ASSESSMENT AND IMPROVEMENT OF GENE TRANSFER INTO HUMAN HEMATOPOIETIC STEM CELLS

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ASSESSMENT AND IMPROVEMENT OF GENE TRANSFER INTO HUMAN HEMATOPOIETIC STEM CELLS

BEPALING EN VERBETERING VAN GENOVERDRACHT IN BLOEDVORMENDE STAMCELLEN VAN DE MENS

PROEFSCHRIFT

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Aan mijn ouders en mijn broer

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 Scope of this Thesis

The application of somatic gene transfer as a potential treatment in human disease has progressed from speculation to reality in a short time [4,20,21,84,85,87,105,117,174]. In May 1989 the first clinical marker gene protocol took place [145], followed by the first gene therapy protocol for the correction of adenosine deaminase deficiency in September 1990 [3,11]. Subsequently, the number of gene marking and gene therapy trials grew exponentially. In 1996 more than 200 clinical protocols were approved worldwide [5] and more than 1,500 patients are now enrolled in gene transfer trials [96]. However, up to now the gene therapy trials have not brought clear clinical benefits for patients [5,31,51,98]. An important explanation for the lack of therapeutic effects is the very low gene transfer efficiency in human cells.

One of the main targets for gene therapy is the hematopoietic system because of well-developed procedures for bone marrow transplantation, the many types and wide distribution of hematopoietic cells in the body and the existence of many diseases and treatments that affect hematopoietic cells. The target cell for gene transfer is the primitive hematopoietic stem cell (HSC), which is present at low frequency in the bone marrow, is responsible for long-term hematopoiesis and gives rise to all hematological and lymphoid lineages. Although many research groups have reported transfer and long-term expression of genes in a significant percentage of murine hematopoietic cells [77,142,177], the achievements in larger outbred animals [81,93,150], nonhuman primates [14,164,165] and humans [17,29,36,44,48,72,86] show only a small percentage of long-term expression in a limited number of recipients.

The aim of this study was to develop protocols which improve gene transfer of human HSC. For this purpose suitable assays were developed to determine the frequency and proliferative capacity of different classes of human HSC. Subsequently, culture conditions for human HSC were studied. As a result, using newly developed human HSC assays and optimized culture conditions, improved human HSC gene transfer protocols could be proposed. In the presented studies we have opted for clinically relevant and acceptable protocols. This included the use of target cells which are most frequently used in clinical autologous stem cell transplantation, namely mobilized peripheral blood stem cells (PBSC) [161] and serum-free transfection cultures in which target cells and virus-producing cells were separated.

1.2 Hematopoietic Stem Cells

The hematopoietic system is a cell renewal system which continuously produces mature hematopoietic cells with a limited life-span. The life-long formation of all blood cell lineages originates from a small pool of primitive HSC residing in the bone marrow [1]. HSC can be mobilized from the bone marrow to the peripheral blood after cytotoxic chemotherapy treatment and cytokine administration

[43,47,52,55,56,76,80,88,110,111,140,151,152]. In clinical practice both bone marrow cells and mobilized PBSC are used for HSC transplantation purposes. Allogeneic HSC transplantation is performed to treat patients with primary immunodeficiency diseases, aplastic anemia and genetic diseases. The application of either allogeneic or autologous HSC transplantation also permits marrow ablative radio- and chemotherapy for the treatment of hematological malignancies and solid tumors [124,158-161].

In murine transplantation studies a hierarchy of stem cell subsets has been demonstrated. Heterogeneity in stem cells was observed using fractionated bone marrow grafts [183], bone marrow cells pretreated with cell cycle specific drugs [16,67-70,146-148,170], spleen colony forming cells [94,109,182], physically separated stem cells [8,10,116,126-131], genetically marked transplants [63,74,79], irradiated bone marrow donors [42,101,102,135] or different host conditioning regimens [40,41]. Only the most primitive stem cells are responsible for long-term engraftment. A single primitive stem cell can contribute to the *in vivo* hematopoiesis for more than a year. In contrast, more mature stem cells subsets have only a short-term (weeks to months) repopulating activity *in vivo*, while committed progenitor cells of both mice and humans which can form colonies in semi-solid medium *in vitro* probably do not contribute to *in vivo* hematopoietic engraftment after transplantation [60,187].

1.3 In Vitro Assays for Primitive Hematopoietic Stem Cells

In vivo experiments in humans are ethically restricted and *in vivo* experiments in animals are limited for ethical, practical and financial reasons. To study long-term hematopoiesis *in vitro* long-term stroma-dependent culture systems have been developed [38,53]. In those cultures bone marrow cells are maintained for many weeks. During the culture an adherent stromal layer is formed in which HSC are localized. The long-term culture system has been exploited to establish *in vitro* assays for the quantification of primitive HSC, i.e., the murine cobblestone area forming cell (CAFC) assay [132] and the human long-term culture-initiating cell (LTC-IC) assay [157]. Both assays allow measurements of the frequencies of HSC in hematopoietic samples on a preformed stromal feeder layer using a limiting-dilution approach.

Murine cobblestone area forming cell assay

In the murine CAFC assay the formation of phase-dark hematopoietic clones under the stromal layer is used as an endpoint [132]. This visual endpoint correlates with colony forming cell (CFC) production, which is used in the LTC-IC assay. Extensive correlation studies using physically separated stem cell subsets in four different murine transplantation models have shown that the same hierarchy of stem cell subsets which have been disclosed in murine transplantation studies can be identified in the CAFC assay [40-42,132-137,166,168,169]. This implies that early appearing CAFC represent a more

mature stem cell subset than late appearing CAFC do. From these studies it became apparent that the CAFC frequency at day 10 strongly correlates with the number of spleen colony forming cells day 12, an *in vivo* assay of short-term repopulating stem cells. Primitive HSC with long-term repopulating abilities *in vivo* are assessed at day 28 or later in the CAFC assay.

Human long-term culture-initiating cell assay
In the human LTC-IC assay the frequencies of primitive HSC, which are able to produce CFC cells after 5 to 8 weeks of LTC, can be assessed [157]. It has been shown that LTC-IC and CFC represent different cellular entities and they can be physically separated [156]. LTC-IC are 4-hydroperoxycyclophosphamide resistant cells while CFC are sensitive to this drug [181]. In analogy with murine primitive HSC, LTC-IC are rhodamine-123 dull cells [163]. Further characterization showed that LTC-IC have a low orthogonal light scatter with a high CD34 expression and a low expression of HLA-DR, CD33, CD38, CD45RA and CD71 [6,73,89,90,156,157].

1.4 Somatic Gene Therapy

Gene transfer involves the delivery, to target cells, of an expression cassette made up of one or more genes and the sequences controlling their expression. As a result, this clinical strategy modifies the genetic repertoire of somatic cells for therapeutic purposes or genetic marking. Gene marking studies have been performed using bacterial antibiotic resistance genes, which allow the genetically modified cells to be identified [17,18,29,36,44,48,145]. Because the marking genes have no function other than to permit selection of modified cells *in vitro*, the trials using marker genes have been designed to demonstrate the feasibility of human gene transfer, to uncover biologic principles relevant to human disease and to evaluate safety. Gene therapy has amongst others been proposed for the correction of monogenic disorders, delivery of therapeutic agents, enhancement of immune responses to tumors, generation of tumor vaccines, insertion of suicide genes into neoplastic cells, modification of tumor cells by suppressor genes or anti-oncogenes, conferral of chemotherapy resistance to normal cells and insertion of genes to inhibit human immunodeficiency virus-1 replication. Successful gene transfer into humans has been shown by the transfer of genes into a large range of target cells such as lymphocytes, neoplastic cells of hematopoietic lineages and of solid tumors, fibroblasts, hepatocytes, airway epithelium cells, muscle cells, keratinocytes, endothelial cells, dendritic cells and HSC.

1.5 Retroviral Gene Delivery Vectors

The most commonly used and the most efficient vehicle for stable integration of genes into human primary somatic cells up to now is an amphotropic murine retrovirus [106,172]. Retroviruses have a wide target cell range and the genetic information is integrated into the host cell DNA which ensures transfer of the introduced DNA into the progeny of the target cell. Hence, these vectors are ideal for transferring genes into a rapidly dividing cell populations (e.g. tumor cells or lymphocytes) or HSC which are capable of extensive proliferation giving rise to all hematopoietic and lymphoid lineages. However, the main disadvantage of retroviral vectors is that most retroviruses are unable to infect non-dividing cells, because integration of virus DNA depends on mitosis [107,143]. This explains the low efficiency of transfer into quiescent, primitive HSC. Provided that the retroviral vector is replication-defective, the vector appears to be nontoxic. However, replication-competent vectors can potentially damage genes leading to oncogenesis [39].

The gene transfer procedure is performed with retroviruses from which many of the structural and replicative viral genes have been replaced by one or more genes of interest, driven either by the retroviral promotor in the 5' long terminal repeat or by an internal promotor. Viral replication functions are provided by packaging cells that contain the missing retrovirus genes and thus reproduce and package a vector with an expression cassette that is not replication-competent [97,104]. In humans the amphotropic retrovirus enters the target cell via a specific receptor, the human amphotropic retrovirus receptor [78,108,141,171,186]. In the cytoplasm, the reverse transcriptase carried by the vector converts the vector RNA into proviral DNA that is integrated into the target cell genome, where the expression cassette makes its product.

1.6 Gene Transfer in Human Primitive Hematopoietic Stem Cells

There are efficient methods available to transduce murine primitive HSC *in vitro* using high-titre ecotropic retroviral vectors and following activation of proliferation of stem cells with cytokines. However, attempts to reproduce the same high efficiency of amphotropic retroviral gene transfer into human primitive HSC have met little success. In man, following gene transfection and subsequent transplantation only a small fraction of repopulating HSC appear to be transduced. This is the case even though protocols similar to those which are successful in murine models, are employed [17,29,36,44,48,72,86]. As a result, currently investigators focus on technical and biological aspects that might lead to an improvement of human HSC gene transfer [123].

Colocalization of retroviruses and target cells

In most clinical transfection procedures HSC are exposed to a cell-free supernatant of a retrovirus producing cell line. Direct contact between target cell

and virus producing cell is prevented to exclude contamination of virus producing cells in the HSC graft. Using supernatant transfection strategies the approximation between cells and virus depends on Brownian motion [24] and viral half-life [32].

The colocalization of HSC and virus particles can be promoted through coating of the culture surface with carboxy-terminal fibronectin fragments [61,82,113,115,178,179]. Under the latter facilitating conditions, retroviral gene transfer efficiencies are enhanced and percentages of HSC transduction reach values that are comparable to those following co-cultivation transfections. Other methods which have been shown to enhance the probability of target cell - retroviral particle contact are centrifugation and fluid flow strategies [7,22,25].

Electrostatic repulsive force resulting from the negative charges on the lipid bilayers of the target cell and the virus particle inhibits further approximation between HSC and retroviral vector. Therefore, the binding of viruses on the cell surface can be promoted by neutralizing this repulsion between the opposing bilayers by polycations such as polybrene and protamine sulphate [26]. Indeed, gene transfer efficiency rises as a function of increasing polybrene concentrations [125,162]. Because polybrene is toxic at higher concentrations or long exposure periods, protamine sulphate is successfully used as a less toxic alternative [28].

Amphotropic retrovirus receptor expression

The identification of the amphotropic retrovirus receptor [78,108,171] and the relation between retroviral gene transfer efficiency and the level of amphotropic retrovirus receptor expression on the target cells [141,186] has focussed the attention to the expression level of this receptor on human HSC. Because suitable antibodies directed against the amphotropic retrovirus receptor are not available, experimental data are limited.

It has been shown that the level of mRNA encoding for the amphotropic retrovirus receptor in mouse and human HSC is low [121]. Furthermore, it has been suggested that the mRNA level correlates with the efficiency of retroviral transfection. The lowest level of amphotropic retrovirus receptor mRNA level was found in primitive CD34-positive/CD38-negative (CD34+/CD38-) HSC, which were comparatively most resistant to retroviral transfection. CD34+/CD38+ committed progenitor cells, on the other hand, show higher levels of amphotropic retrovirus receptor mRNA level and are more susceptible to gene transfer.

The level of binding of virus particles to target cells has been determined using a monoclonal antibody, which recognizes an epitope on the retroviral envelope. Target cells are incubated with retrovirus containing medium, followed by a labeling procedure with this antibody. Virus particle binding is used as an indirect measure of receptor expression and/or affinity. It was shown that following culture of CD34+ bone marrow cells in the presence of the cytokines interleukin-3 (IL-3), stem cell factor and IL-6, amphotropic retrovirus binding could be upregulated. Virus binding to more primitive CD34+/CD38- cells was

only observed following culture of the cells with those cytokines [30].

Cell cycle status

To obtain stable expression of the transfected gene in the target cell and all its daughter cells the gene must integrate in the DNA of the target cell. Because most human primitive HSC are in G_0 [62,75,83,144] and stable retroviral integration occurs almost exclusively in actively dividing cells [107,143] several techniques have been used to induce cell cycling of primitive HSC and thereby increase the efficiency of retrovirus-mediated gene transfer.

In animal studies, it has been shown that prior treatment of animals with 5-fluorouracil increases both the number of cycling stem cells [64] and improves retroviral gene transfer into those cells [13,175]. Also in humans it has been shown that an increased number of bone marrow derived HSC enter the active cell cycle after cytotoxic chemotherapy [83]. The latter regenerating bone marrow has also successfully been used for gene marking studies [17]. However, it has also been shown that the 5-fluorouracil prestimulation effect is not always present and may be replaced by cytokine-stimulation [46].

In vitro stimulation with recombinant cytokines can also induce HSC to cycle [75,83]. Indeed, the resulting higher proliferative status of HSC improves the retroviral gene transfer efficiency [12,118,119]. In this respect, the most commonly used cytokines are IL-3, stem cell factor and IL-6. It has also been shown that these three cytokines have positive effects on the recovery of HSC following culture. However, cytokine stimulation of HSC may also have disadvantages and diminish the abilities of stem cells for engraftment *in vivo*. Even short incubations of murine HSC with several cytokine combinations inhibit the lodgement of those cells in hematopoietic organs and the ability to repopulate the hematopoietic system of irradiated recipients [167].

Stromal cell support

A supporting stromal layer has also been shown to improve the transfection of primitive HSC [33,60,112,120,185]. The mechanism of the stromal support has not been elucidated. Several possible explanations have been proposed. Firstly, enhanced transfection by stromal support may result from the local elaboration of cytokines required to increase cycling of quiescent HSC. Secondly, HSC may directly attach to extracellular matrix molecules, which may lead to proliferation of HSC or upregulation of amphotropic retrovirus receptor expression. Thirdly, target cells and virus particles may colocalize on the stroma similar to the effect of adherence of retroviral viruses to stromal extracellular matrix elements.

Besides the improved transfection efficiency, there are also indications that stromal support during transfection might improve the recovery of primitive HSC and maintains their ability to engraft in vivo [33,60,120].

1.7 Human Hematopoietic Stem Cell Gene Therapy

Up to now, most gene therapy studies of HSC have concentrated on stem cell marking, correction of genetic disorders and conferral of chemotherapy resistance [20,84,85,87].

Gene marking

Initial studies directed towards gene therapy involved transfer of marker genes in autologous transplants of cancer patients. These experiments had two goals: Firstly, to mark and subsequently detect contaminating tumor cells in these transplants [18,36]. Secondly, to determine the transfection efficiency and expression of these genes in normal long-term repopulating stem cells illustrating that stem cell gene therapy may be feasible [18,29,36,44,48]. In these studies, cells from autologous transplants of patients with acute myeloblastic leukemia, neuroblastoma, chronic myelogenous leukemia, acute lymphocytic leukemia, multiple myeloma and breast cancer in clinical remission were transfected with a neomycin phosphotransferase (NEO) gene. Subsequently, tumor relapses of those patients were analyzed for the presence of the marker gene. It was shown in these studies that the marker gene was present in a proportion of the tumor recurrence, demonstrating the contribution of contaminating tumor cells in the transplant to these relapses. These studies might imply that effective tumor cell purging of autologous transplants may be important to reduce the risk of relapse. Furthermore, it appeared from these studies that the infused and genetically marked normal HSC contributed to both short- and long-term hematopoietic recovery. Thus genes have been introduced into *in vivo* engrafting stem cells using a retroviral vector. Analysis of hematopoietic cells using *in vitro* G418-selection showed that there was NEO gene expression in all hematopoietic lineages from one to 18 months after transplantation. Because of the G418-toxicity, it was not possible to verify this feature *in vivo*. While on one hand the gene marking studies are encouraging, on the other hand it should be noted that most transplants contained low or even undetectable numbers of genetically marked cells. This made analysis after transplantation extremely difficult. Current gene marking protocols are developed to study purging technologies of malignant cells [19], *ex vivo* stem cell expansion strategies [66], gene transfer protocol development [60] or the evaluation of different sources of HSC as targets for gene therapy [34,44].

Correction of monogenic disorders

One application of gene transfer could be the correction monogenic disorders by introducing the relevant gene into primitive HSC of the patient. In theory, genetic diseases with a clinical expression restricted to cells of hematopoietic origin might be completely cured by transplantation of genetically corrected primitive HSC. In generalized genetic disorders hematopoietic cells might provide a continuous source of the normal gene product in various sites and tissues of the recipient depending on where cells arrive. As a result, gene therapy of

monogenic disorders could furnish the same benefits as allogeneic bone marrow transplantation for the same diseases, but without the immunological complications. [124]. However, in most disorders the disease-specific gene would need to be transferred into high percentages of the reconstituting HSC and with an appropriate level of gene expression in order to correct the disorder.

Adenosine deaminase (ADA) gene therapy is an approach in which even an low efficiency of gene transfer in primitive HSC might be of potential benefit [15,71,72,85-87]. It was assumed that the genetically corrected cell population would initially be extremely small as a consequence of the low number of transduced HSC and the lack of myeloablative conditioning prior the transplantation. However, with time after transplantation an expansion of transduced cells might be expected due to a selective growth advantage over unmodified cells. Although all patients received enzyme replacement therapy with polyethylene glycol-conjugated ADA, which may have counteracted the selective advantage of ADA gene transduced HSC, in two out of three clinical studies an outgrowth of genetically corrected T cells originating from the genetically corrected HSC has been reported [15,87].

In addition to ADA gene therapy, protocols to transduce HSC with the glucocerebrosidase gene are well advanced [45,184] and protocols to treat other lysosomal storage disorders [149] and Fanconi anemia [50] are in varying stages of development.

Conferral of chemotherapy resistance

Treatment with cytotoxic chemotherapy has resulted in improved survival of cancer patients. For most anticancer drugs the response rates improve with increasing the dose and intensity of the cytotoxic treatment [103,180]. However, myelosuppression is the major dose-limiting toxicity of many anticancer drugs and can lead to delay of further treatment. Therefore, HSC transplants have been used to support sequential high-dose regimens [57]. The transfection of chemotherapy resistance genes into HSC to increase the tolerance against cytotoxic agents could be a strategy to protect the hematological system. As a result, the chemotherapy-resistant-modified hematopoietic cells may allow more intensive anticancer treatment or circumvent the need for drug dose reduction and, potentially, increase cure rates. Studies of cytotoxic drug resistance in tumor cells have revealed several chemotherapy resistance genes which might be exploited for this purpose [84].

Another more general clinical application could be the use of a drug resistance gene as a dominant selectable marker allowing the *in vivo* selection of gene-modified cells over their unmodified companions. In this approach a second therapeutic gene is linked to the drug resistance gene using a bicistronic retroviral vector [54,155]. This application could lead to a strategy of positively selecting gene-modified HSC to a level where therapeutic benefit could be obtained, despite low-efficiency gene transfer.

Murine and human studies have shown that HSC can be transduced *ex vivo* using retroviral vectors containing the multidrug resistance-1 (MDR1) gene [58-

60,92,138,153,154,173], the dihydrofolate reductase gene [9,27,49,91,99, 176], the methylguanine DNA methyltransferase gene [2,100,114,139] or the aldehyde dehydrogenase gene [95]. In all instances, gene transfer resulted in significant drug resistance in the transduced HSC.

The first clinical chemotherapy resistance gene therapy study protocols are based upon the transfer of the MDR1 gene. The product of the MDR1 gene, P-glycoprotein, functions as a drug efflux pump and confers resistance to a wide variety of chemotherapeutic agents including anthracyclines, vinca alkaloids, epipodophyllotoxins, actinomycin D and taxol [23,84]. In murine studies it has been demonstrated that the overexpression of a retrovirally introduced MDR1 gene can indeed protect hematopoietic cells *in vivo* against cytotoxic treatment [58,153]. Clinical trials are in progress to transfer the MDR1 gene into bone marrow cells and mobilized PBSC of autologous grafts from cancer patients [35,37,60,65,122]. Following the transplantation of transduced HSC, the patients will be treated with MDR1-dependent chemotherapeutic agents to assess the feasibility of MDR1 gene therapy.

1.8 Introduction to the Experimental Work

This thesis describes the development of human stem cell assays which are subsequently used to evaluate the effects of *ex vivo* manipulation on HSC from mobilized peripheral blood.

In chapter 2, the human CAFC and long-term culture-colony forming cell (LTC-CFC) assays are presented. These assays permit the assessment of the frequencies of different stem cell subsets and the analysis of the abilities of stem cells to produce CFC. A first attempt is made to validate the human CAFC assay as a primitive stem cell test using physically sorted or 5-fluorouracil treated bone marrow cells.

Chapter 3 shows the CAFC and LTC-CFC analyses of 47 leukapheresis products from 21 cancer patients who received those leukaphereses as autologous transplants. To further validate the CAFC and LTC-CFC assays these data were compared with immunophenotypic analyses of the transplants. In addition, the effect of premobilization treatment on the CAFC number and their CFC producing ability was studied. Finally, the *in vitro* data were compared with the short-term *in vivo* posttransplantation recovery.

As a prelude to gene transfer experiments, the effects of cytokines, stroma-conditioned media and stroma-contact on CAFC and LTC-CFC recovery of CD34-selected PBSC were studied in short-term cultures (Chapters 4 and 5).

In chapter 6, the human CAFC and LTC-CFC assays were used to analyze the fate of HSC from mobilized peripheral blood after a transfection procedure with a retrovirus containing the human MDR1 gene. Improved human HSC gene transfer protocols are described in chapter 7 using a clinically relevant procedure in which mobilized PBSC are transfected on a carboxy-terminal fibronectin fragment coated culture surface in serum-free and cell-free transfection cultures.

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CHAPTER 2

FREQUENCY ANALYSIS OF HUMAN PRIMITIVE HEMATOPOIETIC STEM CELL SUBSETS USING A COBBLESTONE AREA FORMING CELL ASSAY

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ABSTRACT

Stroma-dependent long-term bone marrow cultures (LTBMC) assay the ability of primitive hematopoietic stem cells (HSC) for long-term production of clonable progenitors. We have a developed a limiting dilution type LTBMC assay allowing frequency analysis of transiently repopulating HSC and long-term culture initiating cells (LTC-IC) without the necessity to replate large numbers of wells. Normal or 5-FU treated Ficoll bone marrow cells (BMC), or BMC sorted on CD34 or HLA-DR expression or Rhodamine-123 (Rh123) retention, (input range 40-70,000 CFU-GM + BFU-E/10^5 cells) were plated at limiting dilution on unirradiated adherent layers formed by a novel murine preadipose cell line (FBMD-1). The percentage of wells with at least one phase-dark hematopoietic clone (cobblestone area, CA) beneath the stromal layer was weekly determined for at least 8 weeks and CA-forming cell (CAFC) frequencies were calculated using Poisson statistics. Parallel LTBMC of the same samples were weekly assessed for supernate CFU-GM/BFU-E production. Weekly addition of interleukin-3 (IL-3) and granulocyte colony-stimulating factor (G-CSF) supported a high average clonogenic cell output per CA and dramatically increased CA size, but did not significantly alter the apparent CAFC frequency. The generation of CFU-GM per CA was constant over a period of 6 weeks with weekly means of eight normal bone marrow samples ranging between 5-16. At week 6 the mean CAFC frequency was 29/10^5. Early appearing CAFC were highly sensitive to 5-FU, and were contained over the full Rh123 and HLA-DR fluorescence profile of $CD34^{pos}$ cells, whereas CAFC week 5-8 were predominantly contained in the $CD34^{pos}$ $Rh123^{dull}$ $HLA\text{-}DR^{low}$ fraction in agreement with previously reported LTC-IC characteristics. In conclusion, the CAFC assay enumerates LTC-IC using a direct visual endpoint and allows study of LTC-IC heterogeneity with respect to progenitor cell generation per stem cell clone in various hematologic diseases.

INTRODUCTION

Strategies for HSC transplantation and gene therapy demand *in vitro* assays for pre-transplantation enumeration and functional assessment of primitive HSC. These HSC, which have the ability to initiate stable chimerism *in vivo* following their transplantation, are generally referred to as long-term repopulating stem cells, and can be normally assumed in unmanipulated marrow grafts. However, the necessity for frequency analysis of long-term repopulating stem cells has evolved in recent years along with the availability of a variety of novel techniques that assist the clinician in optimizing bone marrow transplantation. Such techniques include the purging of malignant cells from bone marrow or leukapheresis products, and the depletion of non-relevant cells using physical sorting procedures, which could lead to co-depletion of primitive HSC with concomitant risk of graft failure. Pre-transplantation estimation of long-term repopulating stem cell survival and transfection efficiency may also be desirable

when applying *ex vivo* protocols for somatic gene therapy using HSC in individual patients. In addition, the increased interest in the use of leukapheresis products for peripheral blood stem cell transplantation requires insight in the protocol-specific kinetics of long-term repopulating stem cell mobilization in relation to that of the more mature progenitors, which are routinely assayed in clonogenic cell assays. Finally, basic research on the etiology of hematologic diseases and malignancies benefit from a frequency analysis assay fit for routine use.

In vitro frequency analysis of human HSC and progenitors is often performed in a semi-solid clonogenic cell assay. However, this assay is not specific for the most primitive stem cells with potential for long-term engraftment, or may not even detect such cells unless properly stimulated. The ability of primitive stem cells to produce primitive daughter cells and clonable progenitors over an extended period is therefore assessed in stroma-dependent and -independent LTBMC [1-6]. Because LTBMC is a qualitative method, frequency analysis of stem cell subsets with presumed different proliferative ability is not feasible unless carried out in a limiting dilution set-up. The Vancouver group has reported a similar miniaturized LTBMC, in which the frequency of LTC-IC was determined at week 5 by scoring individual wells for replatable clonogenic cells [4,5]. However, assessment of frequencies at multiple time points would require a large culture effort, which prohibits routine use. Another disadvantage of human LTBMC in general as compared to the murine assay is the fact that establishment of adherent stromal layers requires large BMC inocula, preferably from normal donors, and culture periods of 2-6 weeks [3,7], while the variation between experiments is inevitable due to the use of multiple allogeneic bone marrow donors. In order to eliminate these variables, stromal cell lines derived from mouse marrow have been employed to replace human marrow adherent layer feeders [5,7,8].

There exist ample evidence for heterogeneity of the stem cell compartment in the mouse. Most relevant are the differences with respect to the ability of distinct stem cell subsets for long-term or stable engraftment of irradiated stromal layers or mice. We have shown that the murine CAFC assay is a useful and reliable tool for *in vitro* frequency analysis of these different stem cell subsets [9-16]. The CAFC assay is a miniaturized LTBMC, in which cells are overlaid at limiting dilution on previously established irradiated stromal layers and the percentage of wells with at least one phase-dark hematopoietic cobblestone area (a primitive hematopoietic clone localized under the stromal layer) is determined over a period of about 40 days. CAFC frequencies then can be calculated using Poisson statistics. In the murine CAFC assay the extensive use of physically sorted BMC populations, facilitating comparison of *in vivo* stem cell assays with CAFC data, has revealed that the primitiveness of stem cell subsets is related to the onset and duration of CA formation beneath the stromal layer. Thus, CAFC day 10 frequencies correlate with the proportion of stem cells forming spleen colonies on day 12, while CAFC day 28-42 frequencies are directly related to the number of BMC required to induce 40 percent donor-type

engraftment at 6-15 months in sublethally irradiated mice [14,15]. In the present work we have established conditions for a human CAFC assay that meets the requirements for single hit kinetics and reproducible stromal quality, allowing multiple frequency determinations in time in a single assay that renders information on the proportion of distinct stem cell subsets with transient and permanent engraftment potential.

MATERIALS AND METHODS

Bone marrow cells

BMC were obtained by posterior iliac crest puncture from hematologically normal adults who had given their informed consent. A separate donor was used for each experiment. The BMC were collected in Hanks' balanced salt solution (HBSS; Gibco, Breda, The Netherlands) with heparin, diluted in HBSS and layered over a Ficoll-gradient (1.077 g/cm^2; Nycomed, Oslo, Norway). After centrifugation the mononuclear cells were harvested and washed twice in HBSS. The cells were cryopreserved until use in 7.5% dimethyl sulfoxide (BDH, Poole, United Kingdom) and 20% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, U.S.A.).

Fluorescence-activated cell sorting (FACS)

Rh123 (Eastman Kodak, Rochester, NY, U.S.A.) and CD34 staining was performed by incubating the Ficoll BMC with 0.1 μg/ml Rh123 per ml phosphate-buffered saline (PBS; Gibco) for 30 min at 37°C. The cells were then centrifuged and incubated for 15 min at 37°C in PBS containing 5% FCS in order to allow the cells to efflux loaded Rh123. For CD34 staining, the cells were incubated in a second and third incubation step with αCD34-biotin Mab (CellPro, Bothell, WA, U.S.A.) for 30 min at room temperature and Streptavidin Tri-color (Caltag, San Francisco, CA, U.S.A.) for 30 min on ice, respectively.

For combined HLA-DR and CD34 staining, the BMC were incubated in three steps for 30 min on ice with respectively anti-HLA-DR (Becton Dickinson, San Jose, CA, U.S.A.), FITC-conjugated rabbit anti-mouse antibodies (Dako, Glostrup, Denmark) and anti-CD34-PE (anti-HPCA-2 Mab; Becton Dickinson). Between the incubations cells were washed twice in PBS containing 5% FCS and pellets were resuspended. Cells were washed again and resuspended at a concentration of 2 x 10^6 cells/ml PBS with 100 μg/ml DNAse (Calbiochem, La Jolla, CA, U.S.A.). Sorting was performed using a FACS Vantage (Becton Dickenson). The settings of the sorting windows were as shown in Figure 2.1.

Incubation with 5-fluorouracil

5-Fluorouracil (5-FU; Sigma, St Louis, MO, U.S.A.) was dissolved in PBS (1 mg/ml). Ficoll BMC in Iscove's Modified Dulbecco's Medium (IMDM; Gibco) and 10% FCS were incubated with 5, 15, 25 or 40 μg/ml or without 5-FU for 24 h at 37°C. Subsequently, the cells were washed three times with HBSS.

Hematopoietic growth factors

The following purified recombinant human growth factors were kindly provided: G-CSF (Amgen, Thousand Oaks, CA, U.S.A.), granulocyte-macrophage CSF (GM-CSF; Genetics Institute, Cambridge, MA, U.S.A.), IL-3 (Gist Brocades, Delft, The Netherlands) and stem cell factor (SCF; Immunex, Seattle, WA, U.S.A.). Purified recombinant human erythropoietin (EPO) was purchased from Boehringer (Mannheim, Germany).

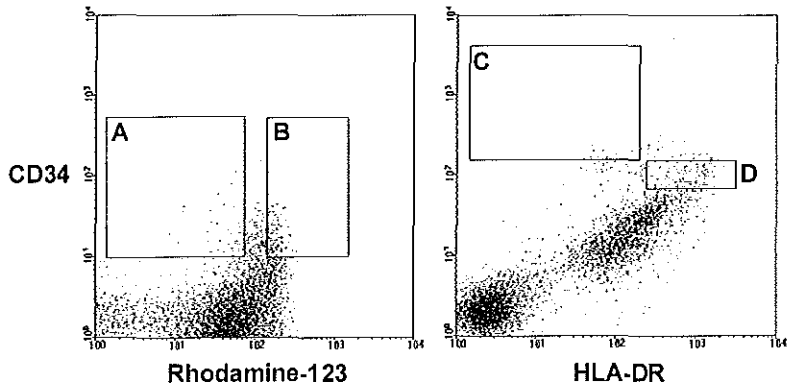

Figure 2.1 Dot plots of log CD34 Tri-Color or PE fluorescence intensity versus log Rh123 or HLA-DR FITC fluorescence intensity. $CD34^{pos}$ cells were sorted into (A) a $Rh123^{dull}$ (20% most dull) and (B) a $Rh123^{bright}$ (30% most bright) fraction. $HLA\text{-}DR^{low}$ cells were sorted from a $CD34^{high\ pos}$ fraction (C) and (D) $HLA\text{-}DR^{bright}$ cells from a $CD34^{medium\ pos}$ fraction.

