (1-31)	3 (2-40)	100 (4-383)	10 (***-76)
PBSCT it (n=4)	337 (200-561)	2.7 (2.3-2.8)	232 (162-320)	24 (18-94)	22 (10-46)	26 (****-67)	14 (6-61)

Data are depicted as median (range).
eg: engrafted patients gf: graft failure patients it: isolated thrombocytopenia patients
$CD34^+$ NC enumeration has not been performed in the BMT setting.
As four (*), three (**), one (***) and two (****) patients generated data below detection level, we assigned the data of these patients to 50% of the detection level value of the assay for those particular patients in the statistical analysis.

Table 3.4 Statistical analysis of in vitro parameters transplanted per kg BW to the various patient populations.

	NC /kg BW	$CD34^+$ NC /kg BW	CFC /kg BW	CAFC wk.2 /kg BW	CAFC wk.6 /kg BW	LTC-CFC wk.2 /kg BW	LTC-CFC wk.6 /kg BW
BMT engrafted vs. graft failure patients	N.S.	-	N.S.	N.S.	N.S.	N.S	0.009
PBSCT engrafted vs. graft failure patients	N.S.	0.004	0.004	0.025	N.S.	0.003	0.002
PBSCT engrafted vs. isol. thr. cyt.* patients	N.S.	N.S.	0.028	N.S.	N.S.	0.002	0.006
PBSCT graft failure vs. isol. thr. cyt.* patients	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

* isolated thrombocytopenia
\- $CD34^+$ NC enumeration has not been performed in the BMT setting.
N.S. not significant
Mann Whitney-U-test has been used for statistical analysis. P-value <0.05 is considered significant. If difference is significant than p-value is indicated.

Threshold value of the progenitor cell content of the graft for successful engraftment in autologous BMT or PBSCT

The minimum of a particular parameter transplanted per kg BW to the engrafted patient group (indicated in Table 3.3) is considered as the threshold below which successful hematological recovery did not occur.

Patients that received less than 2.0 x10^6 $CD34^+$ NC per kg BW in the PBSCT setting showed no successful neutrophil and platelet recovery within the first 3 or 6 months post-transplantation, respectively (Table 3.5). If less than 50,000 CFC in BMT, or 126,000 CFC in PBSCT, were transplanted per kg BW, the patients encountered graft failure or isolated thrombocytopenia. The same is true for the number of CAFC wk.2 transplanted per kg BW, where <5,000 in BMT, or <7,000 in PBSCT per kg BW led to graft failure. Similarly, if less than 1,000 CAFC wk.6 per kg BW were transplanted in the BMT setting no hematological recovery could be observed. CAFC wk.6 had no prognostic value for graft failure development in PBSCT and no clear lower threshold could be identified for this parameter in this transplantation setting. If the number of LTC-CFC wk.2 transplanted per kg BW was below 54,000 in BMT, or below 206,000 in PBSCT, than hematological recovery was not (completely) successful. At least 39,000 LTC-CFC wk.6 in case of BMT and 97,000 in the PBSCT setting were to be transplanted per kg BW for complete hematological recovery. On the basis of these thresholds identified for the various studied parameters, we have indicated in table 3.5 in how many patients their (partial) recovery failure could have been retrospectively predicted. As is clear from tables 3.4 and 3.5, the number of LTC-CFC wk.6 transplanted per kg BW is the only predictive parameter for graft failure in the case of BMT, while it is the best in PBSCT.

Table 3.5 Threshold of the studied parameters transplanted per kg BW below which successful hematological recovery after BMT or PBSCT did not occur.

	NC /kg BW	$CD34^+$ NC /kg BW	CFC /kg BW	CAFC wk.2 /kg BW	CAFC wk.6 /kg BW	LTC-CFC wk.2 /kg BW	LTC-CFC wk.6 /kg BW
BMT	$<21 \times 10^6$	-	<50,000	<5,000	<1,000	<54,000	<39,000
	3/13 gf^{ω}		4/13 gf^{ω}	4/13 gf^{ω}	5/13 gf^{ω}	6/13 gf^{ω}	9/13 gf^{ω}
PBSCT	$<83 \times 10^6$	$<2.0 \times 10^6$	<126,000	<7,000	*	<206,000	<97,000
	1/4 gf^{ω}	3/4 gf^{ω}	3/4 gf^{ω}	2/4 gf^{ω}		3/4 gf^{ω}	4/4 gf^{ω}
	0/4 isol. thr.	0/4 isol. thr.	1/4 isol. thr.	0/4 isol. thr.		4/4 isol. thr.	4/4 isol. thr.
	$cyt.^{\omega}$	$cyt.^{\omega}$	$cyt.^{\omega}$	$cyt.^{\omega}$		$cyt.^{\omega}$	$cyt.^{\omega}$

The minimum of a particular parameter transplanted per kg BW to the engrafted patient group (indicated in Table 3.3) is considered as the threshold below which successful hematological recovery did not occur.

$^{\omega}$ Indicates the number of graft failure (BMT or PBSCT) or isolated thrombocytopenia (PBSCT) patients who suffered from (partial) absence of hematological recovery post-transplant, that could have been predicted using these thresholds.

\- $CD34^+$ NC enumeration has not been performed in the BMT setting.

* No relation between the number transplanted per kg BW and the clinical outcome of the stem cell transplantation.

gf graft failure patients

isol. thr. cyt. isolated thrombocytopenia patients

DISCUSSION

Our data suggest that graft quality, as assessed by the LTC-CFC wk.6 assay, has a highly statistically significant prognostic value for developing sustained graft failure in the autologous BMT and PBSCT setting. Of a series of other possibly relevant parameters studied it is even the only useful prognostic parameter in BMT. In addition, the CFC and LTC-CFC wk.2 and wk.6 allowed identification of the possible development of isolated thrombocytopenia in PBSCT.

In both the BMT and PBSCT setting the suggested stem cell defect in transplants of graft failure and isolated thrombocytopenia patients had a functional rather than a numerical character.

The data in table 3.4 suggest that the number of NC transplanted per kg BW in either transplantation setting is not related to the outcome of the post-transplant hematological reconstitution, while the number of CFC and $CD34^+$ NC grafted per kg BW is, at least in the PBSCT setting. These data are in agreement with data from Amigo et al (4) showing that patients displaying cytopenias, i.e. leukocyte count <4 $x10^9$/L and platelet count <100 $x10^9$/L at 6 months after autologous PBSCT, had received a significantly lower dose of CFU-GM and $CD34^+$ NC than patients without cytopenias.

Our study was not designed to include effects on the engraftment kinetics, however, various authors have previously reported that the speed of neutrophil recovery after transplantation is directly related to the total number of CFU-GM transplanted per kg BW after BMT (5, 6) and to the total number of CFU-GM and $CD34^+$ cells grafted per kg BW after PBSCT (7-12). There does not seem to be a relation between the total number of NC transplanted per kg BW and the speed of post-transplant recovery after BMT (13) or PBSCT (14).

Long-term bone marrow cultures (LTBMC) have been the subject of earlier studies in determining the predictive value of this assay for (un)successful short-term post-transplant recovery. Jackson et al (15) studied the relationship between bone marrow growth in a LTBMC system and the speed of engraftment of the same marrow following autologous transplantation. They found that the number of cells present in the supernatant of the flask culture after 1 week in culture showed a close correlation with time to neutrophil (>0.5 $x10^9$/L within 21 days) and platelet (>50 $x10^9$/L within 28 days) engraftment. Furthermore, they

reported that when the supernatant cell count at one week of culture was below the range of counts established from normal LTBMCs, neutrophil and platelet engraftment was delayed, i.e. >21 days for neutrophil and >28 days for platelet recovery. Gilabert et al (16) showed that absence of formation of an adherent cell layer defined a patient group with poor hematological reconstitution after autologous bone marrow transplantation. Additionally, they observed that the initial CFU-GM content of the graft, total cell number and total CFU-GM values produced in the 6 weeks of LTBMC did not correlate with the speed of hematological recovery. However, the time needed for granulocyte recovery (>0.5 $x10^9$/L) correlated significantly with the presence of CFU-GM in the supernatant of the LTBMC at the fourth week of culture. While these studies suggest that the speed of engraftment may be reflected in LTBMC, our investigation indicates that hemopoietic activity in LTBMCs relates to the potential of a graft to reconstitute a conditioned recipient over at least 3 to 6 months post-grafting.

In our experiments we did not study the ability of the graft samples to form a adherent stromal layer, but used the murine stromal cell line FBMD-1 to form a confluent stromal layer in 96-wells plates or 25T flasks prior to inoculation with hemopoietic cells. This experimental setup allowed us to study possible defective number or functionality of the hemopoietic stem cells proper, rather than indirect stromal effects.

One of the obvious causes for a functional defect of stem cells in the autologous transplantation setting could be chemotherapy. In addition, both chemotherapy and recipient conditioning may infer detrimental and often covert proliferative effects on hemopoietic and stromal cells (17-22). This is supported by the observations that transplants from patients who received chemotherapy prior to transplantation show a decreased ability to produce progenitors in stroma-supported assays (23), a decrease in total number of mobilised progenitors (23-26) and failing or slow platelet and neutrophil recovery when such cells are used for autologous transplantation (5, 6, 8, 12, 13, 27-30). Additionally, after exposure to chemotherapeutic agents, bone marrow stromal cells may have a reduced supportive ability for both myeloid or lymphoid progenitor cell production (31-34). In conclusion, the bone marrow and its environment are likely to be greatly affected by chemotherapy in the autologous transplantation setting and this may have profound effects on post-transplant hematological recovery.

As stated earlier, we did not observe significant differences in the number of chemotherapy cycles administered to the patients in either transplantation setting (Tables 3.1A

and 3.1B). Our observation that the engrafted autologous BMT patients received more chemotherapy cycles containing alkylating drugs than the graft failure patients suggests that graft failure in the BMT setting in our patient population is unlikely to be fully due to chemotherapy-induced damage and may therefore have had a different etiology. Further extension of the patient population and stratification on the basis of the number of cycles and type of chemotherapy is required to allow a more meaningful conclusion.

In addition to chemotherapy, viruses may affect the functionality of stem cells as well. For instance, the cytomegalovirus (CMV) status of the patients seems to be of great importance for successful platelet recovery after autologous or allogeneic transplantation. In acute myeloid leukemia (AML) CMV positivity does not seem to negatively affect the platelet recovery following autologous transplantation suggesting that in this transplantation setting, the stem cell disorder itself adds to the delayed or absent platelet recovery (35).

Isolated thrombocytopenia patients were defined in our study as patients who reached 1.0 $x10^9$/L neutrophils within the first three months but not >100 $x10^9$/L platelets within the first six months after PBSCT. As no signs of autoimmunity directed towards platelets could be observed in the isolated thrombocytopenia patients our data suggest a functional stem cell defect as the cause for the (partial) graft failure. Such a defect may explain the observation that in bone marrow biopsies from patients suffering from isolated thrombocytopenia after transplantation a significantly lower number of megakaryocytes was present as compared to controls, both in the autologous (36) and in the allogeneic (37, 38) transplantation setting. Our data however, do not explain the fact that patients with isolated thrombocytopenia show successful neutrophil recovery. Indeed, the low LTC-CFC wk.6 data of these patients does not differ significantly from that of graft failure patients, suggesting that this stroma-supported assay does not distinguish between the possibly different stem cell defects.

It is clear from table 3.1A that the patient population with graft failure after BMT largely consisted of AML patients. As is known from literature, AML patients are prone for unsuccessful post-transplant recovery after autologous transplantation. Possible explanations are the heavy pre-treatment, a disease-affected unsuitable microenvironment or a quantitative or qualitative stem cell deficiency, which may also be caused by the disease itself. We are aware that the BMT engrafted patients predominantly consists of patients with non-stem cell disorders. Therefore, we can not exclude the possibility that the engraftment circumstances in the control patients are more favorable as compared to the AML patients and thereby

contribute to the differences we have observed between the graft failure and the engrafted patients after BMT. In the PBSCT setting the various patient populations were better matched (Table 3.1B).

This study shows that in vitro assays, especially the LTC-CFC assay, can have prognostic value for post-transplant hematological reconstitution. This notion also contributes to further validation of the LTC-CFC assay as an in vitro estimate of repopulating cells. As a practical approach to clinical graft evaluation, selection of samples to be assayed in the LTC-CFC assay could be done on the basis of a minimal number of $CD34^+$ NC, $CD34^+$ subsets or CFC transplanted per kg BW, although not all graft failure patients will be included using such a procedure.

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CHAPTER 4

COUNTERFLOW CENTRIFUGATION ALLOWS ADDITION OF APPROPRIATE NUMBERS OF T-CELLS TO ALLOGENEIC MARROW AND BLOOD STEM CELL GRAFTS TO PREVENT SEVERE GVHD WITHOUT SUBSTANTIAL LOSS OF MATURE AND IMMATURE PROGENITOR CELLS

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ABSTRACT

Using counterflow centrifugation elutriation (CCE) lymphocytes can be separated from $CD34^+$ populations based on size. Immature progenitors tend to be smaller than mature cells suggesting that CCE introduces loss of stem cells. We compared the separation of 12 CCE manipulated peripheral blood stem cell (PBSC) with 16 CCE manipulated bone marrow (BM) transplants. Cells were separated in twelve fractions (3000-2200 rpm) and the rotor off (RO) fraction. Separation patterns of BM and PBSC were comparable.

B-cells were collected in high speed fractions followed by T- and NK cells. In contrast, progenitors were collected in lower speed fractions. By adding successively T-cell depleted fractions to the RO fraction a BM transplant could be composed containing 0.7 $x10^6$ T-cells/kg and 90%, 89% and 68% recovery of $CD34^+$ nucleated cells (NC), colony-forming unit-granulocyte macrophage (CFU-GM) and burst forming unit-erythroid (BFU-E), respectively. PBSC were separated in 4 CCE runs inducing higher numbers of T-cells in the graft (4.4 $x10^6$/kg) and 54% $CD34^+$ NC, 46% CFU-GM and 37% BFU-E recovery. Time of engraftment was not delayed and no graft failure was observed. The higher number of T-cells was not associated with higher incidence of graft-versus-host-disease (GvHD). Acute GvHD ≥grade III occurred in 0 of 16 BM and 2 of 12 PBSC recipients; extensive chronic GvHD was observed in four of 15 and three of nine recipients, respectively.

To study immature cells in the graft, CD34 subpopulations and cells with long-term in vitro repopulating ability, determined using the cobblestone area forming cell (CAFC) assay, were evaluated in each fraction. The separation patterns in BM and PBSC were comparable. Cells with mature and immature phenotype were enriched in lower speed fractions (mean recovery of 74% $CD34^+/CD13^-/HLA\text{-}DR^-$). The CAFC week (wk.) 2, 4 and 6 were also enriched in these fractions.

These data show that the used CCE procedure is a reliable method to deplete T-cells from stem cell transplants without substantial loss of immature and mature progenitors.

INTRODUCTION

Extensive T-cell depletion of transplants is the most effective way to reduce the incidence and severity of GvHD but is associated with an increased risk of graft failure and relapse after transplantation (1, 2). The last decade many methods of T-cell depletion have been developed. These include monoclonal antibodies (MAb) conjugated to toxins or magnetic beads, or in combination with complement, and soybean lectins and sheep E-rosetting (3-7). The depletion efficacy may vary considerably resulting in an inconsistent low T-cell number in the graft.

Although a decline of T-cells in the graft reduces the severity of GvHD, it is associated with a higher probability of graft rejection (1, 8). This implicates that in the graft the number of immunocompetent lymphocytes of the donor should be in balance with the residual chemo-radiotherapy resistant lymphocytes in the recipient. The outcome of allogeneic transplantation would therefore improve strongly if the number of T-cells/kg body weight (BW) of the patient in the graft could be adapted to each individual patient. Extensive elimination may simultaneously deplete populations such as natural killer (NK) cells that are likely to be involved in the graft-versus-leukemia activity. This is manifested in higher relapse rates. The preferred approach is a technique that allows control of the separation process and that can be adapted to each individual patient.

Separation of cells by CCE is based on sedimentation velocity due to differences in cell-size and density of lymphocytes and hemopoietic progenitor cells (9). CCE has been successfully applied to deplete T-cells in BMT as also pioneered in our center (10-17). The incidence of both graft failure and relapse in recipients of T-cell depleted grafts using CCE compares favorably with other methods of T-cell depletion (10-13). This may be due to the number of remaining lymphocytes in the graft after T-cell depletion resulting in a sufficient anti-leukemia activity (18). Less clinical experience exists with the use of CCE to deplete T-cells from PBSC after mobilization with granulocyte-colony stimulating factor (G-CSF). Recent experimental data showed that the separation of PBSC progenitors is comparable with those of BM progenitors (19, 20).

The capability of engraftment is traditionally based on the number of CFU-GM in the graft. It is assumed that presence of high numbers of CFU-GM in the graft warrants a sufficient content of pluripotent stem cells which is essential for complete long-term repopulation. Clinical trials in which selected $CD34^-$ cells were infused have demonstrated

that this population is responsible for hemopoietic reconstitution (21, 22). Previous studies have documented a correlation between the number of reinfused $CD34^+$ cells and time to hematological recovery (23-25). However, these parameters may be unfit to predict engraftment when the graft is manipulated by purging. Indeed, donor progenitor cells are unlikely to contribute to immediately and permanent engraftment in mouse models (26, 27). In addition, small subpopulations of CD34-positive cells (e.g. $CD34^+/CD33^-$) are assumed to contain virtually all immature progenitor cells that initiate long-term hemopoietic engraftment. Depending on the depletion technique these cells may be eliminated simultaneously with T-cells.

Though CCE results in the recovery of most CFU-GM and $CD34^+$ cells from BM as well from PBSC transplants, some investigators suggested that this technique may remove substantial numbers of immature progenitors from the transplant (19, 28, 29). However, other studies, including preliminary data of our separations, showed contrary results both in BM transplantation (BMT) and PBSC transplantation (PBSCT) (20, 30, 31). These different results might be explained by variations in CCE-equipment and CCE-procedures used in the different studies.

In the present study we investigated the separation process of the Curameé 3000 elutriator that has been extensively applied in our institute to enrich BM and PBSC transplants of HLA-identical sibling donors in approximately 500 stem cell transplantations. The separation profiles as well as the recoveries of lymphocytes and mature and immature progenitor cells of BM and PB were studied. Lymphocyte populations and CD34-positive subpopulations were determined by means of flowcytometric analysis. The proliferative capacity of more mature progenitors was quantified by clonogenic assays for CFU-GM, colony-forming unit-granulocyte-erythrocyte-monocyte-megakayocyte (CFU-GEMM) and BFU-E and of immature progenitors by the CAFC assay (32). We found that T-cells are efficiently separated from other cells without substantial loss of mature and immature progenitor cells.

MATERIALS AND METHODS

BM and PBSC collection and mononuclear cell separation

BM was harvested from 16 healthy HLA-identical sibling donors of a median age of 43 years (range 19-64 years) under complete anesthesia. The BM harvest (approximately 25 $x10^9$ NC) was anti-coagulated by collecting cells in 10% ACD-A and 5 IU/ml heparin. After fat and plasma extraction by centrifugation, mononuclear cells (MNC) were isolated by density floatation separation in Percoll (Pharmacia, Uppsala, Sweden) gradients as described previously (33). BM was mixed with 90% Percoll solution obtaining a density of 1.085 g/l. This suspension was pumped under a 1.073 g/L Percoll layer in 250 ml bottles. After centrifugation, the 1.073 g/l interphase cells were obtained by pushing out the complete gradient by using high dense glucose solution (50% glucose). The cells were washed and loaded in the CCE chambers.

Twelve healthy HLA-identical sibling donors of a median age of 47 years (range 25-56 years) were treated with G-CSF (10 μg/kg BW) for 5 to 6 days prior to apheresis in order to mobilize their stem cells (PBSC). Cells were obtained by harvesting of 5 to 10 L blood equivalents by means of a Fenwal CS3000 cell separator (Baxter Healthcare, Deerfield, IL, USA) on each of two consecutive days. Each day approximately 30 $x10^9$ cells were collected. Contaminating erythrocytes and granulocytes were removed by Percoll gradients (1.073 g/L) in 250 ml bottles. Interphase MNC were collected and further separated in the elutriation chambers.

Counterflow centrifugation elutriation (CCE)

Interphase MNC were separated in a 4-chamber CCE rotor (Curameé 3000 system; Dijkstra Vereenigde BV, Amsterdam, The Netherlands) under constant control of the outflow by using a computerized scatter-monitor device and a speed tuned stroboscopic light as described previously (9). Briefly, cells were loaded up to 2 $x10^9$ cells per chamber under a constant flow rate. Glucose phosphate buffer supplemented with 1% human serum albumin (CLB, Amsterdam, The Netherlands) was used as elutriant. By reducing the rotor speed stepwise approximately 13 consecutive gradually more lymphocyte-depleted and the maximally lymphocyte-depleted RO fraction were collected.

Flowcytometric analysis of CCE fractions

The number of T-cells ($CD3^+$), B-cells ($CD19^+$) and NK cells ($CD3^-/CD56^+$) as well as the number of progenitor/stem cells ($CD34^+$) in each fraction of CCE were determined by using dual color immunofluorescence measured in flowcytometry. One million cells from each fraction were incubated with the following combinations of fluorochrome-labeled MAb, CD3-FITC (UCHT1; Dako A/S, Glostrup, Denmark)/CD19-PE (HD37; Dako A/S), and CD3-FITC/CD56-PE (B159; Coulter-Immunotech, Marseille, France) to recognize T-, B- and NK cells, respectively, and by CD34-PE (class III recognizing MAb 581; Coulter-Immunotech)/CD45-FITC (J33, Coulter-Immunotech) to stain progenitor cells.

In order to study different progenitor cell subpopulations CD34-PE or CD34-PE-Cy5 (Coulter-Immunotech) was combined additionally with CD13-PE or CD13-FITC (WM-47, Dako A/S), CD38-PE (HB-7; Becton Dickinson, San Jose, CA, USA), anti-HLA-DR-FITC (CR3/43; Dako A/S), CD7-FITC (BB-7; IQP, Groningen, The Netherlands) and CD19-PE (HD37; Dako A/S). Cells were incubated for 30 min at 4°C. Remaining erythrocytes were lysed by using FACS lysing solution (Becton Dickinson) during 10 min at room temperature. Cells were washed in PBS supplemented with 1% bovine serum albumin and fixed with 1% paraformaldehyde. Cells were analyzed for immunofluorescence in a Coulter Epics XL flowcytometer (Coulter Corporation, Hialeah, FL, USA). Lymphocyte subpopulations were determined by gating on forward scatter and fluorescence whereas progenitor cells were measured by sequential gating on CD45-FITC against side scatter (SSC) followed by CD34 against SSC (ISHAGE protocol; (34)). For a reliable determination of $CD34^+$ populations at least 100,000 cells were analyzed in the $CD45^+$ gate and at least 100 events were collected within the $CD34^+$ gate.

To study the recoveries of T-cells and progenitor cells after the separation procedures the numbers of $CD3^-$ and $CD34^+$ cells were also assessed in the initial harvest, the lymphocyte and progenitor cell fractions, and the compiled graft after CCE.

Composition of the stem cell grafts

MNC from BM could be depleted of lymphocytes in one CCE run, whereas MNC from PBSC transplants were separated in 2 runs on each of 2 consecutive days. Several T-cell fractions of each run were added to the RO fraction to obtain a graft containing approximately 0.7 $x10^6$ T-cells/kg BW of the BM recipients and 4 $x10^6$ T-cells/kg BW of the PBSC recipients. The lymphocyte fractions leaving the rotor just prior to the RO fraction were used for the T-cell adaptation. With this technique it is permitted to add an appropriate number of T-cells to each graft.

Progenitor cell assays

The number of different progenitor cells in the harvest, CCE fractions and the graft were also assessed by semi-solid clonogenic assays for CFU-GM, BFU-E and CFU-GEMM. To assess CFU-GM cells were plated in 35 mm Petri dishes (Costar, Cambridge, MS, USA) containing Bacto-Agar 0.3% (Difco labs. Detroit, Michigan, USA) and Iscoves medium (IMDM; Gibco, Long Island, NY, USA) supplemented with 20% FCS (Hyclone Labs, Logan, Utah, USA) and recombinant growth factors, IL-3 (Sandoz Pharma, Basel, Switzerland), GM-CSF (Sandoz) and G-CSF (Amgen, Thousand Oaks, CA, USA) (35). To assess BFU-E and CFU-GEMM, cells were plated in 35 mm Petri dishes containing 1.2% methylcellulose (Stem Cell Technology, Vancouver, Canada), 20% FCS, BSA, IL-2, IL-3, 1.5 U erythropoietin (Eprex, Cilag, Brussels, Belgium) and alpha-monothioglycerol. Cultures were performed in duplicate and incubated in a humidified incubator containing 5% CO_2 in air at 37^0 C. After 14 days the cultures were scored for the presence of colonies by using an inverted microscope as described previously (33, 35).

Cobblestone area forming cell (CAFC) assay

To determine the recovery of functional immature progenitor cells after CCE the CAFC assay, as described by Ploemacher et al (32), was performed. Briefly, stromal cells were prepared by seeding 10^3 cells of the FBMD-1 cell line from log-phase cultures into flat bottom 96-well plates (Falcon, Franklin Lakes, NY, USA) in IMDM (Gibco) supplemented with 10% FCS (Summit, Fort Collins, CO, USA), 5% horse serum (Integro, Zaandam, The Netherlands), 10^{-4} mol/l β-mercaptoethanol (Merck, Darmstadt, Germany), 10^{-5} mol/l hydrocorticosone 21-hemisuccinate (Sigma, St. Louis, MO, USA), penicillin (100 U/ml; Gibco) and streptomycin

(100 µg/ml; Gibco). When cultures reached confluency the CCE cell fractions were inoculated onto this stromal layer in a limiting dilution setup with 5 $x10^4$ to 2.5 $x10^6$ cells per well in the first dilution. Twelve two-fold dilutions with 15 replicate wells per dilution were applied for each sample. The same culture medium as used for culturing of the cell line was added, however supplemented with IL-3 (10 ng/ml; Gist Brocades, Delft, The Netherlands) and G-CSF (20 ng/ml; Roche, Mijdrecht, The Netherlands). The percentage of wells with at least one hemopoietic clone of at least five phase-contrast dark cells (i.e. cobblestone area) beneath the stromal layer was determined at week 2, 4 and 6 of culture. The CAFC frequencies were calculated using Poisson statistics (36).

Clinical outcome after stem cell transplantation

Initial engraftment was monitored by daily evaluation of hematological parameters. Engraftment was defined by a white blood cell (WBC) count of >1.0 $x10^9$/L on two consecutive days and a platelet count of >20 $x10^9$/L unsupported. As GvHD prophylaxis patients received Cyclosporine A (CsA) 3 mg/kg/day by continuous infusion from days -1 to +14, followed by 2 mg/kg/day as a continuous intravenous infusion until day 21. Beyond day 21, CsA was administered orally (6 mg/kg/day) until 12 weeks after transplantation. CsA was gradually tapered and stopped 16 weeks post-transplantation. Acute and chronic GvHD were classified from grade I through IV and as limited or extensive, respectively (37, 38). Statistically significant differences were determined by Fisher's exact test or Mann-Whitney U-test analysis.

RESULTS

Cell separation in CCE

Maximally 2.0-2.5 $x10^9$ MNC derived from BM or PBSC from sibling donors were loaded in each CCE chamber and separated. By reducing the rotor speed stepwise, starting at 3000 rpm, cells were collected in 13 fractions including the RO fraction (Figure 4.1). Apart from the RO fraction all other fractions consisted of comparable numbers of cells implicating that the percentage of cells is a reliable parameter for evaluation of the CCE pattern. High speed fractions consisted mainly of B- ($CD19^+$) and T- ($CD3^+$) cells (from 3000 to 2600 rpm). NK

($CD3^-/CD56^+$) cells were enriched in the intermediate fractions (2600 to 2400 rpm). Although low amounts of progenitor cells, expressed as CD34-positive cells, were found in the high speed fractions (3000 to 2700 rpm) most $CD34^+$ cells were collected in the lower speed fractions (≤2500 rpm). The percentage of $CD34^+$ cells was lower in the RO fraction than in the low speed fractions (data not shown). However, due to the high cell numbers of this fraction (approximately 10-times higher than the other fractions) the absolute number of $CD34^+$ cells was the highest in the RO fraction. BM and PBSC separations showed comparable profiles.

The separation pattern of CD34-positive cells was comparable with the profiles of the more and less mature erythroid and myeloid hemopoietic progenitor cells determined in assays of CFU-GM, BFU-E and CFU-GEMM (Figure 4.2).

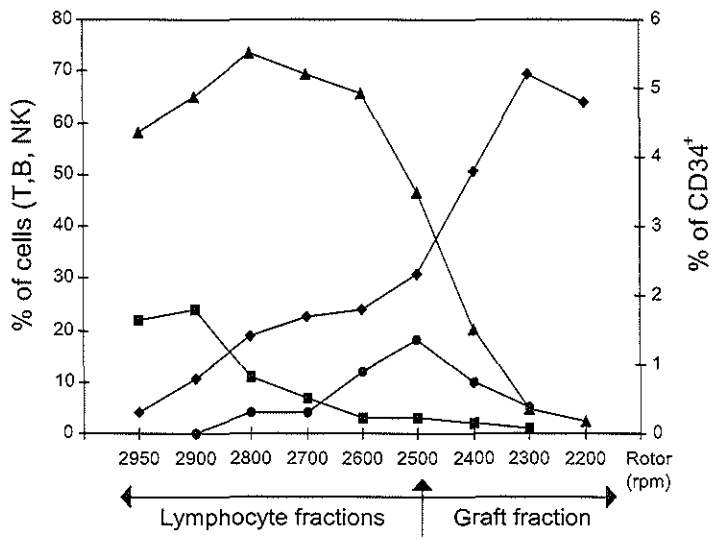

Figure 4.1 CCE fractions were analyzed for the percentage of T- ($CD3^-$) (▲), B- ($CD19^-$) (■), NK ($CD56^+$) (●) and $CD34^+$ (◆) cells. The number of NC per fraction was comparable. The graft was composed by adding cells from lymphocyte fractions with the lowest speed to the RO fraction in order to obtain 0.7 $x10^6$ CD3-positive cells/kg BW. This resulted in a graft containing fractions ≤ 2500 rpm. RO fraction is not depicted.