Methylcellulose cultures

Quantification of CFU-GM and BFU-E was performed using a semisolid (0.88% methylcellulose; Methocel, Stade, Germany) culture medium (IMDM) at 37°C and 5% CO_2. The cultures contained 30% FCS, 0.75% charcoal-treated bovine serum albumin (Sigma) supplemented with human transferrin (0.6 mg/ml; Behringwerke, Marburg, Germany), lecithin (20 μg/ml; Merck, Darmstadt, Germany), sodium-selenite (0.2 ng/ml; Merck) and β-mercapto-ethanol ($5x10^{-5}$ M; Merck), and the cytokines EPO (1 U/ml), IL-3 (12.5 ng/ml), G-CSF (50 ng/ml), GM-CSF (5 ng/ml) and SCF (5 ng/ml) all at final concentrations. Colony-forming units-granulocyte macrophage (CFU-GM) and burst-forming units-erythroid (BFU-E) were counted on day 14 of culture in the same dish.

Stromal feeders

The FBMD-1 stromal cell line was derived from the confluent adherent layer of a 16-day old flask culture established from female adult C57BL/6 murine BMC grown in Fischer's medium (Gibco) with 20% FCS at 33°C in 5% CO_2. The trypsinized cells were subcultured in 75 cm^2 flasks at a split ratio of 1:3 in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 10% FCS and 10^{-5} hydrocortisone 21-hemisuccinate. As determined by PCR, unstimulated FBMD-1 cells express RNA for transforming growth factor-ß, macrophage-CSF, IL-6 and SCF, but not for IL-1, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, GM-CSF, tumor necrosis factor-α or leukemia inhibitory factor. FBMD-1 stromal feeders were used between the 16th and 20th passage and prepared by seeding 10^5 FBMD-1 cells from log-phase cultures into 25 cm^2 culture flasks (Costar, Badhoevedorp, The Netherlands or Falcon, Meylan Cedéx, France) or 10^3 cells per well into 96-well plates (Falcon). Culture plastics destined for establishment of FBMD-1 stromal feeders were incubated overnight with 0.1% gelatin (Sigma) to improve adherence of the stromal layer. The FBMD-1 cells were cultured in α-modified DMEM supplemented with HEPES (3.5 mM; Sigma), glutamine (2 mM; Sigma), sodium-selenite (10^{-7} M), β-mercapto-ethanol (10^{-4} M), 10% FCS, 5% horse serum (HS; Gibco) and hydrocortisone 21-hemisuccinate (10^{-5} M final concentration; Sigma). After 7 to 10 days of incubation at 33°C the stromal layer had reached confluence and were used within the following 2 weeks.

Long-term bone marrow cultures in flasks

Confluent stromal layers of FBMD-1 cells in 25 cm^2 flasks were overlaid with 1-4 x 10^6 Ficoll

BMC. The cells were cultured in α-modified DMEM supplemented with HEPES (3.5 mM), glutamine (2 mM), sodium-selenite (10^{-7} M), β-mercapto-ethanol (10^{-4} M), 12.5% FCS, 12.5% HS and hydrocortisone 21-hemisuccinate (10^{-6} M final concentration). Unless otherwise indicated, IL-3 (10 ng/ml) and G-CSF (20 ng/ml) were added weekly to the cultures. Flask cultures of each bone marrow sample were set up in triplicate and maintained at 33°C for 6 weeks with weekly half-medium changes and therefore removal of only half of the non-adherent (NA) cells. The NA cells, CFU-GM and BFU-E output of individual flask cultures was determined weekly. At the end of 6 weeks the number of CFU-GM and BFU-E in the adherent layer was also determined. To this purpose, the medium was removed from the flasks and replaced by 3 ml of 0.1% trypsin (Gibco) for 5 minutes. The digestion was stopped by adding 1 ml of ice-cold FCS. 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 cell suspension were plated in a semi-solid clonogenic cell assay.

Cobblestone area forming cell assay

Confluent stromal layers of FBMD-1 cells in 96-well plates were overlaid with sorted or unsorted Ficoll BMC in a limiting dilution setup. Input values ranged between 24 and 50,000 nucleated cells per well for Ficoll BMC and between 1 and 2,000 nucleated cells per well for sorted cells. 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 LTBMC in flasks. The percentage of wells with at least one phase-dark hematopoietic clone of at least 5 cells (cobblestone area) beneath the stromal layer was determined weekly for 6-8 weeks and CAFC frequencies were calculated using Poisson statistics as described previously [9].

RESULTS

Growth factors effects on CAFC frequencies and clonogenic cell production in LTBMC

The murine stromal cell line FBMD-1 allowed easy detection of phase-dark CA under the stromal layer (Figure 2.2). Weekly addition of low IL-3 and G-CSF concentrations resulted in a significant increase in average CA size, thereby enhancing the detection level and minimizing scoring time. The apparent frequency of CAFC did not, or only slightly, increase in IL-3 + G-CSF containing cultures (Table 2.1). However, addition of IL-3, with or without EPO, led to clearly increased CA formation but unaltered CFU-GM production from parallel flask LTBMC, resulting in a decreased proliferative potential of CAFC, as indicated by the average number of clonogenic cells produced per CAFC clone. The presence of exogenous IL-3 + GM-CSF also increased CAFC frequencies but significantly decreased the clonogenic cell production in LTBMC so that the average weekly clonogenic cell output per CAFC clone was even more reduced. Because of these findings we adapted the addition of IL-3 + G-CSF as standard condition in our CAFC assay. Apparent CAFC frequencies in the absence of exogenous growth factors were only 30% lower on week 5 and similar on week 8 as compared with the IL-3 + G-CSF containing cultures. These observations indicate that the murine stromal cell line FBMD-1 is permissive for successful clonal expression of LTC-IC and long-term production of clonogenic cells, and that IL-3 + G-CSF merely enhanced the clonogenic cell output per LTC-IC.

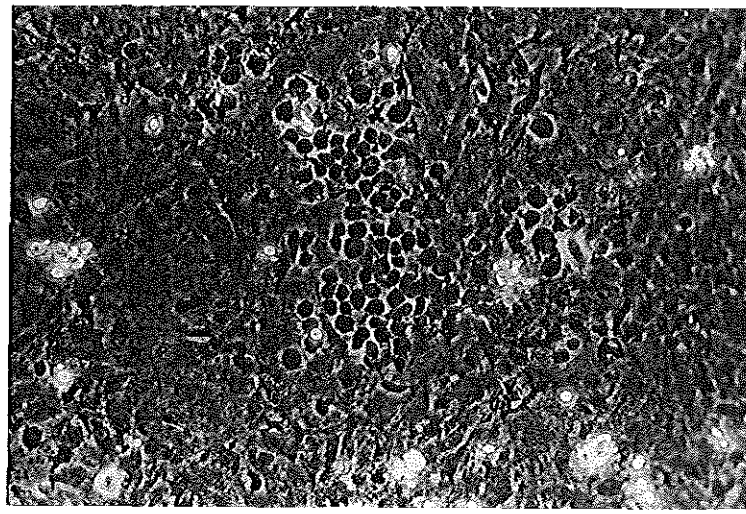

Figure 2.2 Phase contrast micrograph of a human cobblestone area situated under the murine preadipose stromal cell line FBMD-1.

Table 2.1 Effect of Exogenous Cytokines on CAFC Frequencies and Clonogenic Cell Production in Long-Term Bone Marrow Cultures.

Hematopoietic growth factors	Clonogenic cells produced at week 5 (%)[a]	CAFC frequencies (%)[a]		Clonogenic cells produced/ CAFC week 5[b]
		week 5	week 8	
IL-3 + G-CSF (n = 5)	100	100	100	14.7
None (n = 1)	ND	67	99	ND
IL-3 (n = 2)	113	408	265	4.8
IL-3 + GM-CSF (n = 1)	19	302	292	1.6
IL-3 + EPO (n = 1)	80	703	233	1.5

ND, Not determined.

[a], CAFC frequencies and clonogenic cell production are expressed as percentage of cultures in which IL-3 + G-CSF were added. Growth factors were added weekly at concentrations of 5-10 ng IL-3/ml, 20 ng G-CSF/ml, 2 ng GM-CSF/ml or 0.2 U EPO/ml.

[b], Absolute number of CFU-GM and BFU-E produced per 10^5 BMC in flask cultures divided by the week 5 CAFC proportion as determined in parallel limiting dilution assay.

Long-term bone marrow culture of Ficoll bone marrow cells

Figure 2.3 shows the ability of the FBMD-1 stromal feeders to support NA

clonogenic cell and terminal cell output from ten different Ficoll bone marrow samples cultured with exogenous IL-3 and G-CSF. As compared to input values (week 0), continuous high and stable production of NA CFU-GM and nucleated cells was observed. BFU-E generation typically declined during the culture period but remained detectable up to week 6. At week 6 the number of clonogenic cells in the stromal layer was also determined following trypsinization and replating. The mean CFU-GM and BFU-E in the adherent layer of the ten bone marrow samples tested were 320 (1 SEM: 186) CFU-GM and 23 (11) BFU-E per 10^5 input cells (data not shown).

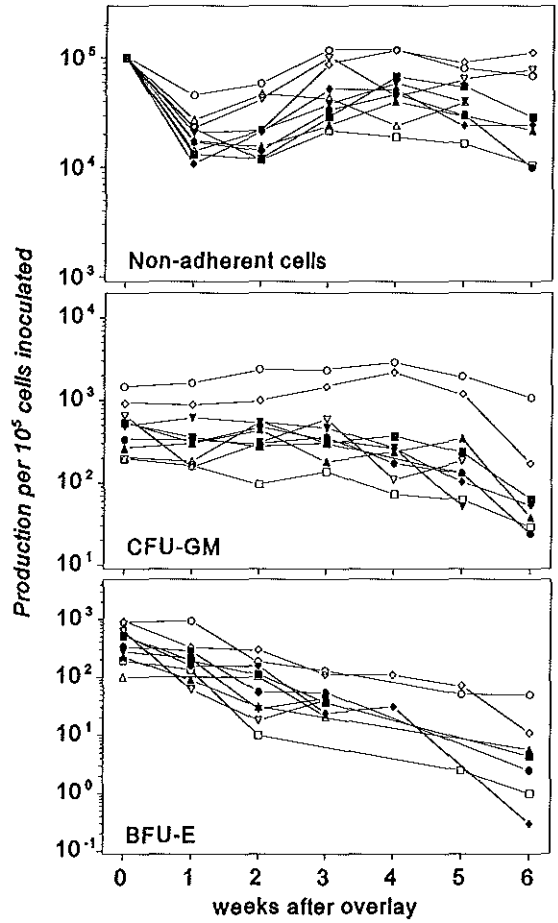

Figure 2.3 Serial non-adherent nucleated cell, CFU-GM and BFU-E production per 10^5 input cells in flask cultures of Ficoll bone marrow from ten normal donors with input values ranging between 1-4x10^6 nc per flask. Data were not corrected for weekly demi-population at medium changes. Each symbol represents one marrow sample. Data on week 0 represent the input values.

CAFC assay of Ficoll bone marrow cells

From eight out of ten Ficoll bone marrow samples used for flask LTBMC experiments (see figure 2.3) weekly CAFC frequencies were determined for at least 8 weeks with weekly addition of IL-3 and G-CSF (Figure 2.4). CAFC frequencies differed between the various samples as was also observed for the clonogenic cell outputs in flask LTBMCs, with a characteristic peak CAFC frequency at week 2-3, and subsequent gradually decreasing CAFC frequencies at later observation points. The CFU-GM and BFU-E in these samples ranged between 195-1430 and 100-875, respectively, per 10^5 and accurately reflected the week 1-3 CAFC frequencies. The mean CAFC frequency (1 SEM) of the eight bone marrow samples studied was 44 (14) per 10^5 cells inoculated as determined at week 5, and 13 (4.2) at week 8. These results are almost identical to previously reported LTC-IC frequencies in Percolled BMC, with a LTC-IC week 5 frequency of 37 per 10^5 cells and 15 per 10^5 cells at week 8 [5].

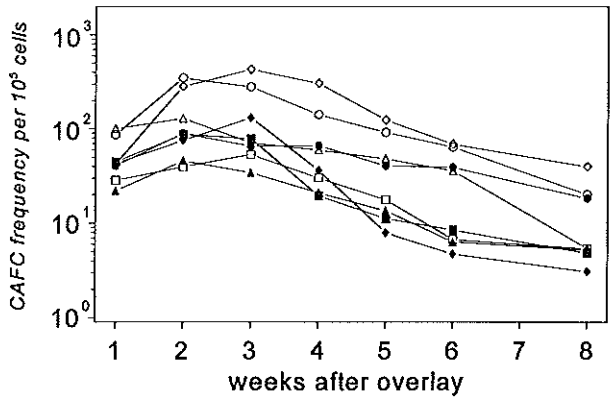

Figure 2.4 Frequency distribution of CAFC week-types in bone marrow from eight normal donors with input values ranging between 24 and 50,000 nc per well as determined using limiting dilution type assay. Twelve dilutions two-fold apart were used for each sample with 15 replicate wells per dilution. Symbols correspond to those used to discriminate samples in figure 2.3.

Single hit kinetics of the CAFC assay

Figure 2.5 shows that the presence of phase contrast dark CAs was strictly determined by the number of BMC overlaid. Typical data sets representing percentages of negative wells at a series of limiting BMC inocula for a single Ficoll BMC sample (Figure 2.5A) and a Ficoll $CD34^{pos}$ $Rh123^{dull}$ sample (Figure 2.5B) over an 8-week culture period are shown. The single hit kinetics of the CAFC assay is clearly illustrated by the observation that linear fitted lines through the weekly date sets extrapolate to the origin of the coordinate system.

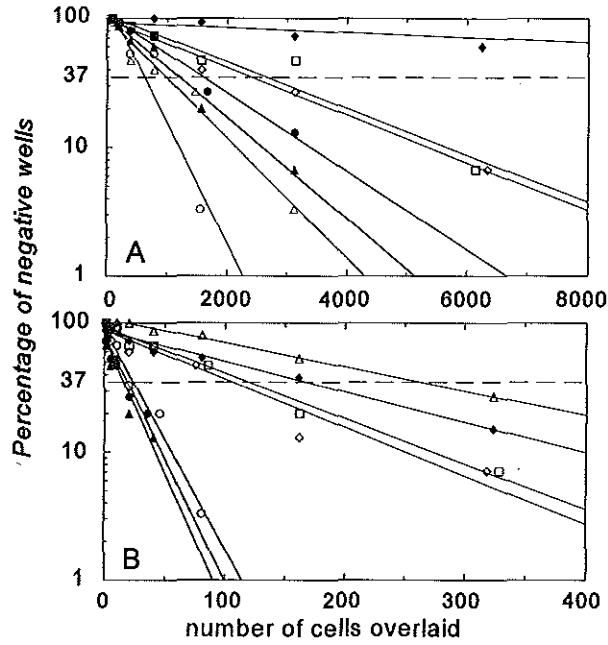

Figure 2.5 The presence of human cobblestone areas on the murine FBMD-1 stroma is strictly determined by the number of BMC overlaid. Typical data sets of percentage negative wells at a series of BMC dilutions for a single Ficoll BMC sample (A) and a Ficolled $CD34^{pos}$ $Rh123^{dull}$ sample (B) over an 8-week culture period. The single hit kinetics of the CAFC assay is clearly illustrated by the observation that linear fitted lines through the weekly data sets extrapolate to the origin of the coordinate system. ▵, week 1; ○, week 2; ▲, week 3; ●, week 4; ◇, week 5; □, week 6; ◆, week 8.

CAFC frequency relates to clonogenic cell production in long-term cultures

Figure 2.6 represents the weekly data sets from eight normal bone marrow samples accumulated during a 6 week culture. The number of NA clonogenic cells (CFU-GM + BFU-E) produced in flask cultures was linearly related to the proportion of CAFC in corresponding samples. Calculation of the generation of either NA cells, CFU-GM or BFU-E per CAFC, as determined in parallel flask cultures and limiting dilution type assays (Figure 2.7), showed a steady weekly CFU-GM production per CAFC clone in combination with decreasing BFU-E values and increasing cell output. These data indicate that the scoring of CAs over a 6-week culture period is a measure of CFU-GM production of the samples overlaid. These observations are in difference with the increasing clonogenic cell output that we previously reported for murine LTBMC [10]. Another difference with the murine CAFC is the relatively high frequency of late human CAFC relative to those of early appearing CAFC and the resulting possibility to maintain active human LTBMC over periods in excess of six weeks.

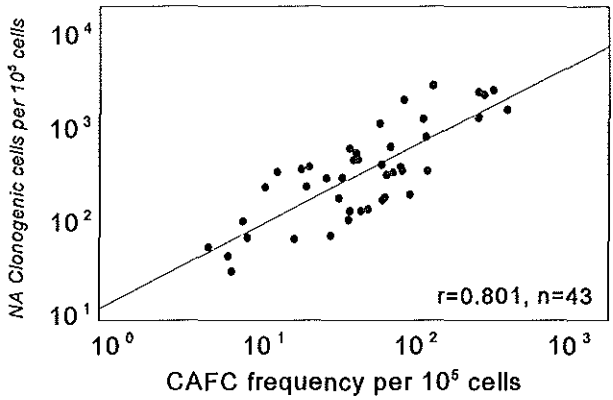

Figure 2.6 The number of non-adherent (NA) clonogenic cells produced in flask long-term cultures is linearly related to the proportion of CAFC in corresponding samples. The figure represents the weekly data sets from eight normal Ficoll bone marrow samples accumulated between week 1 and 6. Data were not corrected for weekly demi-population at medium changes.

The CAFC assay enumerates a series of phenotypically different stem cell subsets

Frequency distribution of a series of CAFC subsets was determined in BMC fractions sorted from Ficoll bone marrow on the basis of light scatter, affinity for CD34 and HLA-DR, and Rh123 retention. The sort gate settings (Figure 2.1) were deliberately chosen as to clearly separate primitive and more mature stem cell subsets rather then characterizing all fractions from a full fluorescence profile. In a series of three experiments 20% of the most $Rh123^{dull}$ and 30% of the most $Rh123^{bright}$ cells were sorted from the $CD34^{pos}$ window (Figure 2.8). CAFC week 5-8 were predominantly, but not exclusively, contained in the $Rh123^{dull}$ region of the $CD34^{pos}$ cells whereas CAFC week 1-2 were distributed among the full Rh123 retention spectrum. These findings agree with previously reported characteristics of LTC-IC by Udomsakdi et al. [17]. However, far more CFU-GM and BFU-E were contained in the lower part of the Rh123 fluorescence profile than among the top $Rh123^{bright}$ cells (Figure 2.9). This seems in difference with the equal distribution of clonogenic cells over the Rh123 profile in bone marrow [17], but is in agreement with the predominance of CFU-GM and BFU-E in the $Rh123^{dull}$ fraction of peripheral blood mononuclear cells [18].

From one donor HLA-DR^{low} $CD34^{high\ pos}$ and HLA-DR^{high} $CD34^{medium\ pos}$ Ficoll BMC were sorted (Figure 2.10). CAFC week 1 and 2 were more enriched in the HLA-DR^{high} $CD34^{medium\ pos}$ fraction, but the majority of CAFC week 4 to 6 was concentrated in the HLA-DR^{low} $CD34^{high\ pos}$ fraction. Plate mapping of wells that were scored positive for CA content during the whole culture period allowed an estimation of the maximum clonogenic potential of the samples studied, and provided insight into the percentage of CAFC that had started clonal expansion

at a certain time point (Table 2.2). While 50% of all CAFC in unsorted Ficoll bone marrow samples, $CD34^{pos}$ $Rh123^{bright}$ and $CD34^{medium\ pos}$ $HLA\text{-}DR^{high}$ cells had initiated clonal amplification between weeks 1 and 2, new CAFC clones were observed even between week 3 and 5 in the $HLA\text{-}DR^{low}$ $CD34^{high\ pos}$ and the $CD34^{pos}$ $Rh123^{dull}$ fraction.

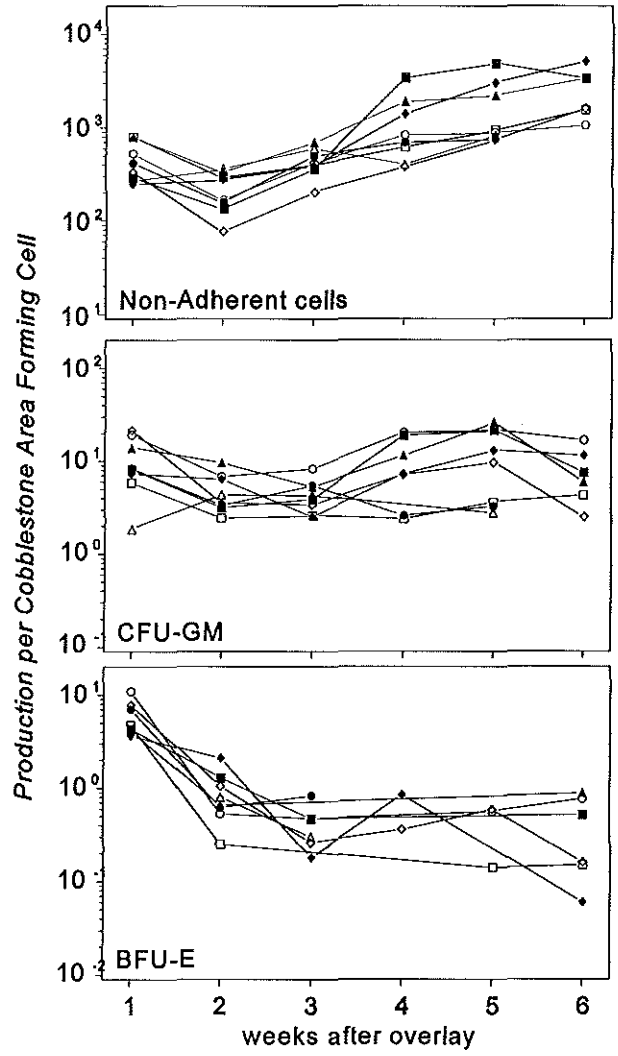

Figure 2.7 Calculated non-adherent cell, CFU-GM and BFU-E production as assessed in flask cultures per CAFC as determined in parallel limiting dilution type cultures. The data show a steady weekly CFU-GM production per CAFC clone in combination with decreasing BFU-E values and increasing cell output.

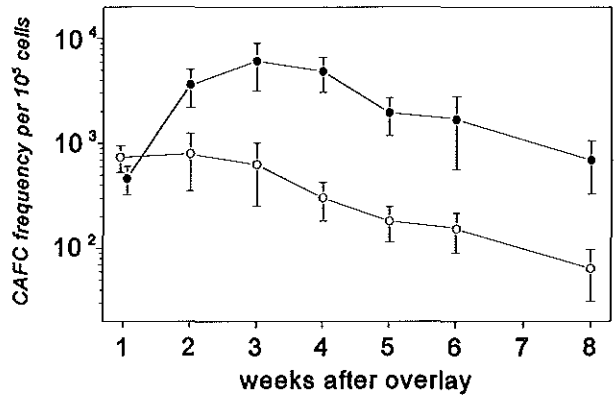

Figure 2.8 Average proportion of CAFC week-types in Rh123^{dull} (●; 20% most dull) and Rh123^{bright} (O; 30% most bright) Ficoll CD34^{pos} BMC from three separate donors (±1SD). The figure shows that late CAFC are predominantly, but not exclusively, contained in the Rh123^{dull} region of the CD34^{pos} cells, whereas early CAFC are distributed among the full Rh123 retention spectrum.

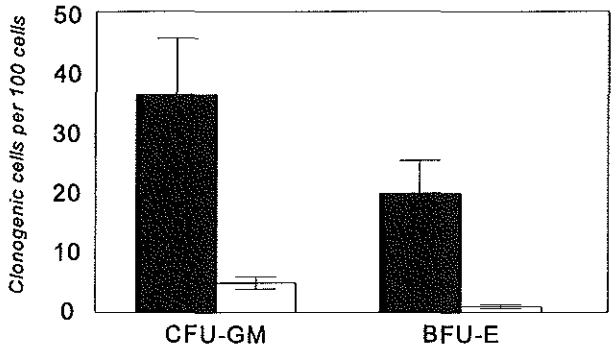

Figure 2.9 Number of CFU-GM and BFU-E determined in the 20% most Rh123^{dull} (■) and 30% top Rh123^{bright} (□) CD34^{pos} populations per 100 sorted cells (±1SD).

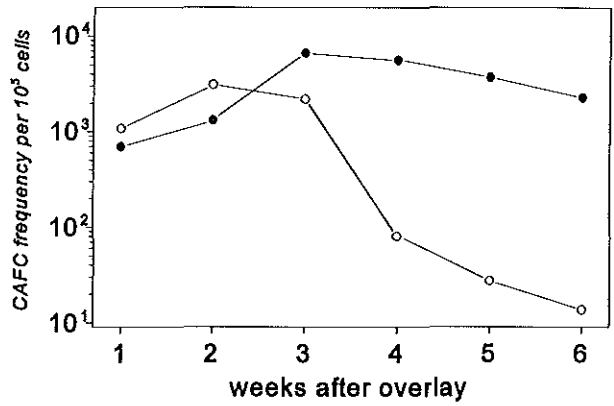

Figure 2.10 Proportion of CAFC week-types in HLA-DR^{low} $CD34^{high\ pos}$ (●) and HLA-DR^{high} $CD34^{medium\ pos}$ (O) Ficoll BMC from a single sample. The distinct frequency distribution of early and late CAFC in the sorted bone marrow samples clearly demonstrates that the CAFC assay allows detection of distinct stem cell subsets with extremely different capacity for initiating long-term stroma-dependent hematopoiesis.

Table 2.2 Kinetics of Maximum Clonogenic Activity of CAFC in Various Bone Marrow Samples as Determined by Plate Mapping.

	Percentage of maximum CAFC frequency	
	50% at week no.	100% at week no.
Ficoll, unsorted	1-2	3
$CD34^{pos}$ $Rh123^{dull}$	2-3	5
$CD34^{pos}$ $Rh123^{bright}$	1-2	4
$CD34^{high\ pos}$ HLA-DR^{low}	2-3	5
$CD34^{medium\ pos}$ HLA-DR^{high}	1-2	3

Data were extracted from all reported CAFC cultures presented in this chapter.

CAFC week-types differ in their resistance to 5-fluorouracil

Resistance to 5-FU has been used to identify and characterize primitive quiescent stem cells among BMC and peripheral blood cells [19]. After a 24 h incubation of Ficoll BMC with 5-FU, frequencies of CFU-GM, BFU-E and CAFC were determined. Of all clonogenic cells detected in the semi-solid assay, 23% of the BFU-E and 13% of the CFU-GM showed some resistance to 40 μg 5-FU /ml (Table 2.3). Only 11-31% of CAFC week 1 and 2 were resistant to 15 to 40 μg 5-FU/ml incubation, as compared to 52-90% of the CAFC week 4 to 6. These results strongly suggest that CAFC frequencies determined on week 4 and later represent more primitive HSC subsets than those at week 1 and 2.

Table 2.3 Effect of Pre-Incubation with Different Doses of 5-Fluorouracil on CFU-GM, BFU-E and CAFC Frequencies in Ficoll Bone Marrow.

	5-fluorouracil concentration (μg/ml)				
Stem cell subset	0	5	15	25	40
CFU-GM	100	83	36	25	13
BFU-E	100	68	45	32	23
CAFC week 1	100	100	25	21	16
CAFC week 2	100	95	31	16	11
CAFC week 3	100	106	57	40	33
CAFC week 4	100	104	90	70	52
CAFC week 5	100	ND	81	80	75
CAFC week 6	100	ND	81	ND	ND

All data are expressed as percentage of progenitor cell recoveries in the groups not containing 5-fluororuacil. ND, Not determined.

DISCUSSION

The present data demonstrate, that the human CAFC assay allows frequency analysis of a series of distinct HSC subsets without the necessity to replate large numbers of single wells. The use of a stromal cell line for establishment of supportive feeders ensures linear hit kinetics of the assay, facilitates easy and rapid preparation of homogenous feeders and minimizes inter-assay variability as may occur when using different allogeneic stromal donors. We show here that the proportion of CAFC in a suspension linearly relates to the ability of the inoculated cells to generate CFU-GM in flask LTBMC using unseparated BMC fractions, indicating that the method does not require previous separation of the samples. Similarly, LTC-IC frequencies have been reported to show a linear relationship with the size of the BMC inoculum, revealing a 1:4 ratio between LTC-IC proportion and clonogenic cell numbers produced in parallel flask cultures [4,5]. This finding has been exploited to calculate LTC-IC frequencies in normal bone marrow from the clonogenic cells produced per flask [20]. However, since cytostatic treatment and protocols for stem cell selection of malignant cell purging are likely to select for HSC subsets with different clonogenic potential even at 5-8 weeks of LTBMC, it seems recommended to determine both CAFC or LTC-IC proportion and ability to generate clonogenic cells in parallel cultures, allowing study of the average clonogenic potential of CAFC. In addition, although frequency analysis of a single sample at multiple time-points is time-consuming, it presents the clinician with a full insight into the proportions of a series of stem cell subsets varying between progenitor cells to HSC with transient and long term repopulating ability.

Sutherland and colleagues have reported that M2-10B4 murine stromal cells

supported maintenance of LTC-IC as effective as standard human marrow layers, and that genetic engineering of these cells to produce IL-3 and G-CSF was able to further enhance the maintenance and differentiation of LTC-IC without a concomitant decline in their proliferative potential as measured by the clonogenic cell output per LTC-IC [5]. The beneficial effect of exogenous gibbon IL-3 on CFU-GM production in human LTBMC has also been reported [21]. Our murine preadipose stromal cell line FBMD-1, which has been selected for identical support of murine stem cell propagation in comparison with primary murine stromal layers (S. Neben and R.E. Ploemacher, unpublished data), also gave excellent and long-term support for human CAFC development irrespective of the presence of exogenous growth factors. In our cultures, the presence of low concentrations of exogenous IL-3 and G-CSF gave no significant increase in the apparent CAFC frequencies at week 5-8 but led to a dramatic increase in CA size which facilitated optimal visual identification at scoring.

The CAFC assay enumerates a series of HSC that differ in their phenotypic and functional characteristics. Although it has not yet been fully established what the characteristics are of human LTC-IC, it is evident that CAFC and LTC-IC week 5-8 share a series of functional and phenotypic properties. Thus, 52-90% of late CAFC are 5-FU resistant as compared with 60% 4-HC resistant LTC-IC [22]. Similarly to LTC-IC [17], CAFC week 5-8 are preferentially, but not exclusively, contained in the $CD34^{pos}$ $Rh123^{dull}$ fraction of Ficolled BMC, rather than in the $CD34^{pos}$ $Rh123^{bright}$ fraction, demonstrating the ability of CAFC/LTC-IC to efficiently efflux Rh123 due to high expression of P-glycoprotein, a product of the MDR1 gene [23]. In addition, both LTC-IC [5] and CAFC week 5-8 can be highly enriched in the $HLA\text{-}DR^{low}$ fraction from the top 2% of the CD34 fluorescence profile. Further, frequencies of CAFC and LTC-IC are strikingly similar. Thus, using a limiting dilution set-up the frequency of LTC-IC week 5 and 8 in Percolled BMC has been previously estimated at about 37 and 15, respectively, per 10^5 nucleated cells, while a highly purified $CD34^{pos}$ $HLA\text{-}DR^{low}$ subpopulation was assessed to contain 1,300-2,300 LTC-IC per 10^5 nucleated cells [5]. In our experiments, the proportion of CAFC week 5 and 8 was 44 and 13, respectively, per 10^5 Ficolled cells, with about 3,700 CAFC week 5 per 10^5 $CD34^{high\ pos}$ $HLA\text{-}DR^{low}$ BMC. The latter frequency represented an estimated 80-fold enrichment for CAFC week 5 over their frequency in Ficoll BMC and a 600-fold enrichment as compared to unseparated BMC. In difference with the phenotype of CAFC/LTC-IC week 5-8, we have observed that early appearing CA have distinct properties, i.e. a) 11-31% of CAFC week 1-2 is 5-FU resistant in agreement with the properties of BFU-E and CFU-GM, whereas b) $CD34^{pos}$ CAFC week 1-2 are found over the full HLA-DR and Rh123 fluorescence profile.

In conclusion, the human CAFC assay exploits a visual endpoint for multiple enumeration of a series of stem cell subsets differing in their sensitivity to 5-FU, expression of CD34, HLA-DR and Rh123, and their ability for transient and long-term engraftment of stromal layers. The striking overlapping characteristics of human and murine CAFC subsets strongly suggests that the human CAFC week 1-2 subset is analogous to the murine spleen colony forming cell, whereas the

human CAFC week 5-8 enumerates primitive stem cells that are responsible for the sustained maintenance of blood cell production or for stable *in vivo* engraftment of hematopoiesis following cytotoxic treatment. The validation of the human CAFC/LTC-IC week 5-8 method as an assay detecting *in vivo* engrafting potential awaits long-term follow-up of sufficient numbers of patients (co)engrafted with genetically marked BMC, which include the phenotypic criteria described for these primitive stem cells.

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CHAPTER 3

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 hematopoietic recovery than bone marrow. The development of different protocols for chemotherapy conditioning, mobilization and *ex vivo* manipulation of PBSC may potentially lead to loss of primitive hematopoietic 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 2 as a tentative indicator of progenitor cells and transiently repopulating HSC ranged from 0.89 to 205 per 10^5 mobilized nucleated cells and the frequencies of more primitive CAFC week 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, bone marrow 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 week 6 per kg body weight.

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 week 6 in LPs. The frequency of CFC, immunophenotypic subsets and CAFC subsets transplanted and the transplant quality as determined in LTC assays was related with 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 hematopoietic 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 hematologic malignancies refractory to standard chemotherapeutic regimens [1-4]. The finding that HSC are mobilized by hematopoietic 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 hematopoietic growth factors have an advantage over bone marrow 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 week 1 to 3) and the 5-fluorouracil resistant LTC-IC (CAFC week 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 bone marrow 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 3.1. Two days after the last course of chemotherapy, G-CSF (Filgrastim, recombinant-methionyl human G-CSF; 5 μg/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 ($CD34^{pos}$) 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 $CD34^{pos}$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 bone marrow cells and during the hematopoietic recovery no relapse of disease was

observed. In 20 patients a complete hematopoietic 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 3.1. Thawed control ampules 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 bone marrow cells the historic data from eight normal bone marrow samples [18] together with four additional unmanipulated bone marrow samples from one normal donor and from one Hodgkin disease and two non-Hodgkin lymphoma patients in remission were used.

Immunofluorescence analysis

CD34 staining was performed by incubating 10^6 nucleated cells (NC) after erythrocyte lysis for 30 minutes with anti-CD34-FITC (anti-HPCA-2 Mab; Becton Dickinson, San Jose, CA). 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). 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 3.1 shows the dot plot analysis of one of the LPs with the windows which were used. CAFC analyses with combined CD34 and CD38 sorted LPs have shown that the highest CAFC week 6 to 8 frequencies are found in the $CD34^{pos}$/CD38-negative fraction. However, the majority of the late CAFC week 6 to 8 were found in the and $CD34^{pos}$/CD38-dim fraction (R.E. Ploemacher et al., unpublished observations). Therefore, the percentage of $CD34^{pos}$/CD38-negative and dim ($CD34^{pos}/CD38^{neg/dim}$) cells was determined using dot plot 1A, window 1. Several authors have shown that the highest frequency of LTC-IC and late CAFC week 5 and 6 are contained in the $CD34^{pos}$ and HLA-DR-low ($CD34^{pos}/HLA\text{-}DR^{low}$) fraction [16,18]. As a result, in dot plot 1B, window 2 the percentage $CD34^{pos}/HLA\text{-}DR^{low}$cells was determined.

Hematopoietic 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), human GM-CSF and murine SCF (Genetics Institute, Cambridge, MA) and human interleukin-3 (IL-3; Gist Brocades, Delft, The Netherlands).

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 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), penicillin (100 U/ml; Gibco), streptomycin (100 µg/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.

Table 3.1 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 $CD34^{pos}$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.

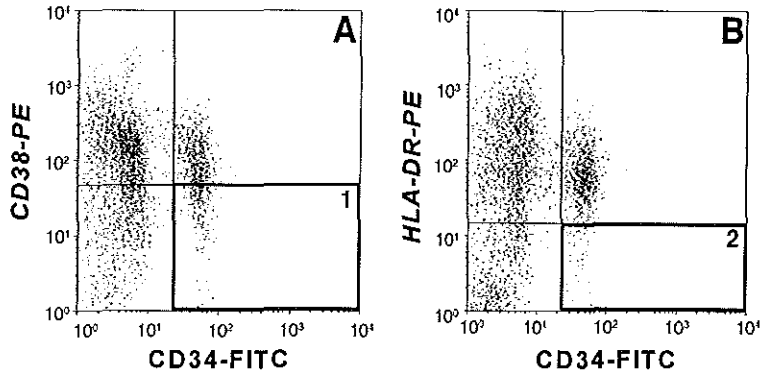

Figure 3.1 Immunophenotyping of leukapheresis products. Dot plot image showing the windows from which the percentages of $CD34^{pos}/CD38^{neg/dim}$cells (Dot plot 1A, window 1) and $CD34^{pos}/HLA\text{-}DR^{low}$cells (Dot plot 1B, window 2) were determined.

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) or 10^3 cells per well into flat-bottom 96-well plates (Falcon, Lincoln Park, NJ). 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 μg/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 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 μg/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 set up 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 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 hematopoietic 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). 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). 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 3.2, 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 and on average two-fold less than were detected in the supernatant of the same flasks at that time. The dashed lines in figure 3.2B 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 bone marrow samples in LTC as previously reported [18].

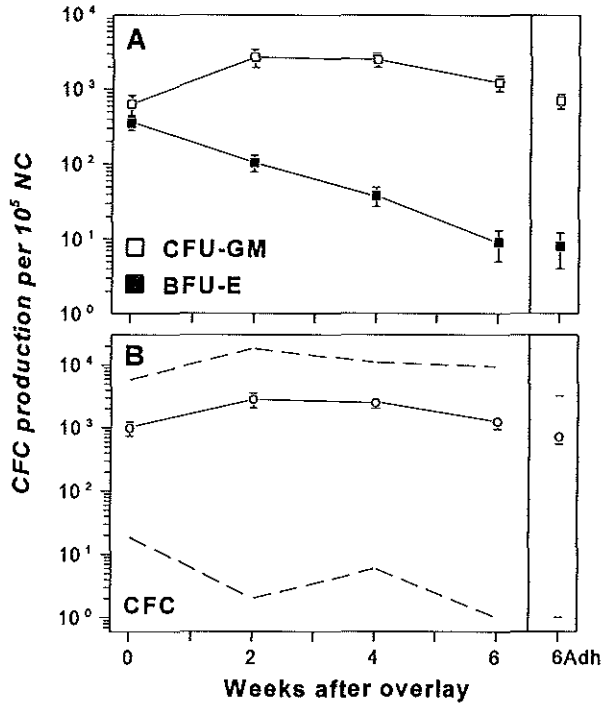

Figure 3.2 Mean (± 1SEM) primary CFU-GM (Figure 3.2A, □), BFU-E (Figure 3.2A, ■) and total colony forming cell (CFC; Figure 3.2B, O) content (week 0), non-adherent CFC production at week 2, 4 and 6 and CFC numbers in the adherent stromal layer at week 6 (6Adh) in flask LTC per 10^5 nucleated cells (NC) of 36 samples containing 47 leukapheresis products from 21 patients. Dashed lines in figure 3.2B 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 3.3A. Similar to the CFC output in flask LTC, the CAFC frequencies differed among the various patients. The frequencies of CAFC week 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 week 6 ranged from 0.37 and 48. However, following a correction of the data for the number of $CD34^{pos}$cells these ranges of values were condensed to less than a 40-fold difference between the observed extremes (Figure 3.3B). On average, lower CAFC week 1 to 6 frequencies were found in LPs as compared to bone marrow samples (Table 3.2). This difference was larger at early weeks (week 2: 3.7-fold) than at later weeks (week 6: 2.3-fold).

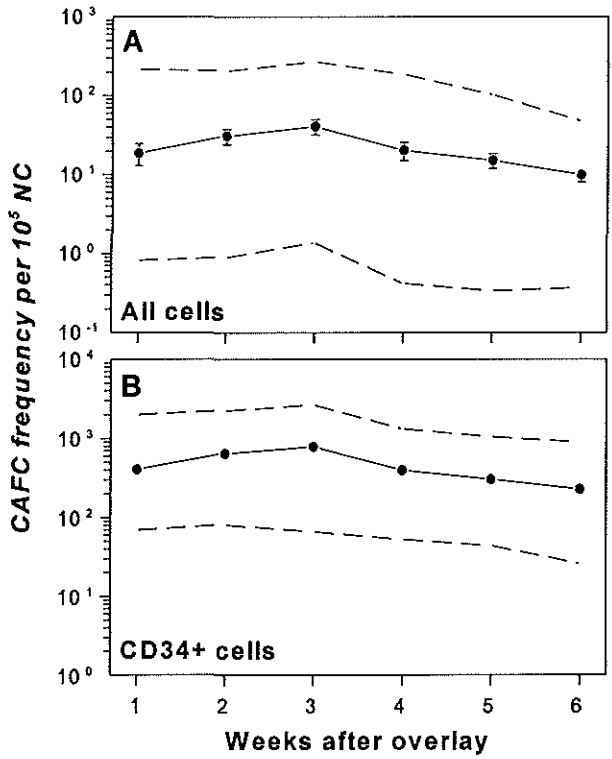

Figure 3.3 Mean (±1SEM) frequency distribution of CAFC week-types per 10^5 all nucleated cells (NC) (Figure 3.3A) and per 10^5 $CD34^{pos}$cells (Figure 3.3B) in 36 samples containing 47 leukapheresis products from 21 patients. Dashed lines show the maximum and minimum CAFC frequency of the 36 samples.

Table 3.2 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 (n=12)	47 (±9)	114 (±28)	110 (±34)	69 (±25)	36 (±10)	23 (±7)
Leukaphereses (n=36)	19 (±6)	31 (±7)	41 (±9)	21 (±5)	15 (±3)	10 (±2)

Quality analysis of LP and bone marrow 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 3.3 shows the average number of NA-CFC produced per CAFC week 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 bone marrow, 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 bone marrow, the total NA- and SA-CFC production in LTC at week 6 was related with the CAFC week 6 frequency (Figure 3.4). In bone marrow any increase in CAFC week 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 bone marrow 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 week 6 harvested (Figure 3.5). LPs with an absolute yield of less than 5,000 CAFC week 6 harvested per kg body weight expressed an average poor stem cell quality (Mean ±1SEM: 74 ±31), while harvests containing a total of 5,000 or more CAFC week 6 per kg body weight 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 3.3 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 week 6
	1	2	3	4	5	6	
Bone Marrow (n=12)	16 (±3)	8 (±2)	12 (±5)	11 (±2)	13 (±2)	9 (±2)	23 (±5)
Leukaphereses (n=36)	ND	73 (±11)	ND	115 (±19)	ND	114 (±23)	188 (±33)

Abbreviations: CAFC, Cobblestone Area Forming Cell; CFC, Colony Forming Cell; NA, Non-Adherent; ND, Not Determined; SA, Stroma-Adherent.

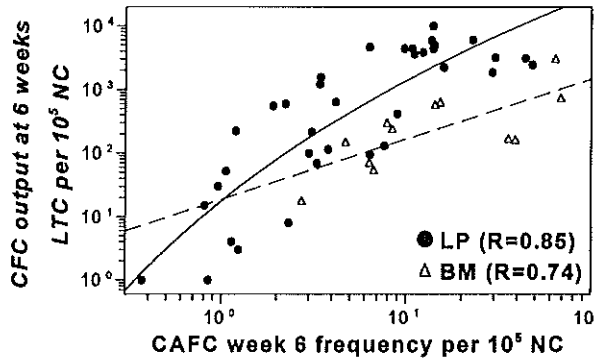

Figure 3.4 Qualitative comparison of leukapheresis products (LP) and bone marrow (BM). The non-adherent and stroma-adherent colony forming cell (CFC) output at 6 weeks long-term culture (LTC) is related to the proportion of CAFC week 6. The figure represents the data sets from 36 LP samples and from 12 bone marrow samples. In bone marrow 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, Nucleared Cells.

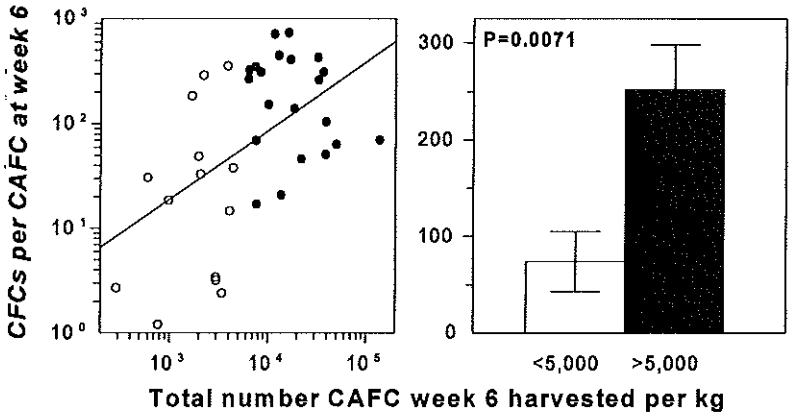

Figure 3.5 The non-adherent and stroma-adherent colony forming cells (CFC) per CAFC at week 6 stem cell quality in leukapheresis products is a function of the total number of CAFC week 6 mobilized. Leukapheresis products with a total CAFC week 6 number per kg body weight below 5,000 (O) had a statistically 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 week 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 3.1). $CD34^{pos}$cell percentages ranged from 0.31% to 18%. The percentage of $CD34^{pos}/CD38^{neg/dim}$cells varied between 0.01% and 3.6% and $CD34^{pos}/HLA\text{-}DR^{low}$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 3.4). CFC frequencies showed a good correlation with $CD34^{pos}$cells ($r_s = 0.82$, $P < 0.0001$), $CD34^{pos}/CD38^{neg/dim}$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, $P \leq 0.0002$).

Table 3.4 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-positive	CD34-pos/ CD38-neg/dim	CD34-pos/ HLA-DR-low	CFC
CFC	0.82	0.86	0.74	X
CAFC week 1	0.73	0.68	0.71	0.79
CAFC week 2	0.72	0.74	0.59*	0.84
CAFC week 3	0.79	0.72	0.68	0.86
CAFC week 4	0.77	0.72	0.68	0.87
CAFC week 5	0.77	0.73	0.71	0.83
CAFC week 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 3.1). The degree of linear association between the number of pre-mobilization chemotherapy cycles and the mean number of CAFC week-types harvested per kg body weight was quantitated by determining the Spearman's rank correlation coefficient. Figure 3.6A shows a negative correlation ($r_s = -0.74$, $P = 0.0001$) between the number of chemotherapy cycles and the number of

CAFC week 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 3.6B shows that the negative correlation increased with later CAFC week-types.

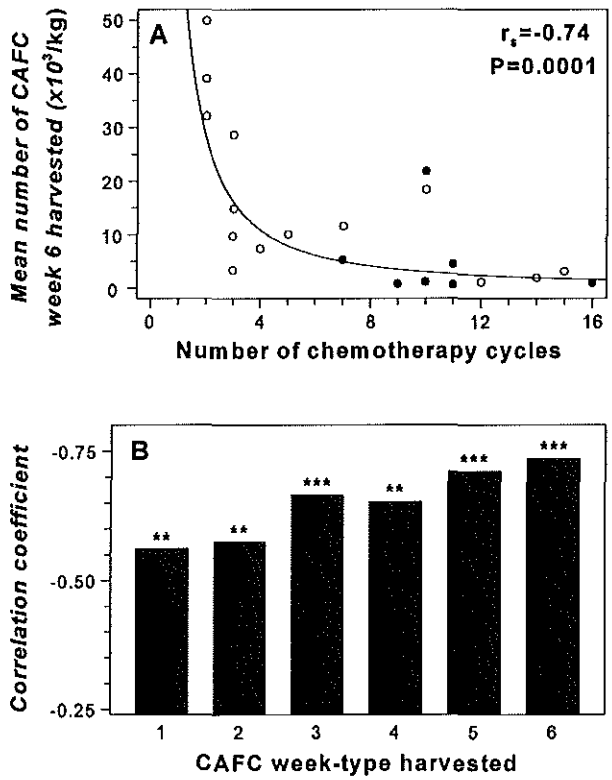

Figure 3.6 Relation between CAFC analysis and pre-mobilization treatment. Figure 3.6A shows the Spearman's rank correlation (r_s=-0.74, P=0.0001) between the number of chemotherapy cycles and the number of CAFC week 6 harvested. Leukaphereses with a low quality of less than 50 colony forming cells per CAFC at week 6 (●) were harvested from patients who had received at least seven chemotherapy cycles. Figure 3.6B 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 body weight. 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, $CD34^{pos}$cells, $CD34^{pos}/CD38^{neg/dim}$cells, $CD34^{pos}/HLA\text{-}DR^{low}$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 $\pm$1SEM: 15 $\pm$1 days, range 11 to 21 days; $n=21$) or platelets >50 x10^9/l (Mean $\pm$1SEM: 19 $\pm$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 3.7. No correlation was found between the number of NC transplanted and the hematopoietic recovery. The number of $CD34^{pos}$cells, $CD34^{pos}/CD38^{neg/dim}$cells, CFC and CAFC week 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 week 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 week 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 week 6 contributes less to early post-transplant recovery than the less primitive CAFC week 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, $P\leq0.0008$).

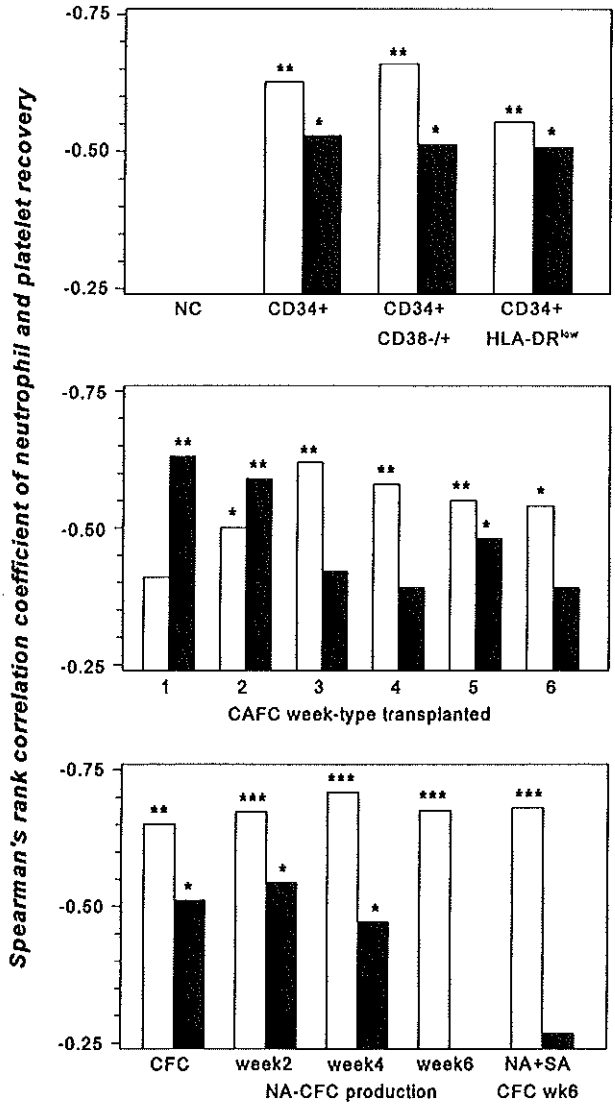

Figure 3.7 Relation of *in vitro* leukapheresis analyses with *in vivo* hematological recovery. The linear associations between the number of nucleated cells (NC), $CD34^{pos}$cells, $CD34^{pos}/CD38^{neg/dim}$cells, $CD34^{pos}/HLA\text{-}DR^{low}$cells, CAFC week-types and colony forming cells (CFC) 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 $>0.5 \times 10^9$/l (□; Mean ±1SEM: 15 ±1 days, range 11 to 21 days; n=21) or platelets $>50 \times 10^9$/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 bone marrow samples the clonogenic potency per HSC shows variation with means of 4 CFC per LTC-IC week 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 bone marrow, the present data show large quantitative and qualitative differences in primitive HSC contained in the analyzed LPs. We observed a mean CAFC week 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 week 6 harvested per kg body weight. 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 week 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 week 6 harvested. Haas et al. recently reported a similar relation between chemotherapy cycles and the number of $CD34^{pos}$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 $CD34^{pos}$cells transplanted. The present data also suggest that a correlation exists between the neutrophil recovery and the number of $CD34^{pos}/CD38^{neg/dim}$cells and CAFC week 3 and 4. The number of CAFC week 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 hematopoietic 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 hematopoietic recovery.

For unexplained reasons post-transplant platelet and neutrophil recoveries of patients transplanted with mobilized PBSC are often more rapid than following bone marrow transplantation [4,10-12]. In our study the more mature CAFC week 1 to 4 subsets show the highest correlation with the speed of early hematological recovery. Although bone marrow contains a higher frequency of CAFC week 1 to 4 than LPs (2.5 to 3.7-fold), the average quality of the CAFC week 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 bone marrow. This may explain the rapid post-transplant hematopoietic 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 4

STROMA-CONDITIONED MEDIA IMPROVE EXPANSION OF HUMAN PRIMITIVE HEMATOPOIETIC STEM CELLS AND PROGENITOR CELLS

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ABSTRACT

It has been reported that stroma-dependent cultures support proliferation of hematopoietic stem cells (HSC). In order to investigate the effect of soluble stromal factors, we developed short-term serum-low liquid cultures in which the effect of stroma-conditioned media (SCM) from the murine FBMD-1, and human L87/4 and L88/5 cell lines was studied on the maintenance and expansion of various human HSC subsets in CD34-selected mobilized peripheral blood stem cells (PBSC) from autologous transplants of lymphoma and multiple myeloma patients. The human cobblestone area forming cell (CAFC) assay was employed to determine the frequencies of both the CAFC week 2 to 4 as tentative indicators of progenitor and transiently repopulating HSC, and the more primitive CAFC week 6 to 8 as indicators of long-term repopulating HSC.

In seven-day liquid cultures containing interleukin-3 (IL-3), stem cell factor (SCF) and IL-6, we recovered 3.0-fold more colony forming cells (CFC) and 1.7 to 1.9-fold more CAFC week 2 and 4. The absolute number of primitive CAFC week 6 and 8 were only maintained (1.1 to 1.4-fold) in these liquid cultures. This modest expansion was significantly improved by the addition of SCM from the FBMD-1, L87/4 or L88/5 cell lines. Output CFC numbers were 6.8, 5.8 and 9.9-fold higher, respectively, than the input values, while absolute CAFC week 2 to 4 numbers were 4.5, 10.2 and 10.2-fold expanded, respectively. The addition of SCM also improved expansion of the more primitive CAFC week 6 to 8 stem cell subsets by 2.2, 4.5 and 4.9-fold, respectively. The addition of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), IL-1β, IL-11 or macrophage inflammatory protein-1α (MIP-1α) to cultures containing IL-3, SCF and IL-6 could not explain the SCM effect and in all these combinations SCM addition further increased the recovery of HSC subsets. Similarly, addition of anti-cytokine antibodies (i.e. α-G-CSF, α-GM-CSF, α-IL-11, α-leukemia inhibitory factor (LIF)) to liquid cultures containing IL-3, SCF, IL-6 and SCM could not neutralize the SCM effect.

These data indicate that SCM significantly enhances expansion of primitive HSC and progenitor cells from CD34-selected PBSC in 7-day cultures and in synergistic combination with multiple cytokines at optimal concentrations. As a result, SCM is an useful component of short-term liquid culture procedures for clinical expansion or manipulation of primitive HSC.

INTRODUCTION

Mobilized PBSC are increasingly used to restore the formation of blood cells after high-dose chemotherapy for solid tumors and hematological cancers [1-5]. For this reason PBSC are important target cells for *ex vivo* HSC manipulation, expansion and gene therapy [6-12]. It has been shown that hematopoietic stroma supports proliferation and more recently it has been reported that it may also enhance retroviral infection of HSC [13-23]. In addition, several

investigators have shown that primary stromal cells can be replaced by cell lines to support HSC cultures [24-28]. The development of "stroma-noncontact" cultures by Verfaillie et al. has demonstrated that direct contact between hematopoietic and stromal cells is not required for proliferation of HSC from normal bone marrow [29]. Furthermore, Verfaillie et al. showed that stromal derived factors synergize with multiple cytokine-induced maintenance and proliferation of HSC in 5-week "stroma-noncontact" cultures [30,31]. However, only in 2-week "stroma-noncontact" cultures and in synergy with multiple cytokines it was possible to expand the absolute number of bone marrow derived primitive HSC [32]. These data indicate that short-term cultures using multiple cytokines and stromal derived factors are most suitable for expansion of primitive HSC. Because it is undesirable to use cultures containing stromal cells or cell lines and high concentrations of serum in clinically protocols for *ex vivo* HSC expansion and manipulation, we prepared conditioned media from various murine and human stromal cell lines in serum-low (2% horse serum) medium. This led to a standardized medium product, which was easier to use than stroma-associated or "stroma-noncontact" cultures.

In murine short-term serum-low cultures we observed after 5 to 7-day liquid culture an average 300-fold expansion of progenitor cells and CAFC day 7 from post 5-fluorouracil bone marrow cells, accompanied with an up to 3.5-fold expansion of *in vivo* long-term repopulating stem cells and CAFC day 35 using SCM together with IL-3, SCF and IL-11 or IL-12 [33]. Similarly, we have now tested the effects of SCM on the generation and recovery of human primitive HSC and progenitor cells in 7-day serum-low liquid cultures. In these experiments CD34-positive (CD34+) selected mobilized PBSC from autologous transplants of lymphoma and multiple myeloma patients were used, because these cells are clinically one of the most relevant target cells for *ex vivo* stem cell expansion and manipulation. Furthermore, SCM was used in combination with multiple cytokine combinations at optimal concentrations, because these cytokines are already employed in clinical *ex vivo* stem cell expansion and manipulation protocols [6-12]. The assessment of different HSC subsets was facilitated by the recently developed human CAFC assay wherein the CAFC week 2 to 4 are used as tentative indicators of progenitor cell activity and transiently repopulating HSC, while frequencies of CAFC week 6 to 8 are interpreted as indicators of more primitive, long-term repopulating stem cells [27,34]. These tools allowed us to study the SCM effect and its synergy with different multiple cytokine combinations on the maintenance and expansion of different human stem cell subsets. In addition, by using different stromal cell lines we were able to compare their colony-stimulating activity with their ability to support HSC maintenance and expansion.

MATERIALS AND METHODS

Peripheral blood stem cells

Fifteen leukapheresis products from eight patients with multiple myeloma, five with non-Hodgkin

lymphoma, one with Burkitt lymphoma and one with Hodgkin disease in remission were used in this study. Before leukapheresis the HSC were mobilized to the blood after several courses of chemotherapy using G-CSF (Filgrastim; Roche, Mijdrecht, The Netherlands) as described before [34]. After cell collection, excess of erythrocytes was removed using buffy coat centrifugation. Fresh or frozen and thawed cells were subjected to CD34-selection to enrich for HSC. For CD34-selection the following methods were used according to the guidelines of the suppliers: Ceprate SC column (CellPro, Bothell, WA, U.S.A.), Dynal CD34 progenitor cell selection system (Dynal, Oslo, Norway) and MACS CD34 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Before CD34-selection using the Dynal system and the MACS kit a density gradient was performed (1.077 g/ml, Lymphoprep; Nycomed, Olso, Norway). After selection the percentage CD34+ cells was determined as described before [34]. Table 4.1 shows the frequency of the different stem cell subsets in the CD34+ selected PBSC before liquid culture as determined using flow cytometry, CFC and CAFC assays.

Table 4.1 Mean Frequency of Different Progenitor and CAFC Subsets in Mobilized Peripheral Blood Stem Cells After CD34-Selection and Before Liquid Culture.

Progenitor or CAFC Subset	Frequency per 100 Nucleated Cells (±1SEM)	Number of Experiments
CD34-Positive Cells	83.3 (±3.1)	15
CFU-GM	13.5 (±2.1)	15
BFU-E	7.7 (±1.0)	15
Total Colony Forming Cells	21.2 (±2.6)	15
CAFC week 2	2.4 (±0.6)	7
CAFC week 4	3.6 (±0.7)	7
CAFC week 6	2.4 (±0.6)	6
CAFC week 8	1.5 (±0.4)	4

Serum-free culture

Serum-free liquid culture experiments were performed in 35 mm bacterial dishes (Greiner, Alphen a/d Rijn, The Netherlands) to prevent strong adherence of the hematopoietic cells to the plastic surface. The serum-free Iscove's modified Dulbecco's medium (IMDM) with Glutamax-1 (Gibco, Breda, The Netherlands) contained 1% bovine serum albumin (A9418; Sigma, St Louis, MO, U.S.A.), penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco), β-mercapto-ethanol (10^{-4} M; Merck, Darmstadt, Germany), bovine insulin (10 μg/ml; Gibco), cholesterol (15 μM; Sigma), linolic acid (15 μM; Merck), iron-satured human transferrin (0.62 g/l; Intergen, Purchase, NY, U.S.A.), cytidine (1 μg/ml; Sigma), adenosine (1 μg/ml; Sigma), uridine (1 μg/ml; Sigma), guanosine (1 μg/ml; Sigma), thymidine (1 μg/ml; Sigma), 2'-deoxycytidine (1 μg/ml; Sigma), 2'-deoxyadenosine (1 μg/ml; Sigma), 2'-deoxyguanosine (1 μg/ml; Sigma). Two thousand to sixty thousand CD34+ cells in 1 ml medium were cultured at 37°C and 5% CO_2. After seven days of culture, the cells were collected from the dishes after scraping with a cell scraper (Greiner) and

rinsing with IMDM. After washing, the cells were resuspended in IMDM and plated in CFC or CAFC assays. Semisolid cultures were performed in the same serum-free medium containing 1% methylcellulose (Methocel, Stade, Germany).

Cytokines and antibodies

For the liquid and semisolid cultures the following purified recombinant human cytokines and monoclonal antibodies were kindly provided and used in the concentrations listed below: GM-CSF (10 ng/ml), IL-6 (2,000 U/ml), IL-11 (50 U/ml), anti-human-G-CSF (α-hu-G-CSF; 1 μg/ml), α-hu-GM-CSF (1 μg/ml), α-hu-IL-11 (1 μg/ml), α-hu-LIF (1 μg/ml), α-murine-G-CSF (α-mu-G-CSF; 1 μg/ml), α-mu-IL-11 (1 μg/ml) and α-mu-LIF (1 μg/ml) from Genetics Institute (Cambridge, MA, U.S.A.), G-CSF (100 ng/ml) and SCF (100 ng/ml) from Amgen (Thousand Oaks, CA, U.S.A.), IL-1β (100 U/ml) from Immunex (Seatle, WA, U.S.A.) and IL-3 (30 ng/ml) from Gist Brocades (Delft, The Netherlands). Purified recombinant human erythropoietin (EPO; 1 U/ml) was purchased from Boehringer (Mannheim, Germany) and, MIP-1α (100 ng/ml), α-mu-GM-CSF (10 μg/ml) and α-porcine-transforming growth factor-β1 (α-TGF-β1; 1 μg/ml) from R&D Systems (Abingdon, United Kingdom).