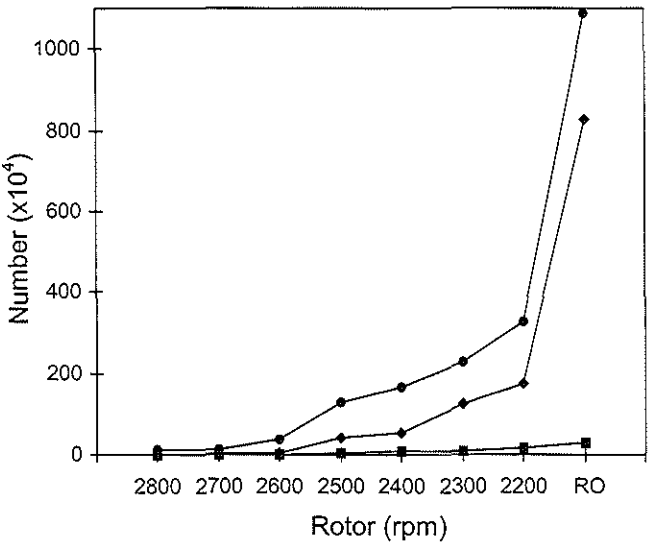

Figure 4.2 CCE fractions were analyzed for the number of progenitor cells determined in semi-solid clonogenic assays of CFU-GM (◆), BFU-E (●) and CFU-GEMM (■) as described in materials and methods. Rotor speed fractions from 2900 to 2200 rpm and RO are studied. Data are the results of three evaluations.

Recoveries of different cell populations after CCE

A mean number of 6.0 ± 0.1 x10^9 BM cells and 36.8 ± 8.6 x10^9 PBSC were processed in one and four CCE runs, respectively. After determination of the number of T-cells in each fraction the graft was composed by adding one or more low speed fractions to the RO fraction in order to obtain a BM graft with a fixed number of 0.7 x10^6 T-cells per kg BW. Due to the combination of fractions derived from 4 CCE runs the number of T-cells in PBSC grafts averages out at 4.4 x10^6 per kg BW (Table 4.1). The recovery of the different cell populations was determined by comparing the graft content with the pooled excluded lymphocyte fractions and the initial MNC population before CCE (Figure 4.3).

The recovery of $CD34^+$ cells, CFU-GM and BFU-E was lower in PBSC grafts than in BM grafts (54%, 46% and 37% versus 90%, 89% and 68%, respectively). However, the recipients of PBSC grafts received significantly more NC, $CD34^+$ cells, CFU-GM and BFU-E

($p<0.001$) (Table 4.1). The cloning efficiency of CFU-GM in BM and PBSC was determined by calculation of the number of $CD34^+$ cells responsible for the formation of one CFU-GM colony. The clonogenic capacity of PBSC appeared to be higher than that of BM (Table 4.1).

The number of infused T-cells per kg BW was also higher in PBSC recipients than in BM recipients (4.4×10^6 versus 0.7×10^6, respectively; $p<0.001$).

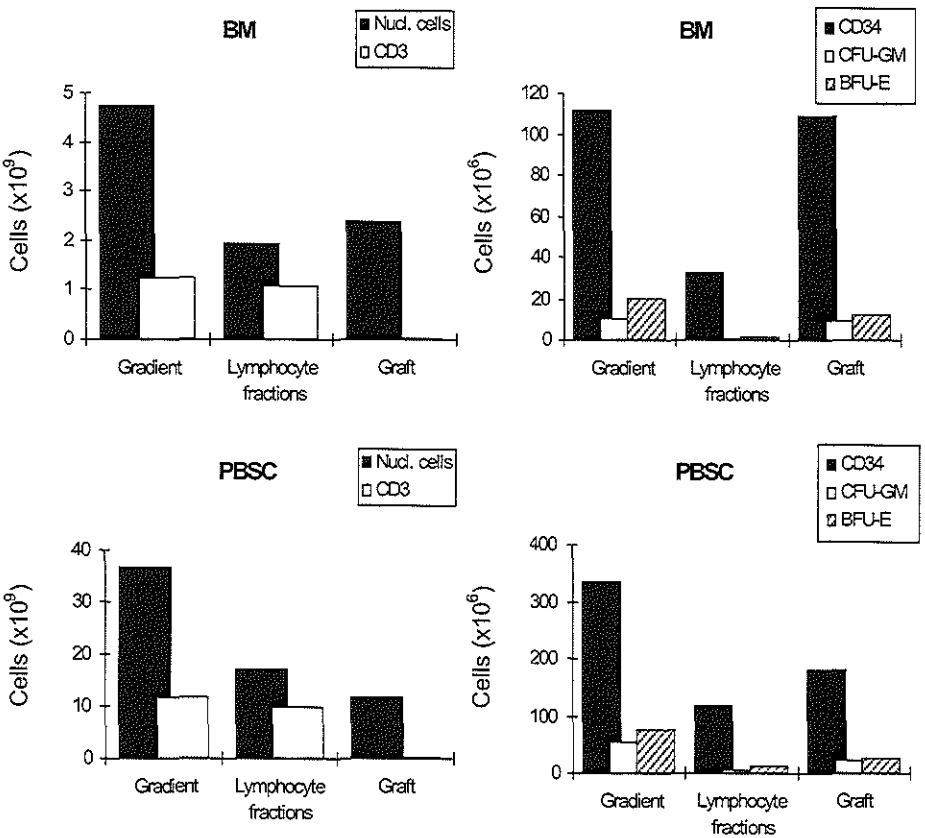

Figure. 4.3 Number of different cell populations in the initial MNC population before CCE, and in the excluded lymphocyte fractions and ultimate graft fractions after CCE of BM and PBSC. Numbers of NC were determined by cell counting. Numbers of T-cells and CD34-positive cells were determined by flowcytometry. Clonogenic progenitor cells of CFU-GM, BFU-E and CFU-GEMM were determined in semi-solid clonogenic assays. Data are the results of the 16 BM and 12 PBSC transplants.

Table 4.1 Composition of BM grafts and PBSC grafts. Median number of different cell populations/kg BW of the patient.

	NC ($x10^8$)	T-cells ($x10^6$)	$CD34^+$ NC ($x10^6$)	CFU-GM ($x10^4$)	BFU-E ($x10^4$)	$CD34^+$ NC/ CFU-GM$^{\varpi}$
BM grafts	0.4	0.7	1.2	10	13	11.4
	(0.2-0.6)	(0.6-0.8)	(0.8-2.1)	(7-18)	(6-18)	
PBSC grafts	1.3	4.4	2.1	28	33	9.4
	(0.8-3.0)	(2.3-6.9)	(1.2-4.0)	(10-76)	(6-97)	
p-value$^{\phi}$	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data are depicted as median (range).
NC nucleated cells
$^{\varpi}$ The cloning efficiency of CD34 is expressed as the number of $CD34^+$ cells responsible for the formation of one CFU-GM colony.
$^{\phi}$ Mann Whitney-U-test has been used for statistical analysis. P-value <0.05 is considered significant.

CCE separation of $CD34^+$ subpopulations

The CCE separation procedure is mainly based on size and density of cells. As a result smaller cells are collected in the high speed fractions. This implicates that small $CD34^+$ subpopulations, that may represent resting and more immature progenitor cells with long-term repopulating capacity, are excluded from graft fractions. As a consequence the incidence of graft failure and secondary graft rejection may be increased after transplantation of grafts that are lymphocyte-depleted using CCE.

To study which fractions mainly contain these immature progenitor cells, the separation patterns of $CD34^+$ subpopulations were investigated using flowcytometry. As expected, flowcytometrical analysis of forward and side scatter of the $CD34^+$ gated cell population revealed that the $CD34^+$ cells found in the higher speed fractions expressed lower forward and side scatter properties than $CD34^+$ cells collected in the lower speed fractions (data not shown). In order to study whether cells collected in high speed fractions were immature progenitors, separation patterns of $CD34^+$ subpopulations were more extensive investigated in 3 CCE

separations of BM and PBSC transplants. Cells derived from the different CCE fractions were labeled with MAbs directed against lineage specific antigens and analyzed in flowcytometry of double- or three-color immunofluorescence (Figure 4.4 and Figure 4.5). Patterns of the investigated $CD34^+$ subpopulations were comparable. Most cells of the $CD34^+$ subpopulations were collected in fractions ≤2500 rpm. Though the RO fraction contained a low percentage of $CD34^+$ cells the absolute number in this fraction appeared to be the highest of all fractions due to the high total number of NC implicating that most progenitors were retained in the ultimate graft. The $CD34^+$ population consisted mainly of mature progenitors represented by $CD38^+$, $HLA\text{-}DR^-$ and $CD13^+$ cells (Figure 4.4). The percentage of lymphocyte progenitors was lower than 0.5% in all fractions and consisted mainly of B-cell progenitors ($CD34^+/CD19^+$). The T-cell progenitors, characterized by $CD34^+/CD7^+$ subpopulations, could not be detected reliably (<0.1%; data not shown).

The percentage of more immature progenitor cell populations, characterized by lacking CD38 and HLA-DR expression, was <0.5% in all fractions of BM and PBSC separations. However, most immature progenitor cells were collected in the low speed fractions and mainly in the RO fraction (Figure 4.5). This profile was most pronounced in PBSC separations. This implicates that a substantial number of immature progenitors were transplanted. Fifty-nine to 85% (mean 74%, 13.2 $x10^6$ cells) of the $CD34^+/CD13^-/HLA\text{-}DR^-$ cells derived from the original BM harvest were retained in the graft. Forty to 72% (mean 55%) of the immature progenitor cells derived from the PBSC harvest were recovered in the graft.

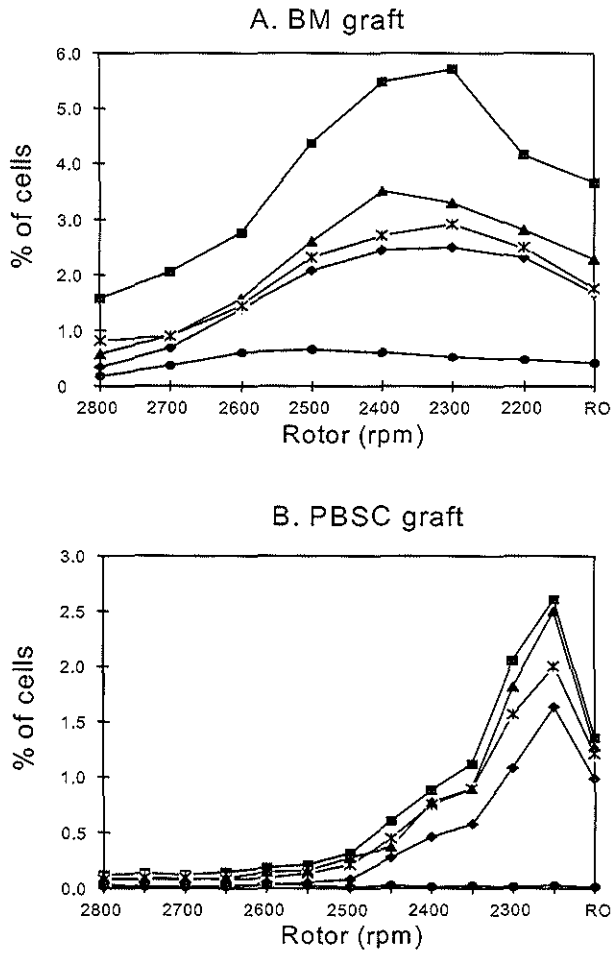

Figure. 4.4 Percentage of different mature progenitor cell populations in the cell fractions after CCE separation of BM (A) and PBSC (B). Subpopulations were determined in flowcytometry by using double immunofluorescence of a combination of CD34-PE and an additional FITC-labelled MAb. The percentage of total $CD34^+$ (■), and $CD34^+/CD13^-$ (◆), $CD34^+/CD38^-$ (✳), $CD34^+/HLA\text{-}DR^-$ (▲) and $CD34^+/CD19^+$ (●) were determined by sequential gating on CD34-PE followed by gating on the second MAb. Rotor speed fractions from 2800 rpm to RO are studied. Data are results of the means of three evaluations.

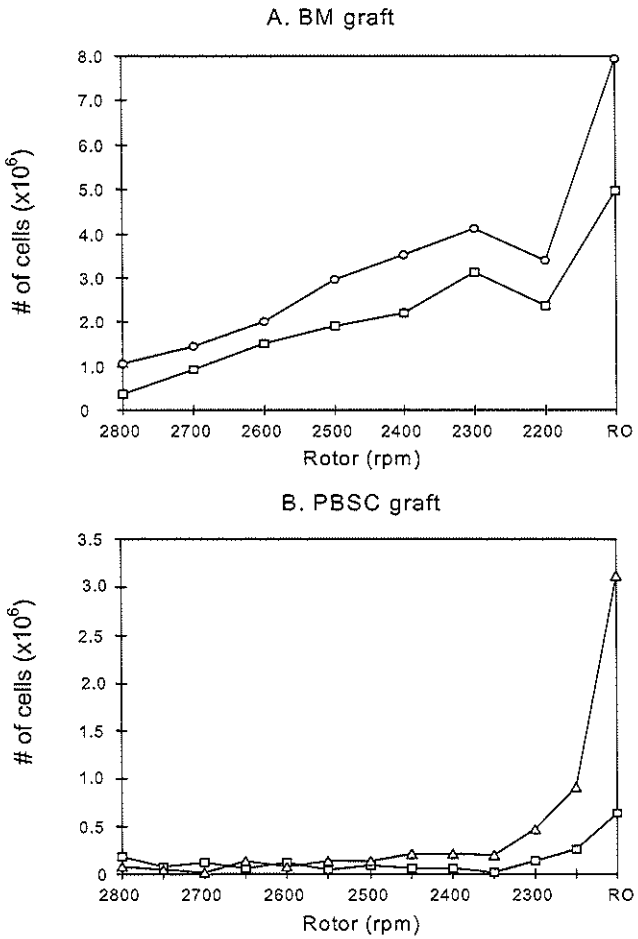

Figure 4.5 The absolute cell numbers of different immature progenitor cell populations in cell fractions after CCE separation of BM (A) and PBSC (B). Subpopulations were determined in flowcytometry by using triple immunofluorescence of CD34-PE-Cy5 and combinations of FITC-labelled and PE-labelled MAbs. The percentage of $CD34^+/CD13^-/HLA\text{-}DR^-$ (□), $CD34^+/CD13^-/HLA\text{-}DR^+$ (O), and $CD34^+/CD38^-$ (△) were determined by sequential gating on CD34 followed by gating on the other MAbs. Rotor speed fractions from 2800 rpm to RO are studied. Data are results of the means of three evaluations.

Potency of CAFC formation in CCE fractions

To study the presence of immature progenitor cells with proliferating capacity CAFC assays were performed. The number of CAFC in the CCE fractions was determined at week 2, 4 and 6, representing different stages of (im)mature cells. The relative number of CAFCs in the different CCE fractions from BM and PBSC are shown in figure 4.6. Due to the variation in frequency between patients a representative result of BM (Figure 4.6A) and PBSC (Figure 4.6B) is depicted. The CAFC wk.4 appeared to be present in a higher quantity than the CAFC wk.2. The most immature cells represented the lowest number. The separation patterns of the different CAFC week-types in BM and PBSC were comparable. Most progenitors were collected in the low speed fractions (<2500 rpm). The fraction with the highest number of CAFCs appeared to vary between the different subsets. Though separation differences were slight, more immature progenitor cells (CAFC wk.6) were collected in a higher speed fraction than the less immature cells (CAFC wk.2). The frequencies of CAFC in the RO fraction was low. However, the absolute numbers were the highest due to the high number of NC.

The evident concentration of immature cells in the low speed fractions becomes more clear comparing the CAFC before CCE and CAFC in the ultimate grafts of BM and PBSC (Figure 4.7). Most immature cells were collected in the graft. Though the total number of CAFC in BM was lower the recovery appeared to be higher in comparison with PBSC (approximately 80% and 60%, respectively).

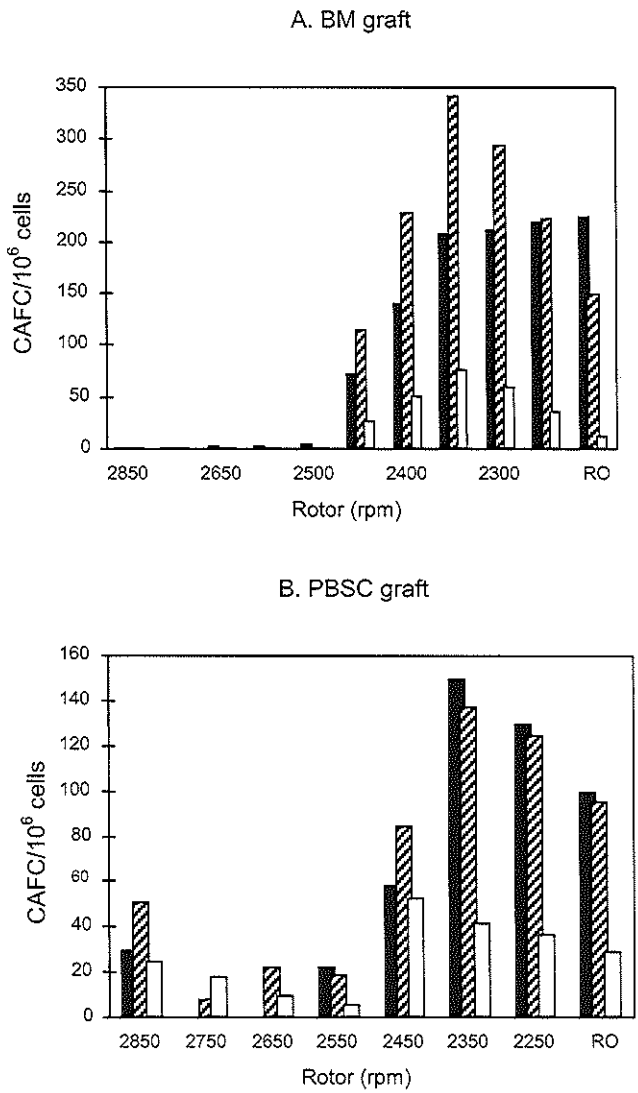

Figure 4.6 The number of CAFC in the different cell fractions after CCE separation. CCE fractions from BM (A) and PBSC (B) were cultured in CAFC assays for 2 (closed bar), 4 (striped bar) and 6 weeks (open bar) and the CAFC were enumerated. The frequency per 10^6 NC is shown. Data derived from each stem cell source are results of one representative evaluation out of three.

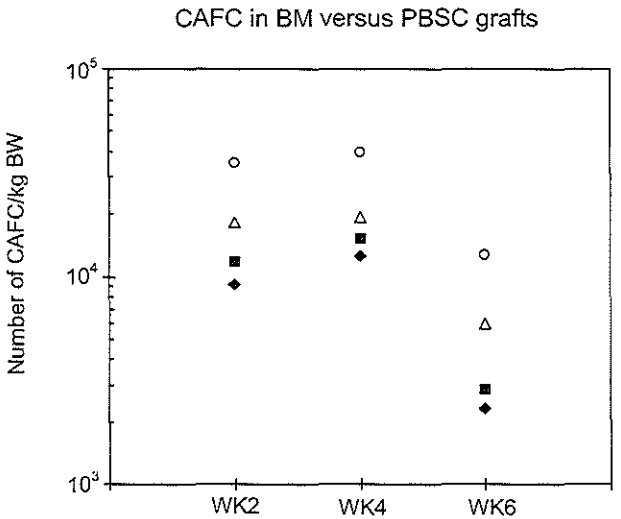

Figure 4.7 The yield of the number of CAFC per kg BW of the patient in the ultimate BM and PBSC grafts after CCE separation. The numbers of CAFC week (wk) 2, 4 and 6 derived from BM (■ harvest, ◆ graft) and PBSC (O harvest, △ graft) before CCE and in the composed graft are compared. Data are the mean values of three evaluations of each stem cell source.

Clinical outcome after transplantation of BM and PBSC

Clinical outcome is given in table 4.2. The median day of WBC engraftment was 19 in BM recipients and 16 in PBSC recipients (p=0.01). All patients engrafted within 26 days. The median day to reach a platelet count of >20 $x10^9$/L without support was 22 after BMT and 14 after PBSCT. One patient did not yet reach this level unsupported.

Though the median numbers of infused NC (0.4 $x10^8$ in BM grafts versus 1.3 $x10^8$ in PBSC grafts) and T-cells (0.7 $x10^6$ in BM grafts versus 4.4 $x10^6$ in PBSC grafts) per kg BW of the patient were significantly lower in BM grafts compared with PBSC grafts (Table 4.1), the incidence and severity of acute GvHD did not differ significantly (Table 4.2). Acute GvHD grade ≥ III occurred in 0 of 16 BM recipients and 2 of 12 PBSC recipients (p=0.17). The onset of acute GvHD was also comparable with a median of 18 and 16 days after BMT and after PBSCT, respectively.

Extensive chronic GvHD developed in 4 of 15 BM recipients and in 3 of 9 patients transplanted with PBSC (p=0.54).

Table 4.2 Clinical outcome after BMT and PBSCT with CCE processed grafts.

	BMT (n=16)	PSCT (n=12)	p-value$^{\phi}$
Median day of Engraftment			
WBC >1 x10^9/L	19 (15-26)$^{\varpi}$	16 (11-24)$^{\varpi}$	0.01
PLT >20 x10^9/L	22 (12-38)$^{\varpi}$	14 (11-NR)$^{\varpi}$	NS
Acute GvHD			
None	6	3	NS
Grade I	6	4	NS
Grade II	4	3	NS
Grade III	0	1	NS
Grade IV	0	1	NS
Chronic GvHD			
NE	1	3	NS
None	4	2	NS
Limited	7	4	NS
Extensive	4	3	NS
Relapse	6	2	NS
Died	4	2	NS
Median months of follow-up	+26 (17-30)	+27 (15-31)	NS

$^{\varpi}$ Data are depicted as median and range.

$^{\phi}$ Mann Whitney-U-test has been used for statistical analysis. P-value <0.05 is considered significant.

NE: not evaluable NR: not reached NS: not significant: WBC: white blood cell count
PLT: platelet count

DISCUSSION

In contrast to various other T-cell depletion techniques, CCE allows titration of an appropriate number of T-cells per kg BW to a graft based on the individual circumstances of each patient without triggering of cells. In this study we compared the CCE separations of 16 BM and 12 PBSC transplants. We showed that CCE using our techniques allows processing of both stem cell sources with sufficient yield of mature and immature progenitor cells. These data are supported by our clinical studies regarding low incidence of graft failure within a normal time of engraftment in almost 500 transplanted patients (10-13). Graft failure occurred in approximately 3% of the HLA-identical sibling transplantations. No graft failure occurred in the patients of the present study groups. This is in contrast with the data of Noga et al (39) who found 10% graft failure and delayed engraftment in his patients after receiving an elutriated allograft. Our patients, however, received a higher T-cell dose per kilogram BW.

Though the number of infused T-cells were significant higher in PBSCT than in BMT, the incidence and severity of acute GvHD did not differ significantly. Due to the CCE separation principle which is based on the cell size and density, it may be speculated that in BMT as well as in PBSCT a number of larger T-cells derived from the low speed fractions are infused that do not possess GvHD-inducing properties but engraftment facilitating activity. This hypothesis is still under investigation.

Despite the low incidence of severe acute and extensive chronic GvHD, we observed a relatively low risk for relapse (10, 12). However, the highest leukemia-free survival rates after BMT are seen in patients with grade I acute GvHD (40). This anti-leukemia activity might also be due to the relatively high number of NK cells remaining in the graft after CCE (approximately 50% of the number in the initial transplant) (18).

We showed that progenitor cells can be enriched in the larger cell CCE fractions. Several investigators, however, have shown in murine and feline models that primitive progenitors can be separated in the smaller cell fractions (41-43) suggesting a loss of immature progenitor cells based on their size and density. Yoder et al (41) demonstrated that different high proliferative potential colony-forming cells (HPP-CFC) can be identified by CCE separation based on growth factor responsiveness but their distribution profile differs between different strains of mice. Different HPP-CFCs were only detected by increasing the number of

CCE fractions and using higher rotor speed. This implicates that comparison of CCE data from different species and different procedures might be complicated.

Herbein et al (19) and Wagner et al (29) demonstrated that less mature $CD34^+$ cells derived from human PBSC not co-expressing CD38 and lacking colony formation were mainly collected in smaller cell fractions. Nevertheless Grimm et al (44) showed that G-CSF mobilized PBSC are heterogeneous in size and density implicating a distribution over all CCE fractions. Chang et al (28) found as a result of experimental CCE separations of BM, a removal of 96% of T-cells accompanied with a loss of 65% of HPP-CFC and long-term culture-initiating cells. Comparable data were found by Wagner at al (29) in experimental PBSC separations. Therefore we have investigated the separation patterns of mature and immature progenitors derived from BM and PBSC by using a Curameé 3000 system containing a multi-chamber rotor. Our results and those from other investigators using the same system (20, 30, 31) are in contrast with the above mentioned authors who all have used the Beckmann JE-5.0 or JE-6B rotor system.

We found that $CD34^+$ cells from BM and PBSC harvests could be effectively enriched in the low speed fractions when applying CCE within a speed range from 3000 to 2200 rpm. These results supported the data obtained in clonogenic assays of CFU-GM, BFU-E and CFU-GEMM. However, the $CD34^+$ cells elutriated in the high speed fractions showed a lower forward and side scatter expression in flowcytometric analysis (data not shown) suggesting a quiescent status of these hemopoietic cells as found by Wagner et al (29). To exclude that this minor population that was collected in the lymphocyte-enriched fractions consisted of the main proportion of immature progenitors, all cell fractions were evaluated in flowcytometry and in CAFC assays. The separation profiles of BM and PBSC appeared to be similar, but the highest concentration of PBSC $CD34^+$ cells tended to occur in lower speed fractions in comparison with BM $CD34^+$ cells. This finding is in agreement with the results of Chang et al (45) who showed a shift to higher flow rate fractions for PBSC $CD34^+$ cells in the Beckmann JE-5.0 elutriator.

Though the cells with the immature phenotype of $CD34^+/CD38^-$ and $CD34^+/CD13^-$ /HLA-DR^- were also present in the lymphocyte-enriched fractions, the highest numbers of these cells were collected in the graft fractions. This was also most pronounced in PBSC. These results were supported by the CAFC assays. Using this assay mature and primitive progenitor cells that represent long-term repopulating cells in vitro can be distinguished and

quantified (45, 46). The evident concentration of immature cells in the low speed fractions becomes clear in the comparison between the CAFC before CCE and CAFC in the ultimate grafts of BM and PBSC (Figure 4.7). Though the yield in BM was lower the recovery of CAFC appeared to be higher in comparison with PBSC (approximately 80% and 60%, respectively).

Differences between our results and those of Chang et al (28) regarding the elutriation profile of immature progenitor cells might be due to the different equipments used (Curameé versus Beckmann JE-5, respectively). However, the loss of HPP-CFC and LTC-IC described by Chang et al is not reflected in clinical studies in which the same equipment was used (14, 17).

The procedure of CCE strongly influences the separation profile. Variation of rotor speed in stead of increasing the flow rate is recommendable because reduction of rotor speed is far better adjustable then is flow rate (Plas et al, unpublished data). A number of differences exists between our procedure and those applied by Chang et al. Firstly, Chang et al elutriated the BM cell populations under a constant centrifugal speed and increasing flow rates. In the clinical setting (14, 17), however, a constant flow with decreasing centrifugal speed was used. Secondly, a higher starting speed rate, as used in our setting, advantageously affects the separation process (41). We loaded the CCE rotor and started the procedure at 3000 rpm. Thirdly, the cell load, the volume and the shape of the separation chamber was also different. Lutz et al (47) showed that a chamber which is calculated according to the hydrodynamic boundaries of the CCE separation improves the separation selectivity. Chang et al processed relatively small amounts of cells while in the clinical settings larger numbers of cells were separated (up to 2 $x10^9$ cells/chamber in our setting). Nevertheless, Kwekkeboom et al (31) also found a concentration of progenitor cells in the lower speed fractions when relatively small numbers of PBSC were loaded.

These data confirm that CCE is a reliable method to deplete T-cells from BM transplants without substantial loss of immature and mature progenitor cells. Furthermore CCE might be considered a feasible method to process PBSC transplants. Though the number of cells that must be processed in PBSCT is much higher than in BMT the separation patterns of progenitor cells are comparable. However, due to the higher number of T-cells and the lower concentration of $CD34^+$ cells in the initial harvest, more CCE runs have to be performed resulting in a laborious procedure. Nevertheless, CCE allows compiling a graft with a

sufficient number of stem cells to ensure engraftment and with an appropriate number of T-cells adjusted to each patient individually. This results in a low incidence of graft failure and a relatively low incidence of severe GvHD.

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CHAPTER 5

TRANSPLANTATION OF HUMAN UMBILICAL CORD BLOOD CELLS IN MACROPHAGE-DEPLETED SCID MICE: EVIDENCE FOR ACCESSORY CELL INVOLVEMENT IN EXPANSION OF IMMATURE $CD34^+/CD38^-$ CELLS

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ABSTRACT

In vivo expansion and multilineage outgrowth of human immature hemopoietic cell subsets from umbilical cord blood (UCB) were studied by transplantation into hereditary immunodeficient (SCID) mice. The mice were preconditioned with liposome-encapsulated dichloromethylene diphosphonate (Cl_2MDP) to deplete macrophages and 3.5 Gy total body irradiation (TBI). As measured by immunophenotyping, this procedure resulted in high levels of human $CD45^+$ cells in SCID mouse bone marrow (BM) 5 weeks after transplantation, similar to the levels of human cells observed in nonobese diabetic/SCID (NOD/SCID) mice preconditioned with TBI. Grafts containing ~10^7 unfractionated cells, ~10^5 purified $CD34^+$ cells or 5 x10^3 purified $CD34^+/CD38^-$ cells yielded equivalent numbers of human $CD45^+$ cells in the SCID mouse BM, which contained human $CD34^+$ cells, monocytes, granulocytes, erythroid cells and B-lymphocytes at different stages of maturation. Low numbers of human glycophorin A positive (GpA^+) erythroid cells and $CD41^+$ platelets were observed in the peripheral blood (PB) of engrafted mice. $CD34^+/CD38^+$ cells (5 x10^4/mouse) failed to engraft, while $CD34^-$ cells (10^7/mouse) displayed only low levels of chimerism, mainly due to mature T-lymphocytes.

Transplantation of graded numbers of UCB cells resulted in a proportional increase of the percentages of $CD45^+$ and $CD34^+$ cells produced in SCID mouse BM. In contrast, the number of immature, $CD34^+/CD38^-$ cells produced in vivo showed a second-order relation to $CD34^+$ graft size, and mice engrafted with purified $CD34^+/CD38^-$ grafts produced 10-fold fewer $CD34^+$ cells without detectable $CD34^+/CD38^-$ cells than mice transplanted with equivalent numbers of unfractionated or purified $CD34^+$ cells. These results indicate that SCID repopulating $CD34^+/CD38^-$ cells require $CD34^+/CD38^+$ accessory cell support for survival and expansion of immature cells, but not for production of mature multilineage progeny in SCID mouse BM. These accessory cells are present in the purified, nonrepopulating $CD34^+/CD38^+$ subset as was directly proven by the ability of this fraction to restore the maintenance and expansion of immature $CD34^+/CD38^-$ cells in vivo when added to purified $CD34^+/CD38^-$ grafts. The possibility to distinguish between maintenance and outgrowth of immature repopulating cells in SCID mice will facilitate further studies on the regulatory functions of accessory cells, growth factors (GF) and other stimuli. Such information will be essential to design efficient stem cell expansion procedures for clinical use.

INTRODUCTION

Traditional sources of hemopoietic stem and progenitor cells for transplantation include autologous and allogeneic bone marrow and mobilized PB. Recently human UCB has been shown to be a realistic alternative source of stem cells (1, 2). UCB contains cells of all hemopoietic lineages including cells that can produce colony-forming unit granulocyte-macrophage (CFU-GM) after extended long-term stromal cell supported culture. Most of these long-term culture-initiating cells are found in the small subset of $CD34^+/CD38^-$ cells (3). The ability to cryopreserve, select, and expand progenitors without loss of proliferative capacity (4) makes UCB an appropriate material to identify immature hemopoietic cell subsets involved in hemopoiesis in vivo, select appropriate GF combinations and culture conditions to maintain and expand stem cells in vitro, and design optimal gene transfer conditions aimed at efficient and stable transduction of transplantable stem cells (5).

Hereditary immunodeficient SCID and NOD/SCID mice are useful recipients to assess human stem cell capacities in a transplantation assay and appear particularly suitable to assess the outgrowth of purified UCB cell subsets and the effects of ex vivo manipulation on hemopoietic capacities after transplantation. Several approaches for engrafting immunodeficient mice with normal or leukemic human hemopoietic cells have been described. The most frequently used systems involve injection of mobilized human PB, BM (6) or UCB cells in sublethally irradiated mice (7, 8), electively followed by human GF treatment (9-12) and/or cotransplantation with nonrepopulating $CD34^-$ accessory cells (13), human BM long-term culture-derived stromal cells or rodent cell lines that produce human GF's (14). Transgenic SCID mice expressing the genes for human IL-3, GM-CSF and SCF have also been used to promote human cell engraftment (15), while human fetal liver, thymus (16, 17) and/or bone fragment (18) implantation has been used to create a human microenvironment in the mouse.

Injection with human cytokines or other additional treatment is not required to establish high-level human cell engraftment after transplantation of human UCB cells in immunodeficient mice, which suggested that neonatal cells either respond differently to the murine microenvironment or provide their own cytokines in a paracrine fashion (7, 8). However, analysis of the hemopoietic potential of UCB cells in SCID is limited by the large number of cells required to achieve significant engraftment levels, possibly because of low

seeding efficiencies of stem cells or elimination of transplanted cells by natural killer (NK) cells or the mononuclear phagocytic system, which are intact in SCID mice. More reproducible and higher levels of engraftment with smaller graft sizes have been achieved with NOD/SCID mice, which has been attributed to the lack of functional macrophages, NK cells and complement activity in this mouse strain (19). Specific elimination of phagocytic cells in spleen and liver of SCID mice can be achieved within 24 hours after a single intravenous (i.v.) injection of Cl_2MDP containing liposomes (20-22). As shown recently for human acute myeloid leukemia (AML) and UCB cells, macrophage-depleted SCID mice supported the production of similar levels of human cells from 10-fold fewer transplanted cells as compared to SCID mice conditioned with TBI alone. For AML cells, preconditioning of SCID mice with liposomes led to similar levels of engraftment as observed in NOD/SCID mice which suggested that macrophages have a prominent role in eliminating injected human cells in SCID mice (20).

The present study was undertaken to quantitatively analyze the maintenance and outgrowth of distinct UCB immature cell subsets in macrophage-depleted SCID mice and to assess the hemopoietic cell lineages produced.

MATERIAL AND METHODS

Human umbilical cord blood (UCB) cells

UCB samples were obtained after informed consent in conformity with legal regulations in The Netherlands from placentas of full-term normal pregnancies. Mononucleated cells were isolated by Ficoll density gradient centrifugation (1.077 g/cm^2, Nycomed Pharma AS, Oslo, Norway), and were cryopreserved in 10% dimethylsulphoxide, 20% heat-inactivated fetal calf serum (FCS) and 70% Hanks Balanced Salt Solution (HBSS; Gibco, Breda, The Netherlands) at -196°C as described (23). After thawing by stepwise dilution in HBSS containing 2% FCS, the cells were washed with HBSS containing 1% FCS and used for flowcytometric analysis, transplantation into SCID mice (unfractionated grafts) or subset purification.

Subset purification

Purification of $CD34^+$ cells was performed by positive selection using Variomacs Immunomagnetic Separation System as described (24) (CLB, Amsterdam, The Netherlands). The percentage $CD34^+$ cells in the unseparated population (unfractionated UCB) and in the purified $CD34^+$ and $CD34^-$ fractions was determined by FACS-analysis. For isolation of $CD34^+/CD38^+$, $CD34^+/CD38^{+/-}$ and $CD34^+/CD38^-$ subsets, purified $CD34^+$ cells were stained with fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE) conjugated antibodies against human CD34 and CD38 (CD34-FITC, CD38-PE, Becton Dickinson) for 30', on ice in HBN (HBBS, 2% (wt/vol) FCS, 0.05% (wt/vol) sodium-azide) containing 2% (vol/vol) normal human serum (NHS). After incubation, the cells were washed twice, resuspended in HBSS and sorted using a FACS Vantage flowcytometer (Becton Dickinson, San Jose, CA, USA).

Transplantation of UCB cells in immunodeficient mice

Female, specified pathogen-free (SPF) CB-17-scid/scid (SCID) mice, 6 to 9 weeks of age, were obtained from Harlan, CPB, Austerlitz, The Netherlands. NOD/LtSz-scid/scid mice (NOD/SCID) were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). The mice were housed under SPF conditions in a laminar air flow unit and supplied with sterile food and acidified drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) ad libitum. The plasma Ig levels of the mice were determined with an ELISA using a sheep anti-mouse antibody reacting with mouse IgG (Boehringer Mannheim Biochemica, Penzberg, Germany) and animals with plasma Ig levels over 40 µg/ml were excluded (25). To deplete macrophages, the SCID-mice were injected i.v. into a lateral tail vein with 200 µl liposome stock solution containing Cl_2MDP (a gift of Boehringer Mannheim GmbH, Mannheim, Germany) one day prior to transplantation of hemopoietic cells (26). In previous studies (20) with human acute leukemia and UCB cells this approach required ten-fold fewer cells for uniform engraftment than in SCID mice conditioned with TBI alone. All mice received TBI at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 µl HBSS containing 0.1% BSA (Sigma) and injected i.v. into a lateral tail vein. Transplanted cell numbers were 10^7 (unfractionated and $CD34^-$ cells), 10^5 ($CD34^+$ cells), 5 x10^4 ($CD34^+/CD38^+$ cells) and 5 x10^3 ($CD34^+/CD38^-$ cells) unless stated otherwise in the results.

In vitro colony assay

Unfractionated and purified $CD34^+$ and $CD34^-$ grafts as well as chimeric mouse BM samples were assayed for the presence of GM-CFU and erythroid burst-forming units (BFU-E) by in vitro colony formation in viscous methylcellulose culture medium as previously described (27-29). Briefly, unfractionated and $CD34^-$ cells were plated at a concentration of 25,000 per 35 mm Petri dish (Becton Dickinson), $CD34^+$ purified grafts at 1,000 per dish and chimeric mouse BM at 50,000 per dish. Culture medium consisted of 1 ml Dulbecco's medium (Gibco, Gaithersburg, MD, USA), containing 0.8% (wt/vol) methylcellulose, 5% (vol/vol) FCS, and further supplemented with 1.5% (wt/vol) BSA, 10 mg/ml insulin, 0.3 mg/ml human transferrin, 15 mmol/L ß-mercaptoethanol, 0.1 mmol/L sodium selenite, 1 mg/ml nucleosides, 15 µmol/L linoleic acid, 15 µmol/L cholesterol, 100 U/ml penicillin, and 50 mg/ml streptomycin. For BFU-E, cultures were supplemented with 0.2 mmol/L bovine hemin (Sigma), 200 ng/ml human SCF and 4 U/ml (25 µg/ml) human recombinant erythropoietin (Behringwerke AG, Marburg, Germany). For CFU-GM, cultures were supplemented with 5 ng/ml human recombinant GM-CSF (Behringwerke AG), 200 ng/ml SCF and 30 ng/ml human recombinant IL-3. The cultures were maintained in a humidified atmosphere of 10% CO_2 at 37°C for 14 days, after which the colonies were counted. Data of duplicate dishes were expressed as average number of colonies per 10^5 cells plated.

Tissue collections and analysis

Mice were examined at a single time point, 35 days after transplantation, to enable meaningful comparisons between experiments, because individual hemopoietic subsets show differences in engraftment kinetics in immunodeficient mice (12). Mice were killed by CO_2 inhalation followed by cervical dislocation in accordance with institutional animal research regulations. From each mouse, both femurs were collected and BM cell suspensions were prepared by flushing. After counting, the cells were cultured in colony assays and analyzed by flowcytometry to determine the percentage of human cells in the mouse bone marrow. Cells were suspended in HBSS containing 2% (vol/vol) FCS, 0.05% (wt/vol) sodium azide, 2% (vol/vol) human serum and 2% (vol/vol) mouse serum and stained for 30' at 4°C with the pan-leukocyte surface marker CD45-FITC antibody and with CD33-PE antibody. Positive samples were further analyzed by incubation with FITC and PE labeled mouse monoclonal antibodies to human CD34, CD19, CD16, CD15, CD38, CD33, CD56, CD4, CD8 (Becton Dickinson

Immunocytometry Systems, San Jose, CA, USA), and GpA, CD3, CD71 (Dako A/S, Copenhagen, Denmark). Parallel samples were incubated with isotype matched control antibodies. Cell samples of nontransplanted mice were stained as negative controls. Fluorescence was measured using a FACScan flowcytometer and Lysis II software (Becton Dickinson, USA). Dead cells were excluded by adding 1 μg/ml propidium iodide (PI) and gating for PI^- cells in the FL3 channel. For all samples 10,000 events were collected in a gate for PI^- cells. To quantitate $CD34^+$ subsets in selected samples, 1,000 - 10,000 events were also collected in a gate that included all viable human $CD34^+$ cells. $CD34^-$ and $CD34^+/CD38^-$ expansion were calculated on the assumption that one femur contains 8.5% of all bone marrow cells (30).

In a number of experiments, PB was collected weekly from the tail vein and analyzed for the presence of human GpA^- erythrocytes and $CD41^+$ platelets, by flowcytometry. Blood samples were collected in EDTA coated tubes and stained with CD41-FITC (PharMingen, San Diego, USA) and GpA-FITC, respectively (Dako A/S) in HBSS with 2% (vol/vol) FCS, 0.05% (wt/vol) sodium azide, 2% (vol/vol) human serum 2% (vol/vol) mouse serum and 2 g/L EDTA for 30' at 4°C. Cell samples of nontransplanted mice and human blood cells were stained as controls.

Statistical and regression analysis

Results are expressed as individual data or as arithmetic mean ± standard deviation. The regression analysis of the percentage of human $CD45^+$, $CD34^+$ and $CD34^+/CD38^-$ cells in the chimeric BM as a function of the number of $CD34^+$ cells transplanted was done by plotting the data on a double logarithmic scale and calculating the regression using the general formula $y = axb$. By this method, an exponent b=1 proves first order (single-hit) kinetics, i.e., direct proportionality (linearity) of chimeric cell numbers and cells transplanted, while an exponent b=2 demonstrates second order (two-hit) kinetics. The frequency of repopulating cells in the SCID mice was approximated using Poisson statistics.

RESULTS

Chimeric bone marrow analysis

Chimerism in SCID mouse bone marrow was assessed by flowcytometric analysis 35 days after UCB transplantation. Typical results of chimeric bone marrow stained with CD45-FITC vs. CD33-PE and CD45-FITC vs. CD34-PE are shown in figure 5.1A and 5.1B, respectively. The percentage of $CD45^+$ cells was used as a measure for engraftment levels of human cells in the mouse BM. Only mice with percentages larger than 1% $CD45^+$ cells were considered to be engrafted. Positive staining for any of these markers was not found in nontransplanted mice (Figure 5.1C and data not shown), demonstrating the specificity of the antibodies for human cells. As shown in figure 5.1A and 5.1B, the $CD45^-$ cells were heterogeneous with respect to CD33 and CD34 expression.

Parallel groups of mice were injected with unfractionated mononucleated UCB cells or with purified $CD34^+$ or $CD34^-$ cells (Figure 5.2) in SCID mice conditioned with either TBI or TBI and macrophage depletion, or in TBI conditioned NOD/SCID mice. Transplantation of unfractionated mononucleated UCB cells into macrophage-depleted SCID mice resulted in more prominent engraftment levels compared to SCID mice conditioned with TBI alone. After transplantation with 10^7 unfractionated or 10^5 purified $CD34^+$ cells, the macrophage-depleted SCID mice showed similar levels of chimerism as NOD/SCID mice preconditioned with TBI. $CD34^-$ cells (10^7 cells transplanted) did not result in high levels of chimerism in either mouse strain (Figure 5.2).

As shown in table 5.1, transplantation of 10^7 unfractionated cells from 5 different UCB samples resulted in high levels of chimerism in all mice (n=22) transplanted. Transplantation of 10^5 purified $CD34^+$ cells also resulted in high levels of human cells in 35 out of 38 mice, whereas mice transplanted with 10^7 $CD34^-$ cells showed only low levels of engraftment in 5 out of 18 mice transplanted. These results demonstrate that relatively low numbers of purified $CD34^+$ UCB cells are capable of proliferation in the macrophage-depleted SCID mouse microenvironment without the support of accessory cells or addition of hemopoietic GF. The $CD34^+$ cells were further separated into a $CD38^+$ subset (~50% of $CD34^+$ cells containing >90% of clonogenic progenitors) and a $CD38^-$ subset (~ 5% of the $CD34^+$ population, enriched for immature, multipotent progenitors (31)) and transplanted into preconditioned SCID mice; cell numbers were 5 $x10^4$ and 5 $x10^3$, respectively. The $CD34^+/CD38^-$ subset showed high

levels of engraftment in 4/6 mice with chimerism levels similar to those obtained with 20-fold larger numbers of $CD34^+$ cells and 200-fold larger numbers of unfractionated UCB (Table 5.1). Despite the 10-fold larger cell numbers, only 1 of 4 mice engrafted with sorted $CD34^+/CD38^+$ cells at the low level of 1.7%. These results show that the ability to repopulate SCID mice resides exclusively in the $CD34^+/CD38^-$ immature population.

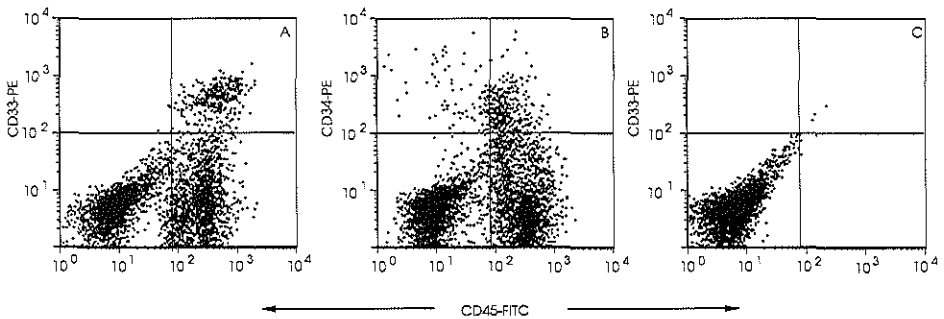

Figure 5.1 Flowcytometric analysis of chimeric mouse BM stained with CD45-FITC vs CD33-PE (A), and CD45-FITC vs CD34-PE (B). BM of nontransplanted mice showed no staining with the CD45-FITC or CD33-PE antibody (C).

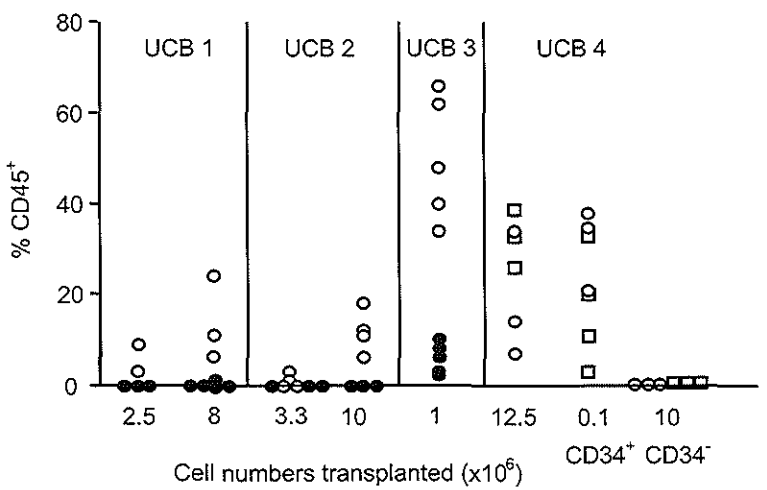

Figure 5.2 Engraftment levels of 4 different UCB samples in SCID and NOD/SCID mice. The percentages $CD45^+$ cells are shown for individual SCID mice preconditioned with TBI and Cl_2MDP (O), SCID mice preconditioned by TBI alone (●) and NOD/SCID mice preconditioned by TBI alone (□).

Table 5.1 Engraftment of UCB cells in preconditioned SCID mice.

Graft	Graft size*	Chimeric mice$^{\Psi}$ /injected mice	% Chimerism ($CD45^+$ cells)	Characteristics of chimersm			
				$CD34^+$ cells		$CD34^+/CD38^-$ cells	
				Cells /mouse BM ($x10^6$)	Expansion factor	Cells /mouse BM ($x10^4$)	Expansion Factor
Unfractionated	10^7	22/22	34.5 ±19.3$^{\varpi}$	1.1 ±1.4°	12.9 ±19.2	1.84 ±2.7	2.19 ±4.92
$CD34^-$	10^7	5/18	7.8 ±8.6	0.1 ±0.1	4.0 ±3.7	0	0
$CD34^+$	10^5	35/38	20.4 ±16.3	1.1 ±0.9	14.1 ±18.6	6.9 ±13.2	10.8 ±16.6$^{\delta}$
$CD34^+/CD38^+$	$5 x10^4$	1/4	1.7	0	0	0	0
$CD34^+/CD38^-$	$5 x10^3$	4/6	18.4 ±8.7	0.1 ±0.1	18.6 ±5.2	0	0$^{\delta}$

* Percentage $CD34^+$ cells in the graft: unfractionated UCB, 0.7% to 3.1%; $CD34^-$, 77% to 83%; and $CD34^-$, 0.1% to 0.6%.

Ψ Mice are considered chimeric at more than 1% $CD45^+$ cells.

ϖ Mean (±SD) of chimeric mice, results of 5 UCB.

$\circ$ $CD34^+$ BM cells, calculated on the assumption that 1 femur represents 8.5% of all BM cells (30).

δ Significantly different from unfractionated graft (p=0.03, Fisher's exact test).

Multilineage outgrowth of UCB cells

BM cells of chimeric mice were cultured in standard methylcellulose culture under conditions of stimulation with recombinant human GF which selectively favour the outgrowth of human monomyeloid and erythroid progenitors and failed to stimulate mouse progenitors. Comparison of clonogenic cell numbers in 15 chimeric mice with the numbers of colony-forming cells in the grafts showed a median expansion of 2.7-fold (range: 0-11) and 1.7-fold (range: 0-13) for CFU-GM and BFU-E numbers, respectively, as measured 35 days after transplantation. Since these progenitor cell populations have a high turn-over rate, this observation demonstrates that monomyelocytic and erythroid progenitors are produced from more immature progenitors in the mouse hemopoietic environment.

The composition of the human cell population in the BM of chimeric mice was assessed by flowcytometry using a panel of lineage specific markers (Figure 5.3). The percentage of cells in each subset identified was expressed relative to the percentage cells stained with the panleukocyte marker CD45 (Figure 5.4). Mice transplanted with 10^7 unfractionated UCB cells showed multilineage outgrowth (Figure 5.4A). The most prominent population (25-50% of the human $CD45^+$ cells) consisted of B-lymphoid cells, which contained immature $CD19^+/CD20^-$ as well as mature $CD19^+/CD20^+$ cells (Figures 5.3F, 5.4). $CD15^-/CD33^+$ monocytes, $CD15^+/CD33^{-/-}$ granulocytes, and $CD15^-/CD33^+$ immature myelomonocytic cells were present at percentages ranging between 6 and 16% of the human cells (Figure 5.3B, 5.4). $GpA^+/CD71^{++}$ erythroblasts and, occasionally, $GpA^+/CD71^-$ mature red blood cells (not visible in Figure 5.3D) were present in low numbers. In keeping with the presence of CD71 on activated non-erythroid cells, the large population of $CD71^+/GpA^-$ cells (Figure 5.3D) contained cells of multiple lineages (32). The composition of the BM of mice transplanted with $CD34^+$ (Figure 5.4B) or $CD34^+/CD38^-$ cells (Figure 5.4C) was similar to that of mice transplanted with unfractionated UCB. The few mice that showed detectable chimerism after transplantation of $CD34^-$ cells also had outgrowth of low numbers of myeloid, erythroid and B-lymphoid cells, which were possibly derived from the low numbers of $CD34^+$ cells (0.1-0.6%) still present in the fraction. However, greater than 50% of the cells growing in these mice consisted of mature $CD3^+$ T-lymphocytes, which also expressed CD4 or CD8. $CD3^+$ cells were also identified in mice transplanted with unfractionated, $CD34^+$ or $CD34^+/CD38^-$ cell subsets, but these $CD3^+$ cells neither expressed CD4 nor CD8 (Figures 5.3, 5.4A-C). These cells may represent a subset of NK cells as CD3 is expressed on some $CD56^+$ cells (33) and $CD56^+$ cells were also identified in low numbers in chimeric mice, including those transplanted with purified $CD34^+/CD38^-$ cells (Figures 5.3C, 5.4C). The large population of $CD3^-$ cells which expressed CD4 or CD8 (Fig 3E), most likely consisted of $CD4^+$ monocytes.

In spite of large numbers of human cells in the BM of SCID mice, very few human cells were detected in the leukocyte fraction of PB, spleen and thymus (data not shown). Whole tail vein blood samples of $CD34^+$ transplanted mice collected at various time points after transplantation, contained human GpA^+ erythrocytes at very low levels (~0.1%) which could only be detected if very large cell numbers ($>10^5$) were analyzed (Figure 5.5C). The largest quantities (0.1-0.2%) were found 2 weeks after transplantation. From the third week on the level decreased and became undetectable in the fifth week. Human $CD41^+$ platelets could

also be detected in the mouse PB and followed a similar time course as the erythroid cells, with peak levels of 0.5% in the second week (Figure 5.5B).

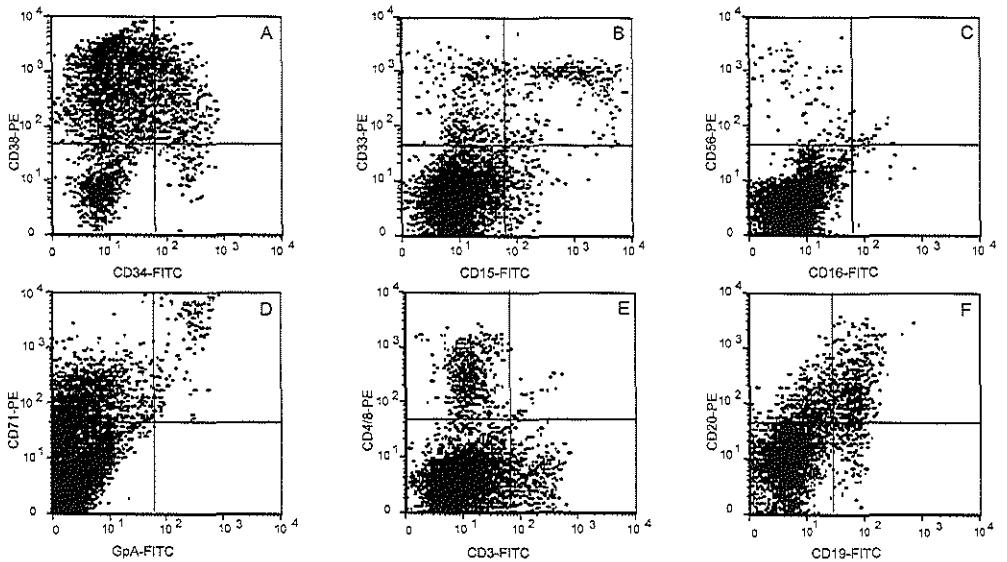

Figure 5.3 Immunophenotyping of chimeric mouse BM. BM (>10% $CD45^+$) was stained with a panel of antibodies specific against different human blood cell lineages. Flowcytometry profiles of a representative mouse are shown for CD34 vs CD38 (A), CD15 vs CD33 (B), CD16 vs CD56 (C), GpA vs CD71 (D), CD3 vs CD4 and CD8 (E) and CD19 vs CD20 (F) expression, respectively.

Evidence for accessory cell requirement for immature cell expansion but not for outgrowth of human UCB cells in SCID mice

The UCB cell number required for engraftment was analyzed by injection of graded numbers of unfractionated or $CD34^+$ cells. Transplantation of 2 $x10^3$ $CD34^+$ cells resulted in a low, but measurable level of chimerism of 1.4% $CD45^+$ cells (Figure 5.6). The level of chimerism increased proportionally with cell dose, reaching ~60% human $CD45^+$ cells after injection of 2 $x10^5$ purified $CD34^+$ cells. Engraftment after transplantation of unfractionated mononuclear UCB cells and purified $CD34^+$ cells followed similar proportional patterns with exponents of 0.8 and 1, respectively (Figure 5.6). Also the percentage of human $CD34^+$ cells detected in

SCID mouse BM after 35 days showed a linear relation with graft size (Figure 5.6). These results demonstrate that the outgrowth of human UCB cells in the SCID mouse BM does not require the support from accessory cells present in either the $CD34^+$ or $CD34^-$ UCB fractions. Figure 5.3A shows that the $CD34^+$ cells produced in SCID mouse BM were heterogeneous with respect to CD38 expression and included cells with low CD38 expression, which suggested that very immature cells were maintained and/or expanded in the mouse microenvironment. As shown in figure 5.6, the production of cells with an immature $CD34^+/CD38^-$ phenotype showed a much steeper dependence on the number of $CD34^+$ transplanted, with a exponent of 2, demonstrating second order (two-hit) kinetics. $CD34^+/CD38^-$ cells were not detected in BM of mice transplanted with purified $CD34^+/CD38^-$ cells (Table 5.1, Figure 5.8A) while, in addition, the numbers of $CD34^+$ cells in these mice were 10-fold lower than in mice transplanted with unfractionated or $CD34^+$ grafts, despite similar levels of $CD45^+$ cells (Table 5.1). Taken together, the nonlinear relation between graft size and percentage $CD34^+/CD38^-$ cells after 35 days, the lower number of $CD34^+$ cells and absence of $CD34^+/CD38^-$ cells in mice transplanted with purified $CD34^+/CD38^-$ grafts demonstrate that immature $CD34^+/CD38^-$ cells can be maintained in the mouse microenvironment, but only with the support of accessory cells.

Figure 5.7 shows the actual expansion of $CD34^+/CD38^-$ cells in BM of the 32 (from 69) chimeric mice in which such cells were detectable. The expansion ranged between 0.2 and 22.1 with a median expansion of 3-fold for unfractionated mononucleated UCB cells and between 1.6 and 63.1 with a median value of 7-fold after transplantation of $CD34^+$ grafts. This difference is statistically not significant.

Direct proof of an accessory role of $CD34^+/CD38^+$ cells in the maintenance of the transplanted $CD34^+/CD38^-$ population in vivo was obtained by transplantation of $CD34^+/CD38^-$ cells, supplemented with $CD34^+/CD38^+$ cells. Dot plots of CD38 vs CD34 expression, collected in a gate for $CD34^+$ cells, show that transplantation of 5 $x10^3$ $CD34^+/CD38^-$ cells results in production of $CD34^+$ cells, which are all $CD38^+$ (Figure 5.8A). After transplantation of 5 $x10^3$ $CD34^+/CD38^-$ cells to which 25 $x10^3$ $CD34^+/CD38^+$ cells were added, $CD34^+/CD38^-$ cells were clearly produced in the mouse BM (Figure 5.8B) at frequencies similar to those observed in mice transplanted with 10^5 $CD34^+$ cells (Figure 5.8C). Also in this experiment, transplantation of 5 $x10^4$ $CD34^+/CD38^+$ cells alone did not result in human cell engraftment (similar to the data presented in Table 5.1). Sorted $CD34^+/CD38^{+/-}$

cells (corresponding to 20% of the $CD34^+$ cells) also repopulated transplanted SCID mice with propagation of immature $CD34^+/CD38^-$ cells, which can be explained by the presence of repopulating and accessory cells in this subset (Figure 5.8D).

Repopulating cell frequency

The maintenance or expansion of $CD34^+/CD38^-$ cells in SCID mice might be considered as a more significant characteristic of the capacity of repopulating stem cells than the ability to produce mature progeny. Taking into account that the seeding efficiency of repopulating cells in transplanted SCID mice is unknown and the support provided by accessory cells may be suboptimal, a lower limit for the frequency of cells with the ability to maintain or expand the numbers of $CD34^+/CD38^-$ cells was estimated using the pooled data of 69 mice engrafted with graded doses of unfractionated or purified $CD34^+$ cells from 5 different UCB samples (Table 5.2). By using Poisson statistics, a value of 1 repopulating cell per 70,000 $CD34^+$ cells was estimated (95% confidence limits: 54,000 - 102,000). This would correspond to 1 repopulating cell per ~7 $x10^6$ unfractionated UCB cells and 1 per 3,500 $CD34^+/CD38^-$ cells.