Stroma-conditioned media

Confluent layers were grown of the stromal cell lines FBMD-1, L87/4 and L88/5 [26,27,35]. The cells were cultured in IMDM with Glutamax-1 supplemented with 10% fetal calf serum (Hyclone, Logan, UT, U.S.A.), penicillin (100 U/ml), streptomycin (100 μg/ml) and β-mercapto-ethanol (10^{-4} M). The FBMD-1 cells were maintained at 33°C and 10% CO_2 and the L87/4 and L88/5 cells at 37°C and 10% CO_2. When the layers were confluent, the medium was removed and rinsed twice with IMDM. Serum-free medium as described above supplemented with 2% horse serum (Gibco) was added to the confluent stromal layers and conditioned for seven days. The SCM were harvested, the non-adherent cells were removed by centrifugation and the media were stored at -20°C until use. Control medium was prepared by parallel incubations without the stromal cell lines. To block the negative effect of TGF-β1 produced by the stromal cell lines on the recovery of HSC, α-TGF-β1 was added to the SCM and control medium as a standard culture condition [27,35-37]. In the cultures, 50% SCM or control medium was used.

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 semisolid CFC assay containing EPO, G-CSF, GM-CSF, IL-3 and SCF as described before [34]. CFU-GM and BFU-E were counted on day 14 of culture in the same dish.

Cobblestone area forming cell assay

Limiting dilution CAFC assays using stromal feeders from the FBMD-1 murine stromal cell line were prepared and maintained as described before [27,34]. Briefly, the cells were cultured at 33°C and 10% CO_2 in CAFC medium. IL-3 (10 ng/ml) and G-CSF (20 ng/ml) were added weekly to the cultures. CAFC assays were performed on confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates. CD34+ cells were overlaid in a limiting dilution setup with input values ranging between one and 2,000 CD34+ cells per well. Twelve dilutions two-fold apart were used for each sample with 15 replicate wells per dilution. The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells (i.e. cobblestone area) beneath the stromal layer was determined at week 2, 4, 6 and 8 and CAFC frequencies were calculated using Poisson statistics as described previously [18,21].

Statistical analysis

Data were analyzed using GraphPad Instat (GraphPad Software, San Diego, CA, U.S.A.). The means of two populations were compared using a paired Student's *t* test. The two-sided P value is determined testing the null hypothesis that the SCM groups are equal to the no SCM group. Only experiments with n greater than 3 were analyzed and marked with an asterisk if P was

smaller than 0.05.

RESULTS

Progenitor cell expansion in defined medium

The first set of experiments was aimed to characterize optimal cytokine combinations for recovery of hematopoietic progenitors from seven-day liquid cultures of CD34+ PBSC. From figure 4.1 it is apparent that IL-3, SCF and IL-6 synergized to increase the recovery of CFC, while the combination of IL-3/SCF/IL-6/G-CSF/GM-CSF gave the greatest expansion of CFC in these liquid cultures. There was no further improvement of this expansion with the addition of IL-11 (Figure 4.1) or IL-12 (Data not shown) as we have reported in the murine system [37-39].

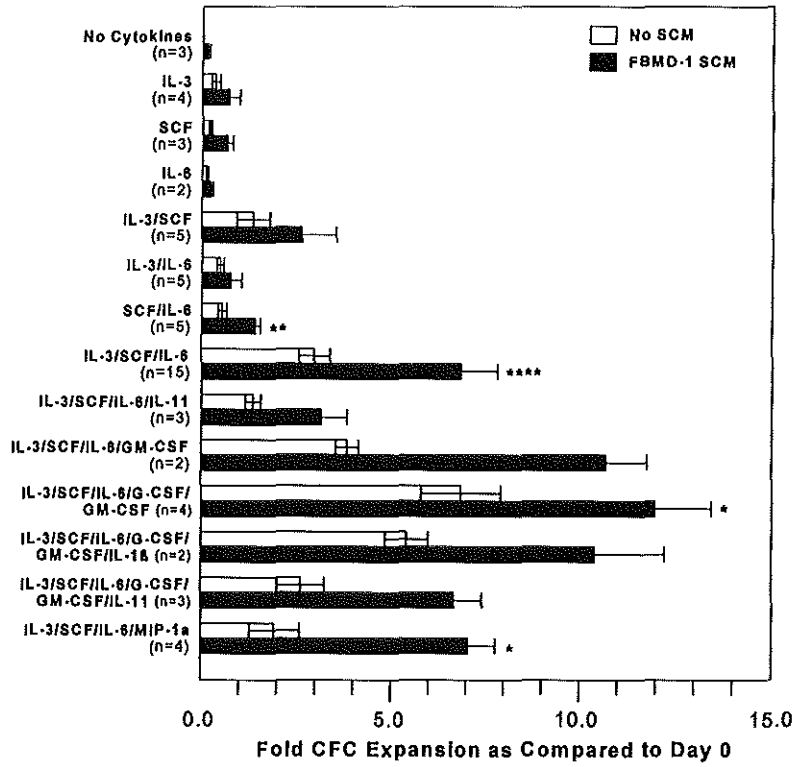

Figure 4.1 The effect of murine FBMD-1 stroma-conditioned medium (SCM) on colony forming cell (CFC) output (±1SEM) in seven-day serum-low liquid cultures of CD34+ peripheral blood stem cells. Comparison between no SCM and with FBMD-1 SCM: *, $P<0.05$; **, $P<0.01$; ****, $P<0.0001$.

Effect of stroma-conditioned media on progenitor cell expansion

Subsequently, we studied the effect of SCM on CFC expansion in serum-low liquid cultures. Because the stromal cell lines produce TGF-β1, we added TGF-β1-neutralizing antibodies to the SCM and the control experiments as a standard culture condition. The addition of FBMD-1 SCM significantly increased CFC expansion in cultures containing at least SCF and IL-6 and resulted in on average 2.5-fold increased expansion as compared to 7-day liquid cultures without FBMD-1 SCM (Figure 4.1). We investigated whether cytokines known for their effects on HSC, i.e. G-CSF, GM-CSF, IL-1β, IL-3, IL-11, MIP-1α or combinations of these could explain this SCM effect. Irrespective of the modulating effects of additional cytokines on CFC expansion in the presence of SCF and IL-6, FBMD-1 SCM always increased the expansion.

To compare the effect of FBMD-1 SCM with SCM of two human stromal cell lines (i.e. L87/4 and L88/5), CFC expansion was done in liquid cultures containing either no cytokines or various combinations of cytokines in the absence or presence of these SCM. Both SCM increased the CFC production in serum-low cultures with different multi-cytokine combinations tested (Figure 4.2). Even in cultures without any other cytokines L88/5 SCM resulted in a 91% CFC maintenance. In cultures containing IL-3, SCF, IL-6, G-SCF, GM-CSF, IL-11 and additional L88/5 SCM there was a 13.8-fold CFC expansion as compared to CFC content before culture.

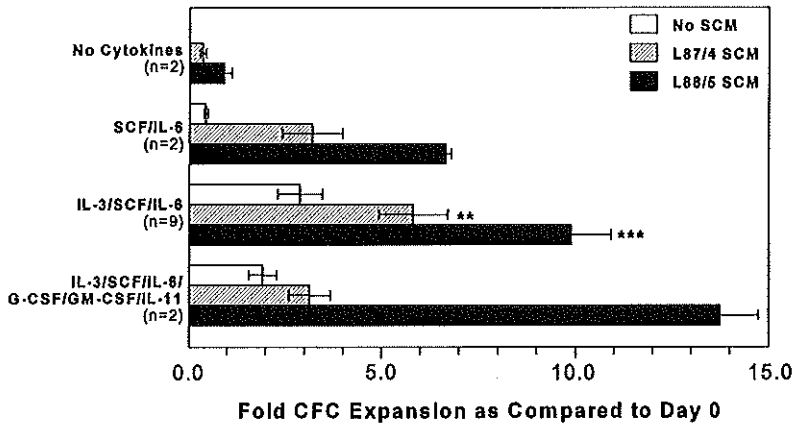

Figure 4.2 The effect of two human stroma-conditioned media (SCM) (i.e. L87/4 and L88/5) on colony forming cell (CFC) expansion (±1SEM) is shown in seven-day serum-low liquid cultures of CD34+ peripheral blood stem cells in the absence or presence of various cytokine combinations. Comparison between no SCM and with SCM: **, $P<0.01$; ***, $P<0.001$.

To investigate whether the effect of SCM could be explained by known stromal cell derived cytokines we added species-specific antibodies against G-CSF, GM-

CSF, IL-11 and LIF to cultures otherwise stimulated by SCM and IL-3/SCF/IL-6. Table 4.2 shows that these antibodies could not abrogate the effect of SCM addition, indicating that the cytokines G-CSF, GM-CSF, IL-11 and LIF were not responsible for the SCM-enhanced CFC recovery. In addition, IL-3, SCF and IL-6 were unlikely to mediate the SCM effect because all cultures contained plateau levels of these cytokines.

Table 4.2 Effect of Antibodies Against G-CSF, GM-CSF, IL-11 and LIF on Stroma-Conditioned Media (SCM) Mediated Colony Forming Cell (CFC) Expansion in Liquid Cultures Stimulated with IL-3, SCF and IL-6.

	Fold CFC Expansion as Compared to Day 0 (±1SEM)					
	Stroma-Conditioned Media					
	None	FBMD-1	None	L87/4	None	L88/5
	n=5		n=4		n=4	
No Antibodies	1.6 (±0.5)	5.0* (±0.6)	1.2 (±0.4)	4.8* (±1.5)	1.2 (±0.4)	10.8* (±1.7)
With Antibodies	1.5 (±0.4)	3.7* (±0.6)	1.3 (±0.3)	5.0* (±1.2)	1.3 (±0.3)	8.2* (±1.7)

Comparison between no SCM and with SCM: *, $P<0.05$

Effect of stroma-conditioned media on primary colony formation

We investigated the presence of colony-stimulating and synergistic activities in SCM by studying primary colony formation of CD34+ PBSC in serum-low semisolid culture medium in the presence or absence of the various SCM and cytokine combinations. From these experiments it appeared that L87/4 and L88/5 SCM, but not FBMD-1 SCM, contain colony stimulating factors (Figure 4.3A+4.3B, Upper panels). The two human SCM also showed a strong synergistic effect on colony formation in the presence of IL-3, SCF and IL-6, whereas FBMD-1 SCM mostly stimulated cluster formation (Figure 4.3A+4.3B, Middle panels). The lower panels of figure 4.3 show that all three SCM dramatically increased the number of myeloid and erythroid colonies in cultures with a six cytokine (IL-3/SCF/IL-6/G-CSF/GM-CSF/EPO) combination. The observation that FBMD-1 SCM was similarly active as the two human SCM in this respect suggests that the activities in FBMD-1 act in synergy with multiple cytokines. Moreover, colonies grown in the presence of SCM were larger. Our observations that the various SCM dramatically enhance the number and size of colonies indicates that the SCM activities trigger more progenitors to proliferate and to produce larger clones. This suggests that the SCM activities have an effect on relatively early progenitor cells, while the two human SCM affect more

differentiated progeny as well.

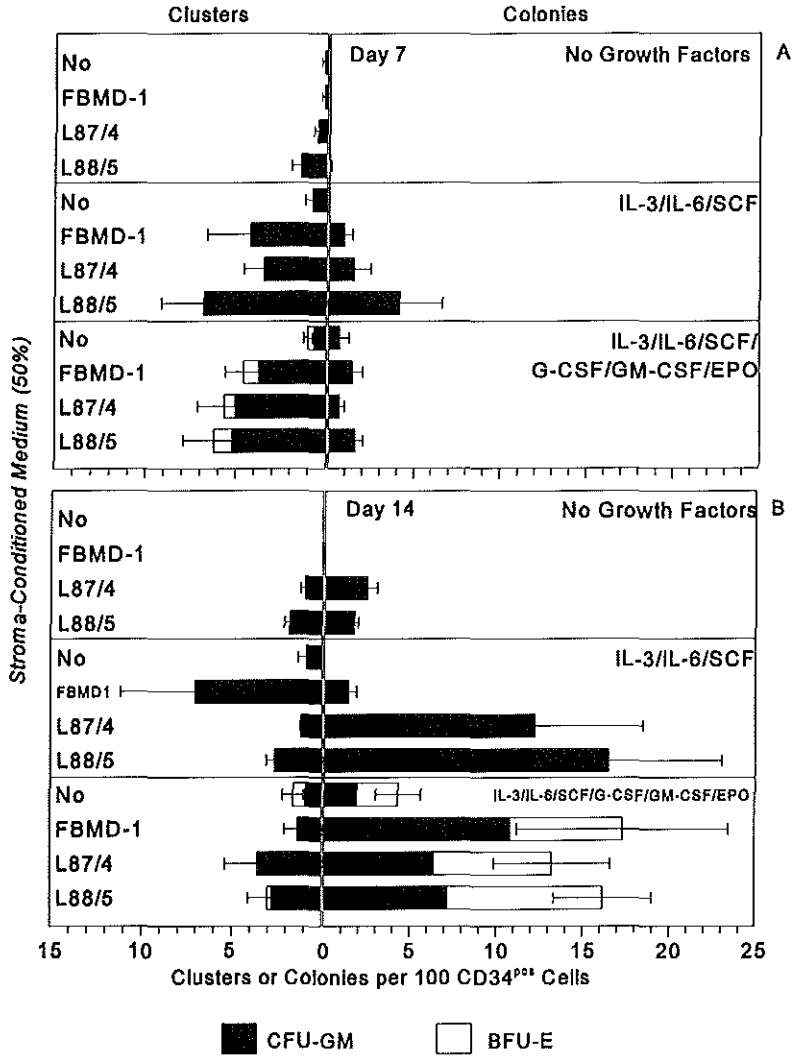

Figure 4.3 The effect of stroma-conditioned media (SCM) on primary colony formation by CD34+ peripheral blood stem cells in serum-low semisolid culture medium (n=4; ±1SEM). In the upper panels of section A and B the direct colony stimulating effect of L87/4 and L88/5 SCM is demonstrated. The middle panels of section A and B shows the strong synergistic effect of all three SCM on colony formation in the presence of IL-3, SCF and IL-6. The lower panels of section A and B illustrate the SCM effect on colony formation in cultures with a six cytokine combination.

Effect of stroma-conditioned media on the expansion of different CAFC subsets. To study the effect of SCM on the different stem cell subsets the human CAFC assay was used (Figure 4.4). In seven-day liquid cultures containing IL-3, SCF and IL-6, 1.7 and 1.9-fold more CAFC week 2 and 4, respectively, were recovered. The absolute number of primitive CAFC week 6 and 8 were only maintained (1.4 and 1.1-fold, respectively) in liquid cultures containing IL-3, SCF and IL-6. This expansion was significantly improved by the addition of SCM from the FBMD-1, L87/4 or L88/5 cell lines. CAFC week 2 to 4 were 4.5 (range 1.0 to 8.0), 10.2 (range 2.0 to 23.6) and 10.2-fold (range 2.0 to 22.8) expanded, respectively. Also the expansion of the more primitive CAFC week 6 to 8 stem cell subsets was improved by the addition of SCM. In liquid cultures with FBMD-1, L87/4 and L88/5 SCM there was a 2.2 (range 1.0 to 3.5), 4.5 (range 0.8 to 8.1) and 4.9-fold (range 1.0 to 9.7) expansion, respectively, while we observed maintenance of CAFC week 6 and 8 in the absence of SCM. Although the results in figure 4.4 show sometimes large ranges, the effect of SCM was always present in each individual experiment.

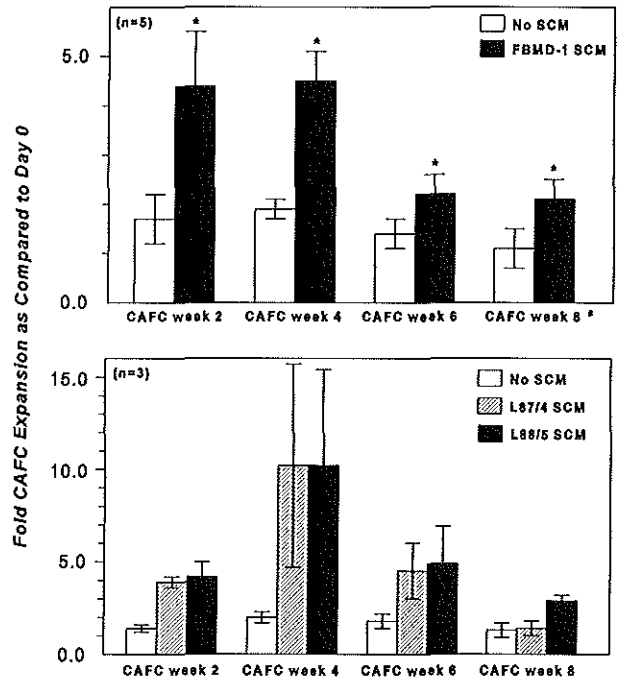

Figure 4.4 The effect of stroma-conditioned media (SCM) on the expansion (±1SEM) of different CAFC week-types in seven-day serum-low liquid cultures of CD34+ peripheral blood stem cells containing IL-3, SCF and IL-6. Comparison between no SCM and with SCM: *, $P<0.05$. #, $n=4$.

Experiments with liquid cultures containing IL-3, SCF and IL-6 showed that the addition of known cytokines (i.e. G-CSF, GM-CSF, IL-11) could not explain the FBMD-1 SCM effect, because in all these combinations there was a reproducible and clear SCM effect (Table 4.3). Similarly, addition of species-specific anti-cytokine antibodies (i.e. α-G-CSF, α-GM-CSF, α-IL-11 α-LIF) to liquid cultures containing IL-3, SCF, IL-6 and FBMD-1, L87/4 and L88/5 SCM could not neutralize the SCM effect (Table 4.4). Due to inter-experimental variation the overall expansion of CAFC week 4 to 8 seemed lower in the presence of these antibodies. However, the effect of SCM did not disappear with the antibodies and the ratio 'SCM/No SCM' was even higher in the 'with antibodies' groups.

Table 4.3 Effect of FBMD-1 Stroma-Conditioned Medium on the Expansion of Different CAFC Week-Types in Liquid Culture (n = 1).

	Fold CAFC Expansion as Compared to Day 0 (± 1SEM)			
	Cytokine Stimulation			
	IL-3/SCF/IL-6/G-CSF/ GM-CSF		IL-3/SCF/IL-6/G-CSF/ GM-CSF/IL-11	
	Stroma-Conditioned Media		Stroma-Conditioned Media	
	None	FBMD-1	None	FBMD-1
CAFC week 2	3.9	5.9	4.2	11.5
CAFC week 4	3.2	9.0	1.5	3.6
CAFC week 6	1.2	3.6	0.5	1.9

Table 4.4 Effect of Antibodies Against G-CSF, GM-CSF, IL-11 and LIF on Stroma-Conditioned Media (SCM) Mediated CAFC Week-Type Expansion in Liquid Cultures Stimulated with IL-3, SCF and IL-6.

CAFC Subset	Antibodies (Ab)	Fold CAFC Expansion as Compared to Day 0 (±SEM)					
		Stroma-Conditioned Media					
		None	FBMD-1	None	L87/4	None	L88/5
CAFC week 2	No Ab	1.7 (±0.5)	4.4* (±1.1)	1.4 (±0.2)	3.9 (±0.3)	1.4 (±0.2)	4.2 (±0.8)
		n=5		n=3		n=3	
	With Ab	1.6 (±1.1)	5.6 (±1.3)	1.6 (±0.1)	5.8 (±0.2)	1.7 (±0.3)	6.3 (±1.2)
		n=3		n=2		n=2	
CAFC week 4	No Ab	1.9 (±0.2)	4.5* (±0.6)	2.0 (±0.3)	10.2 (±5.5)	2.0 (±0.3)	10.2 (±5.2)
		n=5		n=3		n=3	
	With Ab	0.8 (±0.4)	3.1 (±1.3)	1.0 (±0.3)	4.7 (±1.5)	0.6	3.3
		n=3		n=2		n=1	
CAFC week 6	No Ab	1.4 (±0.3)	2.2* (±0.4)	1.8 (±0.4)	4.5 (±1.5)	1.8 (±0.4)	4.9 (±2.0)
		n=5		n=3		n=3	
	With Ab	0.6 (±0.1)	1.4 (±0.1)	0.5	1.5	0.5	1.4
		n=2		n=1		n=1	
CAFC week 8	No Ab	1.1 (±0.4)	2.1* (±0.4)	1.3 (±0.4)	1.4 (±0.4)	1.3 (±0.4)	2.9 (±0.3)
		n=4		n=3		n=3	
	With Ab	0.6	1.1	0.4	1.1	0.4	1.0
		n=1		n=1		n=1	

Comparison between no SCM and with SCM: *, $P<0.05$

DISCUSSION

In this study the propagation of different human stem cell subsets and progenitors in a short-term serum-low liquid culture system was investigated. The addition of stromal cell line conditioned media to these short-term cultures significantly improved the expansion of both short-term and long-term culture initiating cells. We documented a 10.2-fold expansion of progenitor cells (CAFC week 2 to 4) and a 4.9-fold expansion of primitive CAFC week 6 to 8 in serum-low liquid cultures of CD34+ PBSC stimulated with IL-3, SCF, IL-6 and L88/5 SCM. This SCM-supported expansion of primitive CAFC week 6 and 8 in static cultures was a major improvement as compared similar cultures of long-term culture-initiating cells or late CAFC from CD34-selected PBSC [6,12]. However, it matches with data from continuous perfusion cultures of unselected PBSC [7,10,12] and is in line with the results from 2-week "stroma-noncontact" cultures of normal bone marrow cells [32].

In our attempt to further improve culture conditions, we focused on the stromal micro-environment, because it has been shown that stroma-dependent and "stroma-noncontact" cultures support proliferation and expansion of primitive HSC [13-18,20,21,23-32]. We investigated the effect of serum-low SCM on the expansion of different stem cell subsets in liquid cultures. The addition of SCM from the FBMD-1, L87/4 or L88/5 cell lines improved the expansion of a broad spectrum of progenitor and stem cells, including that of the most primitive HSC. This improved expansion could not be explained by a series of known cytokines because the effect was observed in all cytokine combinations using IL-3, SCF, IL-6, G-CSF, GM-CSF, IL-1β, IL-11 and MIP-1α and also in the presence of neutralizing antibodies for G-CSF, GM-CSF, IL-11, LIF and TGF-β1. Because SCM was a cell-free supernatant, SCM could also be useful in a clinical setting for improvement of *ex vivo* culture conditions.

Our data indicate that the SCM affects primary colony formation, the expansion of progenitor cells and the proliferation/survival of more primitive HSC. Other investigators have shown similar results in stromal cell co-culture and in "stroma-noncontact" culture systems [24,27-32]. Interestingly, this stromal cell activity synergized with a range of cytokines and was still present using multiple cytokine combinations at optimal concentrations. Because we have observed an absolute expansion of CAFC week 6 and 8 numbers, our results suggest that the SCM activity not only supports maintenance of primitive stem cells but is also active at the level of triggering resting stem cells into cycle and stimulating proliferation rather than providing differentiation signals. The nature of the synergistic effect of SCM is still unidentified and certainly not similar to that of a series of known cytokines. The possibility exists that this synergistic SCM effect is no cytokine but a stromal cell product that is shed off the stromal surface e.g. glycosaminoglycans (GAGs). The effect of GAGs on HSC was originally suggested in the 1970's in studies of the hematopoietic microenvironment [40-42]. Also the effect of "stroma-noncontact" cultures on stem cell recovery has been explained as an effect of known cytokines enhanced by extracellular matrix

molecules [29,30]. Recently, Gupta et al. have shown that heparan sulphate GAGs derived from the murine stromal cell line M2-10B4 indeed improves the maintenance of primitive HSC [43]. Therefore, the synergistic effect of SCM could reflect the cytokine-presenting capacity of those extracellular matrix molecules, since it has been shown that GAGs and heparan sulphate can bind growth factors and present these factors to the HSC [44,45].

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CHAPTER 5

STROMA-CONTACT PREVENTS LOSS OF HEMATOPOIETIC STEM CELL QUALITY DURING EX VIVO EXPANSION OF CD34-POSITIVE MOBILIZED PERIPHERAL BLOOD STEM CELLS

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Blood, In press

ABSTRACT

Stroma-supported long-term cultures (LTC) allow estimation of stem cell quality by simultaneous enumeration of hematopoietic stem cell (HSC) frequencies in a graft using the cobblestone area forming cell (CAFC) assay, and the ability of the graft to generate progenitors in flask LTC (LTC-CFC). We have recently observed that the number and quality of mobilized peripheral blood stem cells (PBSC) was low in patients having received multiple rounds of chemotherapy. Moreover, grafts with low numbers of HSC and poor HSC quality had a high probability to cause graft failure upon their autologous infusion. As *ex vivo* culture of stem cells has been suggested to present an attractive tool to improve hematological recovery or reduce graft size, we have studied the possibility that such propagation may affect stem cell quality.

In order to do so, we have assessed the recovery of different stem cell subsets in CD34-positive PBSC after a 7-day serum-free liquid culture using CAFC and LTC-CFC assays. A numerical expansion of stem cell subsets was observed in the presence of interleukin-3 (IL-3), stem cell factor (SCF) and IL-6, while stroma-contact, stromal soluble factors or combined addition of FLT3-ligand (FL) and thrombopoietin (TPO) improved this parameter. In contrast, *ex vivo* culture severely reduced the ability of the graft to produce progenitors in LTC while stromal soluble factors partly abrogated this quality loss. The best conservation of graft quality was observed when the PBSC were cultured in stroma-contact. These data suggest that *ex vivo* propagation of PBSC may allow numerical expansion of various stem cell subsets, however, at the expense of their quality. In addition, cytokine-driven PBSC cultures require stroma for optimal maintenance of graft quality.

INTRODUCTION

Mobilized PBSC are increasingly used to restore the formation of blood cells after high-dose chemotherapy for solid tumors and hematological cancers [1-3]. More recently, PBSC from cancer patients are cultured *ex vivo* [4-6] because expanded HSC may possibly reduce the time to hematopoietic recovery after their transplantation. Furthermore, the use of smaller transplants may lead to a reduction of contaminating tumor cells. Although clinical *ex vivo* expansion trials have already started, many questions are still unanswered. Firstly, although many investigators have shown that the total number of CD34-positive (CD34+) cells, progenitors and primitive stem cells in PBSC can be expanded *in vitro* [4-10], there has been no report of improved hematopoietic recovery using such *in vitro* propagated grafts [4-6]. Secondly, due to the non-myeloablative conditioning regimens and/or co-transplantation of unmanipulated HSC in these studies it is also not apparent if primitive stem cells are still capable of long-term engraftment after *ex vivo* culture. *Ex vivo* propagation studies in mice have shown both loss of *in vivo* engraftment [11] and an increased ability of cultured

cells to repopulate irradiated hosts [12]. Diminished engraftment *in vivo* may result from a reduced ability of stem cells to home to the bone marrow. Indeed, we have recently shown that short incubations of murine stem cells with several cytokine combinations diminish their lodgement in hematopoietic organs and hence their ability to repopulate the hematopoietic system of irradiated recipients [13]. In addition, loss of primitive stem cell quality may also lead to a reduced *in vivo* repopulating ability. Previously, we have studied 47 mobilized PBSC harvests of 21 autologous transplantation cancer patients and shown that poorly mobilized PBSC harvests contain a low number of primitive HSC (CAFC week 6), and also produce less progenitors per primitive stem cell in stroma-supported LTC-CFC [14]. This poor primitive stem cell quality was related with the number of cytoreductive pre-treatment rounds administered to the patients. In addition, we have observed low CAFC week 6 numbers and low primitive stem cell quality in the original autologous transplant of patients that failed to engraft within six months after transplantation [15].

It has been shown that primary stromal feeder layers and stromal cell lines support the culture of HSC [16-19]. In 2- or 5-week cultures without exogenous cytokines, the Verfaillie group has demonstrated that primitive stem cell (LTC-initiating cell; LTC-IC) recovery and colony forming cell (CFC) production in LTC was improved when normal bone marrow derived HSC were propagated in stroma-noncontact cultures as compared to stroma-contact [20-23]. This improvement was explained by proliferation inhibition of CFC and LTC-IC during direct stroma-contact possibly via adhesion of the fibronectin receptor to stroma [22,23]. The stroma-noncontact cultures were further improved by the addition of IL-3 and macrophage inflammatory protein-1α (MIP-1α) to the medium and simplified by using stroma-conditioned medium (SCM) instead of stroma-noncontact transwell inserts [24]. However, Koller et al. have shown that cytokine-driven LTC-IC expansion can only be achieved with the use of a stromal feeder layer [25,26].

In contrast to studies on normal bone marrow derived HSC, only a limited number of studies are dedicated to *ex vivo* expansion of LTC-IC or CAFC week 6 from clinically relevant mobilized peripheral blood from cancer patients. In 7- to 12-day static liquid cultures of CD34-selected mobilized PBSC a maintenance of LTC-IC or CAFC week 6 has been reported [7,9,10]. Two- to 20-fold expansion of LTC-IC or CAFC week 6 from mobilized PBSC was only observed in cultures containing stromal factors and/or accessory cells and in perfusion bioreactors [8-10]. However, those studies did not provide information about the HSC quality.

In the present study we have focussed on the effect of *ex vivo* propagation on both the number and quality of HSC, because, in our view, numerical expansion of HSC can only be effective if their quality is not reduced at the same time. We used CD34-selected mobilized PBSC from myeloma and lymphoma patients in our experiments because this HSC source is also used in most clinical *ex vivo* expansion studies [4-6]. In 7-day serum-free cultures supplemented with IL-3, SCF and IL-6 with or without FL and TPO, the effect of SCM addition from various stromal cell lines was tested in comparison with direct contact with a

murine stromal cell layer and stroma-noncontact cultures on the recovery of progenitors cell and primitive stem cell numbers and their quality. The assessment of different HSC subsets was done using the human CAFC assay wherein the CAFC week 2 to 4 are tentative indicators of progenitor cell activity and transiently repopulating HSC, while CAFC week 6 is interpreted as indicator of more primitive, long-term repopulating stem cells [14,18]. In parallel flask-LTC the CFC production was determined in the corresponding weeks as an estimate of total graft quality. LTC-CFC production and CAFC frequency allowed us to assess the individual primitive stem cell quality.

MATERIALS AND METHODS

Mobilized peripheral blood

Nine leukapheresis products from four patients with non-Hodgkin lymphoma, four with multiple myeloma and one with Burkitt lymphoma in remission were used in this study. Before leukapheresis the HSC were mobilized to the blood after several courses of chemotherapy using granulocyte colony-stimulating factor (Filgrastim, recombinant-methionyl human G-CSF; Roche, Mijdrecht, The Netherlands) as described before [14]. After cell collection, excess of erythrocytes was removed using buffy coat centrifugation. Fresh or frozen and thawed leukaphereses were subjected to CD34-selection to enrich for HSC. For CD34-selection the following methods were used according to the guidelines of the suppliers: Ceprate SC column (CellPro, Bothell, WA), Dynal CD34 progenitor cell selection system (Dynal, Oslo, Norway) and MACS CD34 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Before CD34-selection using the Dynal system and the MACS kit a density gradient was performed (1.077 g/ml, Lymphoprep; Nycomed, Olso, Norway). After selection the percentage CD34+ cells was determined as described before [14]. Table 5.1 shows the frequency of the different stem cell subsets in the CD34+ selected PBSC before *ex vivo* culturing as determined using flow cytometry, CFC and CAFC assays.

Table 5.1 Mean Frequency of Different Progenitor and CAFC Subsets in Mobilized Peripheral Blood Stem Cells Before *Ex Vivo* Culture.

Progenitor or CAFC Subset	Frequency per 100 Cells (±1SEM)	Number of Experiments
CD34-Positive	84 (±3)	9
Colony Forming Cells	22 (±3)	9
CAFC week 2	3.0 (±0.7)	9
CAFC week 4	4.6 (±1.0)	9
CAFC week 6	2.7 (±1.0)	9

Cytokines
The following purified recombinant cytokines were kindly provided: human granulocyte-macrophage-CSF (GM-CSF), human IL-6 and murine SCF from Genetics Institute (Cambridge, MA), human FL, human G-CSF and human SCF from Amgen (Thousand Oaks, CA), human IL-3 from Gist Brocades (Delft, The Netherlands) and human TPO from Genentech (South San Francisco, CA).

Serum-free liquid culture
Serum-free liquid culture experiments were performed in 35 mm bacterial dishes (Greiner, Alphen a/d Rijn, The Netherlands) to prevent strong adherence of the hematopoietic cells to the plastic surface. The serum-free Iscove's modified Dulbecco's medium with Glutamax-1 (IMDM; Gibco, Breda, The Netherlands) contained 1% bovine serum albumin (A9418; Sigma, St Louis, MO), penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco), 10^{-4} M β-mercapto-ethanol (Merck, Darmstadt, Germany), bovine insulin (10 μg/ml; Gibco), 15 μM cholesterol (Sigma), 15 μM linolic acid (Merck), iron-satured human transferrin (0.62 g/l; Intergen, Purchase, NY), cytidine (1 μg/ml; Sigma), adenosine (1 μg/ml; Sigma), uridine (1 μg/ml; Sigma), guanosine (1 μg/ml; Sigma), thymidine (1 μg/ml; Sigma), 2'-deoxycytidine (1 μg/ml; Sigma), 2'-deoxyadenosine (1 μg/ml; Sigma), 2'-deoxyguanosine (1 μg/ml; Sigma). Forty thousand CD34+ PBSC in two ml serum-free medium supplemented with IL-3 (25 ng/ml), human SCF (100 ng/ml), IL-6 (100 ng/ml) and anti-human-transforming growth factor-β1 (αTGFβ1; 1.0 μg/ml; R&D Systems, Abingdon, United Kingdom) with or without FL (100 ng/ml) and TPO (10 ng/ml) were cultured at 37°C and 10% CO_2. After seven days of culture, the cells were collected from the dishes after scraping with a cell scraper (Greiner) and rinsing with IMDM. After washing, the cells were resuspended in IMDM and plated in CFC, CAFC and flask-LTC assays.

Stroma-conditioned media
Confluent layers were grown of the stromal cell lines FBMD-1, L87/4 and L88/5 [18,19,27]. The cells were cultured in IMDM supplemented with 10% fetal calf serum (FCS; Summit, Fort Collins, CO), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10^{-4} M β-mercapto-ethanol. The FBMD-1 cells were maintained at 33°C and 10% CO_2 and the L87/4 and L88/5 cells at 37°C and 10% CO_2. When the layers were confluent, the medium was removed and rinsed twice with IMDM. Serum-free medium was added to the confluent stromal layers and conditioned for seven days. The SCM were harvested, the non-adherent (NA) cells were removed by centrifugation and the media were stored at -20°C until use. Control medium was prepared by parallel incubations without the stromal cell lines. In the cultures, 50% SCM or control medium was used.

Stroma-contact cultures
In 35 mm tissue culture dishes (Falcon, Franklin Lakes, NJ) confluent layers were grown of the stromal cell line FBMD-1. When the layers were confluent, 40,000 CD34+ PBSC were cultured on these stromal feeders in the same medium and under the same conditions as used for the serum-free liquid cultures. After seven days of culture, the NA cells were collected from the dish and after two rinses with IMDM replaced by 1 ml of 0.1% trypsin (Gibco) for 5 minutes. The digestion was stopped by adding 1 ml of ice-cold FCS and the dish was scraped with a cell scraper to include all adherent cells. The NA and adherent cells were pooled and after washing the cells were resuspended in IMDM and plated in CFC, CAFC and flask-LTC assays.

Stroma-noncontact cultures
In 6-well plates (Costar, Cambridge, MA) confluent layers were grown of the stromal cell line FBMD-1. When the layers was confluent, 40,000 CD34+ PBSC were cultured in a collagen-coated membrane transwell insert (0.4 μm pore size; Costar) placed above the FBMD-1 stromal layer in the same medium and under the same conditions as used for the serum-free liquid cultures. After seven days of culture, all cells were collected from the transwell insert and after washing the cells were resuspended in IMDM and plated in CFC, CAFC and flask-LTC assays.

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 semisolid CFC assay containing erythropoietin (Boehringer, Mannheim, Germany), G-CSF, GM-CSF, IL-3 and murine SCF as described before [14]. CFU-GM and BFU-E were counted on day 14 of culture in the same dish.

Long-term cultures in flasks
Confluent stromal layers of FBMD-1 cells in 25 cm^2 flasks (Costar) were overlaid with 30,000 CD34+ PBSC or the output of 30,000 cultured CD34+ PBSC. The cells were cultured in IMDM supplemented with 10% FCS, 5% horse serum (Integro, Zaandam, The Netherlands), 10^{-5} M hydrocortisone 21-hemisuccinate (Sigma), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10^{-4} M β-mercapto-ethanol. IL-3 (10 ng/ml) and G-CSF (20 ng/ml) were added weekly to the cultures. Flask cultures were set up in duplicate and maintained at 33°C and 10% CO_2 for six weeks with weekly half-medium changes and therefore removal of half of the 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 IMDM replaced by 3 ml of 0.1% trypsin for 5 minutes. The digestion was stopped by adding 1 ml of ice-cold FCS and the flasks were scraped with a cell scraper to include all 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 cell suspension were plated in a semisolid CFC assay.

Cobblestone area forming cell assay
Limiting dilution CAFC assays were performed on confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates (Falcon). The cultures were maintained under the same conditions as the LTC in flasks. CD34+ PBSC were overlaid in a limiting dilution setup. Twelve dilutions two-fold apart were used for each sample with 15 replicate wells per dilution. The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells (i.e. cobblestone area) beneath the stromal layer was determined at week 2, 4 and 6 and CAFC frequencies were calculated using Poisson statistics.

Statistical analysis
Data were analyzed using GraphPad Instat (GraphPad Software, San Diego, CA). The means of two populations were compared using a paired Student's *t* test.

RESULTS

Expansion of progenitor and primitive stem cells in liquid cultures

In 7-day serum-free liquid cultures containing IL-3, SCF, IL-6 and αTGFβ1 (3/S/6/αT), we were able to expand progenitor cells (CFC and CAFC week 2 to 4) 2.6-fold and to maintain primitive stem cells (CAFC week 6: 1.2-fold expansion) (Figure 5.1A). As reported recently, the expansion of HSC could significantly be improved when SCM from the FBMD-1, L87/4 or L88/5 stromal cell lines was added to the liquid cultures [10]. Using L88/5 SCM, progenitor cells and primitive stem cells were 7.9-fold and 3.5-fold expanded, respectively (Figure 5.1A).

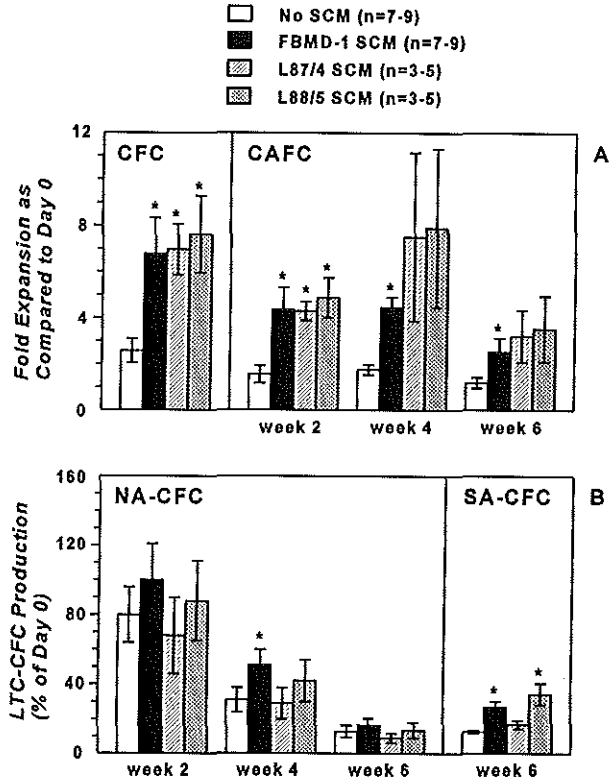

Figure 5.1 The effect of 7-day serum-free liquid cultures containing IL-3, SCF, IL-6 and αTGFβ1 with or without stroma-conditioned media (SCM) on (A) the expansion of different stem cell subsets (colony forming cells (CFC) and CAFC week 2 to 6) and (B) the ability of stem cells to produce non-adherent (NA) CFC in parallel flask long-term cultures (LTC) for six weeks. At week 6 also the stroma-adherent (SA) CFC content was determined. Comparison between no SCM and with SCM: *, $P<0.05$.

Graft quality of unexpanded CD34+ mobilized peripheral blood stem cells

To determine the *in vitro* graft quality of unexpanded CD34+ PBSC, flask-LTC were performed in parallel to the CAFC assay. In stroma-dependent flask-LTC the ability to produce NA-CFC was measured at week 2, 4 and 6. At week 6 also the number of stroma-adherent (SA) CFC was assessed. Throughout the culture period the total NA-CFC production ranged between 21 and 36 per 100 CD34+ input cells and at week 6 the adherent layer still contained 12 CFC per 100 input cells (Table 5.2). These results show that unmanipulated CD34+ PBSC were able to produce a relatively constant number of progenitors for at least six weeks of culture.

Table 5.2 Total Long-Term Culture Colony Forming Cell Production of Mobilized Peripheral Blood Stem Cells Before *Ex Vivo* Expansion.

	Total LTC-CFC Production per 100 Input Cells (± 1SEM)	Number of Experiments
NA-CFC at Week 2	36 (± 12)	7
NA-CFC at Week 4	30 (± 8)	7
NA-CFC at Week 6	21 (± 6)	8
SA-CFC at Week 6	12 (± 5)	8
NA + SA CFC at Week 6	33 (± 8)	8

LTC, Long-Term Culture; CFC, Colony Forming Cell; NA, Non-Adherent; SA, Stroma-Adherent.

Loss of graft quality of expanded CD34+ mobilized peripheral blood stem cells after liquid culture

Using the same setup for liquid cultures as described above, the *in vitro* graft quality of CD34+ PBSC was determined following a 7-day serum-free liquid culture. In contrast to the expansion of HSC numbers (Figure 5.1A), the ability of stem cells to produce LTC-CFC was diminished as compared to the input cells (Figure 5.1B). CD34+ PBSC that had been cultured in the presence of 3/S/6/αT produced 80% NA-CFC at week 2 as compared to unexpanded CD34+ PBSC. At later weeks there was a further reduction of NA-CFC production (week 4: 31%; week 6: 13%). At week 6 also the SA-CFC content was only a fraction of the control CD34+ PBSC (13%). The addition of FBMD-1 and L88/5 SCM showed only a modest improvement of the graft quality (NA-CFC week 2: 100% and 88%; NA-CFC week 4: 51% and 42%; NA-CFC week 6: 16% and 14%; SA-CFC week 6: 27% and 34%, respectively, as compared to unexpanded cells). L87/4 SCM did not influence the graft quality.

Expansion of progenitor and primitive stem cells in stroma-contact and stroma-noncontact cultures

To investigate whether direct contact with stroma cells would similarly improve the recovery and quality of primitive stem cells as did soluble stromal factors, we studied the fate of HSC when cultured for 7-days in serum-free medium containing 3/S/6/αT in FBMD-1 SCM, in direct contact with a FBMD-1 stromal layer and in FBMD-1 stroma-noncontact (Figure 5.2A). SCM and stroma-contact showed no significant differences in their effect of the numerical expansion of progenitors and primitive stem cells and both significantly improved generation of progenitor cells (CFC and CAFC week 2 to 4) as compared to stroma-noncontact cultures.

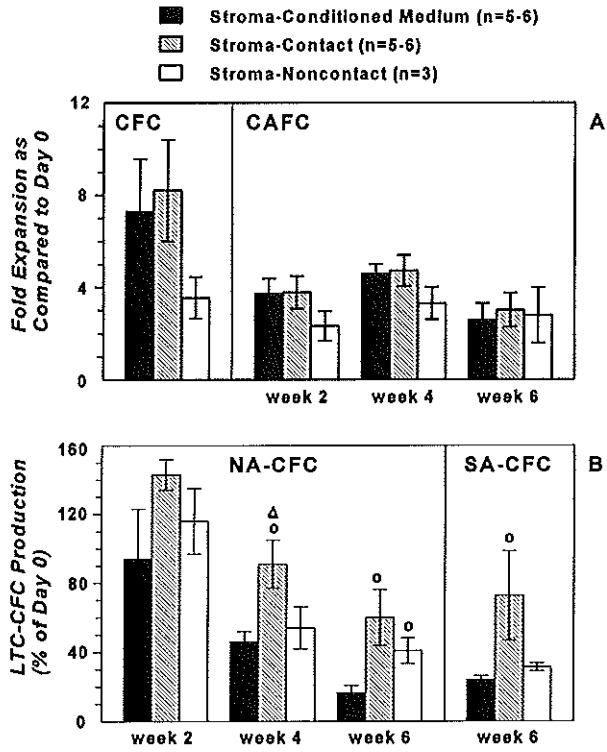

Figure 5.2 The effect of FBMD-1 stroma-conditioned media (SCM), FBMD-1 stroma-contact and FBMD-1 stroma-noncontact on (A) the expansion of different stem cell subsets (colony forming cells (CFC) and CAFC week 2 to 6) and (B) the ability of stem cells to produce non-adherent (NA) CFC in parallel flask long-term cultures (LTC) for six weeks in 7-day serum-free liquid cultures containing IL-3, SCF, IL-6 and αTGFβ1. At week 6 also the stroma-adherent (SA) CFC content was determined. Comparison between FBMD-1 SCM and FBMD-1 stroma-contact or stroma-noncontact: °, $P<0.05$. Comparison between FBMD-1 stroma-noncontact and FBMD-1 stroma-contact: $^{\Delta}$, $P<0.05$.

Stroma-contact prevents loss of primitive stem cell quality in expansion cultures

CD34+ PBSC cultured in FBMD-1 stroma-contact showed a significantly improved graft quality as compared to FBMD-1 SCM and stroma-noncontact cultures (Figure 5.2B). At week 2 and 4 the stroma-contact expanded CD34+ PBSC produced 143% and 91% NA-CFC, respectively, as compared to the input CD34+ PBSC. The NA-CFC and SA-CFC at week 6 of stroma-contact expanded cells were still 60% and 73%, respectively, as compared to unexpanded cells.

In table 5.3 the primitive stem cell quality in week 6 LTC is summarized. In 7-day serum-free liquid cultures containing 3/S/6/αT there was a dramatic loss of graft quality (13% of input) as expressed in NA+SA LTC-CFC at week 6 (Table 5.3, second column).The addition of FBMD-1 SCM could only slightly prevent

this quality loss (21% versus 13%). In FBMD-1 stroma-noncontact cultures quality of primitive stem cells was more preserved (38% versus 13%), while FBMD-1 stroma-contact cultures proved to be the best in preventing the loss of graft quality (66% versus 13%). By calculating the mean number of LTC-CFC produced in week 6 flask-LTC per CAFC week 6, we were able to estimate the average individual primitive stem cell quality of expanded CD34+ PBSC (Table 5.3, third column). Although the CD34+ PBSC which had been propagated in FBMD-1 stroma-contact had also the best average LTC-CFC per CAFC at week 6, in all culture conditions there was extensive loss (6- to 13-fold) of individual primitive stem cell quality as compared to unexpanded CD34+ PBSC.

Table 5.3 CAFC Percentage, NA+SA LTC-CFC Production and Mean LTC-CFC per CAFC at Week 6 of Mobilized Peripheral Blood Stem Cells Before and After *Ex Vivo* Expansion.

7-Day Culture Condition	CAFC Week 6 Expansion (% of input; ±1SEM)	LTC-CFC Week 6 Graft Quality (% of input; ±1SEM)	LTC-CFC per CAFC at Week 6 (±1SEM)
Before Expansion	100	100	20 (±6) (n=8)
IL-3/SCF/IL-6/ αTGFβ1	119 (±23) (n=8)	13 (±2) (n=7)	2.0 (±0.7) (n=7)
+FBMD-1 SCM	252 (±59)* (n=8)	21 (±3)* (n=7)	2.3 (±1.0) (n=7)
+L87/4 SCM	321 (±113) (n=5)	14 (±2) (n=4)	1.6 (±0.8) (n=4)
+L88/5 SCM	352 (±143) (n=5)	22 (±1)* (n=4)	2.5 (±1.1) (n=4)
+FBMD-1 Contact	301 (±73)* (n=6)	66 (±15)*° (n=5)	3.3 (±1.7) (n=5)
+FBMD-1 Noncontact	278 (±120) (n=3)	38 (±5)*° (n=3)	1.7 (±0.6) (n=3)

CAFC, Cobblestone Area Forming Cell; CFC, Colony Forming Cell; LTC, Long-Term Culture; NA, Non-Adherent; SA, Stroma-Adherent; SCM, Stroma-Conditioned Medium. Comparison between IL-3/SCF/IL-6/αTGFβ1 and IL-3/SCF/IL-6/αTGFβ1 with SCM, stroma-contact or stroma-noncontact: *, $P<0.05$. Comparison between FBMD-1 SCM and FBMD-1 stroma-contact or stroma-noncontact: °, $P<0.05$.

FL and TPO further improve the numerical expansion and quality maintenance of both progenitor and primitive stem cells

To test whether the effects of stroma and stroma-elaborated activities was due to the recently cloned cytokines FL and TPO, we performed experiments in which FL and TPO were added to the 7-day cultures in the presence of 3/S/6/αT with or without FBMD-1 SCM or FBMD-1 stroma-contact. The combination of IL-3, SCF, IL-6, αTGFβ1, FL and TPO with FBMD-1 stroma-contact led to a 21.1- and 4.9-fold expansion of CAFC week 2 and 6, respectively (Table 5.4). Addition of FL/TPO to 7-day FBMD-1 stroma-contact cultures also further enhanced the non-adherent CFC production in LTC leading to a complete maintenance of LTC-CFC quality (Table 5.5). As a result, inclusion of FL/TPO improved the recovery of all progenitor and stem cell subsets tested, and their ability to generate CFC. Remarkably, the inclusion of FBMD-1 SCM or FBMD-1 stroma-contact still gave further improvement of these parameters, indicating that the stroma-related effects described here were not mediated by FL and/or TPO.

Table 5.4 CFC and CAFC Week-Type Expansion of Mobilized Peripheral Blood Stem Cells After *Ex Vivo* Expansion With or Without the Addition of FL and TPO.

7-Day Culture Condition	Fold Expansion as Compared to Day 0			
	CFC	CAFC Week 2	CAFC Week 4	CAFC Week 6
IL-3/SCF/IL-6/αTGFβ1	0.9	0.7	1.0	0.5
+FL/TPO	4.4	5.5	2.7	2.1
3/S/6/αT/FBMD-1 SCM	2.4	1.6	2.0	0.9
+FL/TPO	10.2	13.7	4.1	3.4
3/S/6/αT/FBMD-1 Contact	4.6	2.1	2.1	1.7
+FL/TPO	15.9	21.1	6.7	4.9

3/S/6/αT, IL-3/SCF/IL-6/αTGFβ1; CAFC, Cobblestone Area Forming Cell; CFC, Colony Forming Cell; SCM, Stroma-Conditioned Medium.

Table 5.5 Non-Adherent LTC-CFC Production of Mobilized Peripheral Blood Stem Cells After *Ex Vivo* Expansion With or Without the Addition of FL and TPO

7-Day Culture Condition	Non-Adherent LTC-CFC Production (% of Day 0)		
	Week 2	Week 4	Week 6
IL-3/SCF/IL-6/αTGFβ1	9	8	22
+FL/TPO	34	10	20
3/S/6/αT/FBMD-1 SCM	7	8	37
+FL/TPO	24	41	118
3/S/6/αT/FBMD-1 Contact	19	88	61
+FL/TPO	39	43	144

3/S/6/αT, IL-3/SCF/IL-6/αTGFβ1; LTC-CFC, Long-Term Culture-Colony Forming Cell; SCM, Stroma-Conditioned Medium.

DISCUSSION

At least three different groups have reported the transplantation of CD34-selected mobilized PBSC following a period of *in vitro* culture in an attempt to expand repopulating cell numbers [4-6]. Using 8- to 12-day liquid culture systems with various cytokine combinations, 28 patients were transplanted with *ex vivo* propagated CD34+ cells. These studies showed an up to 332-fold expansion of progenitor cells in the PBSC transplants, however, the expanded grafts did not significantly improve hematological recovery. This suggests that a numerical expansion of progenitors may not be of relevance for short-term hematopoietic recovery post-transplantation. Indeed, this notion is supported by a murine study showing that committed progenitors play no role in short-term *in vivo* repopulation [28].

In the present study we investigated the recovery of both progenitors and primitive stem cells in CD34+ PBSC after *ex vivo* propagation in more detail. We showed an expansion of both progenitor and primitive stem cell numbers, which was consistent with previous reports [4-10]. As reported before, the effect of soluble stromal factors significantly improved this expansion [10]. Although we found an expansion of CFC and various CAFC subsets in these cultures, the grafts ability to generate progenitors was only maintained in week 2 flask-LTC, while the cultured cells produced dramatically less progenitors at later weeks. This showed that an absolute numerical expansion of progenitors and primitive stem cells may have occurred at the expense of the ability of primitive HSC to generate CFC in long-term stroma-supported cultures, or alternatively, that not all primitive HSC survived. This situation could explain the inability of expanded grafts to improve hematological recovery in conditioned recipients.

Although the clinical relevance of a reduced ability of expanded primitive stem cells to produce CFC at later weeks is not fully clear, we feel that this observation is more than just "an *in vitro* artifact". Our group has recently observed that autologous grafts that were unable to lead to significant hematopoietic repopulation of patients at three months post-transplantation contained low CAFC week 6 numbers, while their ability to generate CFC in LTC was very low [15]. In addition, we have reported that only few CAFC week 6 are mobilized in patients that have received intense cytotoxic chemotherapy, and that their quality is reduced [14].

In the second part of this study we have investigated factors which could improve the expansion of primitive stem cells (CAFC week 6) and maintain their quality (LTC-CFC at week 6). We have found that both soluble stromal factors and stroma-contact increase the expansion of CAFC week 6 in the presence of cytokines (IL-3/SCF/IL-6) and αTGFβ1. The improved generation of CAFC week 6 is probably the combined result of their proliferation and conservation. The Verfaillie group has shown that the maintenance or expansion of LTC-IC in 2-week stroma-noncontact cultures supplemented with cytokines (IL-3/MIP-1α) is the result of extensive proliferation of a small fraction of the input LTC-IC [29]. In addition, the same research group using 2- or 5-week stroma-noncontact cultures without addition of cytokines has reported an inhibitory effect of stroma-contact on CFC and LTC-IC proliferation as compared to stroma-noncontact cultures [22,23]. In our study we do not observe inhibitory effects of stroma-contact on CAFC week 6 expansion. This can be explained by the addition of cytokines (IL-3/SCF/IL-6) to both stroma-contact and stroma-noncontact cultures in our experiments, which may have overruled this stroma-contact mediated proliferation block [23]. Furthermore, the addition of neutralizing antibodies directed against TGFβ1 could have further abrogated the proliferation inhibition of stroma-contact, because TGFβ1 is an important inhibitor of primitive stem cell proliferation [30] and is produced by the FBMD-1 stromal cell line [18]. In addition to the favorable effect of stroma-contact for numerical CAFC expansion, direct stroma-contact appears to be required for conservation of total graft quality (LTC-CFC week 6). Because soluble stromal factors only partly protect against quality loss of HSC, it may be argued that the concentration of SCM in our cultures may have been too low, however, stroma-noncontact cultures gave comparable results as SCM-containing cultures. As a result, our data support the observations of Koller et al. in that stromal cells exert a favorable effect on bone marrow derived LTC-IC expansion in 2-week cultures containing IL-3, SCF, GM-CSF and erythropoietin [25,26]. In the light of the recent observations from other investigators that *in vitro* expanded progenitor and primitive stem cell grafts do not improve time to hematological recovery, it seems indeed pertinent to include stromal elements in bioreactors for cytokine-driven *ex vivo* expansion of HSC contained in mobilized peripheral blood [31].

A recent study of Petzer et al. reports that in 10-day liquid cultures IL-3, SCF, FL and TPO are the most important cytokines for the expansion of LTC-IC from CD34+/CD38-negative bone marrow cells [32]. In addition, it has been

shown that in these cultures there is no loss of CFC-producing ability [33]. Therefore, we performed additional experiments in which FL and TPO were added to the expansion cultures. Indeed, the addition of FL/TPO to expansion cultures improved the recovery of progenitors and primitive stem cells and that this FL/TPO effect was still present in the presence of soluble stromal factors and direct stromal contact. In addition, FL/TPO together with stromal factors further improved the LTC-CFC production at week 6 resulting in a complete maintenance of primitive stem cell quality. These observation strongly support the addition of FL and TPO to stroma-dependent expansion strategies.

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CHAPTER 6

FREQUENCY ANALYSIS OF MULTIDRUG RESISTANCE-1 GENE TRANSFER INTO HUMAN PRIMITIVE HEMATOPOIETIC STEM CELLS USING THE COBBLESTONE AREA FORMING CELL ASSAY AND DETECTION OF VECTOR-MEDIATED P-GLYCOPROTEIN EXPRESSION BY RHODAMINE-123

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ABSTRACT

Transfer of the multidrug resistance-1 (MDR1) gene into hematopoietic stem cells (HSC) may reduce myelotoxicity of MDR1-related cytotoxic agents and therefore allow dose intensification. Mobilized peripheral blood stem cells (PBSC) can be obtained in ample quantity and are a suitable target cell population. CD34-selected PBSC samples (n = 6) were transduced with cell-free supernatant (SNT) of a cell line producing recombinant retrovirus containing the human MDR1 gene. Limiting-dilution long-term cultures (LTC) were employed which allow to continuously monitor stroma-adherent cobblestone areas (CA) and to compare their frequency in a 5-log range over time. According to Poisson statistics, proviral DNA was contained in 22% of unselected cobblestone area forming cells (CAFC) week 6 which represent primitive HSC. In comparison, 1.0% ±0.44% (mean ±SEM) of CAFC week 6 were expressing P-glycoprotein at sufficient levels to convey vincristine-resistance, suggesting low expression of the retroviral vector or splicing of the vector-drived mRNA in HSC. Next we analyzed lineage-committed progenitors. The proviral DNA was detectable in 20% to 66% of colony-forming units granulocyte-macrophage (CFU-GM) while corresponding percentages (25% to 52%) of CD34+ PBSC were in the S/G_2M phase of the cell cycle at the end of the transduction period. The proportion of vincristine-resistant CFU-GM was similar to the CAFC data and no significant differences were found between various MDR1-SNT transduction schedules, while MDR1 co-cultivation, which served as positive control, yielded significantly higher proportions of resistant colonies (5.3% ±1.4%, interleukin-3 (IL-3), 96 hours, $p < 0.05$). Assessment of Rh123 efflux in the myelo-monocytic progeny of MDR1 transduced cells mirrored the CFU-GM assay results in the SNT and co-cultivation groups. Less culture effort was required in the Rh123 assay and functional characterization of the transferred P-glycoprotein was possible using cyclosporin A. Further development towards an effective MDR1 gene therapy should be facilitated by the CAFC assay which allows to estimate the retroviral gene transfer frequency into primitive HSC and by the Rh123 assay which permits tractable side by side assessments of numerous MDR1 transduction protocols or different MDR1-SNT lots.

OVERVIEW SUMMARY

Transfer of the MDR1 gene to hematopoietic cells for myeloprotection against cytostatic agents is a new and rapidly developing field in cancer gene therapy. The requirements to be met by the MDR1 gene delivery system include:

1. Efficient transfer into relatively large numbers of HSC
2. Stable expression of the MDR1 gene allowing long-term protection of hematopoietic cells
3. High functional expression of the MDR1 gene
   Stroma-dependent LTC assay the ability of primitive HSC for long-term

production of clonable progenitors. We used a limiting-dilution type LTC assay which allows frequency analysis of transiently repopulating HSC and LTC-initiating cells (LTC-IC) without the necessity to replate large numbers of wells. Addition of drug-selection and molecular analysis of primitive stem cell progeny enabled us to characterize MDR1 gene transfer and expression in human primitive mobilized PBSC.

The fluorescent dye Rh123 allows for quantitation of P-glycoprotein activity on a single cell basis. The high proportion (32% to 33%) of CD34+ cells that are naturally $Rh123^{dull}$ precluded the use of this assay directly after transduction. When CD34+ PBSC are, however, cultured in the presence of stem cell factor (SCF), IL-3, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-CSF (G-CSF), a population of granulo-monocytic cells evolves that display a $Rh123^{bright}$ phenotype while cells expressing vector-derived P-glycoprotein remain $Rh123^{dull}$. This permitted us to functionally test the MDR1 gene in the mature progeny of transduced HSC.

INTRODUCTION

The response of hematological malignancies and solid tumors to cytotoxic therapy appears to be dependent on the dose-intensity [1,2]. Since myelosuppression is the main side effect of cytostatic agents, PBSC have been used to support sequential high-dose regimens [3]. Mobilization of PBSC may become difficult in the course of the disease because of a chemotherapy-related depletion [4]. Transfer of cytostatic drug resistance genes into PBSC at an early stage of the disease may therefore delay stem cell exhaustion.

MDR1 is a candidate gene, which codes for an ATP-dependent transmembrane efflux pump (P-glycoprotein) of 170 kDa that extrudes many lipophilic metabolites and xenobiotics [5]. In murine studies transfer of the MDR1 gene into HSC with long-term repopulating potential could be shown by a maintained chemoprotection of up to six serially transplanted cohorts of mice [6]. In humans, stroma-dependent LTC are considered a valid assay-system to assess the long-term repopulating ability of a graft [7]. Several groups were able to show MDR1 gene transfer into LTC-IC [8,9]: Plating of non-adherent bone marrow cells from five to six-week old stroma-dependent LTC in CFU-GM assays resulted in 13% to 25% of colonies containing the MDR1 gene. However, these data may not reflect the transduction efficiency of the most primitive stem cells, because the number of clonogenic cells formed per LTC-IC is highly variable [7]. For this purpose we used a recently developed human limiting-dilution CAFC assay which allowed quantification of MDR1-transduced stem cells with short- and long-term repopulating ability [10,11]. It was possible to demonstrate a high gene transfer rate and sustained expression of the retrovirally-transferred MDR1 gene in primitive HSC. In MDR1 transduced lineage-committed cells the assessment of Rh123 dye efflux allowed a less labor-intense and equally sensitive detection of functional P-glycoprotein than CFU-GM assays with

vincristine.

MATERIALS AND METHODS

MDR1 retrovirus preparation
A cell line producing amphotropic recombinant retrovirus containing the wild-type human MDR1 gene cloned into a modified amphotropic retroviral vector [12] and driven by the long terminal repeat (LTR) of Moloney Murine Leukemia Virus (MoMuLV) was used [13]. Producer cells expressing high levels of P-glycoprotein were selected in vincristine (70 nM; Sigma, St Louis, MO). Cells were propagated at 37°C, 10% CO_2 in Dulbecco's modified Eagle's media (DMEM; Gibco, Grand Island, NY), 10% fetal calf serum (FCS; Hyclone, Logan, UT) and 100 U/ml penicillin (Gibco) and 100 mg/ml streptomycin (Gibco).

Harvesting of retroviral supernatant
Retroviral producer cells were grown to 80-90% confluency in T175 flasks. Virus harvesting and transduction was performed in a medium selected for supporting HSC growth as previously described [14]. Supernatants were then filtered through a 0.45 mm filter (Millipore, Molsheim, France) and frozen in liquid nitrogen until use.

Selection of CD34 positive cells
Mobilized PBSC were obtained in the recovery phase after G-CSF-supported (Filgrastim; Amgen, Thousand Oaks, CA) chemotherapy from one patient with non-Hodgkin lymphoma, two patients with breast cancer and two patients with multiple myeloma or after G-CSF alone from an allogeneic PBSC donor after informed consent. Frozen PBSC samples from the lymphoma and breast cancer patients were thawed, mononuclear cells were obtained using Ficoll-Hypaque (Sigma) centrifugation and CD34-selection was performed using small-scale Ceprate LC columns (CellPro, Bothell, WA). From the other donors, fresh cells were used after CD34-selection on large-scale Ceprate SC columns (CellPro).

Fluorescence-activated cell sorting (FACS) analysis for CD34+ cell determination
Five to fifty x 10^4 cells were incubated at 4°C for 30 min in 0.1 ml PBS and 1% bovine serum albumine (BSA) with 5 μl of a PE-conjugated anti-CD34 antibody (HPCA-2; Becton Dickinson, Mountain View, CA) and 5 μl of a FITC-conjugated anti-CD45 antibody (HLe1; Becton Dickinson). Isotype-identical control antibodies were used (Becton Dickinson). After washing, cells were acquired on a FACSort flow cytometer (Becton Dickinson). Cells were analyzed with the Lysis II software program (Becton Dickinson). The percentage of CD34+ cells was calculated as the ratio of CD34+ cells to CD45+ cells and multiplied by 100.