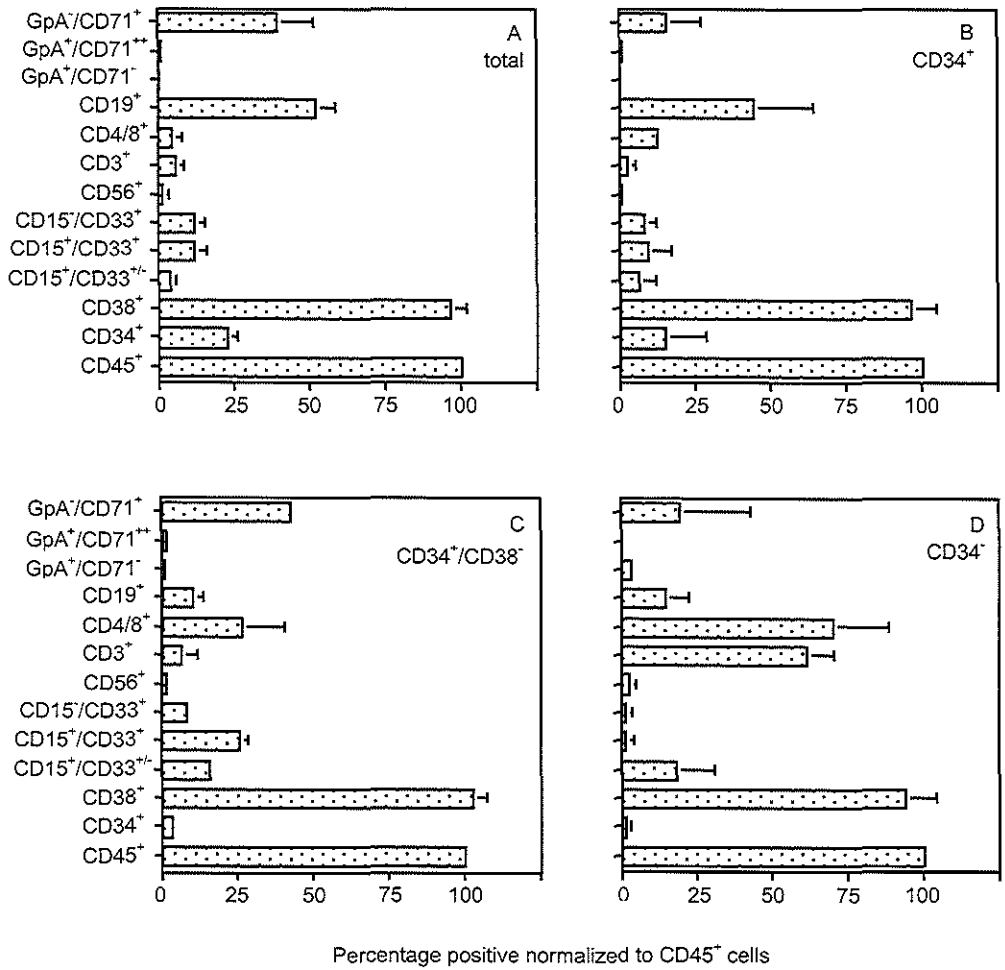

Figure 5.4 Composition of the human $CD45^+$ cell population in chimeric SCID mice stained for the human markers shown in figure 5.3. Results (average±SD) of 23 mice in total, transplanted with unfractionated UCB (A), purified $CD34^+$ (B), $CD34^+/CD38^-$ (C) and $CD34^-$ (D) grafts derived from 5 UCB samples. Percentage of cells in each subset was expressed relative to the percentage $CD45^+$ cells present in the BM of each mouse. The percentage chimerism ranged between 10 and 40% for the data shown in (A) through (C), and between 1 and 15% for the data shown in (D).

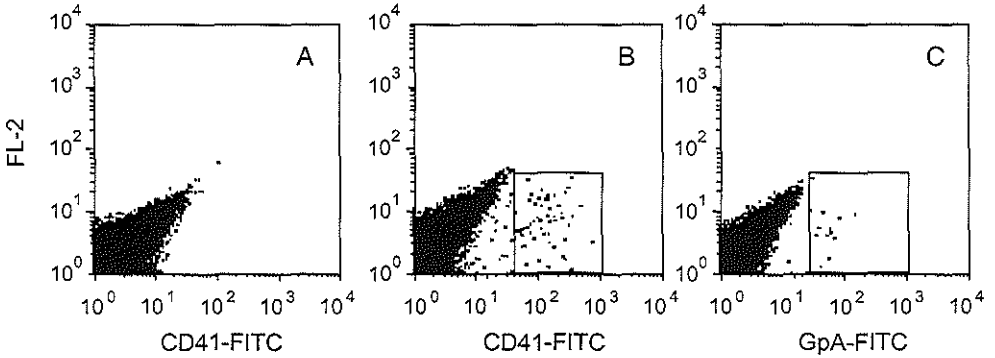

Figure 5.5 Circulating $CD41^+$ platelets and GpA^+ erythrocytes in the peripheral blood of $CD34^+$ transplanted SCID mice. Blood was collected in the presence of 2 g/L EDTA and stained immediately with CD41-FITC (B) and GpA-FITC (C). (A) shows the blood of a nontransplanted mouse stained with CD41-FITC.

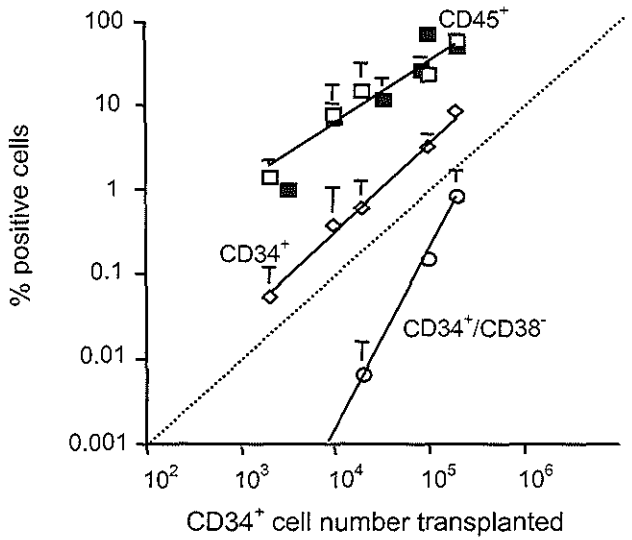

Figure 5.6 Relation between the number of $CD34^+$ cells transplanted and percentage of human $CD45^+$ (□), $CD34^+$ (◊) and immature $CD34^+/CD38^-$ (O) cells detected in SCID mouse BM after 5 weeks. Results show the mean±SD for 3 mice per data point. For comparison, the numbers of $CD45^+$ cells detected in BM of mice transplanted with graded number of unfractionated cells are also shown (■).

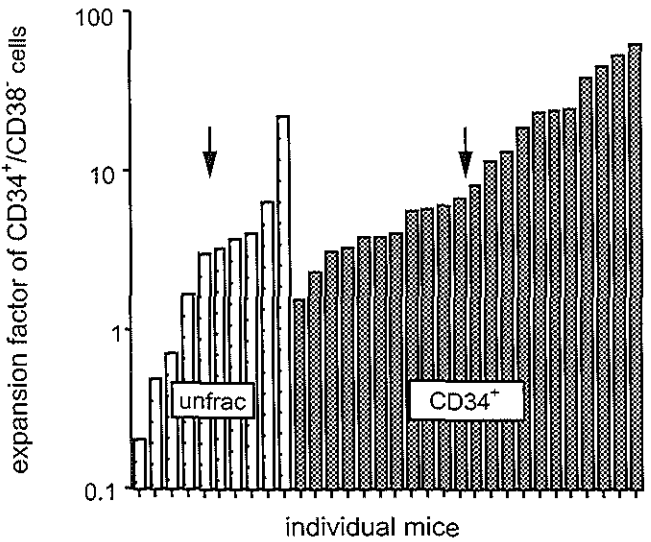

Figure 5.7 Expansion of $CD34^+/CD38^-$ cells after transplantation of unfractionated or $CD34^+$ cells. Data for 32 mice which showed detectable $CD34^+/CD38^-$ cells from a group of 69 chimeric mice (>1% $CD45^+$ cells). Twenty-eight mice were transplanted with unfractionated (unfract) cells and 41 mice with $CD34^-$ grafts from 5 different UCB samples. The arrow shows the median expansion factor of $CD34^-/CD38^-$ cells in each group.

Table 5.2 Frequency analysis of repopulating cells.

Graft*	$CD34^+$ cell no. transplanted	Mice positive for $CD34^+/CD38^-$ (n)	Mice negative for $CD34^+/CD38^-$ (n)	% negative
$CD34^+$	1,660	0	2	100
	8,300	0	2	100
	16,600	1	1	50
	80,000	10	12	55
	104,097	8	2	20
	166,000	3	0	0
Total	3,200	0	2	100
	9,600	0	3	100
	32,000	0	4	100
	60,000	2	3	60
	70,000	3	3	50
	111,000	2	1	33
	198,900	3	2	40

* Only chimeric SCID mice (>1% $CD45^+$ cells by flowcytometry) were included in the analysis. n= 5 UCB, 69 mice transplanted.

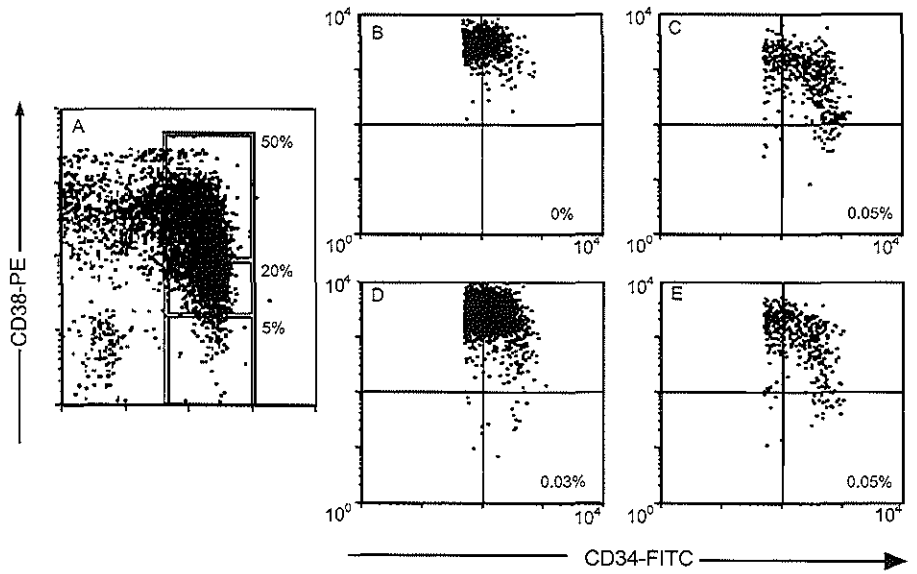

Figure 5.8 Distribution of human CD34 and CD38 in chimeric mouse BM after transplantation of $CD34^-$ subsets, sorted as defined by the windows in (A). (B) through (E) provide the results 35 days after transplantation of 5 $x10^3$ $CD34^+/CD38^-$ cells (B), 5 $x10^3$ $CD34^+/CD38^-$ + 25 $x10^3$ $CD34^+/CD38^+$ cells (C), 10^5 $CD34^-$ cells (D) or 25 $x10^3$ $CD34^+/CD38^{+/-}$ (E). One thousand to tenthousand events were collected in a window containing $CD34^+$ cells only. Quadrants were set to indicate $CD34^+/CD38^+$ and $CD34^-/CD38^-$ cells. The percentages indicate the frequency of human $CD34^+/CD38^-$ cells in mouse BM. $CD34^-/CD38^+$ cells did not engraft (data not shown). The dissociation of outgrowth of $CD45^+$ cells and maintenance of expansion of $CD34^+/CD38^-$ cells is also in this experiment indicated by the CD45 percentages, i.e., 25.1% for (B) without $CD34^+/CD38^-$ cells and 5.5% for (C), 46.7% for (D) and 2,9% for (E) with similar frequencies of $CD34^+/CD38^-$ cells.

DISCUSSION

Engraftment of UCB in SCID mice preconditioned by 3.5 Gy TBI and injection of CL_2MDP liposomes was more prominent than in SCID mice conditioned with TBI alone and similar to that observed in NOD/SCID mice. The macrophage-depleted SCID mice supported the multilineage outgrowth of unfractionated UCB, purified $CD34^+$ cells and the immature subset of $CD34^+/CD38^-$ UCB cells, with production of B-lymphocytes, monocytes, granulocytes,

erythroid cells, NK cells and platelets as well as production of $CD34^+$ cells, including phenotypically immature $CD34^+/CD38^-$ cells. Small numbers of purified $CD34^+/CD38^-$ cells also engrafted efficiently with chimerism levels similar to those observed in accessory cell and/or GF supported NOD/SCID mice (13), whereas $CD34^+/CD38^-$ cells did not engraft, indicating the SCID repopulating potential resides exclusively in the $CD34^+/CD38^-$ subset.

The detection of $CD34^+/CD38^+$ cells in SCID mouse BM is consistent with the finding that $CD34^+/CD38^-$ cells recovered from the BM of engrafted SCID and NOD/SCID mice have retained the capacity to produce clonogenic progeny in long-term culture and to differentiate into myeloid and lymphoid cells in single cell/well cultures (34, 35). Results showing that human cells from mouse-human chimeric NOD/SCID BM may engraft secondary recipients, also suggest that repopulating stem cells are maintained in the BM of immunodeficient mice (36). Taken together, these data demonstrate that immature $CD34^+/CD38^-$ UCB cells can survive and expand in transplanted immunodeficient mice.

The level of expansion of the immature $CD34^+/CD38^-$ subset in chimeric mouse bone marrow, but not the multilineage production of more differentiated progeny, appeared to be dependent on accessory cells. This is most clearly demonstrated by the second order (two-hit) kinetics of the relation between graft size and the numbers of immature $CD34^+/CD38^-$ cells produced in the SCID mouse BM in contrast to the directly proportional relation between graft size and the numbers of mature $CD45^+$ cells and the $CD34^+$ population as a whole (Figure 5.6). Additional data show that engraftment levels and types of human cells produced in the bone marrow of mice transplanted with 5 $x10^3$ $CD34^+/CD38^-$ cell were similar to those obtained with 20-fold more $CD34^+$ cells or 2,000-fold larger numbers of unfractionated mononucleated UCB cells, which also demonstrated that accessory cells or exogenous GFs are not needed for multilineage outgrowth of immature human cells in immunodeficient mice. In contrast, the observation that SCID mice transplanted with $CD34^+/CD38^-$ grafts produced 10-fold fewer $CD34^+$ cells and no detectable $CD34^+/CD38^-$ cells, despite equal numbers of $CD45^+$ cells, than mice transplanted with unfractionated or $CD34^+$ grafts with equivalent numbers of $CD34^+/CD38^-$ cells (Table 5.1), provides additional evidence for an involvement of accessory cells in the maintenance and expansion of immature UCB cells in the SCID mouse microenvironment. Because mice transplanted with unfractionated mononucleated UCB cells did not show larger numbers of $CD34^+$ cells (Table 5.1) or more extensive expansion of $CD34^+/CD38^-$ cells (Figure 5.7) than mice transplanted with purified $CD34^+$ cells, we postulate

that the accessory cells needed for the support of immature UCB cells are present in the $CD34^+$ population. Formal proof was obtained by injection of $CD34^+/CD38^-$ cells supplemented with $CD34^+/CD38^+$ cells (Figure 5.8). Whereas transplantation of $CD34^+/CD38^-$ cells alone did not result in the maintenance of these cells, the addition of 50% $CD34^+/CD38^+$, a fraction that by itself did not result in substantial chimerism, restored the propagation of $CD34^+/CD38^-$ cells in engrafted mice (Figure 5.8).

One possible function of the accessory cells UCB cells might be to prevent elimination of the small numbers of $CD34^+/CD38^-$ cells by residual immune-reactivity in the SCID mouse by providing an excess of human cells. However, because small numbers of $CD34^+/CD38^-$ cells produced equal numbers of mature progeny in the macrophage-depleted SCID mice than much larger unfractionated or $CD34^+$ grafts (Table 5.1), it is unlikely that such a mechanism plays a prominent role in promoting $CD34^+/CD38^-$ cell engraftment. It is more likely that accessory cells provide essential GFs or other stimuli needed for the self-renewal of immature human cells which are not provided by the mouse microenvironment. $CD34^+$ UCB cells and their immediate progeny have been demonstrated to produce various GF, including IL-3, G-CSF and GM-CSF, which stimulate in vitro colony formation by UCB in an autocrine or paracrine fashion (37). A role of accessory cell derived GF in the maintenance of immature cells is also suggested by the supportive role of a cocktail of Epo, SF, IL-3 and GM-CSF for expansion of human cells in NOD/SCID mice transplanted with high numbers of unfractionated human BM cells, which was only observed late after transplantation, when the number of human cells were reduced (12). Further studies are required to examine to what extent optimal combinations of these or other GF's can replace accessory cells in maintaining and expanding $CD34^+/CD38^-$ cells in immunodeficient mice.

Estimation, by Poisson statistics, of the frequency of original UCB cells that can maintain or expand $CD34^+/CD38^-$ cell numbers during the five weeks engraftment period yielded a value of 1 in 70,000 $CD34^+$ cells (corresponding to 1 in 3,500 $CD34^+/CD38^-$ cells). This value is lower, but in the same order of magnitude than the 1 in 600 SCID repopulating $CD34^+/CD38^-$ cells which has been calculated on the basis of the frequency of transplanted NOD/SCID mice with detectable numbers of human cells in the BM as assessed by Southern blots (38). The difference is most likely due to the criteria chosen in that the ability to expand $CD34^+/CD38^-$ cells is a more stringent parameter for engraftment of immature cells than the production of mature progeny at a level of as low as 0.05% human cells detected by DNA

blotting analysis (38). Such low engraftment levels can in principle be derived from contaminating mature cells, as demonstrated in our study by the low, but detectable (>0.5% of mouse BM), engraftment with mature T-cells in some mice transplanted with purified $CD34^-$ cells. The ability to maintain or expand $CD34^+/CD38^-$ cell numbers in SCID mouse BM is probably more characteristic for repopulating stem cells than production of mature progeny per se, because it may reflect an essential hemopoietic stem cells feature, i.e., the ability to maintain its own numbers in vivo.

The differences in repopulating cell frequencies might also be due to the co-transplantation of accessory cells and/or administration of GF in the NOD/SCID mouse model which may have promoted human cell engraftment (38). Although it is clear that $CD34^+/CD38^-$ cells still represent a heterogeneous cell population with only a minority of cells capable of hemopoietic reconstitution, all frequency estimates of the SCID mouse repopulating human cells likely underestimate the frequency of human repopulating cells and should be treated with caution. In particular, the seeding efficiency of these cells has not been assessed yet, while the efficacy of the growth stimuli provided by the xenogeneic environment, accessory cells in the transplant or exogenous GF administration might very well be suboptimal. Studies into the kinetics of human BM cell engraftment in immunodeficient mice have shown that the number of immature, $CD34^+/Thy\text{-}1^+$ cells that can be detected in the mouse BM two days after transplantation is at least two logs lower than input numbers, suggesting that only a very small fraction of the immature human cells develop in these mice (12).

The present study provides evidence for differential regulation of the expansion as opposed to multilineage outgrowth of immature human hemopoietic stem cells in transplanted SCID mice. The possibility to distinguish experimentally between these essential functions in the SCID mouse transplantation assay now opens an experimental approach to examine the effects of various GF's, cell subsets and other agonists on the self-renewal of human immature stem cell subsets. This information will be essential to design and test conditions for ex vivo activation and expansion of immature hemopoietic cells and for various experimental purposes, such as required for the development of efficient gene transfer protocols into hemopoietic cells with retention of repopulating ability.

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CHAPTER 6

HIGHLY EFFICIENT TRANSDUCTION OF THE GREEN FLUORESCENT PROTEIN GENE IN HUMAN UMBILICAL CORD BLOOD STEM CELLS CAPABLE OF COBBLESTONE AREA FORMATION IN LONG-TERM CULTURES AND MULTILINEAGE ENGRAFTMENT OF IMMUNODEFICIENT MICE

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ABSTRACT

Purified $CD34^+$ and $CD34^+/CD38^-$ human umbilical cord blood (UCB) cells were transduced with the recombinant variant of Moloney murine leukemia virus (MoMLV) MFG-EGFP or with SF-EGFP in which enhanced green fluorescent protein (EGFP) expression is driven by a hybrid promoter of the spleen focus-forming virus (SFFV) and the murine embryonic stem cell virus (MESV). Infectious MFG-EGFP virus was produced by an amphotropic virus producer cell line (GP+*env*Am12). SF-EGFP was produced in the PG13 cell line pseudotyped for the gibbon ape leukemia virus (GaLV) envelope proteins. Using a 2-day growth factor prestimulation, followed by a 2-day, fibronectin fragment CH-296 supported, transduction, $CD34^+$ and $CD34^+/CD38^-$ UCB subsets were efficiently transduced using either vector. The use of the SF-EGFP/PG13 retroviral packaging cell combination consistently resulted in 2-fold higher levels of EGFP-expressing cells than the MFG-EGFP/Am12 combination. Transplantation of 10^5 input equivalent transduced $CD34^+$ or 5 $x10^3$ input equivalent $CD34^+/CD38^-$ UCB cells in nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice resulted in median engraftment percentages of 8% and 5%, respectively, which showed that the in vivo repopulating ability of the cells had been retained. In addition, mice engrafted after transplantation of transduced $CD34^+$ cells using the MFG-EGFP/Am12 or the SF-EGFP/PG13 combination expressed EGFP with median values of 2% and 23% of human $CD45^+$ cells, respectively, which demonstrated that the SCID repopulating cells were successfully transduced. $EGFP^+$ cells were found in all human hemopoietic lineages produced in NOD/SCID mice including human progenitors with in vitro clonogenic ability. EGFP expressing cells were also detected in the human cobblestone area forming cell (CAFC) assay at 2-6 weeks of culture on the murine stromal cell line FBMD-1. During the transduction procedure the absolute numbers of CAFC week (wk.) 6 increased 5- to 10-fold. The transduction efficiency of this progenitor cell subset was similar to the fraction of $EGFP^+$ human cells in the bone marrow (BM) of the NOD/SCID mice transplanted with MFG-EGFP/Am12 or SF-EGFP/PG13 transduced $CD34^+$ cells, i.e. 6% and 27%, respectively. The study thus demonstrates that purified $CD34^+$ and highly purified $CD34^+/CD38^-$ UCB cells can be transduced efficiently with preservation of repopulating ability. The SF-EGFP/PG13 vector/packaging cell combination was much more effective in transducing repopulating cells than the MFG-EGFP/Am12 combination.

INTRODUCTION

Efficient procedures for gene transfer into human immature hemopoietic cells with repopulating capacities following transplantation may in principle open new avenues for the treatment of a variety of hereditary and acquired diseases. Retroviral mediated gene transfer to such cells, which is attractive by its simplicity and efficiency, has, however met with considerable difficulty, which is only partly understood (1, 2). The availability of a rapid selectable marker, such as the green fluorescent protein (GFP), is thought to be of pivotal importance to study major variables influencing the efficiency of gene transfer, as well as to track the progeny of transduced cells following transplantation. In the present study we evaluated the use of the enhanced (E) recombinant variant of GFP to label immature human umbilical cord blood cells, using outgrowth in NOD/SCID mice (1, 3, 4) as well as CAFCs (5, 6) as assays for immature cells with considerable hemopoietic reconstitution capacity.

The CAFC assay and the long-term culture-initiating cell (LTC-IC) assay allow for frequency analysis of cells capable of long-term repopulation in vitro (5, 7). Murine studies have shown that the CAFC scored at week 2 are related to CFU-S day 12, while CAFC wk.5 strongly correlate with long-term repopulating cells in vivo (6, 8). In human hemopoiesis the rare population with the primitive phenotype of $CD34^+/CD38^-$ is highly enriched for CAFC wk.6. The primitive nature of CAFC wk.6 is further illustrated by enrichment following incubation with 5-Fluorouracil (5-FU), a cytotoxic drug for proliferating cells. The CAFC wk.2 however, are absent in the $CD34^+/CD38^-$ population and more than 1 log reduced after 5-FU treatment. Based on these results the CAFC wk.6 have been proposed to be representative for cells with long-term repopulating ability in vivo in the human situation (9). On this basis, this assay is considered suitable to assess the effect of manipulation of human hemopoietic progenitor cell populations, such as by gene transfer protocols (10, 11).

The efficiency of gene transfer to stem cells is limited by the inability of most retroviral vectors to integrate DNA into the cellular genome of quiescent cells (12-15). Stimulation of stem cell cycling with hemopoietic growth factors (HGF) such as IL-3, IL-6, stem cell factor (SCF) or Flt3-L (16) prior to and during virus exposure would seem to be essential to promote transduction (17, 18) but may result in loss of repopulating ability of transduced cells as a result of differentiation (16, 19). In addition, colocalization of target cells and virus on dishes

coated with the recombinant fibronectin-fragment CH-296 has been shown to further increase gene transfer efficiency (20, 21).

For transduction of human hemopoietic cells murine retroviruses based on the MoMLV are most commonly used. However, expression of functional receptors for the MoMLV envelope protein is presumably low and pseudotyping the vector with the GaLV envelope protein resulted in higher transduction efficiencies in hemopoietic progenitor cells (22-24), which has been attributed to a higher expression of functional pseudotyped GaLV receptor (Pit-1) by the immature hemopoietic cells (22, 24) than the amphotropic retroviral receptor (Pit-2) (23, 25-28). A study in which $CD34^-$ cells were transduced by the GaLV-pseudotyped retroviral vector revealed that $CD34^+$ cells were efficiently transduced (21-33% transduction) as determined by culture in a colony-forming cell assay (2). It is not known to what extent the relative transduction inefficiency of the MoMLV type viruses is caused by a low Pit-2 expression on immature stem cells or by inefficient activation and provirus integration in quiescent cells. Transplantation of $CD34^+$ or $CD34^+/CD38^-$ transduced cells in immunodeficient beige/nude/xid (bnx) mice showed that 8 of 10 mice transplanted with $CD34^+$ transduced cells contained the retrovirally transduced bacterial neomycin phosphotransferase resistance (neo) gene whereas only 2 of 14 mice that had received $CD34^+/CD38^-$ cells contained low levels of transduced cells (2). The ability to engraft the BM of NOD/SCID mice and provide multilineage outgrowth, which resides exclusively in the $CD34^+/CD38^-$ population (3), has been described as unsuccessful, in contrast to the LTC-IC or CAFC wk.6 which were transduced with efficiencies ranging between 10 to 70% (1). These differences led to the suggestion that NOD/SCID repopulating cells are distinct from the LTC-IC or CAFC wk.6 (1). However, recent data obtained with vectors that contained the neo-gene show that transplantation of retrovirally transduced $CD34^+$ UCB cells in NOD/SCID mice result in transduced human hemopoiesis in the NOD/SCID BM with transduction levels similar to those obtained for LTC-IC (29).

Use of the GFP gene from the jellyfish *Aequorea victoria* as a retrovirally transduced marker allows rapid identification of transduced cells by fluorescence microscopy, flowcytometry or culture in real time without additional staining steps in contrast to other genetic markers such as the neo-gene (30-32) and the bacterial ß-galactosidase gene (LacZ) (33-36). As wtGFP produces a weak (but stable) green fluorescence signal, several GFP variants, such as EGFP, have been created which are better suited for detection of expression

by fluorescence microscopy and flowcytometry (37, 38). Studies with murine cells have shown that cells with the ability of in vivo reconstitution can be transduced with EGFP (39). High expression levels of EGFP could be detected in mouse BM, peripheral blood, spleen and thymus for a current observation period of 6 months after transplantation and were retained in secondary recipient mice, indicating that long-term repopulating stem cells can be successfully transduced [Wognum, A.W., et al., in press]. Human cell lines and purified $CD34^+$ cells were also transduced using EGFP containing vectors (28). Therefore, retroviral vectors containing EGFP genes can be used to transduce a variety of cells which can then be easily detected in vitro as well as in vivo.

To initiate an analysis directed at optimal vectors and transduction procedures, the MFG-EGFP retroviral vector produced by an amphotropic packaging cell line and the SF-EGFP vector pseudotyped for the GaLV envelope protein were used to transduce immature cell subsets in human UCB. The potential of these vector/packaging cell combinations for transduction of purified $CD34^+$ and $CD34^+/CD38^-$ UCB subsets were compared by assessing the ability of transduced cells to produce EGFP-positive cobblestone areas in the CAFC assay and to contribute to multilineage human hemopoiesis in NOD/SCID mice.

MATERIALS AND METHODS

Human umbilical cord blood (UCB) cells

UCB samples were obtained from placentas of full-term normal pregnancies after informed consent in conformity with legal regulations in The Netherlands. Mononucleated cells were isolated by Ficoll density gradient centrifugation (1.077 g/cm^2, Nycomed Pharma AS, Oslo, Norway), and were cryopreserved in 10% dimethylsulphoxide, 20% heat-inactivated fetal calf serum (FCS) and 70% Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) at -196°C before use, as described (40). After thawing by stepwise dilution in HBSS containing 2% FCS, the cells were washed with HBSS containing 1% FCS and used for gene transduction experiments.

Viral vectors and packaging cell lines

The amphotropic retroviral producer cell line, MFG-EGFP, was obtained by a 20 hour incubation of GP+*env*Am12 under standard culture conditions with supernatants containing ecotropic retrovirus from the GP+E-86/MFG-EGFP cell line and hexadimethrine bromide at 4 μg/ml (Sigma, St. Louis, MO, USA) as described (38). The pseudotyped retroviral producer cell line PG13/EGFP7 was developed by transducing the PG13 packaging cell line (kindly provided by D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA, USA) with 0.45 μm filtered supernatant from PA317/EGFP cell cultures (28). EGFP expression was analyzed by flowcytometry and bright single cells were sorted on 96-well plates by using an EPICS Elite ESP flowcytometer coupled to an autoclone device (both from Coulter, Miami, FL, USA). Single clones were cultured as previously described (28). The sorted clones were additionally selected for high virus titer. The viral titer of both the amphotropic and the pseudotyped producer cell line was in the order of 10^6 infectious particles per ml as determined by supernatant titration on cultured murine NIH 3T3 cells and human HeLa cells, respectively. Absence of replication-competent virus was verified by failure to transfer GFP-expression from a transduced cell population to a secondary population. Additionally, for the SF-EGFP/PG13 vector/packaging cell combination pseudotransduction was tested on HeLa cells and found absent.

Subset purification

Purification of $CD34^+$ cells was performed by positive selection using Variomacs Immunomagnetic Separation System as described (41) (CLB, Amsterdam, the Netherlands). The percentage of $CD34^+$ cells in the unseparated population (low density UCB) and in the purified $CD34^+$ and $CD34^-$ fractions was determined by FACS-analysis. For isolation of $CD34^+/CD38^-$ subsets, purified $CD34^+$ cells were stained with fluorescein isothiocyanate (FITC) and R-phycoerythrin (PE) conjugated antibodies against human CD34 and CD38 (CD34-FITC, CD38-PE, Becton Dickinson) for 30', on ice in HBN (HBBS with 2% (wt/vol) BSA (Sigma), 0.05% (wt/vol) sodium-azide (Merck, Darmstadt, Germany) and 2% (vol/vol) normal human serum (NHS). After incubation, the cells were washed twice, resuspended in HBSS and $CD34^+/CD38^-$ cells, the window set at 5% of the $CD34^+$ population with the lowest CD38 expression levels (Figure 6.1) were sorted using a FACS Vantage flowcytometer (Becton Dickinson, San Jose, CA, USA).

Retroviral transduction of UCB subsets

Supernatants containing recombinant retrovirus were generated by culturing approximately 80% confluent producer cells for 12 hours in culture medium consisting of a serum-free enriched version of Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD) (3, 39, 42). Media for all cultures routinely included 100 U/ml of penicillin and 100 µg/ml of streptomycin. The cultures were maintained at 37°C with 10% CO_2 (measured every 15' with read-outs between 9.5% and 10%) in a humidified atmosphere. The culture supernatant was subsequently procured and passed through a 0.45-µm filter. To enhance the transfection efficiency, Falcon 1008 (35 mm) bacteriological culture dishes were coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) at a concentration of 10 µg/cm^2 as described previously (21). UCB subsets (CD34$^+$ or CD34$^+$/CD38$^-$) were prestimulated for 2 days in either medium consisting of enriched Dulbecco's medium (Gibco, Gaithersburg, MD), or CellGro®SCGM (Boehringer Ingelheim, Heidelberg, Germany). Different combinations of human recombinant HGF were added to the culture medium; IL-3 (20 ng/ml; Gist-brocades NV, Delft, The Netherlands), IL-6 (100 ng/ml; Ares-Serono SA, Genève, Switzerland, thrombopoietin (TPO; 10 ng/ml, kindly provided by Genentech, South San Francisco, CA, USA), stem cell factor (SCF; 100 ng/ml and Flt3-L (50 ng/ml, the latter two kindly provided by Amgen, Thousand Oaks, CA, USA). The HGF combination FLT3-L, TPO, IL-6 and SCF was used during the transduction procedure; in some initial experiments, as indicated in the legend of the figures and tables, the IL-3, IL-6, SCF combination was used. Before adding purified cord blood subsets to the fibronectin-coated dishes, the CH-296 fibronectin fragment was preincubated with supernatant containing the amphotropic MFG-EGFP or the pseudotyped SP-EGFP vector for 1 hour at 37°C (20, 21). Subsequently, nucleated cells were resuspended in the vector-containing supernatant supplemented with hemopoietic growth factors, and added to the dishes. Over a period of 2 days, culture supernatant was once replaced completely by resuspending non-adherent cells into fresh retrovirus supernatant and HGF. Finally the cells were harvested and used for FACS analysis, human granulocyte-macrophage colony-forming unit (CFU-GM) and burst-forming unit-erythroid (BFU-E) assays, CAFC assay, and transplantation into NOD/SCID mice.

Flowcytometry

Cell samples were analyzed using a FACSCalibur flowcytometer (Becton Dickinson) as previously described (38, 39). Immunophenotyping of EGFP transduced cells was performed by staining with Peridinin chlorophyll protein (PercP) labeled anti-CD45 and cyanin-5-conjugated anti-CD34 (Cy5; Amersham, Buckinghamshire, UK) or PE conjugated monoclonal antibodies against CD38, CD2, CD4, CD8, CD19, CD20, CD56, CD33 (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Mice were considered engrafted if the percentage $CD45^+$ cells exceeded 1%.

Transplantation of transduced UCB subsets in immunodeficient mice

Specific pathogen-free (SPF) NOD/LtSz-scid/scid (NOD/SCID) mice, 6 to 9 weeks of age, were bred and housed under SPF conditions in a laminar air flow unit and supplied with sterile food and acidified drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) ad libitum. Housing, care and all animal experimentation were done in conformity with legal regulations in The Netherlands, which include approval by a local ethical committee. All mice received total body irradiation (TBI) at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 µl HBSS containing 0.1% BSA and injected intravenous into a lateral tail vein. Transplanted cell numbers were 10^5 $CD34^+$ cells and 5 $x10^3$ $CD34^+/CD38^-$ cells. 35 Days after transplantation the mice were killed by CO_2 inhalation followed by cervical dislocation, both femurs isolated and BM cell suspensions prepared by flushing. After counting, the cells were cultured in colony assays and analyzed by flowcytometry to determine the percentage of human $EGFP^+$ cells in the mouse BM.

In vitro colony assay

Purified UCB cells, EGFP transduced cells and chimeric mouse BM samples were assayed for the presence of human CFU-GM and BFU-E by in vitro colony formation in viscous methylcellulose culture medium as previously described (3, 42-44). The number of colonies was determined after 14 days of culture in a humidified atmosphere of 10% CO_2 at 37°C. $EGFP^+$ colonies were scored under excitation by ultraviolet light.

Stromal feeders and cobblestone area forming cell (CAFC) assay

The contact inhibited FBMD-1 murine stromal cell line was used as described before (5). After seven to ten days of culture at 33°C and 10% CO_2 the stromal layers had reached confluence and were overlaid with nontransduced or transduced $CD34^+$ or $CD34^+/CD38^-$ UCB cells within the subsequent week. Confluent stromal layers of FBMD-1 cells in flat-bottom 96-wells plates were overlaid with UCB cells in a limiting dilution setup. Input values of the $CD34^+/CD38^-$ population and the $CD34^-$ were 25 nucleated cells and 500 nucleated cells per well in the first dilution, respectively. Twelve two-fold serial dilutions were used for each sample with 15 replicate wells per dilution. The cells were cultured at 33°C and 10% CO_2 for six weeks with weekly half-medium changes. The percentage of wells with at least one phase-dark hemopoietic clone of at least five cells (i.e., a cobblestone area) beneath the stromal layer was determined weekly with an inverted microscope. Green fluorescent cobblestone areas were screened in the same way but with an UV-light excitation source. Frequencies of total and green-fluorescent CAFC were calculated by using Poisson statistics as described previously (6). During the period of culture no transfer of the EGFP gene to the stromal layer has been observed.

Statistical analysis

Data are expressed as median (range). Statistical comparisons were performed according to Mann Whitney U-test. P values of <0.05, two tailed, were considered significant.

RESULTS

Transduction efficiencies in purified cells with MFG-EGFP and SF-EGFP vectors

Purified $CD34^+$ and $CD34^+/CD38^-$ UCB cells (Figure 6.1) were prestimulated for two days and subsequently transduced with either MFG-EGFP/Am12 or SF-EGFP/PG13 vector/packaging cell combination, during two days of exposure to virus-containing supernatants in fibronectin fragment-coated bacterial dishes. Transduction efficiencies obtained by infection using the amphotropic MFG-EGFP producer cell line were compared to those obtained with the pseudotyped SF-EGFP cell line. The percentage $EGFP^+$ cells was assessed by flowcytometry (Figure 6.2). The percentage $EGFP^+$ cells of the purified $CD34^+$ population transduced with

the SF-EGFP/PG13 vector/packaging cell combination (median 75% EGFP$^+$) was more than 2-fold higher as compared to MFG-EGFP/Am12 transduced CD34$^+$ cells (median 30%) (Table 6.1). Sorted CD34$^+$/CD38$^-$ cells were also transduced at a higher frequency using the SF-EGFP/PG13 combination (62%) than after transduction with the MFG-EGFP/Am12 combination (19%). On average, transduction frequencies were lower in the purified CD34$^+$/CD38$^-$ cells than in the CD34$^+$ cell fraction, but only for the MFG-EGFP/Am12 transduced cells the difference was statistically significant. The level of transduction of the CD34$^+$/CD38$^-$ subset within the purified CD34$^+$ population obtained with the SF-EGFP/PG13 vector/packaging cell combination was more than 2.5-fold higher than with the MFG-EGFP/Am12 combination. The differences in transduction efficiency between the two vector/packaging cell combinations in these cell populations were significant ($p<0.025$).

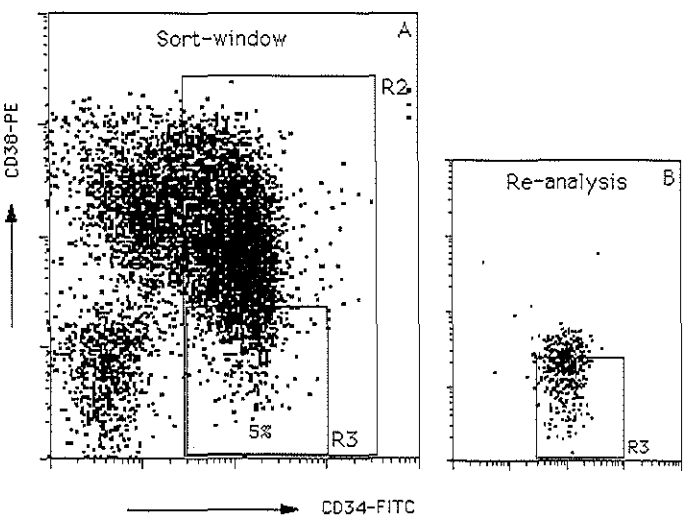

Figure 6.1 Flowcytometric profile used to define and sort the CD34$^+$/CD38$^-$ cell population (A). The window R3 was used to define CD34$^+$/CD38$^-$ cells for sorting and contains 5% of the CD34$^+$ population (as defined by window R2) with the lowest CD38 antigen expression. Re-analysis of the sorted cells is shown in (B).

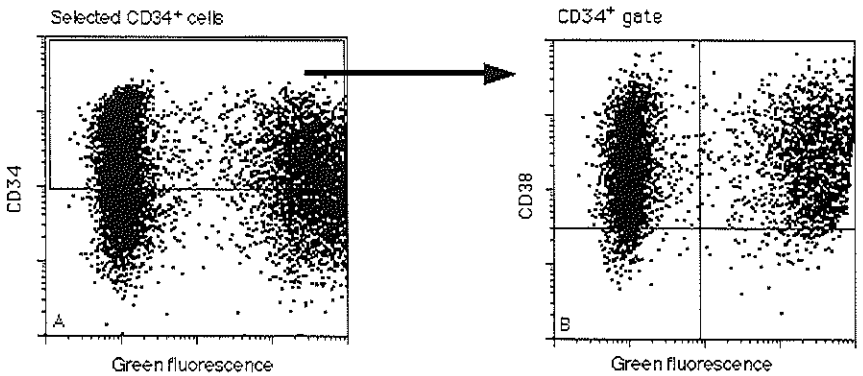

Figure 6.2 Flowcytometric analysis of a representative transfection of purified $CD34^+$ cells with the amphotropic MFG-EGFP retroviral vector after 2 days prestimulation and 2 days supernatant infection in the presence of IL-3, IL-6 and SCF. This particular transduction resulted in efficiencies of 30% within the $CD34^+$ population (A). In (B) $CD34^+$ cells were gated and the CD38 distribution of the EGFP transduced cells was studied. Also $CD34^+/CD38^-$ cells expressed the EGFP gene with efficiencies similar to the total $CD34^+$ population (30% $EGFP^+$).

Table 6.1 EGFP expression of UCB subsets.

vector/ packaging cell line	Purified $CD34^+$ cells	p-value*	$CD34^-/CD38^-$ population within purified $CD34^+$	p-value^	purified $CD34^+/CD38^-$ cells	p-value#
MFG-EGFP/Am12	30 (8-51) (n=13)	p>.05	25 (15-55) (n=9)	p>.05	19 (8-21) (n=4)	0.02
SF-EGFP/PG13	75 (53-84) (n=7)	p>.05	66 (58-81) (n=5)	p>.05	62 (21-71) (n=4)	0.12
p-value$^\Psi$	0.0001		0.003		0.02	

Results are expressed as percentages of $EGFP^+$ cells and depicted as median (range).
The Mann-Whitney U-test has been used for statistical analysis.

* Comparison of the median of purified CD 34^+ cells and $CD34^+/CD38^-$ subset within the purified $CD34^+$ population.

^ Comparison of the median of $CD34^+/CD38^-$ subset within the purified $CD34^+$ population and purified $CD34^+CD38^-$ cells.

\# Comparison of the median of purified CD 34^+ cells and purified $CD34^-/CD38^-$ cells.

Ψ Comparison of MFG-EGFP and SF-EGFP transduced cells.

Transduction efficiency of CAFC subsets

The ability of transduced cells to form cobblestone areas was evaluated in long-term culture supported by FBMD-1 stromal cells. $EGFP^+$ cobblestone areas were identified by fluorescence microscopy (Figure 6.3). The absolute numbers of CAFC at different culture periods increased as a result of the transduction procedure without significant differences between the target cells or vector used (Table 6.2). The absolute number of CAFC wk.2 in the MFG-EGFP/Am12 transduced $CD34^+$ UCB cells increased 5-fold, for the SF-EGFP/PG13 transduced $CD34^+$ UCB cells the increase was 7-fold. The CAFC wk.6 expanded 10-fold and 5-fold, respectively. For the $CD34^+/CD38^-$ UCB cells, similar results were obtained, 6-fold and 10-fold of CAFC wk.6 after MFG-EGFP/Am12 and SF-EGFP/PG13 transduction, respectively. Consistent with the immaturity of the $CD34^+/CD38^-$ cell population, CAFC wk.2 could not be detected in the $CD34^+/CD38^-$ cell fraction prior to transduction. These data show that the transduction protocol that has been used causes a modest expansion of both CAFC wk.2 and wk.6.

The transduction efficiency of the CAFC wk.2 in MFG-EGFP/Am12 transduced $CD34^+$ cells ranged between 23 and 30% with a median value of 26% and in SF-EGFP/PG13 transduced $CD34^+$ cells the median value was 60% (46-74%) (Table 6.2). The transduction efficiency of the CAFC wk.6 in MFG-EGFP/Am12 transduced $CD34^+$ cells ranged between 0% and 11% with a median of 6% $EFGP^+$ cobblestone areas. CAFC wk.6 in SF-EGFP/PG13 transduced $CD34^+$ cells showed as high as 27% transduction. CAFC wk.6 in SF-EGFP/PG13 transduced $CD34^+/CD38^-$ cells showed a similar level of 25% transduction efficiency. Notably, highly purified $CD34^+/CD38^-$ cells transduced with the amphotropic cell line did not produce $EGFP^+$ cobblestone areas wk.6. These experiments clearly demonstrated the superiority of SF-EGFP/PG13 over MFG-EGFP/Am12 in transducing late appearing CAFC, in concordance with the results obtained in phenotypically identified immature $CD34^+$ subsets.

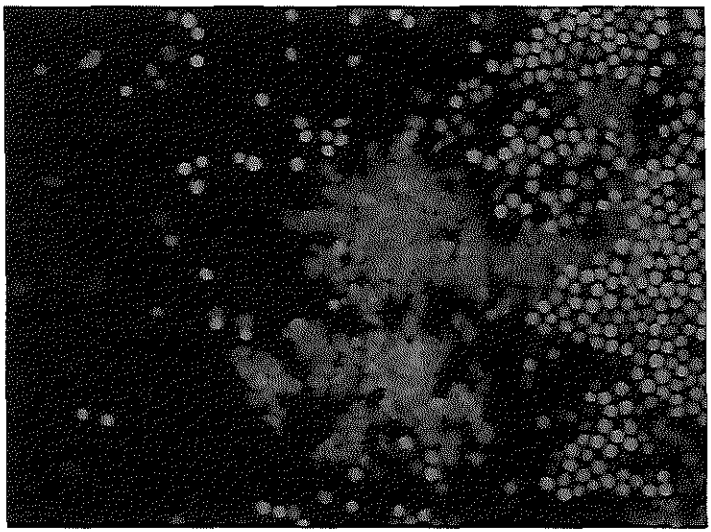

Figure 6.3 Fluorescence microscopic image of a representative EGFP$^+$ cobblestone area. The bright green cells are the mature cells on top of the stromal layer and the dim green cells represent the EGFP$^+$ cobblestone area.

Table 6.2 Absolute numbers of CAFC week 2 and week 6 and percentages of green fluorescent cobblestone areas after transduction of 10^6 selected UCB CD34$^+$ cells or 35 x10^3 CD34$^+$/CD38$^-$ cells with the vectors MFG-EGFP or SF-EGFP.

	CAFC wk2				CAFC wk6			
	CD34$^+$	%	CD34$^+$/CD38$^-$	%	CD34$^+$	%	CD34$^+$/CD38$^-$	%
Before transduction	42 x10^3	-	N.D.	-	4 x10^3	-	0.3 x10^3	-
MFG-EGFP/Am12	218 x10^3*	26	2 x$10^{3\#}$	15	41 x10^3*	6	2 x$10^{3\#}$	N.D.
SF-EGFP/PG13	315 x10^3*	60	2 x$10^{3\#}$	24	22 x10^3*	27	3 x$10^{3\#}$	25

%: percentage of green fluorescent cobblestone areas expressed as median.
N.D. not detectable; * n=2; # n=1

Repopulation of transduced subsets in NOD/SCID mice

In parallel with analysis of cobblestone formation the ability of transduced cells to reconstitute hemopoiesis in vivo was examined by transplantation of the equivalent of 10^5 noncultured $CD34^+$ cells into sublethally irradiated NOD/SCID mice. After 35 days the level of chimerism and the percentage of $EGFP^+$ cells in mouse bone marrow were determined by flowcytometry (Table 6.3). Similar levels of engraftment were found in mice transplanted with noncultured or cultured $CD34^+$ cells. Following transplantation of noncultured $CD34^+$ cells human cells were detected in all mice (n=11) (median: 54% (6-64%) $CD45^+$ cells). $EGFP^+$ cells were found in 6 of 10 repopulated chimeric mice transplanted with MFG-EGFP/Am12-transduced $CD34^+$ cells with a median percentage of $EGFP^+$ cells of 2% (Table 6.3). $CD34^+$ cells transduced using the SF-EGFP/PG13 vector produced higher levels of $EGFP^+$ cells (median: 23%) in the human population in all 4 mice transplanted. These data demonstrated that the repopulating cells in the $CD34^+$ population can be transduced effectively and produce $EGFP^+$ progeny in transplanted NOD/SCID mice. In addition, SF-EGFP/PG13 was much more efficient in transducing the repopulating cells than MFG-EGFP/Am12.

Transplantation of noncultured $CD34^+/CD38^-$ cells and transduced $CD34^+/CD38^-$ resulted in chimerism levels of median 10% (6-29%) for the noncultured cells and 8% (3-12%) and 6% (4-9%) for the MFG-EGFP/Am12 or SF-EGFP/PG13 transduced cells, respectively. In contrast to the results with purified $CD34^+$ cells, $CD34^+/CD38^-$ cells transduced with MFG-EGFP/Am12 were not able to repopulate mouse BM with EGFP-expressing cells, although all 4 mice engrafted with human cells (Table 6.2); this parallels the absence of EGFP expressing CAFC wk.6 in $CD34^+/CD38^-$ cells transduced with MFG-EGFP/Am12. Only 1 of 3 mice engrafted with SF-EGFP/PG13 transduced $CD34^+/CD38^-$ cells. $EGFP^+$ could only be detected in 3% of the $CD45^-$ cells produced. This is in contrast to the results with the $CD34^+$ cells in that apparently most repopulating cells in the highly purified $CD34^+/CD38^-$ subset were not transduced efficiently or the transduced cells displayed a significant reduction in their engraftment potential compared to the cells which were not transduced during the procedure. Nevertheless, SF-EGFP/PG13 was also in these experiments apparently more efficient than MFG-EGFP/Am12.

Table 6.3 Repopulation of EGFP transduced UCB subsets in NOD/SCID mice and CAFC assay.

vector/packaging cell line	UCB subset	transduction efficiency	EGFP$^+$/ chimeric mice*	chimerism in NOD/SCID	EGFP$^+$ on CD45$^+$ cells	CAFC wk.6
		% EGFP		% CD45	%	% EGFP
MFG-EGFP/Am12	CD34$^+$	$^\phi$31 (29-51)	6/10	12 (2-65)	2 (0-18)	6 (0-11)
SF-EGFP/PG13	CD34$^+$	$^\phi$66	4/4	8 (3-12)	23 (2-41)	27 (26-27)
p-value		-	-	p>.05	0.032	0.12
MFG-EGFP/Am12	CD34$^+$/ CD38$^-$	$^\phi$5	0/4	5 (1-24)	0	N.D.
SF-EGFP/PG13	CD34$^+$/ CD38$^-$	$^\phi$21	1/3	6 (4-9)	3	$^\phi$25
p-value		-	-	p>.05	p>.05	-

Results are depicted as median (range) of 2 or 3 experiments.
For statistical analysis the Mann-Whitney U-test has been used.
N.D. not detectable
$^\phi$ insufficient data to perform statistical analysis.
* all transplanted mice engrafted with >1% CD45$^+$ cells.

Multilineage outgrowth of EGFP transduced CD34$^+$ cells

The composition of the EGFP$^+$ human cell population in 2 mice was assessed by flowcytometry using a panel of lineage specific markers (Figure 6.4). EGFP$^+$ cells of the myeloid lineage (CD33; 31-39%, CD11b$^+$; 19.7-25%, CD4; 30-45%), T-lymphoid (CD2; 20-22%), B-lymphoid (CD20; 16-23%), and NK cells (CD56; 1%) were found in mice transplanted with EGFP transduced CD34$^+$ cells. Also immature EGFP$^+$/CD34$^+$ cells were present in the mouse BM (1.1-6.8%) (Figure 6.5). Transduced cells and chimeric mice BM were also cultured in standard methylcellulose medium under conditions which selectively favor the outgrowth of human monomyeloid and erythroid progenitors and fail to stimulate mouse progenitors. In both the graft and the chimeric mice BM, EGFP$^+$ CFU-GM (15/39 in the graft and 3/23 in the mouse BM) and BFU-E (23/40 in the graft and 5/25 in the mouse BM) were identified by flowcytometry of isolated colonies or fluorescence microscopy of whole cultures.

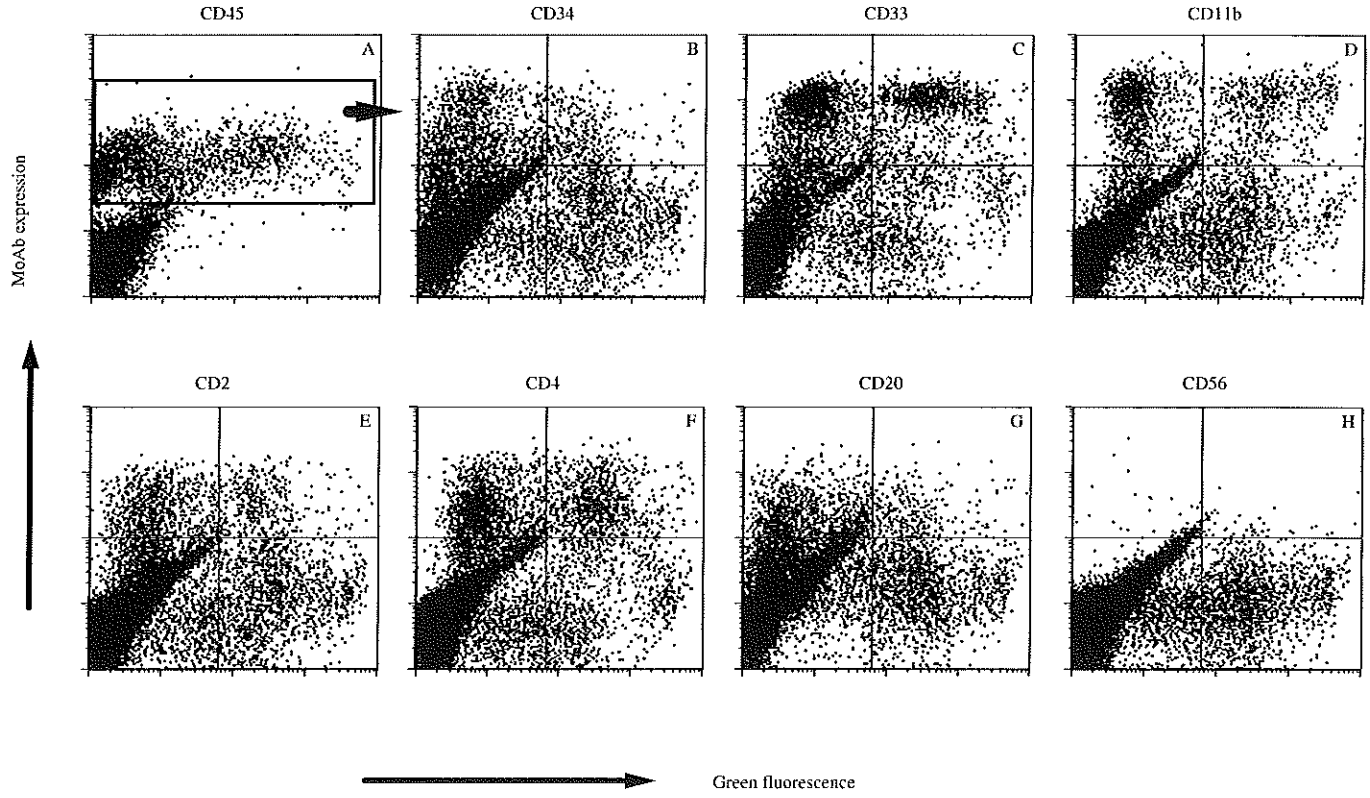

Figure 6.4 Representative immunophenotyping of chimeric NOD/SCID mouse BM 35 days after transplantation of transduced with IL-3, IL-6 and SCF stimulated $CD34^+$ UCB cells. BM (>10% $CD45^+$) was stained with a panel of antibodies specific against different human blood cell lineages and CD45 as a marker for human cells. Figure A shows the bright green autofluorescence on the X-axes vs CD45. The window represents all human, $CD45^+$ cells. The other dotplots shown, are gated cells in this $CD45^+$ window representing only human cells. Representative examples are shown for EGFP vs CD34 (B), EGFP vs CD33 (C) EGFP vs CD11b, (D), EGFP vs CD2 (E), EGFP vs CD4 (F), EGFP vs CD20 (G) and EGFP vs CD56 (H).

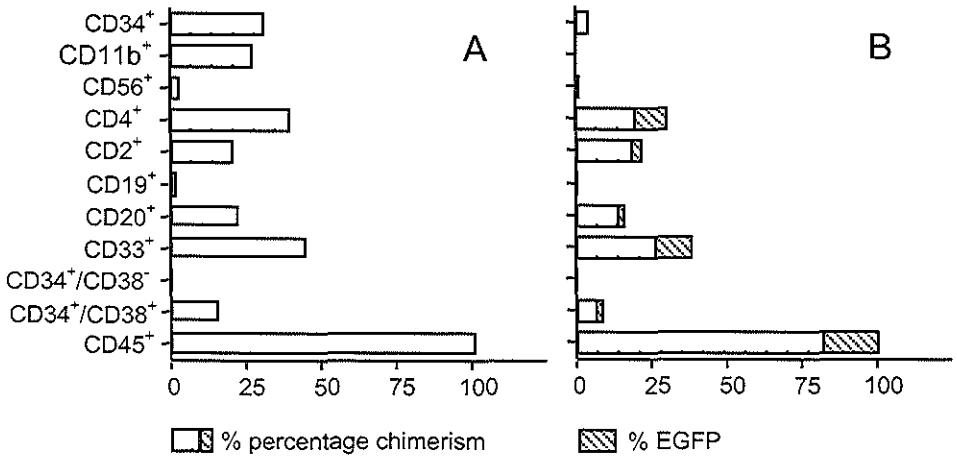

Figure 6.5 Representative chimerism and EGFP expression levels in chimeric NOD/SCID mouse BM 35 days after transplantation of transduced $CD34^+$ UCB cells, relative to the numbers of human ($CD45^+$) cells found.

DISCUSSION

The versatile use of EGFP as a selectable marker of retroviral-mediated gene transfer in hemopoietic cells provides a basis to further optimize retroviral gene transfer to human repopulating stem cells and to evaluate the role of hemopoietic growth factors in activation and expansion of immature hemopoietic cells. This study focused on the development of optimal conditions for gene transfer to human $CD34^+$ and $CD34^+/CD38^-$ UCB cells with the ability to reconstitute hemopoiesis in NOD/SCID mice and produce cobblestone areas for prolonged periods in stroma-supported long-term cultures.

Comparison of transduction frequencies of immunophenotypically characterized immature cells and those of SCID repopulating cells and CAFC may both demonstrate the relationship of these cell types as well as point to essential differences. In general, there was concordance between these assays, in that the GaLV-pseudotyped retroviral vector (SF-EGFP) transduction was much more efficient than the amphotropic retroviral vector (MFG-EGFP) transduction. Also, transduction frequencies of the immature $CD34^+/CD38^-$ subset within the $CD34^+$ population related well to those obtained following transplantation of NOD/SCID mice

and CAFC wk.6. In addition, the study revealed that repopulating cells in the highly purified $CD34^+/CD38^-$ cells were resistant to transduction in the absence of the $CD38^+$ subset, particularly notable for MFG-EGFP/Am12 as demonstrated by the finding that the EGFP transduced $CD34^+/CD38^-$ subset in general failed to produce $EGFP^+$ progeny in NOD/SCID mice. One mouse transplanted with SF-EGFP/PG13 transduced sorted $CD34^+/CD38^-$ cells displayed 3% human $EGFP^+$ cells, one order of magnitude less than the frequency of $EGFP^+$ CAFC wk.6 in the same sample.