Transduction of CD34+ cells
Three transduction schedules were compared. Cells (1 x 10^6/ml) were either directly transduced over 48 hours or prestimulated in growth factors for 48 hours and transduced for 48 hours or directly transduced over 96 hours. All groups received four times retroviral supernatant. Polybrene was added with every supernatant change to maintain a concentration of 1 mg/ml. Three different growth factor combinations were used: IL-3 (50 ng/ml), SCF (100 ng/ml) plus IL-3 (50 ng/ml), SCF (100 ng/ml) plus IL-3 (20 ng/ml) plus IL-6 (50 ng/ml). As positive controls, irradiated MDR1 retroviral producer cells were co-cultivated with CD34-selected PBSC over 96 hours at identical cell and growth factor concentrations as described above. Molecular biological techniques and cell biological assays were employed to monitor the presence and function of the MDR1 gene in hematopoietic cells at different stages of primitivity.

Colony-forming units granulocyte-macrophage assay
CD34-selected cells were plated in duplicate at 3 - 5 x 10^3/ml in 1 ml methylcellulose medium as

previously described [14]. Fifty ng/ml IL-3 and 25 ng/ml GM-CSF (Boehringer Mannheim, Mannheim, Germany) were added. Screening for MDR1 overexpressing progenitor cells was performed with vincristine which is an efficient substrate for the P-glycoprotein. Freshly thawed vincristine was added to the dishes to yield final concentrations of 0, 10, 20, 30, 40 and 50 nM. At all concentrations, cells were plated in duplicate. The plates were incubated at 37°C in 10% CO_2. Hematopoietic colonies were scored after 12-14 days. MDR1-transduced, mock-transduced and primary material were compared. Some colonies were picked and analyzed as described below.

Liquid culture and rhodamin efflux assay

Two to five x 10^4 transduced CD34+ PBSC were incubated for 8-10 days (37°C, 10% CO_2) in a cytokine cocktail containing 10 ng/ml of each SCF, IL-3, IL-6, G-CSF and GM-CSF. α-modified DMEM containing 30% FCS was used as culture medium. Under these conditions, the cultured PBSC differentiated into $Rh123^{bright}$ CD11b+/CD15+ and CD11b+/CD15- cells [15] which were previously shown to represent precursors and mature cells of the granulo-monocytic lineage [16]. MDR1 gene function in these cells was measured by their ability to exclude Rh123, resulting in a $Rh123^{dull}$ phenotype. To do so, transduced and cultured cells were washed and suspended in PBS containing 5% FCS. Rh123 (Eastman Kodak, Rochester, NY) was added to give a final concentration of 0.2 mg/ml. After an incubation period of 30 min at 37°C, cells were centrifuged, resuspended and incubated for 15 min at 37°C in PBS with 5% FCS in order to allow the cells to efflux loaded Rh123. Subsequently, the cells were stained with a PE-conjugated anti-CD45 antibody (KC56.RD1, Coulter, Mijdrecht, The Netherlands) for 30 min. at 4°C in PBS with 1% BSA. Cells were washed and resuspended in 500 ml of PBS. Dead cells were excluded from the analysis by propidium iodide staining (Sigma, final concentration 1 mg/ml). In some experiments, separate controls were run which contained the P-glycoprotein inhibitor cyclosporin A at a concentration of 1.5 mM during all incubation steps. Acquisition and analysis were performed on a FACSort flow cytometer as described above.

Cobblestone area forming cell assay

Confluent stromal layers of the preadipocyte FBMD-1 cell line in 96-well plates were overlaid with CD34-selected primary or transduced PBSC in a limiting dilution set-up as described [10]. Input values ranged from 2000 - 1 cells/well. Twelve dilutions 2-fold apart were used for each sample with 15 replicate wells per dilution. The cells were cultured at 33°C in α-modified DMEM supplemented with HEPES (3.5 mM; Sigma), glutamine (2 mM; Sigma), sodium selenite (10^{-7} M; Merck, Darmstadt, Germany), 2-mercaptoethanol (10^{-4} M; Merck), 20% horse serum (Gibco) and hydrocortisone 21-hemisuccinate (10^{-5} M final concentration; Sigma). Half of the medium was changed weekly. IL-3 (10 ng/ml) and G-CSF (20 ng/ml) were added weekly to the cultures. The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells (cobblestone area) beneath the stromal layer was determined at weeks 2, 3, 5 and 6 after overlay and CAFC frequencies were calculated using Poisson statistics as described previously [11]. For detection of P-glycoprotein overexpression in stroma-attached hematopoietic clones, CAFC assays were performed in the presence of 20 nM vincristine. The murine stromal feeder cell line was resistant to up to 200 nM of vincristine in pilot experiments.

Long-term culture in flasks

Confluent stromal layers of FBMD-1 cells in 25 cm^2 flasks were overlaid with 2-3 x 10^4 CD34-selected primary or transduced PBSC. The cells were cultured in the same medium and under the same conditions as the CAFC assays. At week 3 half of the non-adherent cell fraction was plated in CFU-GM assays. At the end of a 5-week culture period the number of CFU-GM of the pooled non-adherent and adherent fractions was determined. To this purpose, the medium was removed from the flasks and replaced by 3 ml of 0.1% trypsin (Gibco) for 5 min. The digestion was stopped by adding 1 ml of ice-cold FCS. A single cell suspension was made by sieving the cell suspension through a 100 mm nylon filter. The cell suspension was taken up in α-modified DMEM and plated in duplicate in a CFU-GM assay as described above. The high clonogenicity of

the CD34+ PBSC required an additional 1: 10 dilution of the week 3 and week 5 cells.

Polymerase chain reaction
CFU-GM grown in semisolid media for 12 to 14 days without selection pressure and containing 50-1000 cells were individually picked, washed in 0.5 ml PBS and stored as dry pellets at -80°C until further analysis. Similarly, after 6 weeks of culture, all wells of a CAFC assay were rinsed with 200 ml PBS and then trypsinized. Non-adherent and adherent cells were collected from each individual well, washed again in 0.5 ml PBS and stored at -80°C as dry pellets. For cell lysis, pellets were incubated in 50 ml of nonionic detergent lysis buffer (0.5% NP40, 0.5% Tween 20, 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatine, 2.5 mM $MgCl_2$) containing proteinase K (60 mg/ml) at 56°C for 1 hour. Lysates were then heated at 95°C for 5 min to inactivate the proteinase K. PCR was performed on 10 ml lysates in a total volume of 50 ml with 0.25 U of SuperTaq polymerase (HT Biotechnology, Cambridge, United Kingdom) in an optimized reaction mix (final concentration: 200 mM each of 2'-deoxyadenosine-5'-triphosphate (Pharmacia, Roosendaal, The Netherlands), 2'-deoxycytidine-5'-triphosphate (Pharmacia), 2'-deoxyguanosine-5'-triphosphate (Pharmacia), 2'-deoxythymidine-5'-triphosphate (Pharmacia), 10 mM Tris pH 8.3, 50 mM KCl, 0.01% gelatine, 1.3 mM $MgCl_2$, 0.2 mM each of sense and antisense primers). Primers were chosen to selectively amplify the retrovirally transduced MDR1 gene. In the first PCR round the sense strand primer was located at the 3' end of MDR1 gene (primer 1, residues 3411 - 3435 counted from the start codon) while the antisense primer was complementary to the 3' retroviral LTR (primer 2). After a second round with a sense primer further downstream the MDR1 gene (primer 3, residues 3546 - 3567) and antisense primer 2, a 728 base-pair fragment was obtained. Amplification conditions were as follows (TRIO thermocycler, Biometra, Göttingen, Germany): 95°C for 5 minutes, then 40 cycles of 94°C for 45 seconds, 68°C for 45 seconds, 72 C for 1 minute, followed by extension at 72 C for 10 minutes. Ten microliters of each reaction were separated on 1% agarose gel (Pronarose, Hispanagar, Burgos, Spain) and visualized in ultraviolet light by ethidium bromide staining.

Cell cycle analysis
For nuclear DNA staining, 7-aminoactinomycin D (7-AAD, Molecular probes, Eugene, OR) was chosen. Simultaneously, CD34+ cells could be detected by the PE-conjugated HPCA-2 antibody which gives an approximately 3-fold higher fluorescence intensity than the FITC-conjugated HPCA-2 [17]. After CD34 staining of primary and transduced cells, fixation and permeabilization were performed according to a protocol of Corver et al. [18]. Cells were washed twice with ice-cold PBS. Half a ml of ice-cold 2% phosphate-buffered paraformaldehyde, containing 80 mg/ml lysolecithin (Sigma), was added drop-wise to 0.5 ml cell suspension under constant swirling. After 5 min on ice, the reaction was blocked by adding 2.0 ml PBS/1% BSA. Cells were washed once with PBS/BSA and resuspended in PBS/BSA containing a final concentration of 8 mg/ml 7-AAD. Cells were analyzed at least 30 min after adding 7-AAD (room temperature, light-protected) on a FACSort flow cytometer. Doublets were excluded using the FL3-Area and FL3-Width parameters of the doublet discrimination module. In a histogram display, CD34 positive single cells were analyzed for cell cycle distribution. Two regions were set: one encompassing the G_0/G_1 cell population and a second one for proliferating cells falling into the S/G M compartment of the cell cycle.

Statistical analysis
The paired student's t-test was used to test for statistically significant differences in P-glycoprotein expression or growth potential of HSC following retroviral transduction. Data are presented as mean values ± SEM.

RESULTS

MDR1 transduction of CD34-selected peripheral blood stem cells

The CD34-selected PBSC samples contained 69% ±14% CD34+ cells (n=6). The MDR1 retrovirus-containing supernatant (MDR1-SNT) had a titer of 5 x 10^4 infectious particles per ml as tested on MDR1-SNT transduced and vincristine-selected (70 nM) NIH/3T3 fibroblasts in an end point titration assay.

PBSC were assessed for MDR1 provirus integration and P-glycoprotein overexpression following retroviral transduction. To determine the transduction efficiency, 50 CFU-GM not grown under vincristine-selection were picked from the IL-3/48-hour SNT groups of each of 3 experiments. Twenty to sixty-six percent of the colonies contained the MDR1 provirus as assessed by a retrovirus-specific nested PCR (Figure 6.1, Table 6.1), confirming efficient transfer of the MDR1 gene into lineage-committed hematopoietic progenitor cells with the retroviral vector used. An extracellular/intracellular double-staining protocol allowed cell cycle analysis of the CD34+ PBSC. The mobilized PBSC were primarily in the resting phase of the cell cycle. Incubation in hematopoietic growth factors induced cell cycle progression from 4% ±1% in the S/G_2M phase to 25% or above after 48 hours (Table 6.1). As expected, MDR1 gene integration and the proportion of CD34+ cells in cell cycle following incubation in hematopoietic growth factors were correlated [19].

Table 6.1 Correlation of Cell Cycle Activation and Transduction of the MDR1 Retroviral Vector in Lineage-Committed Mobilized Peripheral Blood Stem Cells.

Experiment number	CD34+ cells in S/G_2M	PCR-positive CFU-GM[a]
1	25%	20%
2	31%	32%
3	52%	66%

[a] Fifty different CFU-GM were analyzed in each experiment.

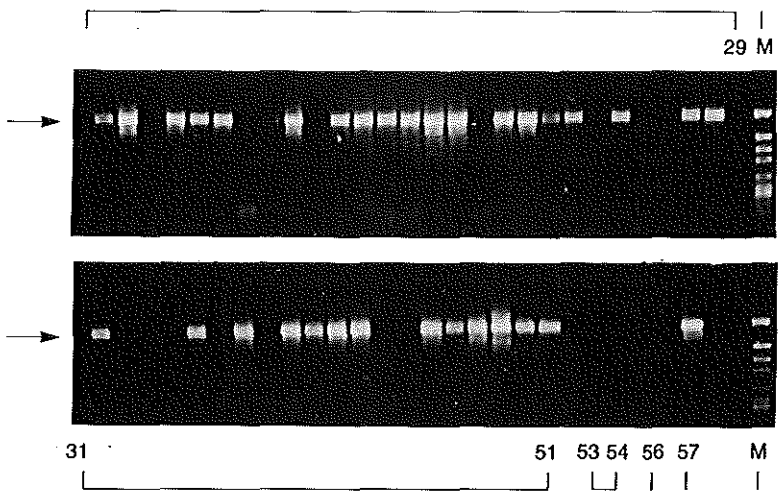

Figure 6.1 MDR1-PCR of CFU-GM picked after 48 hours transduction of CD34+ peripheral blood stem cells in IL-3. Each lane represents a single CFU-GM. Using provirus-specific primers DNA was amplified only in colonies that had integrated the MDR1 gene while untransduced colonies remained PCR negative. Lanes 1 - 29 and lanes 31 - 51 contain MDR1 transduced CFU-GM grown without vincristine-selection. Lanes 53 and 54 contain mock-transduced CFU-GM. Lane 56 is a lysis buffer control. Lane 57 contains the positive control. A size marker (M) was included for the upper and lower lanes. The arrows on the left indicate the expected 728 base-pair fragment.

Next, different cytokine combinations were added during MDR1 gene transfer and the effects on P-glycoprotein induction were compared. The proportion of MDR1 transduced and vincristine-resistant CFU-GM was determined at the drug concentration where the growth of control colonies had ceased, e.g. in the experiment given in table 6.2 at 30 nM vincristine. The cytokine combinations IL-3, SCF/IL-3 or SCF/IL-6/IL-3 added for 48 hour or 96 hour periods of retroviral supernatant transduction yielded a mean of 0.3% to 1.6% vincristine-resistant CFU-GM with no significant difference between MDR1-SNT groups (Figure 6.2). This was considerably lower than the gene transduction frequency to CFU-GM. Co-cultivation of CD34+ PBSC on retroviral MDR1 producer cells resulted in higher proportions of vincristine-resistant CFU-GM than following MDR1-SNT transduction in all experiments; statistical significance was reached in the IL-3 group ($p<0.05$). As already 20% to 66% of the CFU-GM contained the MDR1 gene following SNT transduction, the higher proportion of resistant colonies following co-cultivation may have been due to multiple provirus integrations per progenitor cell.

Table 6.2 Growth of CFU-GM in Increasing Concentrations of Vincristine Following MDR1-Supernatant (SNT) or Mock-Transduction for 48 Hours in the Presence of Interleukin-3.

Vincristine (nM)	MDR1-SNT (number of CFU-GM[a])	Medium without SNT (number of CFU-GM[a])
0	167	201
10	163	125
20	14	10
30	8	0
40	1	0
50	0	0

[a] A total of 5 x 10^3 CD34-selected PBSC plated per dish, mean values of duplicate dishes.

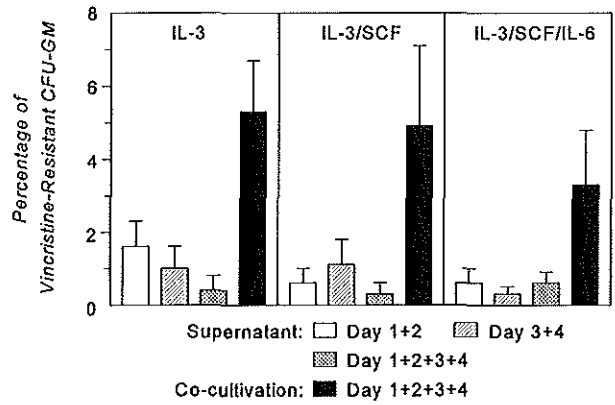

Figure 6.2 Effect of three different cytokine combinations and four different MDR1-transduction schedules on the induction of vincristine resistance in CFU-GM. Control values are zero as the proportion of MDR1 transduced and vincristine-resistant CFU-GM was determined at the drug concentration where the growth of control colonies had ceased, e.g. in the experiment given in table 6.2 at 30 nM vincristine.

The fluorescent dye Rh123 allows quantitation of P-glycoprotein activity on a single cell basis. The high proportion (32% to 33%) of CD34+ cells that are naturally $Rh123^{dull}$ precluded the use of the Rh123 assay directly after transduction. However, when infected CD34+ PBSC are cultured in the presence of SCF, IL-3, IL-6, GM-CSF and G-CSF, a population of CD15 and/or CD11b expressing granulo-monocytic cells evolves [15,16] that display a $Rh123^{bright}$ phenotype [20]. This permitted us to functionally test the MDR1 gene in the mature progeny of transduced progenitor cells (Figure 6.3). MDR1-

transduced cells had an approximately 50-100 fold decreased mean Rh123 fluorescence intensity compared to mock-transduced controls (Figure 6.3). Addition of cyclosporin A was able to abrogate Rh123 exclusion from MDR1-transduced cells (Figure 6.3) which proved that $Rh123^{dull}$ events originated from P-glycoprotein-expression [21]. Data were expressed as the number of $Rh123^{dull}$ cells generated by 10^5 transduced CD34+ PBSC during the liquid culture period (Figure 6.4). Relevant differences in the number of $Rh123^{dull}$ cells were not detected between the various cytokine combinations or MDR1-SNT transduction schedules which mirrored the results of the CFU-GM assays with vincristine. Co-cultivation resulted in higher numbers of $Rh123^{dull}$ cells than SNT in all experiments and again the IL-3 group was the only one to reach statistical significance ($p<0.05$). These comparable results were obtained with less culture effort in the Rh123 assay (two dishes per group) than in the CFU-GM assay (12 dishes per group) and a high number of transduced cells could be analyzed in a shorter time period than CFU-GM be counted in drug-titration experiments, thus advocating the Rh123 assay for the side by side assessment of numerous MDR1 transduction protocols or different MDR1-SNT lots.

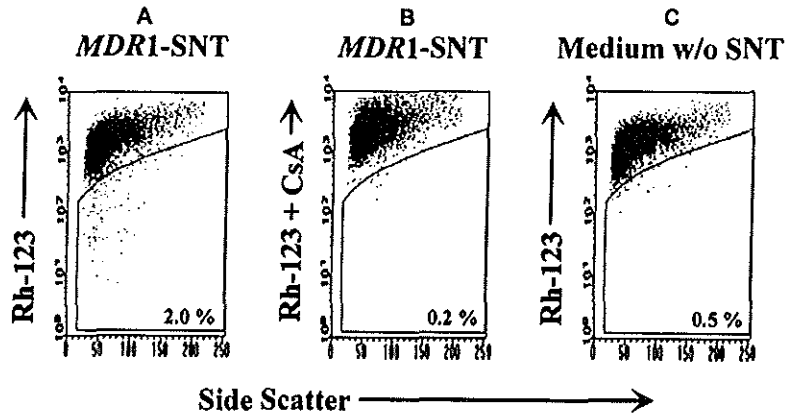

Figure 6.3 Rh123 exclusion analysis in the progeny of cultured CD34+ peripheral blood stem cells following 48 hours transduction in MDR1-SNT in the presence of IL-3 (A). Rh-exclusion could be abrogated by the P-glycoprotein inhibitor cyclosporin A (B). Mock-infection with medium without retroviral SNT resulted in a similarly low background of $Rh123^{dull}$ cells (C)

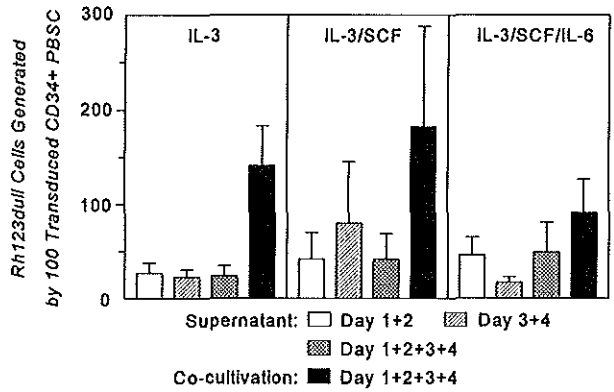

Figure 6.4 Effect of three different cytokine combinations and four different MDR1-transduction schedules on Rh123 exclusion in the progeny of cultured CD34+ peripheral blood stem cells. Values of the matched mock-transduced controls were below 10 $Rh123^{dull}$ cells (data not shown).

Frequency analysis of MDR1 gene transduction and expression in CAFC

The assays described above looked at lineage-committed progenitor cells, while the CAFC assay, which is a limiting-dilution stroma-dependent LTC, allows analysis of different stem cell subsets. As the different MDR1-SNT transduction conditions had yielded comparable results in the CFU-GM and Rh123 experiments, we chose one group, 48 hour transduction in the presence of IL-3, for the MDR1 gene transduction and expression studies using the CAFC assay. After MDR1-SNT transduction, the cells overlaid in the CAFC assay and cultured for six weeks. In this experiment 73 CD34-selected PBSC contained one CAFC week 6. Next we determined the MDR1 gene transfer frequency by PCR in the same CAFC assay. After 6 weeks of culture all wells from 12 dilutions of the MDR1-SNT CAFC assay not grown under vincristine-selection were trypsinized and DNA was extracted. A provirus-specific PCR showed all wells of dilution 1 (2000 input cells/well) to contain the transferred MDR1 gene. In further dilutions, the number of PCR-negative wells increased and wells were uniformly negative from dilution 9 on (146 wells assayed). Stroma-adherent CAFC could not be separated from non-adherent cells before trypsinization. However, the pooled week 6 cells are considered to represent progeny of primitive HSC Ils as 60/92 wells containing at least one CAFC week 6 were MDR1-positive compared to only 6/34 wells that did not contain week 6 CAFC but had week 2-5 CAFC at an earlier time point. Wells which did not have CAFC activity during the culture period were uniformly PCR-negative (n = 20). Among 336 CD34-selected and MDR1-transduced PBSC one produced a PCR-positive CAFC week 6 positive well. When the frequency of 1 CAFC week 6 per 73 input cells was compared with the frequency of one PCR-positive and simultaneously CAFC week 6 containing well per 336 input cells, 22% of primitive HSC contained the proviral MDR1 gene.

For comparison of CAFC frequencies obtained after different culture periods in one assay, the yield of CAFC was calculated per 10^5 input cells [10]. CAFC assays were set up with and without vincristine-selection (Figure 6.5). After MDR1-SNT transduction in IL-3 for 48 hours, 2.3% ±1.5% (n = 4) CAFC week 2 resistant to 20 nM of vincristine were found while 1.0% ±0.44% of more primitive CAFC week 6 survived the selection conditions (Figure 6.5). This was considerably lower than the frequency of primitive stem cells containing the transferred MDR1 gene and reminiscent of results obtained in the CFU-GM assays (Table 6.1, Figure 6.2). Compared to mock-transduced control CA which were not observed above the detection threshold of 1 CAFC per 10^5 input cells the frequency of MDR1-transduced and vincristine-resistant CAFC was at least 10- to 57-fold higher (Figure 6.5). On the other hand, the frequency of CAFC not cultured in the drug was comparable between the MDR1 and control groups (data not shown), suggesting that MDR1 retroviral transduction was not toxic for HSC.

These results indicate a high transduction efficiency of primitive HSC with low level yet maintained provirus expression with the MDR1-SNT protocol used.

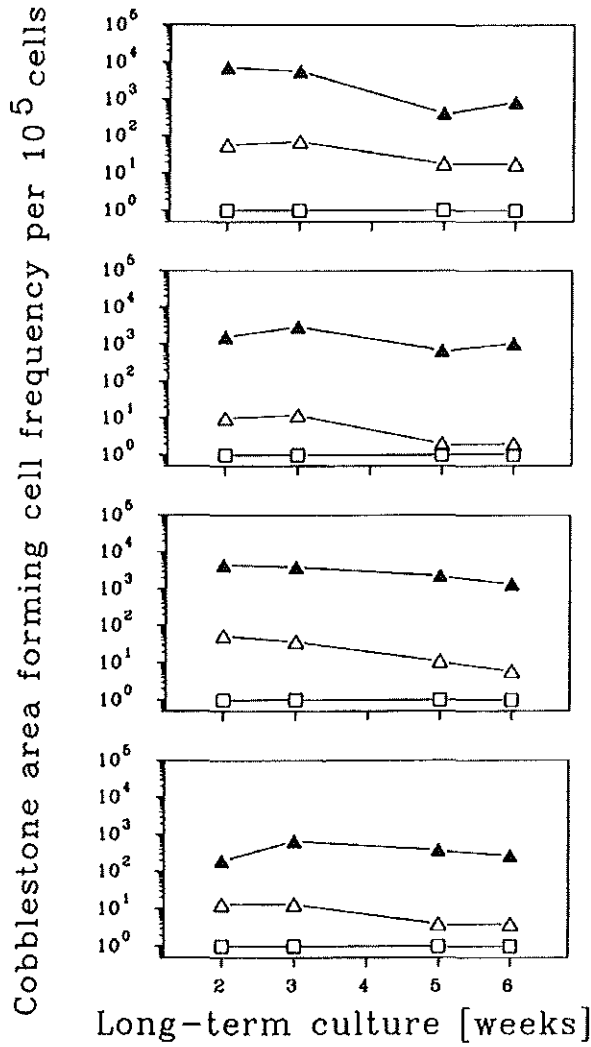

Figure 6.5 Sustained P-glycoprotein expression in early and late CAFC after retroviral MDR1 gene transfer. CD34-selected peripheral blood stem cells were transduced over a period of 48 hours in IL-3 (D) with four retroviral supernatant changes and then cultured in the absence (▲) or presence (△) of 20 nM vincristine for 6 weeks. CAFC were scored on weeks 2, 3, 5 and 6. In matched mock-infected controls vincristine-resistant CAFC were not observed above the detection threshold (□). Data of four experiments using independent peripheral blood stem cell samples are given.

Frequency and proliferative capability of primitive hematopoietic stem cells after cytokine incubation

A further issue which can be addressed with the CAFC assay are cytokine-associated gains or losses of stem cell subsets which may occur during transduction. Others have expressed CAFC frequencies obtained after cytokine-mediated expansion of mobilized PBSC relative to the CAFC frequency of the untreated PBSC [22]. In figure 6.6 (panels A,D,G) relative CAFC numbers following incubation in one of the three cytokine cocktails for 48 or 96 hours are plotted. Early week 2 or week 3 CAFC that may be a reflection of short-term reconstituting cells were significantly expanded in the IL-3 group (48 hours, CAFC week 3, $p<0.05$; 96 hours, CAFC week 2, $p<0.05$). Late week 5 and week 6 CAFC were at best conserved and even depleted in some groups (48 hours, IL-3: $p<0.05$ for CAFC week 5 and week 6).

Sutherland et al. [7] introduced ratios of colony forming cells and LTC-IC detectable at the end of a 5-week culture period to characterize the proliferative capability of mobilized peripheral blood LTC-IC. We employed CAFC in place of LTC-IC as direct visual end point for the enumeration of HSC. CFU-GM counts produced in week 5 replates of flask LTC were divided by the number of CAFC produced in limiting-dilution cultures set-up in parallel (CFU-GM/CAFC). The primary CD34-selected PBSC were heterogenous in their proliferative capacity, ranging from 5 to 34 CFU-GM/CAFC week 5 (median 8). For comparison of the different treatment groups, the proliferative capability following cytokine-incubation was divided by the CFU-GM/CAFC value of the matched untreated material (Figure 6.6, panels B,C,E,F,H,I). SCF-containing cocktails (96 hours, incubation; $p\leq0.05$) but not IL-3 alone (48 hours or 96 hours incubation) significantly reduced the proliferative potential of CAFC week 5. In view of the comparable proportions of P-glycoprotein overexpression following MDR1-SNT transduction in either of the cytokine combinations (Figure 6.2, Figure 6.4), IL-3 addition for 96 hours appeared preferable since it did not reduce the frequency of late CAFC as observed in the 48 hour IL-3 group, nor did it significantly reduce the proliferative capability of week 5 CAFC as found after 96 hour incubation in SCF-containing combinations.

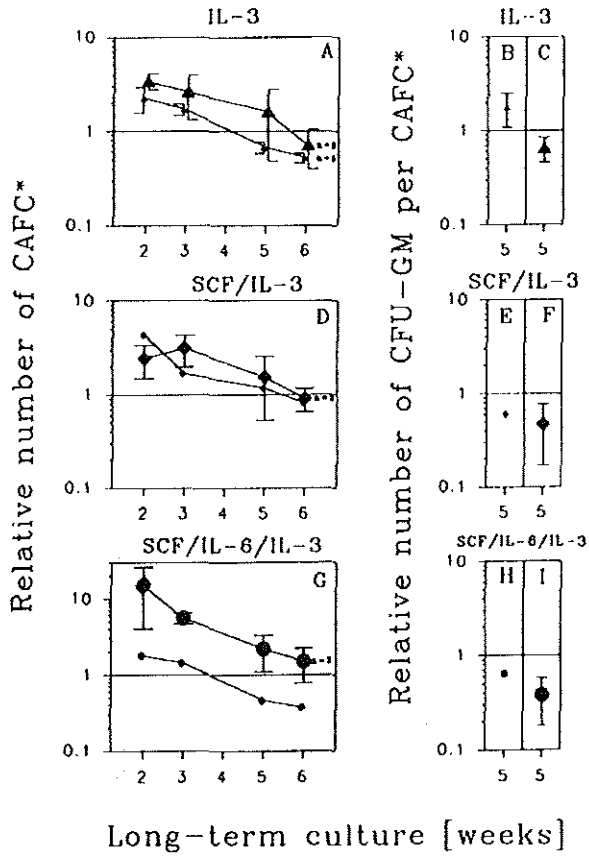

Figure 6.6 Frequency and clonogenic potential analysis of different CAFC week-types. Incubation of CD34+ peripheral blood stem cells with IL-3 (▲), SCF/IL-3 (◆) or SCF/IL-3/IL-6 (●) during a 48 hour (small symbols) or 96-hour (large symbols) period for retroviral transduction. The CAFC frequency along the stroma-culture period is depicted relative to values obtained with matched untreated peripheral blood stem cells (panels A,D,G). The proliferative capability or clonogenic potential of late CAFC was assessed by relating CAFC numbers to CFU-GM counts from week 5 replates of flask long-term culture set-up in parallel to the limiting-dilution plates (panels B,C,E,F,H,I). Lines represent mean values of independent experiments ±SEM. * Values of primary CD34-selected peripheral blood stem cells were set to 1.

DISCUSSION

The most important observation of this paper is the ability of the human CAFC assay to give an estimate of the gene transfer efficiency in HSC *in vitro*. This may reflect the long-term myeloprotection potential after transplantation of MDR1 transduced PBSC. Previous reports suggested integration of a retrovirally-transferred MDR1 gene in human LTC-IC [8,9]. Quantitative analysis of transduction in primitive HSC has not been reported yet. The limiting-dilution CAFC assay is a miniaturized stroma-dependent LTC in which phase-dark hematopoietic clones were followed over a culture period of 6 weeks. In the murine system CAFC appearing at day 10 of culture are ascribed transient repopulating properties whereas late, day 28 to 42 CAFC are indicative of long-term reconstituting ability [11,23]. Several groups have recently adapted this system for human material [10,24,25]. In humans, the CAFC subsets still await biological validation by e.g. gene marking studies.

Mobilized PBSC contained a high proportion of primitive, late CAFC (Figure 6.5) which is in line with previous observations that reported 1 CAFC week 6 per 76 to 443 CD34+ PBSC [24]. Following MDR1 transduction and culturing in the CAFC assay for 6 weeks, a large fraction of 22% of primitive CAFC week 6 cells had integrated the transduced gene in their DNA. Estimations of MDR1 gene transfer into LTC-IC that were not based on limiting-dilution techniques had yielded similar results [8,9]. To screen for P-glycoprotein overexpression in MDR1 transduced PBSC, vincristine was added to the CAFC cultures. The mean proportion of 1% vincristine-resistant CAFC week 6 was comparatively low, suggesting inefficient transcriptional activation or repression of the MoMuLV LTR [26,27]. The retroviral vector employed for this study was driven by the MoMuLV LTR and included a mutated primer binding site. However, recent reports also point to an expression limiting viral enhancer region in the U3 region of the MoMuLV LTR [28]. Furthermore, suboptimal expression of MDR1 is also caused by cryptic splice sites in the human wild-type MDR1 gene which may lead to aberrant splicing of up to 60% of the vector-derived messenger RNA [29]. This observation was confirmed in our group with the vector used in this study (unpublished results). Currently we are developing retroviral vectors expressing mutated MDR1 genes to optimize induction of MDR in hematopoietic cells.