The more prominent transduction efficiency of the green fluorescent protein gene into purified and highly purified immature UCB cells to the GaLV-pseudotyped SF-EGFP when compared with the MFG-EGFP/Am12 retroviral packaging cell combination is consistent with earlier studies where transduction of human hemopoietic progenitors was more efficient with a retroviral vector that uses the GaLV receptor (22, 23, 25, 26). The lower transduction percentage obtained with the amphotropic vector may thus be primarily attributed to the low or absent expression of the amphotropic envelope-receptor on the target cells (45, 46). This was particularly corroborated by the absence of EGFP expression in MFG-EGFP/Am12 transduced sorted $CD34^+/CD38^-$ cells, both in the week 6 CAFC assay and following transplantation into NOD/SCID mice. Alternatively, UCB cells may be more efficiently transduced by the SF-EGFP/PG13 vector/packaging cell combination due to the use of the SFFV/MESV hybrid promoter which has been designed to overcome transcriptional inefficiency and silencing associated with retroviral gene transfer into myeloid progenitors and hemopoietic stem cells (47). Other variables that obviously need to be further analyzed include differences in titer and the ability and efficiency of the vectors to transduce EGFP in hemopoietic cells. The titers of the two vectors used were comparable, but tested in different assays. The colocalization of vector and cells during transduction, using the CH-296 fibronectin fragment (21), makes it unlikely that differences in titer have heavily influenced the results. This is the more so since preparative experiments (not shown) with the MFG-EGFP/Am12 retroviral vector revealed that additional charges of the virus supernatant in the transduction protocol did not result in higher transduction frequencies, which indicated that the transduction system is sufficiently saturated with virus. Also Hanenberg et al (48) concluded that the amount of retroviral particles present in the supernatant was not a limiting factor for transduction of $CD34^+$ BM cells on CH-296-coated plates. The higher efficiency of the SF-EGFP/PG13 combination when

compared to the MFG-EGFP/Am12 combination should therefore not be considered as being due to supernatant virus titer differences.

The observation that repopulating cells in the $CD34^+$ population can be transduced efficiently and produce transduced multilineage progeny in transplanted NOD/SCID mice, whereas repopulating cells in the highly purified $CD34^+/CD38^-$ subset are either not transduced effectively or do not develop in vivo is of considerable interest for elucidation of mechanisms involved in successful transduction of immature hemopoietic cells. The transduction efficiency of the $CD34^+/CD38^-$ tended to be lower than that of the $CD34^+$ cells (2), and was significantly so for the MFG-EGFP/Am12 combination, which may be related to the low or absent expression of the amphotropic receptor. Since repopulating cells are exclusively present in the small $CD34^+/CD38^-$ population, and $CD34^+/CD38^+$ cells do not effectively engraft, the low levels of gene expression in the chimeric NOD/SCID BM after grafting transduced $CD34^+/CD38^-$ cells may indicate that the growth factors used during prestimulation and virus infection were not sufficiently effective for activation and stable virus integration of the NOD/SCID repopulating cells. The much higher frequency of EGFP expressing cells in the BM of NOD/SCID mice after transplantation of transduced stem cells from the less pure $CD34^+$ fraction may indicate that stimuli provided by accessory $CD34^+$ cells were responsible for the more efficient transduction of repopulating $CD34^+/CD38^-$ within the $CD34^+$ cell fraction. Alternatively, these accessory cells may be needed to maintain the repopulating ability of stem cells during the transduction procedure of 4 days, e.g. by preventing differentiation, or to promote the expansion and outgrowth of transduced stem cells after transplantation. We speculate that these accessory cells may be related to the accessory $CD34^+/CD38^+$ cells which are involved in the maintenance and expansion of $CD34^+/CD38^-$ cells in immunodeficient mice transplanted with nontransduced human UCB subsets (3). Further identification of these accessory $CD34^+$ cells and elucidation of the active principle may therefore be both relevant for stem cell expansion physiology and for the design of successful gene transfer strategies for immature hemopoietic cells.

The absolute numbers of CAFC produced after week 2 and week 6 of culture show a modest increase after transduction with the MFG-EGFP or SF-EGFP vectors. The frequency of $EGFP^+$CAFC wk.6 in SF-EGFP or the MFG-EGFP transduced $CD34^+$ UCB cells was similar to levels of $EGFP^+/CD45^+$ cells found in NOD/SCID mice. The reason for the 10-fold discrepancy between the levels of transduction of the CAFC wk.6 and the very low numbers of

$EGFP^+/CD45^+$ in NOD/SCID BM after transplantation of the SF-EGFP/PG13 transduced $CD34^+/CD38^-$ population is not clear. Studies with the murine ADA vector similarly yielded very low numbers of gene-marked human cells in the NOD/SCID mouse BM, in contrast to higher numbers of transduced LTC-IC and colony-forming cells, which was interpreted as evidence that the latter cell types are functionally distinct from NOD/SCID repopulating cells (1). However, this distinction might be artificial if effectively transduced $CD34^+/CD38^-$ require the described $CD34^+$ accessory cells for in vivo maintenance and expansion but not for in vitro cobblestone area forming ability.

We conclude that retroviral mediated EGFP transduction in umbilical cord blood cells, in combination with functional assays for repopulating cells, is a rapid tool to study essential gene transfer variables such as vector tropism and transduction conditions. In addition, the use of the GaLV-pseudotyped retroviral vector SF-EGFP resulted in highly efficient gene transfer in both late CAFC and NOD/SCID repopulating cells, the latter presently the most immature subset of human $CD34^+/CD38^-$ cells that can be approached by a functional assay. These results justify the expectation, that the imminent analysis of variables promoting genetic marking of primitive, transplantable hemopoietic cells, such as further optimized transduction conditions and vector constructs, lead to protocols for clinically relevant levels of therapeutic gene transfer.

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CHAPTER 7

SEEDING EFFICIENCY OF PRIMITIVE HUMAN HEMOPOIETIC CELLS IN NOD/SCID MICE: IMPLICATIONS FOR STEM CELL FREQUENCY ASSESSMENT

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ABSTRACT

The nonobese diabetic/severe combined immune deficiency (NOD/SCID) mouse repopulating cells (SRC) have been proposed to represent a more primitive human stem cell subset than the cobblestone area forming cell (CAFC) week (wk.) 6 or the long-term culture-initiating cell (LTC-IC) wk.5 on the basis of their difference in frequency, phenotype, transducability and multilineage outgrowth potential in immunodeficient recipients (5). We have assessed the percentage of various progenitor cell populations (colony-forming cells and CAFC subsets) contained in unsorted NOD/SCID bone marrow (BM) nucleated cells (NC), human umbilical cord (UCB) NC, BM NC, peripheral blood stem cells (PBSC) and $CD34^+$ selected UCB NC, seeding in the BM and spleen of NOD/SCID mice within 24 hours after transplantation. The seeding efficiency of NOD/SCID BM CAFC wk.5 was median [range] in the spleen 2.9 [0.7-4.0] and in the total BM 8.7 [2.0-9.2] per cent. For human unsorted UCB NC, BM NC, PBSC and $CD34^+$ UCB cells, the seeding efficiency for CAFC wk.6 in the BM of NOD/SCID mice was 4.4 [3.5-6.3], 0.8 [0.3-1.7], 5.3 [1.4- 13.6] and 4.4 [3.5-6.3] per cent, respectively. Using flowcytometry the percentage $CD34^+$ UCB cells retrieved from the BM of sub- or supralethally irradiated NOD/SCID mice was 2.3 [1.4-2.8] and 2.5 [1.6-2.7], respectively. Since we did not observe any significant differences in the seeding efficiencies of the various stem cell subsets it may be assumed that the SRC seeding efficiency in NOD/SCID mice is similarly low. Our data indicate that the seeding efficiency of a graft can be of great influence when assessing stem cell frequencies in in vivo repopulation assays.

INTRODUCTION

Over the past decade various in vitro and in vivo surrogate assays have been developed to estimate human stem cell frequencies. The CAFC (1, 2) and LTC-IC (3) assays have been proposed to determine and enumerate primitive progenitor cells with long term repopulating ability in vitro, in both the murine and human hemopoietic system. A quantitative in vivo assay for human hemopoietic stem cells has been established by the recent development of the NOD/SCID mouse model (4). In this model, human (stem) cells are infused into the tail vein of a sublethally irradiated NOD/SCID mouse, with optional intra-peritoneal administration of

cytokines, in order to facilitate outgrowth of the stem cells that homed to the BM. Thirty-five days after transplantation the BM cells of the NOD/SCID mice are collected and the percentage of human chimerism is determined using either flowcytometric analysis of the panleukocyte marker CD45 or southern blot analysis for the human chromosome 17 specific-α-satellite probe (4). Stage and/or lineage specific antibodies are used to determine multilineage outgrowth. On the assumption that every infused SRC will generate detectable human engraftment, limiting dilution techniques have been used to establish the frequency of the SRC in umbilical cord blood (UCB), BM and peripheral blood stem cells (PBSC), i.e. UCB 1:930,000 NC; BM 1:3,000,000 NC; PBSC 1:6,000,000 NC (5). Conneally et al (6) obtained similar results in enumerating the frequency of the Competitive Repopulating Units in UCB. However, from syngeneic murine studies it is known that only 18-20% of the infused stem cells home to the total BM and 8-10% lodge in the spleen (7). Additionally, it is still unknown whether all stem cells that reach the BM actually contribute to hemopoietic reconstitution of depleted marrow spaces. The cells that do not reach their niches in hemopoietic tissues are likely to be sequestered in organs with large capillary beds like the liver and lungs (8). And considering the xenogeneic transplantation setting of grafting human cells to NOD/SCID mice, the seeding efficiency could even be lower than in the syngeneic murine situation.

This implicates that the seeding efficiency of a graft can be of great influence when assessing stem cell frequencies in repopulation assays. In order to estimate the seeding efficiency of human stem cells in the NOD/SCID mouse we have assessed the percentages of CAFC subsets and colony-forming cell (CFC) that home to the BM and spleen of the NOD/SCID mouse within the first 24 hours post-infusion. Additionally, we have studied the seeding efficiency of $CD34^+$ selected UCB cells using in vitro culture assays as well as flowcytometric analysis.

MATERIALS AND METHODS

Human cells

In the described experiments cells from different human grafts were used. The UCB samples were obtained from umbilical cords from full term, healthy newborns. The cord blood cells were 1:1 diluted with Hanks' Balanced Salt Solution (HBSS; Gibco, Breda, The Netherlands)

and processed using either a ficoll-gradient (1.077 g/cm^2; Nycomed, Oslo, Norway) or a 3% gelatine solution (Sigma, St Louis, MO, USA). After harvesting the mononuclear cell fraction or the erythrocyte-depleted fraction, respectively, the cells were washed twice in HBSS. The BM NC were obtained by posterior iliac crest puncture from five hematological healthy adults. The mononuclear cell fraction was isolated from the BM cells using a ficoll-gradient as described above. The PBSC were obtained from five patients with Non-Hodgkin Lymphoma and one patient with Multiple Myeloma. The mononucleated cell fraction was isolated using a ficoll-gradient. These patients were in complete remission. The patients, the allogeneic donors and the mothers of the newborns gave their informed consent. The UCB NC, BM NC and PBSC were cryopreserved until use in 10% dimethyl sulfoxide (DMSO; BDH, Poole, United Kingdom) and 20% heat-inactivated fetal calf serum (FCS; Summit Biotechnology, Fort Collins, CO, USA). The unsorted human stem cells were used for transplantation into 9 Gy irradiated NOD/SCID mice, CAFC and CFC cultures.

NOD/SCID mouse bone marrow (BM) cells for transplantation

Male and female specific pathogen-free NOD/LtSz-scid/scid (NOD/SCID) mice, 3 to 5 weeks of age, were obtained from the department of Immunology at the Erasmus University Rotterdam, The Netherlands. The mice were included in experiments at the age of 6 to 9 weeks. To obtain NOD/SCID BM cells for transplantation, in total 15 NOD/SCID mice were killed using CO_2 asphyxiation and both femora and tibiae were removed. The NOD/SCID BM cells were harvested using crunching of the bones in HBSS with 5% FCS. The cells were collected and sieved through a 100-μm sieve. After centrifugation at 1800 rpm for 8 minutes the cells were resuspended in phosphate-buffered saline (PBS; Gibco). The NOD/SCID BM cells were used for transplantation into 9 Gy irradiated NOD/SCID mice, CAFC and CFC cultures.

Isolation of CD34$^+$ umbilical cord blood (UCB) cells

The mononucleated cell fraction of UCB samples was thawed and using the Macs-selection system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) CD34$^+$ cells were isolated according to manufacturer's instructions. The purity ranged from 72 to 99%. The CD34$^+$ UCB cells were used for transplantation into 3.5 or 9 Gy irradiated NOD/SCID mice and flowcytometric analysis.

NOD/SCID repopulating cell (SRC) frequency analysis in UCB

In order to estimate the frequency of the SRC in UCB, 5-21 samples were pooled and intravenously (i.v.) transplanted into sublethally irradiated NOD/SCID mice using 9 cell concentrations ranging from 1,660 to 225,000 $CD34^+$ UCB cells per mouse. Per cell dose the medium number of mice used was 6 [2-34]. After 35 days, the femora were isolated and BM cells harvested. The percentage of multilineage engraftment was determined using immunophenotypic analysis for each individual mouse. Mice containing 1% or more $CD45^+$ cells were considered positive for human engraftment. During the engraftment period, i.e. 35 days post-transplant, the mice were not supplemented with cytokines. The frequency of the SRC in UCB in our laboratory was determined by using Poisson statistics. Part of the repopulation data used has been published by Verstegen et al (9).

Assessment of the seeding efficiency of the transplanted cells

For determining the seeding efficiency in NOD/SCID mice of all five grafts studied we determined the number of CAFC subsets and CFC per graft. Simultaneously, we irradiated two recipient NOD/SCID mice per sample with 9 Gy by a ^{137}Cs source (Gammacell, Atomic Energy of Canada, Ottawa, Canada). This dose was chosen to maximally reduce the recipient hemopoietic activity, allowing the detection of low numbers of human seeded stem cells. Two to five hours after irradiation the mice were transplanted. In the experiments assessing the seeding of $CD34^+$ UCB cells as determined by flowcytometry, the mice were either 3.5 or 9 Gy irradiated. The number of transplanted cells per mouse varied in case of unsorted UCB between 63 and 111 $x10^6$ NC, for BM between 30 and 48 $x10^6$ NC, for PBSC between 30 and 94 $x10^6$ NC and for NOD/SCID BM between 46 $x10^6$ and 63 $x10^6$ NC. The median percentage of $CD34^+$ cells in the unsorted UCB graft was 1.7, in BM 5.3 and in PBSC 1.5. For the $CD34^+$ grafts the cell numbers varied between 375,000 and 800,000 pure $CD34^+$ NC per mouse. In case of transplanting unsorted UCB, BM, PBSC, NOD/SCID BM and $CD34^+$ selected UCB NC 3, 5, 6, 4 and 7 individual samples have been used, respectively. At 22 to 24 hours after transplantation (10, 11), the mice were killed and femora and spleen were isolated. The BM cells were harvested by crunching the bones as described above. The spleen was carefully cut in parts and sieved through a 100 μm-mesh filter.

The cells were then washed and resuspended in CAFC medium or PBS containing 0.5% BSA and 2% normal human serum (NHS; obtained from healthy volunteers) in case of

preparing the cells for immunophenotyping. In the CAFC assay 1/10 femur or 1/50 spleen were plated in the first dilution. For the CFC assay 1/10 and 1/100 femur and 1/50 and 1/500 spleen were plated. The irradiation control was negative in 6 out of 6 experiments. For determining the seeding efficiency to the total BM we assumed that 1 femur contains approximately 6% of the total BM cellularity (12). The BM and spleen seeding efficiencies were calculated on the basis of the number of infused and retrieved CAFC and CFC. When $CD34^+$ UCB cells were infused and the phenotypic seeding efficiency was determined the number of retrieved $CD34^-$ UCB cells (as determined using Flow Count Fluorospheres (Coulter Immunotech, Miami, Florida, USA)) was divided by the number of $CD34^+$ UCB cells infused corrected for the purity of the population. Seeding efficiency is expressed as percentage.

Immunofluorescence analysis

Cells were stained with anti-CD34-FITC or PE, anti-CD45-FITC (Coulter Immunotech, Mijdrecht, The Netherlands) or anti-CD38-PE (Becton Dickinson, San Jose, CA) or FITC (Coulter Immunotech) by incubating 10^5 to 10^6 NC for 30 minutes on ice. The incubations were performed in PBS containing 0.5% bovine serum albumin (BSA; Sigma) and 2% NHS. After the incubation the cells were washed once in PBS and 0.5% BSA and resuspended in 0.3 ml PBS. Analysis was performed using a FACScan (Becton and Dickinson). Ten to twenty-five thousand events were acquired per sample. In case of using flowcytometry for determining the seeding efficiency of $CD34^+$ selected UCB cells in the BM of the NOD/SCID mouse a minimum of 750,000 events were acquired. 7-aminoactinomycin D (7-AAD; Molecular Probes, Eugene, Oregon, USA) was used to exclude the dead cells.

Hemopoietic growth factors

Purified recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) and murine stem cell factor (SCF) were kindly provided by Genetics Institute, Cambridge, MA, USA. Human granulocyte-colony stimulating factor (G-CSF) and human interleukin-3 (IL-3) were gifts from Amgen, Thousand Oaks, CA, USA and Gist Brocades, Delft, The Netherlands, respectively.

Human colony-forming cell assay

Quantification of the number of colony-forming units-granulocyte macrophage (CFU-GM) and burst forming units-erythroid (BFU-E) was performed using a semi-solid (1.2% methylcellulose; Methocel, Stade, Germany) culture medium (Iscove's modified Dulbecco's medium (IMDM); Gibco) at 37°C and 5% CO_2. The cultures contained 30% FCS supplemented with penicillin (100 U/ml; Gibco), streptomycin (100 µg/ml; Gibco), ß-mercapto-ethanol (ßme; 5 x10^{-5} M; Merck, Darmstadt, Germany), erythropoietin (1 U/ml; Boehringher, Mannheim, Germany), IL-3 (15 ng/ml), G-CSF (50 ng/ml), GM-CSF (5 ng/ml) and murine SCF (100 ng/ml) all at final concentrations. CFU-GM and BFU-E consisting of more than 50 cells were counted on day 14 of culture in the same dish.

Murine colony-forming cell assay

The number of colony-forming cells present in the murine BM and spleen cells harvested was determined by plating the cells in semi-solid cultures consisting of 1.2% (wt/vol) methylcellulose (Methocel) in IMDM. The medium was supplemented with 10% (vol/vol) pokeweed mitogen-stimulated mouse spleen-conditioned medium and 20% Horse Serum (HS; Gibco). The cultures were kept at 37°C and 10% CO_2. Colonies consisting of 50 cells and more were counted at day 7 and day 14 of culture.

Stromal feeders

The FBMD-1 murine stromal cell line was used as described earlier (2). In short, stromal feeders were prepared by seeding 10^3 FBMD-1 cells per well into flat-bottom 96-wells plates (Falcon, Lincoln Park, NJ, USA) from log-phase cultures. Culture plastics destined for establishment of FBMD-1 stromal feeders were incubated overnight at 4°C with 0.3% gelatin in demineralized water to improve adherence of the stromal layer. The FBMD-1 cells were cultured in FBMD-1 medium consisting of IMDM with glutamax-1 (Gibco) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), ßme (10^{-4} M), 10% FCS, 5% HS (Integro, Zaandam, The Netherlands) and hydrocortisone 21-hemisuccinate (10^{-5} M; Sigma). After seven to ten days of culture at 33°C and 10% CO_2 the stromal layers had reached confluence and used for the CAFC assay.

Cobblestone area forming cell (CAFC) assay

Confluent stromal layers of FBMD-1 cells in flat-bottom 96-wells plates were overlaid with NOD/SCID BM NC, UCB NC (unsorted or $CD34^+$ selected), BM NC or PBSC in a limiting dilution setup. For the primary CAFC assays of the NOD/SCID BM NC, UCB NC, BM NC and PBSC the input values ranged from 27,000 to 50,000 NC per well and 250 cells per well in case $CD34^+$ UCB cells. The portion of the harvested BM and spleen of the transplanted NOD/SCID mice used in the first dilution of the CAFC assay is indicated above. Twelve dilutions two-fold apart were used for each sample with 15 replicate wells per dilution. The cells were cultured at 33°C and 10% CO_2 for five weeks in case of mouse cells and six weeks for the human CAFC with weekly half-medium changes. For the murine CAFC assay FBMD-1 medium was used and in case of performing the human CAFC assay the medium consisted of FBMD-1 medium supplemented with IL-3 and G-CSF at final concentrations of 10 ng/ml and 20 ng/ml, respectively.

The percentage of wells with at least one phase-dark hemopoietic clone of at least five cells (i.e. cobblestone area) beneath the stromal layer was determined weekly for the mouse CAFC and every two weeks for the human CAFC. Frequencies of the CAFC subsets were calculated using Poisson statistics as described previously (1).

Data analysis

Microsoft Excel 97 and SPSS for Windows Release 7.5.2. were used for data analysis. Data are expressed as median [range]. Statistical comparisons were performed according to Mann Whitney U-test. The two-sided p-value was determined testing the null hypothesis that the two population medians are equal. P-values of <0.05 were considered significant.

RESULTS

SRC frequency in UCB

The SRC frequency as determined by limiting dilution analysis in our laboratory is approximately one per 6.6 $x10^6$ unsorted UCB NC. The threshold for human engraftment used to distinguish positive from negative mice is 1 per cent $CD45^+$ NC in the BM of the NOD/SCID mouse as assessed by flowcytometry.

Low seeding efficiency of NOD/SCID BM cells in NOD/SCID mouse BM and spleen

In order to determine the seeding efficiency of syngeneic hemopoietic cells in the NOD/SCID mouse strain, NOD/SCID BM NC were isolated from 6 to 9-week old NOD/SCID mice and processed according to the seeding efficiency protocol as described in the materials and methods section. The seeding efficiency to the total BM of the NOD/SCID mouse tended to decrease with increasing immaturity of the progenitor cell subset studied (Figure 7.1). The median [range] seeding efficiency to the total BM for CFC day 7 (d7) and d14 was 19.5 [17.6-24.1] and 22.8 [13.1-28.4], respectively. In contrast, the seeding efficiency of CAFC wk.5 was 8.7 [2.0-9.2]. For the seeding efficiency of the progenitor subsets to the murine spleen we did not observe significant differences. The seeding efficiency of d7 CFC and d14 CFC to the spleen was 1.4 [0.5-2.4] and 1.2 [0.9-2.5], respectively. The percentage of CAFC wk.5 lodging to the spleen was 2.9 [0.7-4.0].

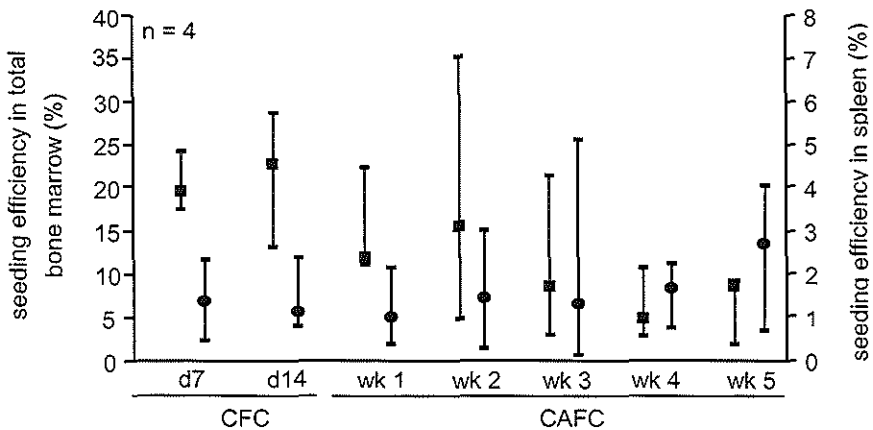

Figure 7.1 Seeding efficiency of unsorted NOD/SCID BM cells in total bone marrow (■) and spleen (●) of NOD/SCID mice. The data represent the median and range of four separate experiments.

Seeding efficiency of unsorted human progenitors in total BM and spleen of NOD/SCID mice

All the progenitor subsets analyzed from unsorted UCB NC showed a lower seeding efficiency to the BM and the spleen than was observed in the NOD/SCID syngeneic setting (Figure 7.2).

The seeding efficiency of d14 CFC and CAFC wk.6 to the total BM of the NOD/SCID mouse was in case of unsorted UCB NC 3.8 [3.8-5.5] and 4.4 [3.5-6.3], in BM NC 0.9 [0.1-1.4] and 0.8 [0.3-1.7] and in PBSC 2.1 [0.1-3.9] and 5.3 [1.4-13.6], respectively. The d14 CFC and CAFC wk.6 seeding efficiency to the spleen was in case of unsorted UCB NC 0.7 [0.6-1.0] and 0.5 [0.4-0.8], for BM NC 0.3 [0.03-0.9] and 0.2 [0.1-0.2] and for PBSC 0.6 [0.1-1.2] and 0.5 [0.1-1.8]. Thus, of all infused progenitors derived from, for example unsorted UCB NC only around 4 of every 100 CAFC infused will home to the total BM and only 5 in every 1000 will lodge to the spleen of the NOD/SCID mouse. The seeding efficiency of the various hemopoietic progenitor cell types from PBSC was comparable to the seeding efficiency of UCB NC, both to total BM and spleen (Figure 7.2). However, PBSC grafts showed larger variability in seeding to BM and spleen as compared to UCB grafts. As with UCB cells, no significant differences could be observed among the subsets assayed.

Also the seeding efficiency of human BM progenitors to the murine BM and the spleen showed no significant differences in seeding efficiency among the various stem cell subsets studied.

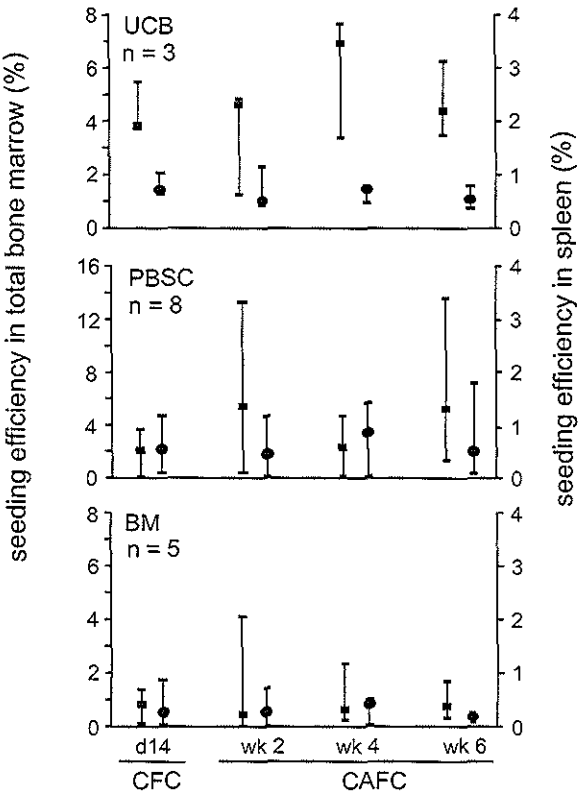

Figure 7.2 Seeding efficiency of human unsorted UCB NC, PBSC and BM NC in total NOD/SCID bone marrow (■) and spleen (●). The data are depicted as median and range. n= number of separate experiments.

Seeding efficiency of CFC and CAFC subsets in CD34$^+$ selected UCB cells

As it may be argued that the seeding efficiency may change in relation to the cell number infused, we have compared the seeding efficiency of CFC and CAFC subsets from CD34$^+$ selected and unsorted UCB cells (Figure 7.3). No significant difference could be observed. As observed with the unsorted grafts, the different progenitor subsets contained in the CD34$^+$ grafts home in comparable percentages to the BM of the NOD/SCID mouse.

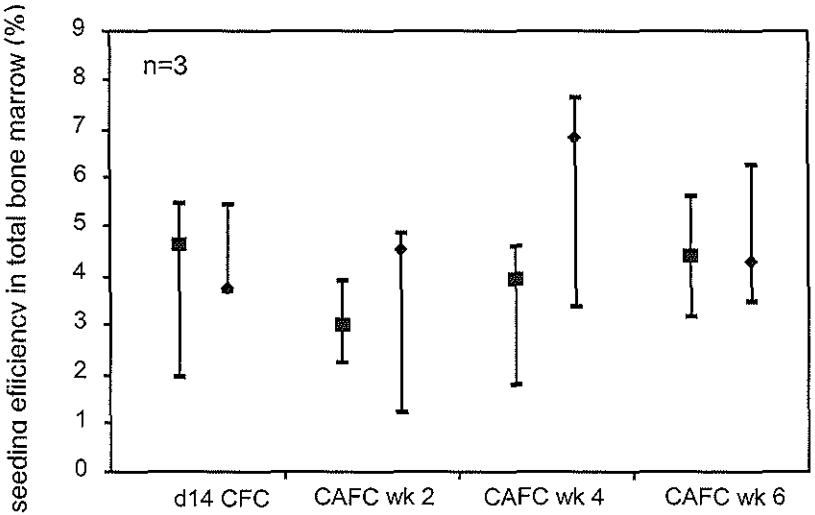

Figure 7.3 Comparison of the seeding efficiency of human $CD34^+$ selected (■) and unsorted (◆) UCB cells in total NOD/SCID bone marrow. The data are depicted as median and range. Three separate experiments for either population were performed.

The effect of 3.5 Gy versus 9 Gy irradiation conditioning on the seeding efficiency of $CD34^+$ UCB cells as determined using flowcytometric analysis

Determination of the repopulating potential of human cells in the NOD/SCID mouse model commonly includes conditioning using a sublethal total body irradiation of 3.5 Gy. In our experiments to determine the seeding efficiency of human grafts in the NOD/SCID mouse we irradiated the mice with a supralethal irradiation of 9 Gy to fully eradicate any measurable constitution by murine progenitors. This dose of irradiation would then assure detection of human progenitor cells exclusively, as we are not able to distinguish murine and human progenitors using the CFC and CAFC assay as used here. In order to assess whether the level of irradiation affected the seeding efficiency of human progenitors in the NOD/SCID mice, we compared the seeding efficiency of $CD34^+$ selected UCB cells as assessed by flowcytometry in 3.5 Gy and 9 Gy irradiated NOD/SCID mice. Figure 7.4 shows the flowcytometric analysis used to determine the number of $CD34^+$ UCB cells retrieved from the NOD/SCID mouse BM

24 hours after transplantation. In dot plot A living, i.e. 7-AAD negative, CD34-PE positive cells are gated and shown in dot plot B. The $CD45^{dim}$ cells possessing large forward light scatter properties were gated, in order to prevent false positive events due to the high autofluorescence of murine BM cells, and shown in a CD45-FITC versus CD34-PE dot plot (C). Dot plot C shows a clear double positive population representing the $CD34^+$ UCB cells that had homed to the NOD/SCID mouse BM.

Figure 7.5 shows that the median seeding efficiency of $CD34^+$ UCB cells in 3.5 Gy irradiated recipients (2.3%) as compared to 9 Gy conditioned NOD/SCID (2.5%) mice as measured by flowcytometry was not significantly different. Furthermore, the seeding efficiency of $CD34^+$ selected UCB cells as determined by the CFC and the CAFC assay is similar to that assessed by flowcytometric analysis except for the CAFC wk.6 ($p=0.04$). However, in $CD34^+$ selected UCB cells the CAFC wk.2 frequency relates to the CAFC wk.6 frequency as 4:1. Therefore, the seeding efficiency of $CD34^+$ UCB cells as determined using flowcytometry will be in the range of the CAFC wk.2 seeding.

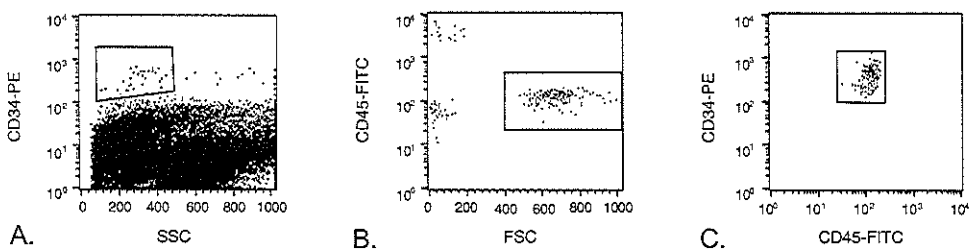

Figure 7.4 Flowcytometric analysis used to determine the number of $CD34^+$ UCB cells retrieved from the NOD/SCID mouse bone marrow 24 hours after transplantation. Dead cells were excluded using 7-AAD. In dot plot A living CD34-PE positive cells are gated and shown in dot plot B. $CD45^{dim}$ cells and possessing large forward light scatter properties are gated and shown in a CD45-FITC versus CD34-PE dot plot (C). Dot plot C clearly shows that all of the gated cells in plot B belong to a clear double positive population representing the $CD34^+$ UCB cells retrieved from the NOD/SCID mouse bone marrow.

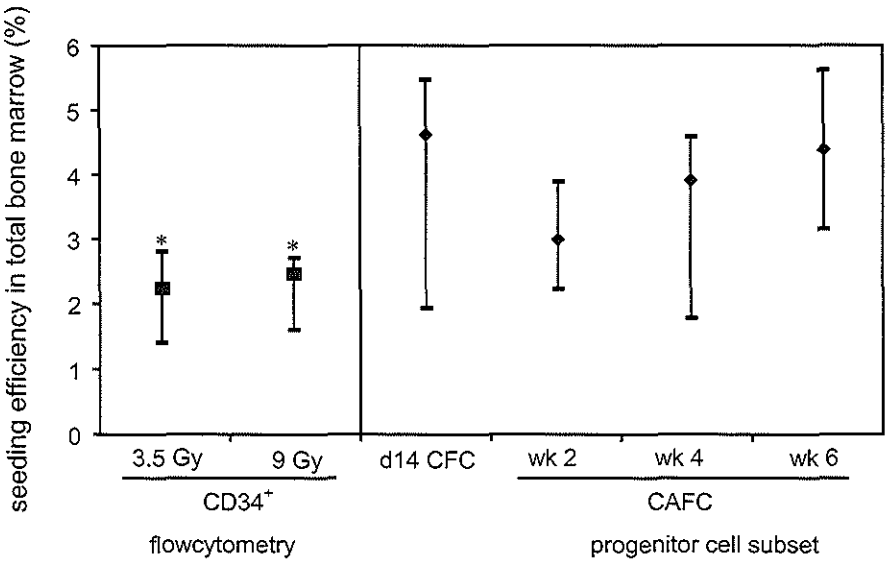

Figure 7.5 Seeding efficiency of human $CD34^+$ UCB cells in total bone marrow of NOD/SCID mice: comparison of flowcytometric analysis with CFC and CAFC data. The data are depicted as median and range of three separate experiments for the CFC and CAFC assays; six experiments for the 3.5 Gy irradiated NOD/SCID mice, and seven experiments for the 9 Gy irradiated NOD/SCID mice.
* = Significantly different from CAFC wk.6.

DISCUSSION

We have demonstrated that the seeding efficiency of human progenitor cells in the BM and spleen of NOD/SCID mice is extremely low. As the different progenitor cell subsets had comparable low seeding rates, it is conceivable by extrapolation that only a few of all infused NOD/SCID repopulating cells will home to the marrow of these mice. Thus, the assumption that every SRC infused will indeed contribute to repopulation should be critically met (5). Rather, our data indicate that the published frequency estimates of human SRC in UCB, BM and PBSC represent a dramatic underestimation. If these frequency estimates were corrected by the respective seeding efficiencies as currently published they would be 18- to 125-fold higher than presently accepted.

We have used 1% $CD45^+$ as a threshold for human engraftment in BM of the NOD/SCID mouse to distinguish positive from negative mice. This threshold is 10- and 20-fold higher than the threshold used by Conneally et al (6) and Wang et al (5), respectively. The lower detection threshold used by Wang et al and Conneally et al may explain why these authors arrived at a higher SRC frequency estimate.

The seeding efficiency of NOD/SCID BM NC to the BM and the spleen of a syngeneic recipient is lower than we have observed in other murine syngeneic transplantation settings (7). Particularly, the seeding efficiency to the spleen is strikingly low. The reason for this discrepancy is as yet unresolved, however, there are several possible explanations for this observation. (A) There could be intrinsic differences of the primitive stem cells between the two mouse strains influencing their homing behavior. (B) The data could indicate that the microenvironment in the NOD/SCID mouse may be less conducive for allowing homing of infused stem cells.

We observed similar seeding efficiencies to the NOD/SCID BM of CFC and CAFC subsets, irrespectively of whether they were contained in $CD34^+$ selected or unsorted UCB cells. These data suggest that $CD34^-$ UCB cells in the unsorted grafts did not effect the homing of the progenitor subsets contained in the $CD34^+$ population.

By using flowcytometry, we were able to compare the seeding efficiency of $CD34^+$ selected UCB cells in 3.5 Gy and 9 Gy irradiated NOD/SCID mice. Furthermore, the seeding efficiency of $CD34^-$ selected UCB cells obtained with immunophenotypic analysis was not significantly different from the data obtained with the CFC and CAFC assays (except for CAFC wk.6). Although the data are highly suggestive, they do not exclude the possibility that $CD34^-$ UCB cells show differences in cloning efficiency in the CFC and CAFC assay after seeding in BM of a 3.5 Gy irradiated mouse as compared to seeding in BM of a 9 Gy irradiated recipient.

Homing of stem and progenitor cells to the BM after i.v. transplantation has been defined as the cells' ability to seek marrow stroma selectively, to subsequently lodge within it and initiate hemopoiesis (13). As investigated in the murine system this process is believed to include two phases. The first phase consists of the recognition of the endothelial cells by the hemopoietic cells possibly through a lectin receptor with galactosyl specificity (14, 15). The second phase is the interaction of galactosyl/mannosyl specific homing receptor to the extra-cellular matrix of the BM (16) and the interaction of the very late antigen-4 (VLA-4) receptor

with vascular-cell-cell adhesion molecule (VCAM) (17-19). Therefore the process of homing seems to be highly specific. In other organs (i.e. liver, lung, kidney) hemopoietic progenitor cells can be temporarily detected after their i.v. transplantation and disappear within 48 hours after transplantation (20). Their sequestration to the latter organs may be determined by other mechanisms, including binding to galactosyl receptors (21).

Also the homing process to the spleen seems to be regulated differently than in the marrow. Recognition of galactosyl/mannosyl residues (16) or the fibronectin receptor (VLA-4) (20) seems not to be involved. The special anatomical structure of the spleen is suggested to be one of the determinants of spleen cell homing.

In our experiments we have injected human cells into sub- and supralethally irradiated NOD/SCID mice. Several publications indicate that the molecules involved in the homing process are highly conserved in evolution (22-24). Therefore, it is likely that the transplanted human cells will home to the BM and spleen of the NOD/SCID mouse in a specific manner.

Our data indicate otherwise. As the total BM of a mouse may be estimated to weigh around 350 mg while a NOD/SCID mouse of 9 weeks old weighs around 18 g, the total BM is around 2% of the total weight of the animal. The weight of the spleen is around 1% of the animal weight. If the homing of the human cells is random, then the seeding efficiency to the total BM will be 2% and to the spleen 1%. However, when we take into account that tissues like fat, skin and brain of the mouse are probably less accessible for progenitor cells, while additionally the capillary beds of the BM and the spleen may be rather extensive, we will expect a slightly higher seeding efficiency than these figures if seeding is a non-specific process. In agreement with the calculated seeding percentages, the median seeding efficiencies of the stem cells in the human grafts studied to the total BM vary between 1 and 7% and to the spleen between 0.2 and 0.9%. These data suggest that human progenitors do not home preferentially to the total BM and spleen of the NOD/SCID mouse but that seeding of human hemopoietic progenitors may predominantly occur on the basis of organ-weight and capillary bed complexity.

The expression of adhesion molecules is different among $CD34^+$ cells derived from UCB and BM as compared to PBSC. PBSC shows lower expression of VLA-4, leukocyte function anigen-1 (LFA-1), inter-cellular adhesion molecule-1 (ICAM-1) and LFA-3 than BM (25, 26). The adhesion molecule expression pattern in UCB NC shows similarities with BM NC, although UCB derived $CD34^+/CD38^-$ cells express higher levels of L-selectin (CD62L)

and LFA-1 (CD11a) than their BM counterparts (27). This would mean that if the homing process of the human cells in the NOD/SCID mouse is specific we might expect comparable seeding efficiencies of UCB and BM NC and a lower seeding of PBSC. In contrast, the seeding efficiencies of UCB NC and PBSC are comparable, while the seeding of BM NC in the BM of the NOD/SCID mouse is very low.

It has been published that expression of the molecules that recognize galactosyl and mannosyl residues changes during differentiation (28). This could cause differences in seeding efficiencies among progenitor and stem cell subsets. However, the fact that we did not observe this, provides us with another strong indication that homing of the human cells in the BM and the spleen of the NOD/SCID mouse is not specific.

Our data suggest that the postulated low frequencies of human long-term repopulating stem cells may largely result from their low seeding efficiency in the BM of NOD/SCID mice. In addition, it should be realized that the proposed SRC frequency in the various human grafts is the resultant of a) seeding efficiency, b) repopulating ability of the transplanted cells, c) exogenous factors facilitating engraftment, d) detection methods, e) detection thresholds and f) definition of a SRC. In the light of this notion the estimated human stem cell frequency could even be higher than the SRC frequency corrected for the seeding efficiency of that particular graft.

It has been recently suggested that the SRC may represent a different and more primitive stem cell subset than are assessed by the LTC-IC and CAFC assay on the basis of a variety of observations (5). Thus, SRC would be more difficult to transduce and occur at far lower frequencies than the LTC-IC or CAFC wk.6. Our recent data on comparable gene expression in CAFC wk.6 and SRC (29) together with our present data, would suggest that the SRC and CAFC wk.6 may differ far less than previously claimed and thus may represent overlapping stem cell populations.

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CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

The increasing ex vivo manipulation of hemopoietic stem cell transplants necessitates the use of in vitro assays able to determine the frequency and the quality of human in vivo repopulating stem cells. Such manipulation includes protocols for gene therapy, ex vivo expansion, dendritic cell augmentation or tumor and T-cell purging. All these conditions may potentially be able to significantly change the number and properties of the most primitive stem cells in the graft at hand and thus may affect the outcome of the transplantation.

The work described in this thesis aims to clarify whether the stroma-supported cobblestone area (CAFC) and flask long-term culture-colony forming cell (LTC-CFC) assays, which were initially developed and validated in the murine hemopoietic system, provide a reliable quantitative and qualitative measure for the human in vivo repopulating stem cells. The CAFC assay allows the assessment of the frequency of progenitor cell subsets in various hemopoietic materials. Additionally, the LTC-CFC assay provides means to assess the quality of the graft by determining the ability of the progenitor cells in the graft to produce progeny.

8.2 How to validate the human stroma-supported CAFC and LTC-CFC assays?

In the dictionary validation is described as 1) legitimate, 2) count, 3) confirmation, 4) approve and 5) equivalent. If this description is applied to the validation of an assay it can be read as a requirement to demonstrate that an assay measures what it is hypothesized to measure. The obvious method to do this is by correlating the results obtained with the assay to be validated with those from an established assay.

How have the murine CAFC and LTC-CFC assays been validated? While studying the cellular hierarchy in the hemopoietic system of the mouse, various transplantation assays were developed. The colony-forming unit in spleen (CFU-S) assay was the first assay (1). This assay detects a heterogeneous population of relatively mature cells, predominantly capable of a limited period of in vivo repopulation. The CFU-S was long considered to be a true hemopoietic stem cell. The development of the marrow repopulating ability (MRA) or pre-CFU-S assay and methods to physically separate bone marrow (BM) cell populations changed this notion. The MRA assay estimates the potential of more primitive cells to populate the BM

of lethally irradiated recipients by measuring their ability to form secondary CFU-S or colony-forming cells (CFC) (2-6). The long-term in vivo repopulation ability (LTRA) assay determines the ability of cells to repopulate the recipient BM for extended periods of time, i.e. longer than 6 months, in primary and secondary recipients (7-11).

The existence of these established in vivo repopulation assays allowed the validation of the murine CAFC and LTC-CFC assay. This was done by selecting murine BM cells on the basis of phenotypic or functional characteristics, e.g. wheat germ agglutinin affinity (12) or Rhodamine-123 retention (13). By subsequently performing the CAFC and LTC-CFC assays in parallel to the murine transplantation assays, good correlations between the in vitro assays and the in vivo models for transient and permanent repopulating ability were established (12-18).

It should be considered that a good correlation between parameters measured in different assays does not necessarily mean that these assays assess the same cell. However, it implicates that the assyas are interchangeable, so that if there is a particular advantage for one assay, e.g. a reduction of the number of experimental animals used, it is legitimate to choose the alternative assay. If subsequently manipulated cells are used, experiments to investigate whether the good correlations are still present need to be undertaken.

To validate the CAFC and LTC-CFC assays for the human hemopoietic system, experiments with a similar setup as the murine setting should be performed. However, transplantation experiments in the mouse can be performed under highly standardized conditions but in humans several uncontrollable parameters need to be considered. First, unlike the inbred mice used in the transplantation experiments, there is a large biological variability between humans. This implies that many patients are needed for correlation studies. Secondly, and also in difference with murine transplantation experiments, healthy individuals are not transplanted. This means that the underlying disease of the patient might affect repopulation characteristics and as a result influence the correlations to be established as well. Thirdly, as humans are not living under specified pathogen-free conditions as most experimental mice, (viral) pathogens might affect the repopulation kinetics in spite of the precautions taken prior to transplantation, like bacterial decontamination or viral (antibody) screening. Finally, transplanting selected cells to lethally or sublethally conditioned human recipients in an experimental setting without cure or clinical improvement as objective is not possible. Therefore, alternative conditions to assess the repopulating ability of human hemopoietic cells

had to be explored. This has resulted in a wide variety of subjects studied with one common goal of validation of the human CAFC and LTC-CFC assays. To this purpose, the CAFC and LTC-CFC subsets have been determined in human mobilized peripheral blood (MPB) and BM grafts, and correlated with clinical parameters reflecting reconstitution of transplanted patients. In addition, data from in vitro assays were compared with those generated in a humanized immunodeficient mouse model (Table 8.1).

Table 8.1 Methods used to validate the human CAFC and the LTC-CFC assays.

Human transplantation setting	Murine transplantation setting
Establishment of the relation between CAFC and LTC-CFC subsets and:	Investigation of two of the proposed differences between the SCID repopulating cell (SRC) and the CAFC week 6:
- the speed of engraftment after autologous BM or MPB transplantation	- frequency
- the probability of failing or delayed engraftment after autologous BM or MPB transplantation	- transducability assessed using retroviral vectors
- the speed of engraftment after transplantation of counterflow centrifugal elutriation (CCE) manipulated allogeneic BM or MPB grafts	
Section 8.3	Section 8.5

8.3 Do the stroma-supported CAFC and LTC-CFC in vitro assays reflect characteristics of the human in vivo repopulating stem cell?

The CAFC and the LTC-CFC assays have been extensively studied in the murine model. It appeared that the frequency of the murine CAFC day 10 and CAFC day 28 correlated

significantly with the frequency of the CFU-S day 12 and the MRA, respectively (15). The frequency of the murine CAFC day 35 correlated extremely well with the number of cells required for 40% donor chimerism at 12 to 15 months post-grafting (12).

To validate the CAFC and LTC-CFC assays for the human hemopoietic system, similar correlations should be established. But as stated before, experimental transplantations are not allowed to be performed on humans. An alternative was found in studying hematological recovery in the first months after hemopoietic stem cell transplantation. During this period, the transplanted cells repopulate the BM of the conditioned recipient and start the production of mature peripheral blood cells. This process is reflected by 1) the contribution of the transplanted cells to hematological recovery of the recipient and 2) the speed of post-transplant hematological recovery.

We have used these parameters to correlate with data from in vitro assays taking into account a number of considerations. a) Repopulation of the BM and subsequent production of differentiated blood cells is a complex process. Various factors, e.g. viral infections, medication, the underlying disease or the graft-versus-host reaction in the allogeneic transplantation setting, can easily affect this process and therefore influence the relation to be established. b) Short-term or long-term endpoints for hematological reconstitution in vivo are defined arbitrarily. And c) regarding the result of these correlation studies, parameter *a* can show a significant correlation with parameter *b* for the group of patients studied but not for the individual patient.

In common clinical practice, graft evaluation is based on determining the number of progenitors transplanted per kg bodyweight (BW) as measured by the number of CFC and $CD34^+$ cells. Various authors have previously reported that the speed of neutrophil recovery after transplantation is directly related to the total number of colony-forming unit-granulocyte macrophage (CFU-GM) transplanted per kg BW after autologous BM transplantation (BMT) (19, 20) and to the total number of CFU-GM and $CD34^+$ cells grafted per kg BW after autologous MPB transplantation (MPBT) (21-29). The total number of nucleated cells (NC) transplanted per kg BW is not indicative for the speed of post-transplant recovery after autologous BMT (30) or MPBT (31).

The data presented in chapter 2 suggest a good correlation between the speed of neutrophil recovery and the number of $CD34^+/CD38^{neg/dim}$ cells and CAFC week (wk.) 3 and 4 grafted per kg BW in the autologous MPB transplantation setting. The number of CAFC wk.1

and 2 transplanted per kg BW showed the best relation with the platelet recovery. An explanation for the latter might be that megakaryocyte precursors show themselves in the CAFC assay around day 11. The number of CAFC wk.6 transplanted per kg BW did not correlate very well with neutrophil or platelet recovery. This suggests that more primitive progenitors do not contribute to early hematological recovery. The data of Ploemacher et al (12) and van der Loo et al (13) on the WGA and Rhodamine 123 sorts on murine BM cells support this hypothesis. However, it is in contrast with the data from Zijlmans et al showing that murine mobilized blood cells with phenotypical and functional primitive characteristics are responsible for early hematological recovery (32).

The use of marked stem cell subsets in the transplants, as has been done in animal studies, (10, 33-35) will reveal which human stem cell subset is predominantly involved in early neutrophil and platelet recovery.

When autologous stem cell transplantation is employed for (hematological) malignancies, the recipient, who is in this transplantation setting also the donor, receives high dose cytotoxic agents for eradication of the diseased cells. Since the 1970-ties it is known that chemotherapeutic agents can cause quantitative and qualitative damage to hemopoietic cells (36-39). Also the bone marrow microenvironment may be damaged leading to a reduced hemopoietic supportive capacity (40-43). In conclusion, the bone marrow and its environment are likely to be greatly affected by chemotherapy, which may have profound effects on hematological recovery in the autologous transplantation setting.

In chapter 2 is shown that repeated administration of chemotherapy indeed can lead to a lower yield and quality of primitive progenitor cells when performing leukapheresis. That the quality of the transplant contributes to hematological recovery is suggested by the high correlation between thespeed of neutrophil recovery and the total transplant quality.

Additional support for this hypothesis is provided by the data described in chapter 3, showing that the total number of LTC-CFC wk.6 transplanted per kg BW is the only discriminative parameter between patients with successful and (partial) unsuccessful hematological recovery after autologous stem cell transplantation. The established graft evaluation parameters, like CD34 and CFC, were not able to retrospectively predict all the patients with (partial) unsuccessful repopulation. In both the autologous BMT and MPBT

setting delayed or absent repopulating activity seems to be caused by a qualitative rather than a quantitative defect of the transplant.

In chapter 4 is shown that in CCE manipulated allogeneic BM and MPB grafts, the repopulating ability co-elutriated with phenotypical and functional mature and immature progenitors. This is illustrated by the low incidence of graft failure in almost 500 transplanted patients (44-47). Furthermore, this study showed that the different CAFC subsets studied could not be physically separated by the CCE procedure.

The data described in chapters 2 through 4 show that the CAFC and the LTC-CFC assays correlate with in vivo reconstitution after transplantation, suggesting they reflect characteristics of the human in vivo repopulating stem cell.

8.4 Which criteria need to be fulfilled by an animal model for human hemopoiesis?

In addition to their use in studying reconstitution after transplantation in the clinical setting, animal models might prove to be helpful in assessing the in vivo repopulating ability of human stem cells. An animal model for human hemopoiesis must allow 1) xenogeneic transplantation and 2) the assessment of stem cell characteristics as measured by a) multilineage outgrowth and b) expansion of the in vivo repopulating stem cell compartment.

Ad 1) and 2a)
In order to cross the xenogeneic transplantation barrier the recipient animal must be severely immunodeficient. A natural and passing period of immunodeficiency is the first trimester of ontogeny. Flake et al (48) and Zanjani et al (49) developed a fetal sheep transplantation assay in which pre-immune fetuses are intra-peritoneally injected with (human) hemopoietic cells. The practical applicability of this model is limited by the high number of unsuccessful transplantations, abortions and low level of human hemopoiesis in the recipient sheep.

The severe combined immunodeficiency (SCID) mouse as well as the nonobese diabetic/SCID (NOD/SCID) mouse models have been successfully used as to study human hemopoiesis. As described in chapter 5, both mouse strains allow transplantation and multilineage outgrowth of human hemopoietic cells. The macrophage-depleted SCID mice supported the multilineage outgrowth of unfractionated, purified $CD34^+$ and the immature

subset of $CD34^+/CD38^-$ umbilical cord blood (UCB) cells, with production of B-lymphocytes, monocytes, granulocytes, erythroid cells, natural killer (NK) cells and platelets as well as $CD34^+$ cells, including phenotypically immature $CD34^+/CD38^-$ cells. However, the level of expansion of the immature $CD34^+/CD38^-$ subset in chimeric mouse BM, but not the multilineage production of more differentiated progeny, appeared to be dependent on $CD34^+/CD38^+$ accessory cells.

Ad 2b)

Serial BM transplantations are essential in determining the expansion ability of long-term in vivo repopulating stem cells. To assess the self-renewal capacity of murine hemopoietic stem cells, BM is transplanted into lethally irradiated mice, and, after engraftment, removed from the reconstituted mice and serially transplanted into another group of lethally irradiated mice. The repopulating ability will not be lost if the interval between the transfers exceeds 6 months (50). This period of time apparently allows slowly proliferating stem cells to regenerate their numbers and reestablish the ratio of repopulating stem cells to committed progenitor cells.

Successful retransplantation of human cells from mouse-human chimeric NOD/SCID BM would indicate survival and expansion of immature human hemopoietic cells. Retransplantation into secondary recipients using high dose (2×10^6) $CD34^+$ selected cells from mouse-human chimeric NOD/SCID mouse BM after $CD34^+$ UCB cell transplantation has, however, not succeeded in our group (Verstegen and van Hennik, unpublished results). Others have reported successful retransplantation of total mouse-human chimeric BM into secondary NOD/SCID recipients (51, 52). An explanation for the discrepancy between their results and ours might be a difference in the setup of the retransplantation experiments. We transplanted a high dosage of $CD34^+$ cells selected from chimeric bone marrow while the other groups transplanted total chimeric bone marrow, containing 2 to 7×10^5 $CD34^+$ NC. This suggests that the human $CD34^-$ cells in the mouse-human chimeric NOD/SCID BM are needed to facilitate the engraftment of the retransplanted cells. Alternatively, the majority of the repopulating ability of the cells in the NOD/SCID BM is no longer confined to the $CD34^+$ population as Sato et al has shown in the syngeneic murine transplantation setting (53).

Recently, Kollet et al (54) reported that only 2 to 5×10^3 $CD34^+$ cells selected from mouse-human chimeric NOD/SCID BM were able to successfully repopulate a secondary

NOD/SCID ß2^{null} recipient. However, the NOD/SCID ß2^{null} mouse has shown to be a better recipient than the NOD/SCID mouse (54).

By using uniquely marked hemopoietic cells in retransplantation experiments the fate of individual cells can be assessed. Data showing that a particular hemopoietic clone can be determined in multiple secondary recipients for extended periods of time after transplanting BM from only one reconstituted primary recipient emphasizes the ability of long-term in vivo repopulating stem cells to actually expand during the course of hematological reconstitution (7, 8).

Genetic modification of human hemopoietic stem cells to cure several hematological and non-hematological diseases has since long been one of the major goals in hemopoietic stem cell research. However, this has met with considerable difficulties. Long-term expression of the transferred gene is most often attempted with retroviral vectors because of the ability of the provirus to stably integrate into the genome of the cell. Integration of the provirus can only take place when the target cells go through cell cycle. However, the target population, the primitive hemopoietic stem cells, is for the most part non-cycling. This can in principle be solved by culturing the cells in the presence of hemopoietic growth factors. However, when primitive cells are stimulated to go through cell cycle this often coincides with loss of stem cell qualities. Therefore, much of the research effort concerning gene therapy has been directed to determining the appropriate conditions for gene transfer into primitive stem cells with preservation of multilineage and long-term repopulation. The CAFC and the NOD/SCID mouse model have shown to be valuable in the evaluation process, as described in chapter 6.

In chapter 6 we have made attempts to transduce primitive progenitors with NOD/SCID BM repopulating ability as well as various cobblestone area forming cell subsets by a 4-day procedure comparing 2 different retroviral vectors. Earlier experiments had learned us that the applied transduction protocol was the most efficient using these vectors.

Comparison of transduction frequencies of immunophenotypically immature cells, e.g. CD34^{+}/CD38^{-}, and those of SCID repopulating cells and CAFC wk.6 may both demonstrate a relationship between these cell types as well as point to essential differences. In general, there was concordance between these assays, in that the gibbon ape leukemia virus (GaLV)-pseudotyped retroviral vector (SF-EGFP) transduction was much more efficient than the amphotropic retroviral vector (MFG-EGFP) transduction. Also, transduction frequencies of the

immature $CD34^+/CD38^-$ subset within the $CD34^+$ population related well to those obtained following transplantation of NOD/SCID mice and CAFC wk.6. In addition, the study revealed that repopulating cells in the highly purified $CD34^+/CD38^-$ cells were resistant to transduction in the absence of the $CD38^+$ subset, particularly notable for MFG-EGFP/Am12, as demonstrated by the finding that the transduced $CD34^+/CD38^-$ subset in general failed to produce transduced progeny in NOD/SCID mice. This may indicate that stimuli provided by accessory $CD34^+$ cells were responsible for the more efficient transduction of repopulating $CD34^+/CD38^-$ within the $CD34^+$ cell fraction. Alternatively, these accessory cells may be needed to maintain the repopulating ability of stem cells during the transduction procedure of 4 days or to promote the expansion and outgrowth of transduced stem cells after transplantation. The accessory $CD34^+/CD38^+$ cells which are involved in the maintenance and expansion of $CD34^+/CD38^-$ cells in immunodeficient mice transplanted with nontransduced human UCB subsets (56) might relate to the alleged accessory cells described here. The reason for the 10-fold discrepancy between the levels of transduction of the CAFC wk.6 and the very low numbers of transduced $CD45^+$ in NOD/SCID BM after transplantation of the SF-EGFP/PG13 transduced $CD34^+/CD38^-$ population may be that effectively transduced $CD34^+/CD38^-$ require the described $CD34^+$ accessory cells for in vivo maintenance and expansion but not for in vitro cobblestone area forming ability.

8.5 Is the SCID repopulating cell (SRC) more primitive than the CAFC week 6?

The SRC has been proposed to be a more immature stem cell than the CAFC wk.6 on the basis of differences in 1) frequency, 2) phenotype, 3) transducability and 4) multilineage outgrowth potential in immunodeficient recipients (Table 8.2) (57). All four tentative differences will be discussed in the light of our recent data in order to establish a more precise characterization of these cells.

Table 8.2 Proposed differences between the SRC and CAFC week 6 (57) in human hemopoietic material.

	SRC	LTC-IC* week 5/ CAFC week 6
Transducability	1 – 2%	10 – 70%
Frequency	1/1 – 6 x10^6 NC	1/4,300 – 10,000 NC
Phenotype[1]	$CD34^+/CD38^-$	$CD34^-/CD38^-$ and $CD34^+/CD38^-$
Mutipotentiality[2]	All lineages	Predominantly myeloid

1 When prolonging the culture time of the long-term culture-initiating cell* (LTC-IC) assay beyond 40 days, only $CD34^+/CD38^-$ cells are able to produce colonies in this extended LTC-IC (ELTC-IC) assay (58, 59).

2 Megakaryocytic, granulocytic, monocytic, macrophage, NK and pre-B cells are produced in the cultures, but T- or erythroid cells are absent. However, when performing switch-cultures (60, 61) or the ML-IC (62) cells present in stroma-supported assays after 5 to 6 weeks of culture are able to give rise to lymphocytic cells.