The CAFC assay was further used to monitor changes in the stem cell composition of the cytokine-incubated and transduced autograft. Early but not late CAFC were expanded after 48 and 96 hours of retroviral transduction in IL-3 (Figure 6.6) which was also found by others [22]. The proliferative capability of LTC-IC week 5 is considered to be a quality parameter of primitive HSC which gives additional infomation to the LTC-IC frequency assessment [7]. We determined the CFU-GM output after 5 weeks of stroma-dependent LTC and compared it to the CAFC week 5 frequency. The addition of SCF to the IL-3 containing transduction cocktail significantly reduced the quality of week 5 CAFC (Figure 6.6).

MDR1 gene transfer in lineage-committed progenitor cells was assayed to complement the CAFC data. MDR1 provirus integration was found in 20% to 66% of CFU-GM transduced by MDR1-SNT which is in the range of results previously reported for retroviral-mediated gene transfer into mobilized PBSC [30]. Corresponding percentages (25% to 52%, Table 6.1) of CD34+ PBSC were in the S/G_2M phase of the cell cycle at the end of the transduction period, suggesting that cell cycle progression is required for integration of murine leukemia virus vectors in the analyzed CD34+ cell population [19]. Since controls for the presence of amplifiable human DNA were not included in the PCR reactions, the transduction frequency of CFU-GM may still be higher than reported here.

Others used clonogenic assays including MDR1-related cytotoxic drugs to compare the transduction efficiency into different hematopoietic cell types [31] or the quality of different MDR1-SNT lots [9]. We cultured MDR1-transduced PBSC in vincristine and found a similar proportion of resistant colonies following co-cultivation on retroviral producer cells as had been reported for MDR1 co-cultivated bone marrow or cord blood [13,31]. MDR1-SNT transduction also resulted in resistant colonies as described before [8,9].

If numerous transduction conditions are to be compared, the CFU-GM assay with drug-titration-ranges spanning six concentrations and duplicate plates for every group requires a high culture and counting effort. We investigated whether the FACS analysis of P-glycoprotein in the progeny of transduced cells could yield equivalent results. In clinical studies of acute leukemia, immunolabelling techniques with the antibodies MRK16, C219 or JSB1 investigating P-glycoprotein presence at the protein level did not provide information about the P-glycoprotein functional status. A Rh123 exclusion assay was the most sensitive test for the complete remission rate [32]. In gene therapy, some analyzed Rh123 efflux directly after MDR1 transduction of CD34+ cells at a time point where a high proportion (32% to 33%) of CD34+ cells are naturally $Rh123^{dull}$ [9]. When CD34+ PBSC are cultured in the presence of SCF, IL-3, IL-6, GM-CSF and G-CSF, a population of CD15 and/or CD11b expressing granulo-monocytic cells evolves [15,16] that display a $Rh123^{bright}$ phenotype [20]. In the progeny of MDR1 transduced PBSC a $Rh123^{bright}$ cell population became detectable that showed an average 50-100 fold reduced Rh123 accumulation compared to controls (Figure 6.3). The Rh123 efflux could be abrogated by the P-glycoprotein inhibitor cyclosporin A [21] which suggested that the MDR activity was due to the transferred gene and not to other transporter proteins [33,34].

Different transduction conditions were assessed and the results of the Rh123 assay mirrored those of the CFU-GM vincristine-titrations (Figure 6.2, Figure 6.4). MDR1 co-cultivation in IL-3 yielded significantly higher proportions of vincristine-resistant CFU-GM or numbers of $Rh123^{dull}$ cells than MDR1-SNT transduction in IL-3. This may have been due to multiple provirus integrations per transduced cell during co-cultivation as following SNT transduction already 20% to 66% of the CFU-GM contained the MDR1 gene. There was no significant

difference between the various MDR1-SNT transduction schedules or cytokine combinations. This may be partially due to the fact that all cytokine combinations contained IL-3 which was reported to be the most relevant cytokine for upregulation of the amphotropic retrovirus receptor on HSC [35]. An additive effect of IL-3, SCF and IL-6 on gene transduction and expression was also not seen in our study in non-human primates [36].

Further development towards an effective MDR1 gene therapy should be facilitated by the possibility to estimate the retroviral gene transfer frequency into primitive HSC using the CAFC assay and by the application of the Rh123 assay for optimizing MDR1 transduction conditions.

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CHAPTER 7

STROMA-CONDITIONED MEDIUM AND SUFFICIENT PRESTIMULATION IMPROVE FIBRONECTIN FRAGMENT MEDIATED RETROVIRAL GENE TRANSFER INTO HUMAN PRIMITIVE MOBILIZED PERIPHERAL BLOOD STEM CELLS THROUGH EFFECTS ON THEIR RECOVERY AND TRANSFECTION EFFICIENCY

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ABSTRACT

Mobilized peripheral blood stem cells (PBSC) are the vehicle of choice for cancer gene therapy. However these stem cells may have a reduced proliferative capacity due to previous cytotoxic chemotherapy treatment of the patient. In addition, primitive hematopoietic stem cells (HSC) from mobilized peripheral blood are almost exclusively quiescent, which makes it hard to induce proliferation *in vitro* and thus to improve stable transfection of introduced genes into a sufficiently large number of primitive stem cells.

In this study the CD34-selected mobilized PBSC from lymphoma and myeloma patients were used as target cells for retroviral-mediated gene transfer using a clinically relevant cell- and serum-free supernatant infection protocol. We have investigated various parameters that may contribute to an improvement of the poor transduction efficiency of the primitive HSC, including prestimulation time, the use of the carboxy-terminal fibronectin fragment CH-296 as well as stromal cell line conditioned media. Retroviral supernatant infection in combination with CH-296 increased significantly the transfection efficiency as compared to supernatant alone and made the use of polycations redundant. Gene transfer of primitive HSC (cobblestone area forming cell (CAFC) week 6) was specifically improved when this procedure was preceded by a five-day pre-culture period as compared to a two-day transfection procedure. The addition of stroma-conditioned media (SCM) during the pre-culture period did not affect the individual CAFC quality or transduction efficiency, but increased greatly the recovery of the total number of transfected and untransfected HSC leading to larger grafts containing higher numbers of transfected stem cells.

INTRODUCTION

Mobilized PBSC are widely used for autologous transplantation in cancer patients and have been proposed as a vehicle for cancer gene therapy using retroviral vectors [1]. However, there is only a limited number of clinically relevant gene transfer studies reporting on the transduction efficiency and recovery of primitive HSC from mobilized peripheral blood of cancer patients [2,3]. One of the gene therapy strategies is to introduce drug resistance genes to prevent myelosuppresion following chemotherapy [3-5]. The most desirable approach in human gene therapy trials is a cell-free virus-containing supernatant infection protocol, but clinical trials using this supernatant approach have demonstrated low levels of transfection into repopulating HSC [2,6-9]. Although mobilized PBSC are clinically the most relevant target cells for cancer gene therapy in adult patients, these cells are likely to be resistant to stable integration of transduced genes. It has been shown that primitive HSC from mobilized peripheral blood are quiescent [10-13] and refractory to cell cycle activation [12,13], however, stable retroviral gene transfer occurs only in cells which are actively replicating at the time of virus infection [14,15].

Recently, it is has been shown that coating of the culture surface with carboxy-terminal fibronectin fragments (FN 30/35 or CH-296) [16,17] significantly improves retroviral supernatant transfection of progenitors and primitive stem cells from normal bone marrow and umbilical cord blood cells by colocalization of virus particles and HSC [18-21]. However, to our knowledge it has not yet been reported whether mobilized CD34-positive (CD34+) PBSC from cancer patients similarly adhere to CH-296 as normal bone marrow and umbilical cord blood cells, and whether this adherence improves retroviral infection and stem cell recovery.

In this study, we investigated the optimization of retroviral neomycin phosphotransferase (NEO) gene supernatant transfection of primitive HSC in mobilized CD34-selected peripheral blood autologous transplants of multiple myeloma and non-Hodgkin lymphoma patients using CH-296 coating. Because previous studies have shown that primitive CD34+CD38-negative HSC of the majority of mobilized peripheral blood samples which have been tested are refractory to cell cycle activation during a two- to three-day of cytokine stimulation [12,13], we also included a pre-culture period of five days. Using this pre-culture protocol the effect of the murine FBMD-1 and human L88/5 SCM on the transfection efficiency and recovery of HSC was investigated, because we have shown previously that the use of SCM considerably improves the recovery of HSC from serum-free liquid cultures stimulated with interleukin-3 (IL-3), stem cell factor (SCF) and IL-6 [22].

The human CAFC assay was used to measure the frequency of different HSC subsets. CAFC week 2 to 4 are tentative indicators of the frequency of progenitor cells and transiently repopulating HSC, while CAFC week 6 frequency is assumed to be an indicator of more primitive, long-term repopulating stem cells [23,24]. By determining the absolute number of CAFC before and after transfection the recovery of various HSC subsets could be assessed. In order to allow parallel CAFC assays under G418-selection, providing the number and percentage of functional NEO gene expressing stem cells, we transfected the supportive stromal cell line FBMD-1 with the NEO gene which transfers G418 resistance. In addition, the colony forming cell (CFC) production in flask long-term cultures (LTC-CFC) was performed as an estimate of total graft quality [23,24]. These assays allowed a detailed quantitative and qualitative analysis of different HSC subsets.

MATERIALS AND METHODS

Mobilized peripheral blood

Cells from leukapheresis products of five non-Hodgkin lymphoma and five multiple myeloma patients were used in this study. Before leukapheresis the HSC were mobilized to the blood after several courses of chemotherapy using granulocyte colony-stimulating factor (Filgrastim, recombinant-methionyl human G-CSF; Roche, Mijdrecht, The Netherlands) as described before [24]. After cell collection, excess of erythrocytes was removed using buffy coat centrifugation. Fresh or frozen and thawed leukapherses were subjected to a density gradient (1.077 g/ml, Lymphoprep; Nycomed, Olso, Norway) following CD34-selection (MACS CD34 isolation kit;

Miltenyi Biotec, Bergisch Gladbach, Germany) to enrich for HSC. After selection the percentage CD34+ cells was determined as described before (range: 78 to 96% CD34+ cells) [24].

Cytokines
The following purified recombinant cytokines were kindly provided: human granulocyte-macrophage-CSF (GM-CSF), human IL-6 and murine SCF from Genetics Institute (Cambridge, MA), human G-CSF and human SCF from Amgen (Thousand Oaks, CA) and human IL-3 from Gist Brocades (Delft, The Netherlands).

Serum-free stroma-conditioned media
Confluent layers were grown of the stromal cell lines FBMD-1 and L88/5 [23,25,26]. The cells were cultured in Iscove's modified Dulbecco's medium with Glutamax-1 (IMDM; Gibco, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; Summit, Fort Collins, CO), penicillin (100 U/ml; Gibco), streptomycin (100 μg/ml; Gibco) and 10^{-4} M β-mercapto-ethanol (Merck, Darmstadt, Germany). The FBMD-1 cells were maintained at 33°C and 10% CO_2 and the L88/5 cells at 37°C and 10% CO_2. When the layers were confluent, the medium was removed and rinsed twice with IMDM. Serum-free medium was added to the confluent stromal layers and conditioned for seven days. This serum-free medium was prepared from IMDM containing 1% bovine serum albumin (BSA; A9418; Sigma, St Louis, MO), penicillin (100 U/ml), streptomycin (100 μg/ml), 10^{-4} M β-mercapto-ethanol, bovine insulin (10 μg/ml; Gibco), 15 μM cholesterol (Sigma), 15 μM linolic acid (Merck), iron-satured human transferrin (0.62 g/l; Intergen, Purchase, NY), cytidine (1 μg/ml; Sigma), adenosine (1 μg/ml; Sigma), uridine (1 μg/ml; Sigma), guanosine (1 μg/ml; Sigma), thymidine (1 μg/ml; Sigma), 2'-deoxycytidine (1 μg/ml; Sigma), 2'-deoxyadenosine (1 μg/ml; Sigma), 2'-deoxyguanosine (1 μg/ml; Sigma). The SCM were harvested and the non-adherent (NA) cells were removed by centrifugation. Subsequently, the SCM were concentrated 20 times using a 30K ultrafiltration (Amicon, Beverly, MA) and stored at -80°C until use. Control experiments indicated that this concentrated SCM contained the same activity as the unconcentrated SCM which has been used in previous experiments (D.A. Breems, unpublished observations) [22].

Serum-free NEO virus supernatant
The retroviral-packaging cell line PA317/MSCVv2.1neo (Gift from Dr. R.G. Hawley; Sunnybrook Health Science Centre and University of Toronto, Toronto, Ontario, Canada) containing a MSCV vector in which the NEO gene is placed under the transcriptional control of an internal phosphoglycerate kinase promotor [27] was after selection in medium (IMDM with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10^{-4} M β-mercapto-ethanol) containing G418 (0.4 mg/ml, active substance; Gibco), 30 μM hypoxanthine (Sigma), 1 μM amethopterin (Sigma) and 20 μM thymidine (Sigma) grown to confluence in medium without selection drugs. When the layers were confluent, the medium was removed and rinsed twice with IMDM. Serum-free medium was added to the confluent stromal layers and conditioned for 18 hours. The NEO virus containing supernatant was harvested and all cells were removed by 0.45 μm filtration (Schleicher and Schuell, Dassel, Germany). The NEO virus was stored at -80°C until use and after thawing the titer was 10^6 CFU/ml using a titration on 3T3 cells.

Serum-free liquid (pre-)culture
Serum-free liquid cultures were performed in 35 mm bacterial dishes (Greiner, Alphen a/d Rijn, The Netherlands) without any coating to prevent strong adherence of the hematopoietic cells to the plastic surface. Eighty thousand CD34+ PBSC in 2 ml serum-free medium supplemented with IL-3 (25 ng/ml), human SCF (100 ng/ml), IL-6 (100 ng/ml) and anti-human-transforming growth factor-β1 (αTGFβ1; 1.0 μg/ml; R&D Systems, Abingdon, United Kingdom) were cultured at 37°C and 10% CO_2. At the end of the culture period, the cells were collected from the dishes after scraping with a cell scraper (Greiner) and rinsing with IMDM.

Retroviral transfection culture
Retroviral transfection was performed in 35 mm bacterial dishes coated with CH-296 (8 $\mu g/cm^2$; Takara Shuzo, Otsu, Shiga, Japan) dissolved in phosphate-buffered saline (Gibco) for two hours at room temperature. Subsequently the dishes were blocked with 2% BSA in phosphate-buffered saline for 30 minutes and washed with Hank's balanced salt solution (Gibco). Eighty thousand CD34+ PBSC were directly or after a serum-free liquid pre-culture period plated in CH-296-coated dishes. During the transfection procedure the CD34+ PBSC were cultured with NEO virus supernatant supplemented with IL-3, SCF, IL-6 and αTGFβ1 (3/S/6/αT) for 48 hours at 37°C and 10% CO_2 unless indicated otherwise. After the first 24 hours the NEO virus supernatant was replaced by fresh supernatant, cytokines and αTGFβ1. The NA cells were returned to the transfection culture after centrifugation. At the end of the transfection period, the NA cells were collected from the dishes and after two rinses with IMDM replaced by 1 ml of 0.1% trypsin-EDTA (Gibco) for 5 minutes. The digestion was stopped by adding 1 ml of ice-cold FCS and the dishes were scraped with a cell scraper to include still adherent cells.

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 semisolid CFC assay containing erythropoietin (Boehringer, Mannheim, Germany), G-CSF, GM-CSF, IL-3 and murine SCF as described before [24]. CFU-GM and BFU-E were counted on day 14 of culture in the same dish. To select for NEO gene expressing CFC G418 (0.5 and 1.0 mg/ml, active substance) was added to the cultures.

Transfection of FBMD-1 cell line with a NEO gene
In order to be able to perform CAFC assays under selective conditions, we prepared a G418-resistant FBMD-1 cell line (FBMD-neo). FBMD-1 cells growing in log-phase were cultured four 18 hours in a serum-free NEO virus supernatant with 10% FCS and polybrene (7.5 mg/ml; Sigma) at 37°C and 10% CO_2. After this bulk transfection, the cells were selected and expanded for 10 days in IMDM supplemented with 10% FCS, 5% horse serum (Integro, Zaandam, The Netherlands), 10^{-5} M hydrocortisone 21-hemisuccinate (Sigma), penicillin (100 U/ml), streptomycin (100 $\mu g/ml$), 10^{-4} M β-mercapto-ethanol and G418 (0.5 mg/ml, active substance) at 33°C and 10% CO_2. Multiple ampules of the FBMD-neo cell line were stored in liquid nitrogen until use. Although control experiments showed that this FBMD-neo cell line had the same stem cell supportive capacity as the FBMD-1 cell line (P.B. Van Hennik and D.A. Breems, unpublished observations), in individual experiments either the FBMD-1 or the FBMD-neo cell line was used.

Long-term cultures in flasks
Confluent stromal layers of FBMD-1 or FBMD-neo cells in 25 cm^2 flasks (Costar, Cambridge, MA) were overlaid with 30,000 CD34+ PBSC or the output of 30,000 transfected CD34+ PBSC. The cells were cultured in IMDM supplemented with 10% FCS, 5% horse serum, 10^{-5} M hydrocortisone 21-hemisuccinate, penicillin (100 U/ml), streptomycin (100 $\mu g/ml$) and 10^{-4} M β-mercapto-ethanol. IL-3 (10 ng/ml) and G-CSF (20 ng/ml) were added weekly to the cultures. Flask-cultures were set up in duplicate and maintained at 33°C and 10% CO_2 for six weeks with weekly half-medium changes and therefore removal of half of the 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 demidepopulations. 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 IMDM replaced by 3 ml of 0.1% trypsin-EDTA for 5 minutes. The digestion was stopped by adding 1 ml of ice-cold FCS and the flasks were scraped with a cell scraper to include still adherent cells. The NA and adherent cell fractions were pooled and 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 cell suspension were plated in a semisolid CFC cell assay.

Cobblestone area forming cell assay
Limiting dilution CAFC assays were performed on confluent stromal layers of FBMD-1 or FBMD-neo cells in flat-bottom 96-well plates (Falcon, Franklin Lakes, NJ). The cultures were maintained under the same conditions as the LTC in flasks. CD34+ PBSC were overlaid in a limiting dilution setup. Twelve dilutions two-fold apart were used for each sample with 15 replicate wells per dilution. The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells (i.e. cobblestone area) beneath the stromal layer was determined at week 2, 4 and 6 and CAFC frequencies were calculated using Poisson statistics. To select for NEO gene expressing CAFC parallel G418 (0.5 mg/ml, active substance) containing CAFC assays were preformed.

Statistical analysis
Data were analyzed using GraphPad Instat (GraphPad Software, San Diego, CA). The means of two populations were compared using a paired Student's *t* test.

RESULTS

Adherence of CD34+ mobilized peripheral blood stem cells to CH-296

Before studying the effect of CH-296 coating on retroviral transfection in CD34+ PBSC, we first investigated adherence of various stem cell subsets to the fibronectin fragment, and checked whether CH-296 adherence did not adversely affect or inhibit progenitor cell recovery after liquid culture. Adherence of CD34+ PBSC to CH-296 was studied by incubation in bacterial dishes coated with CH-296 in serum-free medium at 37°C for 30 to 120 minutes. After the incubation the adherent and NA fraction were assayed separately for the presence of CFC and CAFC week-types. More than 90% of the CFC and CAFC subsets adhered to CH-296 within 30 minutes of incubation (Table 7.1). A control experiment showed that only 4 to 10% of the different stem cell subsets adhered to tissue culture plastic.

Table 7.1 Adherence of Progenitor and Stem Cell Subsets to CH-296 and Tissue Culture Plastic.

CFC or CAFC subset	Percentage Adherent Cells			
	30 min CH-296 (n = 1)	60 min CH-296 (n = 2)	120 min CH-296 (n = 1)	120 min Plastic (n = 1)
CFC	95	94; 99	92	9
CAFC week 2	91	92; 98	90	4
CAFC week 4	90	80; 98	90	7
CAFC week 6	92	85; 96	91	10

CFC, Colony Forming Cell; CAFC, Cobblestone Area Forming Cell.

CH-296 adherence does not influence the recovery of CFC in short-term cultures
To test the effect of CH-296 adherence on the recovery of CFC after two- and seven-day cultures, CD34+ PBSC were plated in BSA-coated or CH-296-coated bacterial dishes with serum-free medium containing 3/S/6/αT with or without FBMD-1 or L88/5 SCM. After two or seven days of culture no effect of CH-296-mediated adherence on the recovery CFC was observed (Figure 7.1). The recently reported positive effect of FBMD-1 and L88/5 SCM on the expansion of CFC after seven days of culture was also not affected by the adherence to CH-296 [22].

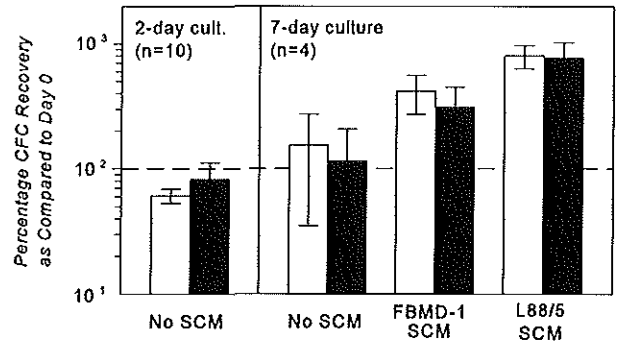

Figure 7.1 The effect of CH-296 adherence on the recovery of colony forming cells (CFC) in short-term cultures. The recovery of CFC is shown after two- and seven-day cultures of CD34+ peripheral blood stem cells plated in BSA-coated (□) or CH-296-coated (■) bacterial dishes with serum-free medium containing IL-3, SCF, IL-6 and αTGFβ1 with or without FBMD-1 or L88/5 stroma-conditioned medium (SCM) as compared to input values at day 0 (dashed line).

CH-296 improves retroviral gene transfer efficiency of CFC
A two-day transfection procedure in which serum-free NEO virus supernatant containing 3/S/6/αT was added at day 0 and day 1 was performed on BSA-coated or CH-296-coated bacterial dishes. At day 2 the percentage G418-resistant (1.0 mg/ml, active substance) CFC was determined. Untransfected control cultures contained <0.1% G418-resistant CFC (data not shown). From transfection cultures on BSA-coated bacterial dishes 1.2% G418-resistant CFC were recovered (Figure 7.2). This percentage was significantly improved to 9.5% using CH-296-coated dishes. Interestingly, the addition of polybrene (7.5 mg/ml) or protamine sulphate (7.5 mg/ml; Novo Nordisk, Zoeterwoude, The Netherlands) could not further improve the transfection efficiency. In the experiments presented in figure 7.2, the stringent selection with 1.0 mg/ml G418 (active substance) may have caused an underestimation of the transfection efficiency. Therefore, a lower G418 concentration (0.5 mg/ml, active substance) was used in the experiments presented in figures 7.4, 7.5 and 7.6.

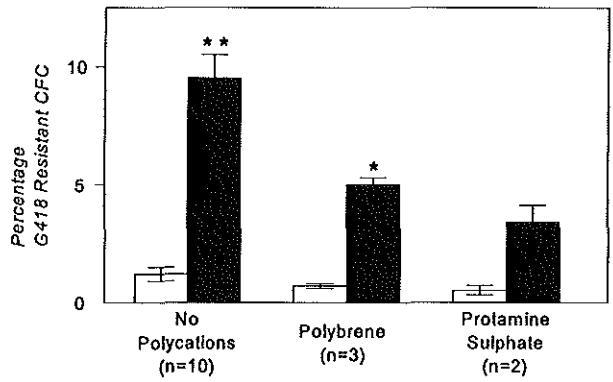

Figure 7.2 The effect of CH-296 adherence on the retroviral gene transfer efficiency of colony forming cells (CFC). The percentage G418-resistant (1.0 mg/ml, active substance) CFC is shown after a two-day transfection procedure of CD34+ peripheral blood stem cells in serum-free medium containing IL-3, SCF, IL-6 and αTGFβ1 on BSA-coated (□) or CH-296-coated (■) bacterial dishes and in the presence or absence of polybrene or protamine sulphate. Comparison between BSA coating and CH-296 coating: *, $P<0.02$; **, $P<0.0001$.

Pre-culture specifically improves retroviral gene transfer into primitive hematopoietic stem cells

Because primitive HSC from mobilized peripheral blood are mostly non-cycling cells, the effect of prolonged culture protocols on retroviral NEO gene transfer in different stem cell subsets was studied. For this purpose four different serum-free transfection protocols containing 3/S/6/αT were developed (Figure 7.3). The first protocol (2T) was a two-day transfection procedure in which serum-free NEO virus supernatant containing 3/S/6/αT was added at day 0 and day 1. This protocol was compared with a second in which a two-day transfection was followed by a five-day liquid culture period (2T5C), a third protocol in which a two-day transfection was preceded by five-day liquid culture period (5C2T) and a last protocol in which a two-day transfection was followed by a three-day culture period and a second two-day transfection (2T3C2T). All four protocols resulted in a comparable transfection percentage of progenitor cells (10.4 to 17.1% G418-resistant CFC and CAFC week 2) (Figure 7.4A). In contrast, the highest transfection efficiency of primitive CAFC week 6 was obtained with the protocol in which a two-day transfection was performed after five-day pre-culture (5C2T: 8.7%) and the protocol of two-day transfection, three-day culture and two-day transfection (2T3C2T: 5.4%), respectively, while the two other protocols with only retroviral supernatant addition in the first two days gave only 0.7% (2T) and 1.1% (2T5C) transfection.

We also studied the effect of these protocols on total recovery of stem cell subsets after transfection (Figure 7.4B). The CFC showed a modest expansion (185 to 261% as compared to day 0) in the transfection protocols with a total length of seven days (2T5C, 5C2T and 2T3C2T). The two-day transfection

protocol (2T) resulted in a loss of total CFC number (61% as compared to day 0). All four transfection protocols showed a lower total CAFC week 2 and 4 recovery as compared to day 0 (week 2: 35 to 70%; week 4: 41 to 69%). The 5C2T protocol had a very low CAFC week 6 recovery (9%), while in the other three protocols the CAFC week 6 recovery ranged from 21 to 38%.

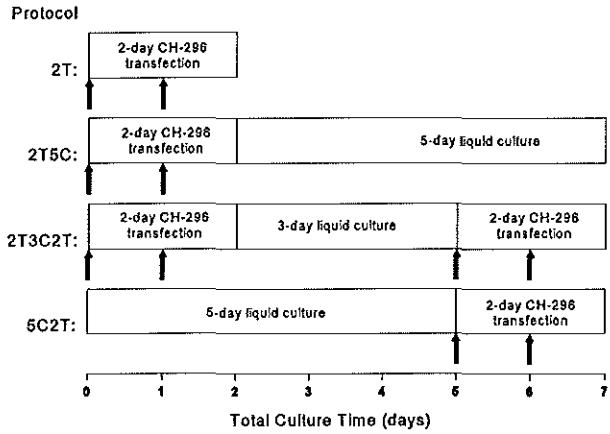

Figure 7.3 Four transfection protocols. 2T: two-day transfection procedure; 2T5C: two-day transfection followed by five-day liquid culture; 2T3C2T: two-day transfection followed by three-day liquid culture and a second two-day transfection; 5C2T: five-day liquid culture followed by two-day transfection. MSCVneo virus supernatant was added once a day (1).

Stroma-conditioned pre-culture improves recovery of hematopoietic stem cells in an ex vivo gene transfer protocol

Because of the low CAFC recovery in the transfection protocols containing 3/S/6/αT, the effect of FBMD-1 or L88/5 SCM was studied. For this purpose we used the 5C2T protocol in which SCM was exclusively added during the five-day pre-culture. This protocol resulted in a poor CAFC week-type recovery without the addition of SCM (week 2: 35%; week 4: 41%; week 6: 9%) (Figure 7.4B). Figure 7.5A shows that the pre-culture with SCM did not improve the transfection percentage of progenitor cells (CFC: 17.3, 18.0 and 11.5%; CAFC week 2: 16.5, 23.0 and 16.8%; No SCM, FBMD-1 SCM and L88/5 SCM, respectively) and that there was only a small improvement of transfection percentage of more primitive HSC (CAFC week 4: 5.9, 12.0 and 9.8%; CAFC week 6: 8.7, 12.5 and 12.3%; No SCM, FBMD-1 SCM and L88/5 SCM, respectively). However, both FBMD-1 and L88/5 SCM pre-culture significantly improved the total recovery of all stem cell subsets during the gene transfer procedure (Figure 7.5B). Figure 7.5C shows that this results in an increased graft size of transfected stem cell subsets as compared to pre-culture without SCM

(CFC: 1.9- and 2.1-fold; CAFC week 2: 6.5- and 9.7-fold; CAFC week 4: 4.2- and 6.2-fold; CAFC week 6: 5.4- and 7.7-fold, FBMD-1 and L88/5 SCM, respectively).

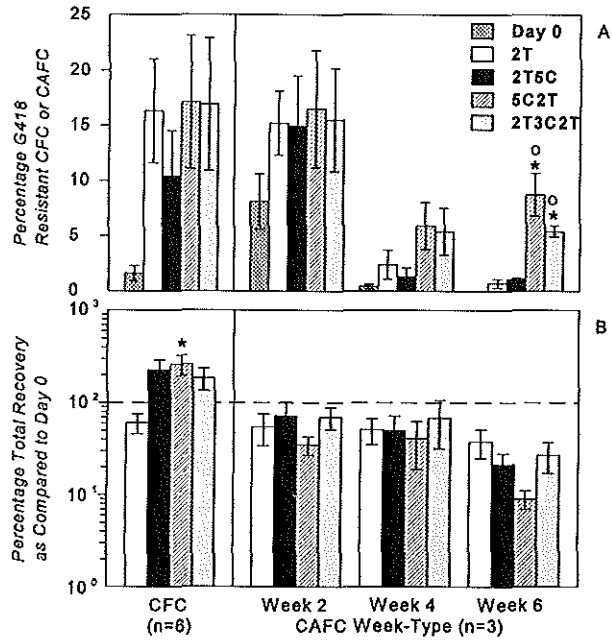

Figure 7.4 The effect of different transfection protocols on retroviral NEO gene transfer in and recovery of different stem cell subsets. (A) The percentage G418-resistant (0.5 mg/ml, active substance) colony forming cells (CFC) and cobblestone area forming cell (CAFC) week-types and (B) the percentage recovery of CFC and CAFC week-types as compared to input values day 0 (dashed line) is shown using four different transfection protocols (2T, 2T5C, 5C2T and 2T3C2T; see Figure 7.3) in serum-free medium containing IL-3, SCF, IL-6 and αTGFβ1. Percentage G418 resistant CFC and CAFC subsets of untreated cells is shown as day 0 value. Comparison between 2T and the other three protocols: *, $P<0.05$. Comparison between 2T5C and the other three protocols: °, $P<0.05$.

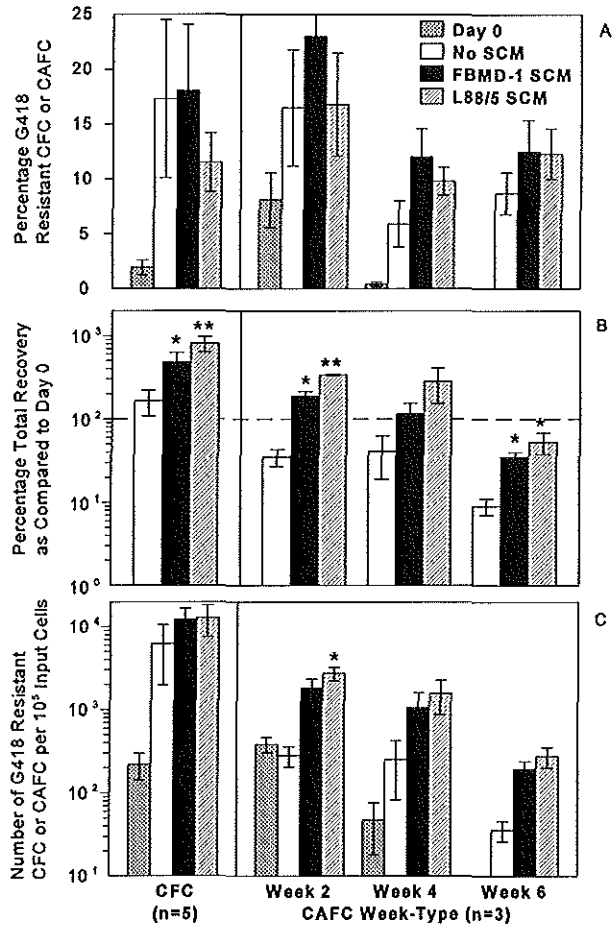

Figure 7.5 The effect of stroma-conditioned media (SCM) on retroviral NEO gene transfer in and recovery of different stem cell subsets. (A) The percentage G418-resistant (0.5 mg/ml, active substance) colony forming cells (CFC) and cobblestone area forming cell (CAFC) week-types, (B) the percentage recovery of CFC and CAFC week-types as compared to input values at day 0 (dashed line) and (C) the total recovery of G418-resistant CFC and CAFC week-types is shown using the 5C2T transfection protocol (see Figure 7.3) in serum-free medium containing IL-3, SCF, IL-6 and αTGFβ1 with or without FBMD-1 or L88/5 SCM. Percentage and number G418 resistant CFC and CAFC subsets of untreated cells is shown as day 0 value. Comparison between no SCM and with SCM: *, $P<0.05$; **, $P<0.01$.