Regarding the first proposed difference between the SRC and CAFC wk.6, transducability, we have shown that the transduction efficiency of the CAFC wk.6 and the SRC contained in $CD34^+$ population is not different using a highly efficient retroviral vector (chapter 6). Conneally et al (63) showed similar results while others were not able to obtain equivalent results (64).

There are several factors contributing to differences in transduction efficiencies. First, whether close proximity of the target cells and the virus particles can be achieved. This is facilitated by recombinant fibronectin fragments with a virusbinding domain adjacent to a cellular binding domain (65-67). Secondly, the efficiency with which a retrovirus can enter a cell. This is largely dependent on the expression level of the specific virus receptor. More primitive hemopoietic cells may have a higher expression level of receptor type A than type B, while on more mature cells it may be the reverse. So when comparing retroviral vectors the expression level for the respective virus receptors on the cells can influence the transduction efficiency. Thirdly, the efficiency with which a retrovirus can integrate into the cellular genome to guarantee long-term expression of the gene may also affect the efficiency of gene transfer. This is dependent on whether the cells go through cell cycle to allow virus integration

into the cellular DNA. Induction of cell cycle in primitive hemopoietic cells often leads to loss of the stem cell characteristics, meaning reduced self-renewal capacity, multipotentiality and long-term in vivo repopulating ability. Fourthly, differences in promoter activity of the retroviral vector will have its effect on the transduction efficiency. Fifthly, the transferred gene may be silenced in vivo but not in vitro.

In order to study gene transfer efficiency into various cell populations on the basis of intrinsic differences of the cell populations these technical limitations of retroviral gene transfer should be overcome. By developing a retroviral vector with a strong promoter that uses a virus receptor that is abundantly expressed on primitive hemopoietic cells of which integration is not dependent on the cell cycle status of the target population, optimal conditions for gene transfer into hemopoietic cells with in vivo repopulating ability might be provided.

The second proposed difference is addressed by the seeding data presented in chapter 7. On the assumption that every infused SRC will generate detectable human engraftment in the NOD/SCID mouse, limiting dilution techniques have been used to establish the frequency of the SRC in UCB, BM and MPB, i.e. UCB 1:930,000 NC; BM 1:3,000,000 NC; MPB 1:6,000,000 NC (57). Conneally et al (68) obtained similar results in enumerating the frequency of the competitive repopulating units in UCB. In contrast, the CAFC wk.6 frequency in UCB is 1:8,333 NC; in BM 1:4,438 NC and in MPB 1:10,000 NC.

Engraftment of human cells in the BM of NOD/SCID mice is the endpoint used to assess the SRC frequency. However, engraftment is the resultant of a) seeding efficiency, i.e. the percentage of injected cells homing to a particular organ, b) the fraction contributing to hematological reconstitution, c) repopulating ability of the transplanted cells, d) exogenous factors facilitating engraftment, e) detection methods, f) detection thresholds, g) definition of a SRC.

We have demonstrated that the seeding efficiencies of human progenitors to the BM and the spleen of the NOD/SCID mouse are very low. As the studied progenitors, i.e. $CD34^+$ cells, CFC and CAFC subsets, had comparable low seeding rates, it is conceivable by extrapolation that only a few of all infused NOD/SCID repopulating cells will home to the BM of these mice. Thus, the assumption that every SRC infused will indeed contribute to repopulation should be critically met (57). Rather, the published frequency estimates of human SRC in

UCB, BM and MPB represent a dramatic underestimation implying that the SRC and the CAFC wk.6 are not that different in terms of frequency.

The third suggested difference between the SRC and CAFC wk.6 is on the basis of phenotype. SRC activity has been proposed to be exclusively contained in the $CD34^+/CD38^-$ population (56, 57), while the CAFC wk.6 and LTC-IC wk.5 are also found in $CD34^+/CD38^+$ cells. When the culture period is prolonged as in the ELTC-IC only the $CD34^+/CD38^-$ cells are able to produce colonies (58, 59). Data that not only $CD34^+/CD38^-$ cells possess NOD/SCID repopulating ability are provided by Bathia et al, showing that the $CD34^-$ cells have SRC activity as well (69).

To elucidate the relation between SRC and CAFC wk.6 additional sorting on phenotypic parameters in parallel to NOD/SCID transplantation and the CAFC assay would need to be performed. Peled et al (55) has shown that the SRC activity in the $CD34^+$ population is confined to the $CD34^+/CD38^-/CXCR4^+$ population. Perhaps the relation between the SRC and the CAFC wk.6 is further clarified when this cell population is more extensively studied.

However, as stated earlier the engraftment of human cells in the NOD/SCID mouse is a complex process influenced by many factors, while the circumstances in the CAFC assay are controllable to a high extent. This implicates that there might always be differences in the frequencies of both parameters that are not necessarily based on intrinsic differences between the SRC and the CAFC wk.6.

The fourth tentative difference is that the CAFC and LTC-IC predominantly facilitate myeloid outgrowth while after transplanting human cells to the NOD/SCID mouse, human myeloid as well as lymphoid lineages can be determined in the murine BM. However, when using the switch-cultures (60, 61) or the ML-IC assay (62), the hemopoietic cells that show myeloid in vitro repopulation after several weeks of culture, comparable to the duration of the CAFC and LTC-IC assay, are able to differentiate into the lymphoid lineage as well.

In conclusion, the SRC and the CAFC wk.6 cell populations might not be as different as defined earlier on the basis of the postulated differences.

8.6 Are the stroma-supported assays clinically applicable?

On the basis of the presented data it may be concluded that the use of the CAFC and LTC-CFC assays is helpful in the clinical setting. However, both assays are technically demanding and not likely to be performed on a routine basis. Consequently, the effect of specific protocol conditions could very well be studied in advance using these stem cell assays, so that the transplantation team can predict the average outcome of such protocols at the moment of transplant manipulation and act accordingly.

8.7 Final conclusions

The aim of the project was to validate the human stroma-supported long-term CAFC and LTC-CFC assays. To this purpose sorted hemopoietic cell fractions would need to be transplanted to human recipients to study short- and long-term in vivo repopulation. However, this kind of transplantation experiments cannot be performed in humans solely for basic stem cell research. Therefore, a broad variety of circumstances and subjects have been used to validate both assays. Each of them separately does not validate the assays, however the conclusions from the various topics together suggest that the CAFC and LTC-CFC are reliable representatives of human in vivo repopulating stem cells.

The conclusions are:

1. Mature CAFC subsets (wk.1–3) show good correlations with the speed of hematological engraftment after autologous transplantation. The CAFC wk.6 does not.
2. The number and quality of the primitive CAFC subset (i.e. CAFC wk.6) that can be mobilized, is negatively affected by chemotherapy.
3. The quality of primitive progenitors, as measured by the LTC-CFC wk.6, has prognostic value for (partial) unsuccessful neutrophil and platelet recovery after autologous stem cell transplantation. The LTC-CFC wk.6 appeared to discriminate the defined patient groups better than the established graft evaluation parameters, like CD34 and CFC. The CAFC wk.6 was not able to distinguish the various patient

groups, indicating that the transplants had a qualitative rather than a quantitative defect of the stem cell compartment.

4. CAFC subsets co-elutriate with the human in vivo repopulating ability.
5. The CAFC wk.6 and the SRC are probably overlapping populations, as the differences on the basis of transducability and frequency do not appear to be as large as assumed earlier while the differences concerning phenotype and multipotentiality can be contested as described above.
6. The CAFC and LTC-CFC assays are highly suitable for evaluating new clinical graft manipulation procedures.

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ABBREVIATIONS

AML	acute myeloid leukemia
BFU-E	burst forming unit-erythroid
BM	bone marrow
BMT	bone marrow transplantation
BSA	bovine serum albumin
BW	bodyweight
CAFC	cobblestone area forming cell assay
CCE	counterflow centrifugal elutriation
CD	cluster of differentiation; used to discriminate cell types
CFC	colony-forming cell (= same as CFU-C)
CFU-C	colony-forming unit in culture (= same as CFC)
CFU-GM	colony-forming unit granulocyte-macrophage
CFU-GEMM	colony-forming unit-granulocyte-erythrocyte-monocyte-megakayocyte
CFU-S	colony-forming unit in spleen
CL_2DMP	di-chloromethylene di-phosphonate
(E)GFP	(enhanced) green fluorescent protein
FACScan	fluorescence activated cell scanning
FBMD-1	flask bone marrow dexter-clone 1; murine stromal cell line
FCS	fetal calf serum
FL	fetal liver
GaLV	gibbon ape leukemia virus
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage-colony stimulating factor
GpA	glycophorin A
GvHD	graft-versus-host disease
HBSS	Hank's buffered saline solution
HC	hydrocortisone
HD	Hodgkin disease
(H)GF	(hemopoietic) growth factors
HLA-DR	activation/differentiation marker
HPP-CFU-C	high proliferative potential colony-forming cell (=same as HPP-CFC)
HS	horse serum
HSC	hemopoietic stem cell
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
Lin^-	lineage marker negative

LP	leukapheresis products (= MPB)
LTC-CFC	long-term culture colony-forming cell
LTC-IC	long-term culture initiating cell
LTRA	long-term repopulating ability
MAb	monoclonal antibody
MESV	murine embryonic stem cell virus
ML-IC	myeloid lymphoid-initiating cell
MNC	mononuclear cells
MoMLV	Moloney murine leukemia virus
MPB	mobilized peripheral blood (= same as LP)
MPBT	mobilized peripheral blood transplantation
MRA	marrow repopulating ability
NA	non-adherent
NC	nucleated cells
NHL	Non-Hodgkin lymphoma
(NOD/)SCID	(nonobese diabetic/) severe combined immunodeficiency
PB	peripheral blood
PBS	phosphate buffered saline
PBSC	peripheral blood stem cell
PWM-MSCM	pokeweed mitogen murine spleen conditioned medium
RO	rotor off
r_s	Spearmann's rank correlation coeficient
SA	stroma-adherent
SCF	stem cell factor
SD	standard deviation
SEM	standard error of the mean
SPF	specified pathogen free
SSFV	spleen focus-forming virus
SRC	SCID repopulating cell
ßme	ß-mercaptoethanol
TBI	total body irradiation
UCB	umbilical cord blood
WBC	white blood cells

SUMMARY

In view of increasing ex vivo manipulation of hemopoietic stem cell transplants when aiming at gene therapy, ex vivo expansion or tumor cell purging, there is a strong need for in vitro assays allowing assessment of the frequency or the quality of human in vivo repopulating stem cells.

Tentative assays are the stroma-supported in vitro cobblestone area forming cell (CAFC) and the long-term culture colony-forming cell (LTC-CFC) assays. The first assay determines the frequency of progenitor subsets in various hemopoietic materials while the second provides means to assess the quality of the graft by measuring its ability to produce progeny.

The aim of this thesis was to clarify whether the CAFC and LTC-CFC assays, which have initially been developed and validated in murine models, provide a reliable quantitative and qualitative measure of human in vivo repopulating stem cells. In the work described in this thesis, the CAFC/LTC-CFC measurements in human hemopoietic material were correlated with clinical parameters as well as with other in vitro assays and data generated in a humanized immunodeficient mouse model.

The most suitable way to validate the human CAFC and LTC-CFC assay is by establishing the relation between the characteristics of a particular human progenitor subset assessed in vitro and its in vivo repopulating ability. To this purpose, sorted hemopoietic cells should be used to perform both in vitro assays in parallel to transplanting conditioned recipients. However, for obvious reasons this kind of experiments is prohibited in humans. Studying hematological recovery in the first months after hemopoietic stem cell transplantation provided an alternative, as shown in chapter 2 through 4.

In chapter 2 and 3 attempts were made to correlate the number of various in vitro parameters transplanted per kg bodyweight and the speed of hematological recovery or the probability of failing or delayed engraftment after autologous bone marrow (BM) or mobilized peripheral blood (MPB) transplantation, BMT and MPBT, respectively. The number of CAFC week 1-3 transplanted per kg bodyweight correlated better with early hematological recovery after MPBT than did the CAFC week 4-6, suggesting that the CAFC week 1-3 are indicators of relatively mature progenitor cells. The data have also shown that repeated administration of chemotherapy can lead to a reduced quality of primitive progenitors as well as a lower yield of primitive progenitor cells when performing leukapheresis. Furthermore, the high correlation between the neutrophil recovery and the total transplant quality indicated that not only the

number of primitive progenitors transplanted per kg bodyweight, but also their quality, contributes to hematological recovery. This observation was sustained by the data described in chapter 3. Where data are presented showing that the ability of a graft to generate colony-forming cells in vitro at week 6 of a stroma-supported culture, rather than the number of primitive progenitors transplanted, has prognostic value for unsuccessful post-transplant hematological recovery in the autologous BM or MPB transplantation setting. These data suggested that (partial) absence of repopulating activity after autologous stem cell transplantation is caused by a qualitative rather than a numerical defect of the stem cell compartment in the transplant. Additionally, these data provided supportive evidence for the validity of the LTC-CFC assay as an in vitro indicator of repopulating ability.

BM and MPB are also used in the allogeneic transplantation setting, where the donor is different from the recipient. Due to the genetic differences between the donor and recipient an immunological reaction is initiated leading to graft-versus-host disease. This potentially life-threatening condition can be abrogated by immune suppressive agents and reduction of the number of effector cells of the immune reaction, the T-cells. One way to reduce the number of T-cells in a transplant is by counterflow centrifugal elutriation (CCE). However, as any kind of graft manipulation may lead to severe loss of stem cells or quality, the number of mature and immature progenitors that were eventually included in the BM or MPB grafts after the CCE procedure, was determined. To this purpose, individual elutriation fractions were evaluated for their content of mature, i.e. $CD34^+$ cells or colony-forming cells, and immature progenitors, i.e. CAFC week 6 or $CD34^+/CD13^-/HLA\text{-}DR^-$ cells. It appeared that the yield of the mature and immature progenitors, the latter as tentative representatives of repopulating cells, in the BM and MPB transplants is sufficient as supported by the clinical studies showing low incidence of graft failure within a normal time of engraftment. These data contributed to further validation of the CAFC assay as an in vitro estimate of repopulating cells.

As repopulation experiments cannot be performed in humans for apparent ethical reasons, an alternative was sought in the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse. These mice are severely immunodeficient allowing for xenotransplantation and multilineage outgrowth of transplanted human cells in the mouse BM. The repopulation ability and outgrowth characteristics of (sub)populations of human umbilical cord blood (UCB) cells in these immunodeficient recipients are described in chapter 5.

It appeared that the macrophage-depleted SCID and the NOD/SCID mouse supported the multilineage outgrowth of human unfractionated, purified $CD34^+$ and the immature subset of $CD34^+/CD38^-$ UCB cells, with production of B-lymphocytes, monocytes, granulocytes,

erythroid cells, NK cells and platelets as well as production of $CD34^+$ cells, including phenotypically immature $CD34^+/CD38^-$ cells. However, the level of expansion of the immature human $CD34^+/CD38^-$ subset in chimeric mouse BM, but not the multilineage production of more differentiated progeny, appeared to be dependent on accessory cells.

It has been postulated that the cells responsible for the repopulation of the bone marrow of the NOD/SCID bone marrow are more primitive than the cells detected by the CAFC assay at week 6 of culture on the basis of difference in 1) transducability, 2) phenotype, 3) frequency and 4) multilineage outgrowth potential in immunodeficient recipients.

In chapter 6 and 7 results of experiments designed to address the tentative differences in frequency and transducability have been described and discussed. Using a highly efficient retroviral vector we have shown in chapter 6 that the transduction efficiency of the cells present in the NOD/SCID BM at 5 weeks after transplantation was comparable to the transduction efficiency of the CAFC week 6 (chapter 6). This contrasts earlier claims by other investigators of extensive differences in sensitivity for gene transfer between the NOD/SCID repopulating cells and CAFC-like progenitors. With regard to the assumed difference in frequency, we have shown that the NOD/SCID repopulating cell seems more rare than the CAFC week 6 because human stem and progenitor cells have a very low seeding efficiency in the BM of the NOD/SCID mouse (chapter 7).

In conclusion, the data in chapter 6 and 7 suggest that the NOD/SCID repopulating cell may show more similarities with the CAFC week 6 on the basis of the proposed differences than had been previously assumed in literature.

The work described in this thesis has provided support to the view that the CAFC and LTC-CFC assay are reliable representatives of the human in vivo repopulating cells. Although it will not be feasible to perform these assays on a routine basis for graft evaluation in the clinical setting, as the assays are time consuming, labor intensive and require a high level of expertise, they are highly suitable to evaluate new graft manipulation procedures.

SAMENVATTING

Door een toename in het ex vivo bewerken van humane bloedvormende (hemopoïetische) stamceltransplantaten ten behoeve van o.a. gentherapie, expansie en tumorcel purging, is er een sterke behoefte aan in vitro assays waarmee het aantal of de kwaliteit van menselijke in vivo repopulerende stamcellen kan worden gemeten.

Daarvoor mogelijk geschikte assays zijn het stroma-afhankelijke 'cobblestone area forming cell' (CAFC) en het 'long-term culture colony-forming cell' (LTC-CFC) assay. Het eerst genoemde assay kan de frequentie van voorlopersubsets in verschillende hemopoïetische materialen bepalen, terwijl het tweede de potentie van voorlopercellen om nakomelingen te produceren kan meten.

Het in dit proefschrift vermelde onderzoek heeft zich er op gericht na te gaan in hoeverre de CAFC en de LTC-CFC assays, die in eerste instantie ontwikkeld en gevalideerd zijn in muismodellen, een betrouwbare kwantitatieve of kwalitatieve maat zijn voor humane in vivo repopulerende stamcellen. Hiertoe zijn CAFC/LTC-CFC metingen in menselijk hemopoïetisch materiaal uitgevoerd en gecorreleerd aan klinische posttransplantatie parameters, alsmede aan andere in vitro assays en data verkregen met een gehumaniseerd immuundeficiënt muismodel.

De meest geschikte manier om het humane CAFC en LTC-CFC assay te valideren is door de relatie te bepalen tussen de karakteristieken van een bepaalde humane voorlopercel gemeten in vitro en zijn in vivo repopulerende capaciteit. Om dit te kunnen doen, moeten gesorteerde hemopoïetische cellen gebruikt worden om beide vitro assays uit te voeren in parallel met transplantatie van geconditioneerde recipiënten. Echter, om voor de hand liggende redenen kunnen deze experimenten niet worden uitgevoerd bij mensen. Het bestuderen van het hematologisch herstel in de eerste maanden na stamceltransplantatie heeft een alternatief geleverd, zoals beschreven in hoofdstuk 2 tot 4.

In hoofdstuk 2 en 3 is gepoogd om voor een aantal verschillende celtypen een relatie te leggen tussen het aantal dat getransplanteerd is per kg lichaamsgewicht en de snelheid van het daaropvolgende hematologisch herstel, dan wel de kans op falende of vertraagde repopulatie na autoloog beenmerg (BM) of gemobiliseerd perifeer bloed (MPB) transplantatie, respectievelijk BMT of MPBT. Het aantal CAFC week 1-3 getransplanteerd per kg lichaamsgewicht correleerde beter met vroeg hematologisch herstel na MPBT dan CAFC week 4-6. Dit suggereert dat de CAFC week 1-3 indicatoren zijn van relatief rijpe voorlopercellen. De data lieten ook zien dat herhaalde chemotherapie kan leiden tot kwaliteitsverlies van onrijpe voorlopercellen en tot een lagere opbrengst van onrijpe voorlopercellen bij leukaferese. De

sterke correlatie tussen neutrofiel herstel en de totale transplantaat-kwaliteit wees erop dat niet alleen het aantal primitieve voorlopers dat wordt getransplanteerd per kg lichaamsgewicht maar ook hun kwaliteit een bijdrage levert aan het hematologisch herstel. Deze waarneming werd ondersteund door de gegevens die zijn beschreven in hoofdstuk 3. Gebleken is dat de potentie van het transplantaat om kolonievormende cellen te genereren in een stroma-afhankelijk assay op week 6 van de kweek een betere prognostische waarde heeft voor niet-succesvol hematologisch herstel na autologe BMT of MPBT dan het aantal voorlopercellen dat wordt getransplanteerd per kg lichaamsgewicht. Deze gegevens suggereerden dat (gedeeltelijke) afwezigheid van repopulerende activiteit na autologe stamceltransplantatie eerder door een kwalitatief dan door een kwantitatief defect van het stamcelcompartiment in het transplantaat wordt veroorzaakt. Tegelijkertijd hebben deze data additioneel ondersteunend bewijs geleverd voor de validiteit van het LTC-CFC assay.

BM en MPB worden ook gebruikt in de allogene transplantatie setting, waar de donor verschillend is van de recipiënt. Door de genetische verschillen tussen de donor en de recipiënt wordt een immunologische reactie geïnitieerd die leidt tot 'graft-versus-host' ziekte. Deze potentieel levensbedreigende ziekte kan worden onderdrukt met immunosupressiva en door vermindering van het aantal effector cellen van deze reactie, de T-cellen, in het transplantaat. Eén manier om het aantal T-cellen in het transplantaat te verminderen is door middel van 'counterflow centrifugal elutriation' (CCE). Echter, omdat manipulatie van een transplantaat kan leiden tot een groot verlies van stamcellen of hun kwaliteit is bestudeerd wat het effect is van CCE op het aantal rijpe en onrijpe voorlopercellen dat uiteindelijk in het BM of MPB transplantaat terechtkomt. Voor dit doel werd in individuele elutriatie fracties het aantal rijpe, i.e. $CD34^-$ cellen of kolonie vormende cellen, en onrijpe voorlopercellen, i.e. CAFC week 6 of $CD34^+/CD13^-/HLA\text{-}DR^-$ cellen, bepaald. Gebleken is, dat het overgrote deel van de initieel aanwezige rijpe en onrijpe voorlopercellen kon worden aangetoond in de transplantaten met onze in vitro assays. Aangezien deze transplantaten in nagenoeg alle gevallen hebben geleid tot een goed herstel van de bloedcelvorming in de ontvangers dragen deze gegevens bij aan de validatie van het CAFC assay als een in vitro maat voor repopulerende stamcellen.

Daar in vivo repopulatie experimenten niet mogen uitgevoerd in mensen om voor de hand liggende redenen is een alternatief gezocht in de 'nonobese diabetic/severe combined immunodeficiency' (NOD/SCID) muis. Deze muizen zijn ernstig immuundeficiënt waardoor xenotransplantatie mogelijk wordt. Daarnaast kunnen de humane getransplanteerde cellen in het muize BM uitgroeien tot de verschillende hemopoïetische gedifferentieerde celtypen. De experimenten waarin de repopulatiecapaciteit en de karakteristieken van de uitgroei na transplantatie van (sub)populaties van menselijke navelstrengbloed cellen in deze

imuundeficiënte recipiënten zijn onderzocht, worden beschreven in hoofdstuk 5. Gebleken is dat de makrofaag-gedepleteerde SCID muis, en de NOD/SCID muis, de uitgroei tot de verschillende hemopoïetische uitgerijpte celtypen van menselijke ongefractioneerde, zuivere $CD34^+$, en de onrijpe subset van $CD34^+/CD38^-$ navelstrengbloed cellen ondersteunde. Hierbij werden B-cellen, monocyten, granulocyten, NK cellen en plaatjes als ook $CD34^-$ cellen, inclusief de fenotypisch onrijpe $CD34^+/CD38^-$ cellen in het muize BM gevonden. De mate van expansie van de onrijpe $CD34^+/CD38^-$ subset in chimeer muis BM blijkt echter afhankelijk te zijn van accessoire cellen, terwijl de productie van meer gedifferentieerde nakomelingen dit niet is.

Men heeft gepostuleerd dat de cellen die het NOD/SCID muis BM kunnen repopuleren primitiever zijn dan de CAFC week 6 op basis van verschil in 1) transduceerbaarheid, 2) fenotype, 3) frequentie en 4) de potentie om uit te groeien in de verschillende hemopoïetische differentiatie richtingen in immuundeficiënte recipiënten. In hoofdstuk 6 en 7 zijn de resultaten beschreven en bediscussieerd van experimenten die ontworpen zijn om de potentiële verschillen in transduceerbaarheid en frequentie te onderzoeken.

Met behulp van een zeer efficiënte retrovirale vector hebben we laten zien dat de transductie-efficiëntie van de humane cellen die aanwezig zijn in het BM van de NOD/SCID muis op 5 weken na de navelstrengbloed transplantatie vergelijkbaar was met het percentage gentransfer in de CAFC week 6 (hoofdstuk 6). Dit is strijdig met het door anderen voorgestelde grote verschil in gevoeligheid voor genoverdracht tussen de NOD/SCID repopulerende cellen en CAFC-achtige voorlopers. Met betrekking tot het, in de literatuur, geponeerde verschil in frequentie hebben we laten zien dat de lage frequentie van de NOD/SCID repopulerende cel ten opzichte van de CAFC week 6 grotendeels verklaard kan worden door de lage 'seeding' efficiëntie van humane stam- en voorlopercellen in het BM van de NOD/SCID muis (hoofdstuk 7).

In conclusie, de data in beide hoofdstukken suggereren dat de NOD/SCID repopulerende cel meer overeenkomsten laat zien met de CAFC week 6 dan eerder werd gepostuleerd in de literatuur.

Het werk dat in dit proefschrift wordt beschreven heeft bijgedragen aan het inzicht dat de CAFC en de LTC-CFC assay waardevolle en betrouwbare vertegenwoordigers zijn van humane in vivo repopulerende stamcellen. Het gebruik van deze assays voor routinematige beoordeling van transplantaten in de kliniek lijkt onrealistisch, omdat daarvoor meestal te weinig tijd is en de assays een grote deskundigheid en arbeidsintensiteit vereisen. De assays zijn echter zeer geschikt om te worden gebruikt bij het evalueren van nieuwe manipulatie procedures van transplantaten.

CURRICULUM VITAE

Paula B. van Hennik

Op 24 juni 1972 ben ik in Arkel, Zuid-Holland geboren. Kort daarna zijn we met het gezin verhuisd naar Warnsveld, Gelderland. Mijn middelbare schoolopleiding is begonnen in 1984 op de Martinet MAVO in Zutphen, waar ik in 1988 mijn diploma behaalde. Vervolgens ben ik gestart in de vierde klas van de HAVO aan de Stedelijke Scholengemeenschap, voorheen Stedelijk Lyceum, te Zutphen. Na de zomervakantie van 1989 ben ik overgestapt naar de vijfde klas van het ongedeelde VWO aan dezelfde scholengemeenschap. In 1991 heb ik het VWO diploma behaald. In die zomer ben ik verhuisd naar Rotterdam om te beginnen met de studie geneeskunde aan de Erasmus Universiteit. De propadeuse heb ik cum laude afgesloten. De doctoraalfase heb ik in november 1995 afgerond met onderzoek uitgevoerd in de groep van Dr. R.E. Ploemacher met als titel 'In vitro indicators of hematological recovery following autologous mobilized peripheral blood stem cell transplantation'. Een maand later ben ik gestart met het promotieonderzoek wat uiteindelijk heeft geleid tot dit proefschrift.

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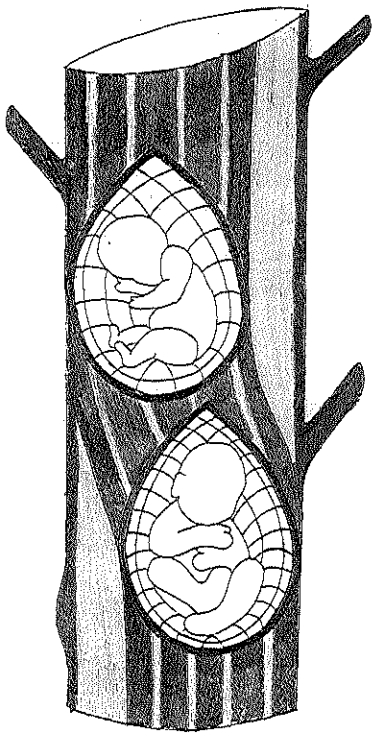
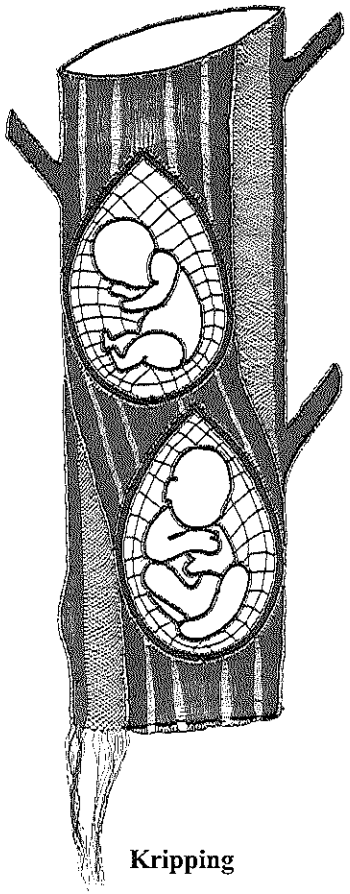
Paula



Ontwerp



Kripping

KANTKLOSWERK

Het idee om mijn oudste zus te vragen 'iets' te ontwerpen voor de kaft van mijn proefschrift, stamt al uit het eerste jaar van mijn AIO periode. We hadden op dat moment beide te maken met de Hematolgie, al was dat op zeer verschillende wijze. Zij volgde in die tijd een opleiding voor kantkloslerares waardoor het voor de hand lag dat zij dat 'iets' voor de kaft zou kantklossen. Dat betekende veel werk, erg veel werk. En ik moet zeggen dat ik blij ben dat ze (altijd heeft gezegd dat ze) het echt leuk vond om te doen.

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Op een bepaald moment tijdens het kantklossen lagen er maar liefst 180 klosjes op het werkkussen. Uit de feiten dat de klosjes een diameter hebben varierend van 0.5 - 0.8 cm met een lengte van 9.5 cm en de diameter van het gebruikte garen slechts 0.05 cm is, blijkt dat ik een koelhoofdige zuster heb.

De kantsoort waar het werk in is uitgevoerd heet Honiton en is rond 1300 onstaan uit een samenspel van Engelse en Vlaamse kantklosvaardigheden in en om Honiton, Devon, Engeland. In 1724 kende deze kantsoort zijn top, maar door het uitvinden van de tulemachine in 1809 en verandering van de mode, daalde de belangstelling. Onder de leden van het Engels koningshuis bleef het echter populair. Koningin Victoria heeft namelijk haar bruidsjurk in deze kantsoort laten uitvoeren en ook de huidige koningin Elisabeth heeft regelmatig bestellingen geplaatst.

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