Stroma-conditioned medium partly abrogates the loss of LTC-CFC

Next to the recovery of various CAFC subsets, the CFC producing ability of the CD34+ PBSC before and after retroviral transfection was determined using

flask-LTC. The NA-CFC output was determined on week 2 and 4 and at week 6 the combined NA and stroma-adherent CFC output was assessed. In addition, the percentage of G418-resistant LTC-CFC was determined. The transduction rate as measured using the CAFC subsets as an endpoint (Figures 7.4A and 7.5A) reflected that as assessed by the LTC-CFC subsets (Figure 7.6A). The total LTC-CFC recovery was lower as compared to the input values (day 0) at all weeks (Figure 7.6B) and also lower than the CAFC recovery (Figures 7.4B and 7.5B). The recovery of LTC-CFC was better in the two-day transfection protocol (2T) (week 2: 39%; week 4: 19%; week 6: 45%) as compared to the protocol with five-day pre-culture (5C2T) (week 2: 20%; week 4: 8%; week 6: 5%) suggesting that a progressive loss of LTC-CFC ability occurred with increasing culture time of the transfection procedure. The addition of FBMD-1 and L88/5 SCM in the five-day pre-culture completely neutralizes the LTC-CFC loss as compared to the two-day transfection protocol at week 2 (57 and 72% as compared to day 0, respectively) and week 4 (28 and 30% as compared to day 0, respectively) and only partially at week 6 (14 and 15% as compared to day 0, respectively).

In order to assess the effect of the transfection protocol on the individual CAFC quality, we calculated the mean number of LTC-CFC produced at week 6 per CAFC week 6. Untreated CD34+ PBSC (day 0) formed 5.2 LTC-CFC per CAFC at week 6 (Table 7.2). After two days of transfection (2T) or five days of liquid culture followed by two days of transfection (5C2T) with or without the addition of SCM there was a three-fold lower individual stem cell quality (range: 1.4 to 1.7 LTC-CFC per CAFC week 6). These observations show that the presently discussed transfection protocol (5C2T) effectively transduces the NEO gene into primitive HSC, however, at the same time causes their numerical and qualitative loss of the graft.

Table 7.2 Mean Number of Flask Long-Term Culture Colony Forming Cells per Cobblestone Area Forming Cell at Week 6 Before and After Retroviral Gene Transfer.

Protocol (see figure 7.3)	LTC-CFC per CAFC Week 6 (±1SEM; n=3)
Day 0	5.2 (±2.0)
2T	1.7 (±0.6)
5C2T (No SCM)	1.5 (±0.5)
5C2T (FBMD-1SCM)	1.4 (±0.6)
5C2T (L88/5 SCM)	1.4 (±0.6)

CFC, Colony Forming Cell; CAFC, Cobblestone Area Forming Cell; LTC, Long-Term Culture; SCM, Stroma-Conditioned Medium.

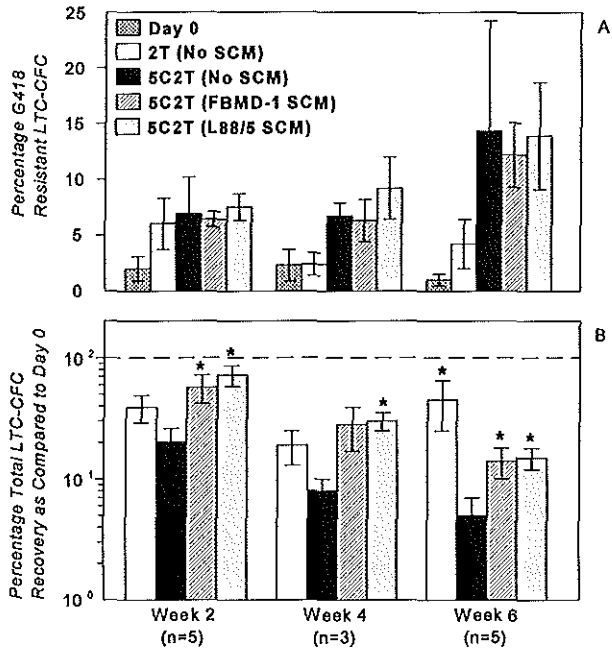

Figure 7.6 The effect of transfection protocol or stroma-conditioned media (SCM) on retroviral NEO gene transfer in and recovery of long-term culture colony forming cell (LTC-CFC) production. (A) The percentage G418-resistant (0.5 mg/ml, active substance) and (B) the percentage recovery of (as compared to input values at day 0: dashed line) non-adherent LTC-CFC week 2 and 4 and combined non-adherent and stroma-adherent LTC-CFC week 6 is shown using the 2T or 5C2T transfection protocols (see Figure 7.3) in serum-free medium containing IL-3, SCF, IL-6 and αTGFβ1 with or without FBMD-1 or L88/5 SCM. Percentage G418 resistant LTC-CFC of untreated cells is shown as day 0 value. Comparison between 5C2T (No SCM) and the other three protocols: *, $P<0.05$.

DISCUSSION

In this study we optimized conditions for retroviral gene transfer into mobilized CD34+ PBSC from cancer patients. As shown before with HSC from normal bone marrow and cord blood [18-21] also mobilized PBSC were more efficiently transfected on carboxy-terminal fibronectin fragment coated dishes. In accordance with Hanenberg et al. this can be explained by colocalization of cells and virus particles, because our study showed that CD34+ PBSC adhere very efficiently to CH-296 and Hanenberg et al. reported that retrovirusses adhere to the heparin binding domain of CH-296 [21]. In addition, we did not observe an effect of CH-296 adherence on the recovery of CFC after two- and seven-day cultures.

In a second set of experiments we showed that five-day pre-culture

specifically improved retroviral gene transfer in primitive PBSC (CAFC week 6) as compared to a two-day transfection protocol. Apparently, the quiescent primitive stem cells in mobilized peripheral blood needed a prolonged cytokine stimulation before cell cycling and subsequent retroviral integration occurred. This was also suggested by reports on the refractiveness of primitive CD34+CD38-negative HSC in mobilized peripheral blood to cell cycle activation during a two- to three-day cytokine stimulation [12,13]. In our study, the transfection efficiency of progenitor cells (CFC and CAFC week 2) were comparable in all four transfection protocols, indicating that transfection efficiencies of progenitor cells have no predictive value for that in more primitive HSC. The seven-day culture and transfection protocols (2T5C, 5C2T and 2T3C2T) in serum-free medium containing 3/S/6/αT resulted in an expansion of CFC, but a loss of all CAFC subsets. This loss of stem cells can partly be explained by the repeated manipulations during the transfection and culture procedures, but could also partly result from suboptimal culture conditions. Indeed, the addition of FBMD-1 or L88/5 SCM to the pre-culture resulted in significantly improved recovery of CFC and all CAFC week-types as was also reported earlier by our group using seven-day expansion cultures of CD34+ PBSC [22]. Although in the present study SCM hardly improved the percentage of G418-resistant HSC, the increase in total recovery of stem cells resulted into an 1.9- to 9.7-fold larger number of transfected stem cell subsets in the graft as compared to cultures without SCM.

Previous studies of our group have shown the importance of stem cell quality assessment. In a selected group of autologous transplantation patients that did not show significant hematopoietic repopulation within six months post-transplant, the original grafts contained low CAFC week 6 numbers, while their ability to generate CFC in LTC was even lower (P.B. Van Hennik et al., manuscript in preparation). In addition, we have reported that few CAFC week 6 are mobilized in patients that have received intense cytotoxic chemotherapy, and that the quality of those stem cells is very low [24]. In the present study, we observed a total LTC-CFC producing ability that ranged from 5 to 45% of input in four different transfection protocols (2T, 5C2T, 5C2T+FBMD-1 SCM, 5C2T+L88/5 SCM). When these LTC-CFC numbers were corrected for the number of CAFC week 6, it appeared that only 1.4 to 1.7 LTC-CFC was generated per CAFC week 6 in all four transfection protocols as compared to 5.2 LTC-CFC/CAFC in the original graft. From these data we may conclude that irrespective of their numerical recovery, the CAFC week 6 always generated the same mean number of LTC-CFC per CAFC suggesting that all transfection protocols lead to identical losses of individual stem cell quality. Our group has made the same observation after expansion cultures of CD34+ PBSC (D.A. Breems et al., manuscript submitted), indicating that this individual proliferative potential of HSC is always affected during *ex vivo* manipulation of PBSC and possibly reflects stem cell aging [28-30].

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CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

The hematopoietic system is one of the main targets for gene therapy. This system is so attractive because only a small pool of primitive hematopoietic stem cells (HSC) is responsible for the life-long formation of all blood cell lineages. As a result, the transfection of primitive HSC would lead to the introduction of the transfected gene in all daughter cells of those stem cells. Up to now, most gene therapy studies of HSC have concentrated on stem cell marking, correction of genetic disorders and conferral of chemotherapy resistance using retroviral vectors. Gene marking studies are performed to study the feasibility of HSC gene therapy, to investigate homing and distribution kinetic patterns of HSC and their progeny, and to detect contaminating tumor cells in HSC transplants of cancer patients. Genetic disorders may be corrected by introducing the relevant gene into the HSC of a patient. HSC from cancer patients can potentially be protected against chemotherapy by introducing resistance genes. However, the success of HSC gene therapy is limited by the low transfection efficiency in those primitive HSC.

The objective of the work presented in this thesis was to improve retroviral gene transfer efficiency in human HSC. Clinically relevant culture and transfection conditions of HSC were studied. HSC were collected from chemotherapy and cytokine-mobilized peripheral blood, because this stem cell source is widely used in autologous HSC transplantation of cancer patients. To monitor optimized gene transfer protocols, assays were developed to assess the quantity and quality of human HSC before and after transfection.

8.2 How to Measure Human Primitive Hematopoietic Stem Cells?

Cobblestone area forming cell and long-term culture assays

The first goal of the project described in this thesis was the development of *in vitro* assays for human primitive HSC, enabling the *in vitro* analysis of human HSC before and after retroviral gene transfer. Stroma-dependent cultures were set up using a murine stromal cell line, the FBMD-1. As a result, the human cobblestone area forming cell (CAFC) assay was introduced as an assay for *in vitro* frequency analysis of different HSC subsets. In addition, using the same stromal cell line a flask long-term culture (LTC) assay was established which allows the determination of the production of colony forming cells (CFC) from a hematopoietic specimen. This parameter has been used as an *in vitro* parameter for stem cell ability. In combination these assays can be applied to calculate the average quality of individual stem cells (Chapter 2). In chapters 6 and 7 these assays have been used to study gene transfer protocols. In addition, the human CAFC and LTC-CFC assays have been very helpful in analyzing stem cell grafts (Chapter 3) and *ex vivo* stem cell expansion cultures (Chapters 4 and 5). Also studies of acute and chronic myeloid leukemia benefited from these assays [1-4]. Furthermore, it was possible using the same setup with rhesus monkey

interleukin-3 (IL-3) to establish rhesus monkey CAFC and LTC-CFC assays [5].

Arguments supporting the validity of CAFC and LTC-CFC assays

The validity of these stroma-based assays for primitive stem cells that are able to induce long-term chimerism *in vivo* has not formally been proven. However, various arguments support their validity.

1. The human CAFC assay has been extrapolated from the murine CAFC assay. Extensive correlation studies using physically separated stem cell subsets in four different murine transplantation models have shown that the established hierarchy of *in vivo* repopulating stem cell subsets is likewise apparent in the murine CAFC assay [6]. This would suggest that in analogy with the murine CAFC assay, also in humans, early appearing CAFC represent transiently repopulating stem cells, while late appearing CAFC have *in vivo* long-term repopulating ability. An as yet unresolved issue is the exact culture time required for the assessment of such primitive stem cells.
2. Using sorted or 5-fluorouracil treated bone marrow cells it has been shown that the human CAFC assay can indeed discriminate between progenitor cells (CAFC week 1 to 3) and more primitive stem cells (CAFC week 5 to 8) (Chapter 2).
3. Other investigators have shown that CD34-positive/Thy-1-positive/Lineage-negative cells from mobilized peripheral blood were enriched for both CAFC week 4 to 7 and for cells with the ability to give multilineage engraftment in human fetal bone transplants in mice with severe combined immunodeficiency disease (SCID) [7].
4. Supportive evidence for the validity of these stroma-supported assays is also supplied by analysis of clinical stem cell transplants (Chapter 3). Mobilized peripheral blood stem cell (PBSC) harvests of intensively pretreated cancer patients contained low numbers of CAFC subsets and the *in vitro* individual stem cell quality of those stem cells was low. In contrast, less heavily pretreated patients produced stem cell grafts with better CAFC yields. Attempts were made to correlate the number of CAFC week-types in grafts with the *in vivo* repopulation abilities of those transplants. The number of CAFC week 1 to 3 transplanted correlated better with early hematopoietic recovery than did the number of CAFC week 4 to 6. This is consistent with the notion that CAFC week 1 to 3 are indicators of relatively mature progenitor cells.
5. Recent studies of autologous transplantation patients show that transplants with a low number of CAFC week 6 and/or reduced LTC-CFC producing ability resulted in graft failure. In contrast, the progenitor cell content (CFC and CAFC week 2) of those transplants was within the normal range, suggesting that late CAFC (week 6) provide a predictive index for repopulating capacities [8].

These circumstantial arguments support the conclusion that the human CAFC assay at successive time points discriminates between different stem cell subsets. However, it has not been definitively established whether the human CAFC assay can measure primitive HSC with *in vivo* long-term repopulating ability. To prove this, one would need transplantation studies using sorted and

marked primitive stem cells subsets, which are difficult, if not impossible, to perform in humans.

The studies in clinical autologous transplantation also indicate the possible clinical relevance of the LTC-CFC assay. In the present thesis, it was shown that CAFC week 6 from intensively pretreated cancer patients have a low CFC producing ability in LTC (Chapter 3). Analysis of graft failure patients showed that in a selected number of cases the number of CAFC week 6 transplanted was in the normal range, but that their CFC producing ability was severely reduced [8]. In chapter 5 also a strong reduction of LTC-CFC producing ability was observed after *ex vivo* propagation of HSC from mobilized peripheral blood. For these reasons, protocols for *ex vivo* manipulation of HSC should not only be analyzed in terms of stem cell numbers, but also using qualitative assays, because reduction of stem cell quality might also be a possible reason for failure of a transplant.

Other human hematopoietic stem cell assays

Two recent reports have questioned the validity of the human LTC-initiating cell (LTC-IC) assay, an assay similar to the human CAFC assay, for the assessment of the transfection efficiency of *in vivo* long-term repopulating stem cells [9,10]. Although these investigators report high transfection efficiencies into human LTC-IC, the first human gene therapy trials report only a small percentage of long-term expression in a limited number of recipients. This implies that the *in vivo* long-term repopulating stem cell is more resistant to retroviral transfection and therefore differs from the *in vitro* LTC-IC.

In the first report it was suggested that by extending the LTC beyond 8 weeks, a more primitive stem cell subset can be observed [9]. This extended LTC-IC (ELTC-IC) was CD34-positive/CD38-negative and expressed a higher CFC-producing potential than did the standard LTC-IC. The transfection percentage of ELTC-IC was also much smaller than that of LTC-IC using a three-day transfection procedure (transfection percentage: LTC-IC: 35.9% and ELTC-IC: 0.7%). However, in our view, the ELTC-IC assay has not been carried out adequately to permit satisfactory interpretation. Every four weeks the cultures were replated on new stromal layers, which may have interfered with the lodgement or quiescence of primitive HSC in the stroma and makes a practical frequency analysis using a limiting dilution setup almost impossible.

Another stem cell assay, which claims to assess a more primitive stem cell subset than the LTC-IC, is the SCID repopulating cell (SRC) assay [10]. This SRC is also representative of a human primitive HSC. It shows the ability of repopulating SCID or non-obese diabetic/SCID (NOD/SCID) mice. Also the SRC is more resistant to retroviral transfection than the LTC-IC (transfection percentage: LTC-IC: 29% and SRC: 0.4%).

However, transfection experiments described in chapter 7 show that the CAFC week 6 is also very resistant to retroviral transfection in a two-day transfection procedure as compared to the CAFC week 2 (transfection percentage: CAFC week 2: 15.2% and CAFC week 6: 0.7%). Therefore, in our

view, the CAFC week 6 can still be considered as a primitive stem cell subset, which is difficult to transfect in short-term (2 to 4 days) transfection procedures and thus comparable to the ELTC-IC or SRC.

CAFC versus SRC assay for frequency assessment of transfected stem cells

As compared to the CAFC assay, the SRC assay has some disadvantages for the frequency analysis of transfected human primitive HSC.

1. The mean CAFC week 6 frequencies of unseparated PBSC (10 per 10^5 nucleated cells) and cord blood (12 per 10^5 nucleated cells) are comparable (Chapter 3) [11]. However, for still unknown reasons mobilized PBSC engraft with a lower efficiency in NOD/SCID mice as compared to umbilical cord blood cells. There is a more than six-fold difference between the SRC frequency of PBSC and cord blood cells, i.e. 0.17 and 1.1 per 10^6 nucleated cells, respectively [12]. This low engraftment of PBSC in (NOD/)SCID mice complicates analysis of gene transfer experiments in these mice using HSC from clinical relevant mobilized peripheral blood samples, which have been studied in this thesis.
2. Frequency analysis of SRC using limiting dilution would require very large numbers of mice [12,13], which makes the SRC assay a practically and financially unattractive quantitative assay.
3. Because the percentages of human stem cells homing to the hematopoietic organs of (NOD/)SCID mice are still unknown, a reduced seeding of retrovirally transfected SRC can hinder the assessment of the percentage transfected SRC. For example, a reduced seeding efficiency of manipulated stem cells to the bone marrow and the spleen has been shown in murine experiments, in which the seeding efficiencies of all HSC subsets were decreased following a three-hour preincubation with various hematopoietic growth factors [14]. In the same experiments, the seeding efficiency of these manipulated stem cells in the CAFC assay was not affected [14]. Moreover, when unmanipulated murine CAFC were overlaid on established stromal layers for two hours or longer, all of the inoculated cells could be retrieved from the stroma and exhibited unchanged cobblestone area formation in secondary stroma-supported cultures [15]. In contrast to the 100% seeding efficiency to the stromal layer, only 20% and 10% of all murine CAFC subsets seed *in vivo* to the total bone marrow and the spleen, respectively [14].

Future perspectives

In spite of extensive validation studies suggesting that the human CAFC and LTC-CFC assays determine a primitive HSC subset, it has yet to be proven that these assays determine the 'true' *in vivo* long-term repopulating stem cell. As a result, further studies are necessary. However, this evidence can probably only be obtained from analyses of HSC transplantation in patients. In transplants, which failed to engraft, one could investigate which CAFC subset was predominantly reduced or one could examine whether the LTC-CFC production was inadequate [8]. Analysis of gene therapy patients could tell which genetically transduced CAFC subset has predictive value for the short-term or

long-term engraftment of genetically transduced blood cells [16].

The clinical application of the CAFC and LTC-CFC is limited, because these stroma-dependent assays are labor intensive and time consuming, and their use requires trained and dedicated personnel. For routine use these assays are unpractical. However, for the evaluation of new and experimental manipulations of HSC grafts these assays could provide valuable information (Chapters 4 to 7). Furthermore, these assays could be of decisive use in selected individual cases of doubts about the stem cell quantity or quality of selected HSC grafts (Chapter 3). This information could be of predictive value for the outcome of the transplantation [8]. One could argue that the number of CD34-positive/CD38-negative cells is a measure for primitive HSC. However, although such measurements are much easier to perform than the CAFC and LTC-CFC techniques, it provides no information about the functional quality of those cells. In addition, the large variability of CD38-expression between HSC samples and the extremely low frequency of CD38-negative cells makes it difficult to identify the 'true' CD38-negative cell.

Although during the last few years many laboratories have used CAFC and LTC assays for stem cell research, it is not easy to standardize these assays between laboratories. In this thesis the assays have been partially standardized by using a stromal cell line and adding exogenous recombinant IL-3 and granulocyte colony-stimulating factor. However, the use of animal serum in the culture medium remains a variable factor. Considerable variations between serum batches of both fetal calf and horse have been observed. Also the concentrations of fetal calf serum and horse serum can influence the assays. The development of a defined serum-free CAFC/LTC medium might overcome the latter problem.

Although the original definition of a cobblestone area by Ploemacher et al. is quite clear (at least five phase-dark hematopoietic cells under the stromal layer) [6,17], other investigators have their own criteria. These criteria may be based upon differing parameters of the size and refractile aspect of the cobblestone areas. In different reports, cobblestone areas have been described with significant variations using the following criteria: (1) more than fifteen closely attached homogeneously formed bright cells [18], (2) a cluster of small, tightly packed, nonrefractory cells greater than fifteen cells [19], (3) three separate foci of more than five small, tightly packed, nonrefractory cells [19] or (4) more than fifty tightly packed refractile cells [7]. It would be useful to apply a common and uniform definition of a cobblestone area.

8.3 How to Improve Clinical Gene Transfer Protocols for Human Mobilized Peripheral Blood Stem Cells?

Optimizing culture conditions

The second goal of this thesis was to improve gene transfer into human primitive HSC from mobilized peripheral blood using a clinically relevant supernatant protocol. This study aimed at improving the supernatant gene transfer protocol which was planned at the time this investigation started, i.e. the clinical multidrug resistance-1 (MDR1) gene therapy trial in University Hospital Rotterdam, The Netherlands (Hoogerbrugge et al., clinical protocol). This protocol was adapted from the gene transfer procedures used in adenosine deaminase deficiency (ADA) gene therapy studies of rhesus monkeys [20] and the ADA gene therapy trial in three ADA patients [21]. In this procedure retroviral supernatant was prepared in medium with 5 to 10% fetal calf serum or serum-free medium containing polybrene and IL-3. During the four-day transfection procedure fresh virus supernatant was added to culture daily. In the present thesis the effects of several transfection conditions have been studied on: (A) the gene transfer percentage[1] and (B) the recovery of the number and quality of different HSC subsets[2] (Table 8.1). These two parameters determine the total number of transfected HSC[3] in the transplant which ultimately will be infused into the patient (Table 8.1, Column C).

1. Cytokines

It has been reported that the cytokine combination IL-3, stem cell factor (SCF) and IL-6 (as compared to IL-3 or IL-3/IL-6) promotes retroviral-mediated gene transfer into progenitors and primitive HSC from human bone marrow [22]. However, in this study using CD34-selected mobilized PBSC in a two- and four-day transfection protocol the transfection percentage was not increased when SCF and IL-6 had been added to the transfection cultures as compared to IL-3 alone (Table 8.1, Row 1, Column A).

Furthermore, the recovery of stem cells had neither been affected by SCF and IL-6 in those two- and four-day cultures (Table 8.1, Row 1, Column B). Apparently, a culture period of only two to four days was insufficient to observe an effect of SCF and IL-6 on CD34-selected mobilized PBSC. Because in seven-day expansion cultures, the addition of SCF and IL-6 to the culture enhanced the recovery of PBSC as compared to the addition of IL-3 alone (Chapter 4).

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[1]transfection percentage = the number of transfected cells divided by the total number cells

[2]stem cell recovery = the number of cells after transfection divided by the number of cells before transfection

[3]total number of transfected cells = transfection percentage times stem cell recovery

Table 8.1 The Effect of Different Transfection Conditions on Transfection Percentage, Stem Cell Recovery and Total Number of Transfected Stem Cells.

	A. Transfection Percentage		*B. Stem Cell Recovery*			*C. Total Number of Transfected Stem Cells*	
Transfection Condition	CFC	CAFC wk 6	CFC	CAFC wk 6	LTC-CFC at wk 6	CFC	CAFC wk 6
1. As compared to 2- and 4-day transfection with IL-3							
+ SCF	↔[6]	ND	↔[0]	↔[6]	↔[0]	↔[0]	ND
+ SCF/IL-6	↔[6]	ND	↔[0]	↔[6]	↔[0]	↔[0]	ND
2. As compared to 2-day transfection with IL-3/SCF/IL-6							
2-day culture + 2-day transfection	↔[6]	ND	↔[0]	ND	ND	↔[0]	ND
4-day transfection	↔[6]	ND	↑[0]	↔[6]	↔[6]	↑[0]	ND
2-day transfection + 5-day culture	↔[7]	↔[7]	↑[7]	↓[7]	↓↓[0]	↑[0]	↔[0]
5-day culture + 2-day transfection	↔[7]	↑↑[7]	↑[7]	↓[7]	↓↓[7]	↑[0]	↑[0]
2-day transfection + 3-day culture + 2-day transfection	↔[7]	↑↑[7]	↑[7]	↓[7]	↓↓[0]	↑[0]	↑[0]
3. As compared to 2-day transfection with IL-3/SCF/IL-6							
+ CH-296 coated dishes	↑↑[7]	ND	↔[7]	ND	ND	↑↑[0]	ND
4. As compared to 2-day transfection with IL-3/SCF/IL-6 on CH-296 coated dishes							
+ Polybrene	↔[7]	ND	↔[7]	ND	ND	↔[0]	ND
+ Protamine Sulphate	↔[7]	ND	↔[7]	ND	ND	↔[0]	ND
5. As compared to 5-day culture + 2-day transfection with IL-3/SCF/IL-6 on CH-296 coated dishes							
+ FBMD-1 SCM	↔[7]	↔[7]	↑↑[7]	↑↑[7]	↑[7]	↑[7]	↑↑[7]
+ L88/5 SCM	↔[7]	↔[7]	↑↑[7]	↑↑[7]	↑[7]	↑[7]	↑↑[7]

CAFC, Cobblestone Area Forming Cell; CFC, Colony Forming Cell; LTC-CFC, Long-Term Culture CFC Production; ND, Not Determined; SCM, Stroma-Conditioned Medium; ↔, No effect; ↑, Positive effect; ↓, Negative effect; [0], Data not shown in this thesis; [6], Data shown in chapter 6; [7], Data shown in chapter 7.

2. Transfection protocols

Primitive HSC from mobilized peripheral blood resist transduction in two- to three-day cytokine stimulation protocols (Chapter 7) [23]. It was found that a five-day pre-culture specifically improved the transfection percentage of primitive PBSC (Table 8.1, Row 2, Column A). This five-day pre-culture may have promoted cell cycle activation of primitive HSC from mobilized peripheral blood which are quiescent [23,24]. Another possibility to explain enhanced gene transfer may be that the amphotropic virus receptor expression was upregulated following the five-day culture period. The four- and seven-day transfection procedures also enhanced the recoveries of progenitor cells as compared to the results of two-day transfection protocols (Table 8.1, Row 2, Column B). However, the seven-day transfections reduced the LTC-CFC producing ability of primitive stem cells. As a result, the total number of transfected progenitors increased due to the augmented recovery while the number of transfected primitive HSC increased due to a higher percentage of transfection (Table 8.1, Row 2, Column C).

3. Carboxy-terminal fibronectin fragments

The transfection percentage in HSC from mobilized peripheral blood could considerably be improved by colocalizing retrovirus and target cells using the carboxy-terminal fibronectin fragment CH-296 (Table 8.1, Row 3, Column A). This was in line with observations by other investigators using normal bone marrow and cord blood cells [25-28]. The recovery of HSC was not negatively influenced by the adherence to CH-296 (Table 8.1, Row 3, Column B).

4. Polycations

The use of CH-296 made the addition of the polycations, polybrene or protamine sulphate, redundant and unnecessary (Table 8.1, Row 4).

5. Stroma-conditioned medium

Other investigators have reported that the use of a stromal layer during transfection did not only improve transduction but also the recovery of primitive HSC and their ability to engraft *in vivo* [29,30]. In the present thesis it was found that the addition of stroma-conditioned medium (SCM) to the five-day pre-cultures leads to an increased recovery of both transduced and untransduced HSC (Table 8.1, Row 5). In contrast, the transfection of HSC did not improve by the addition of SCM, suggesting that stroma-contact is required for the favorable effect on the transfection percentage. A recent study is in line with the speculation that soluble stromal factors and stroma-contact have separable effects in gene transfer [30]. In this report it was found that FLT3-ligand (FL) can partially replace the ability of stroma to allow HSC to engraft in beige/nude/xid mice, while FL could not substitute the ability of stroma to increase gene transfer percentage.

These observations may suggest that the SCM effect is exerted by FL present in SCM. However, the addition of SCM to optimal concentrations of FL

further improved the recovery of HSC from seven-day cultures of CD34-selected PBSC as compared to SCM or FL in optimal concentrations alone (Chapter 5). Other investigators have suggested that the glycosaminoglycan heparan sulphate is responsible for the additive SCM effect [31]. However, this could not be confirmed using liquid cultures, as described in chapters 4 and 5. In those cultures no positive effect of heparan sulphate was apparent (Breems et al., unpublished observations). Unfortunately, attempts to purify the SCM activity failed due to the loss of activity following freeze and thaw procedures (Ploemacher et al., unpublished observations). As a result the nature of the SCM activity is still to be elucidated.

6. Stroma-contact

As described in chapter 5 both SCM and stroma-contact can improve the recovery of the number of HSC. In contrast to SCM, stroma-contact can also neutralize the loss of CFC producing quality (LTC-CFC) during seven-day cultures. In this thesis the effect of stroma-contact on the transfection percentage was not evaluated. Other investigators had shown that the use of a stromal layer can also improve transfection [29,30]. Thus, stroma-contact may be an attractive method for gene transfer improvement. However, the mechanism of stroma-contact on the transfection percentage is still unknown. A possible mechanism could be that stem cells and virus particles are colocalized by an extra-cellular matrix molecule present on stroma. Because it has been shown that carboxy-terminal fibronectin fragments can bind both HSC and virus particles [25-28]. The latter close approximation could also occur on stroma and improve the transfection percentage.

In clinical gene transfer protocols the use of stroma-contact appears more complicated. One would prefer to use autologous stroma but a patient-derived stroma obtained following cytotoxic treatment might be difficult to establish in culture. It should be grown prior to the transfection procedure and requires many cells from a large bone marrow puncture. The use of allogeneic stroma or even murine or human stromal cell lines incurs the risk of contamination of the graft with foreign material, including infectious agents. This might cause immunological reactions or potentially carry the hazard of introduction of exogenous pathogens. The identification and purification of the extra-cellular matrix molecules responsible for the effect of stroma-contact would be an elegant solution to this problem. Whether fibronectin (fragments) or other stromal-derived extra-cellular matrix molecules can also replace the favorable effect of stroma-contact on LTC-CFC recovery would need to be the subject of future study.

7. Conclusions

The current findings led to an improved transfection protocol of five-days pre-culture with IL-3/SCF/IL-6 and L88/5 SCM followed by two-day transfection with IL-3/SCF/IL-6 on CH-296 coated dishes (Chapter 7). As compared with the protocol of two- or four-days supernatant with IL-3 alone, which is used for the

clinical MDR1 gene therapy trial in Rotterdam (Hoogerbrugge et al., clinical protocol), this improved protocol results in 5.1- and 17.6-fold greater transfection percentages of progenitors (CFC) and primitive HSC (CAFC week 6), respectively. The recoveries of CFC and CAFC at week 6 increase by 15.8- and 1.4-fold, respectively. However, the recovery of LTC-CFC at week 6 is 3.0-fold reduced. As a net result, the total number of transfected CFC and CAFC week 6 in the graft increases by a factor of 141- and 21.3-fold, respectively (Table 8.2).

Practically, all the gene transfer protocol improvements proposed in chapter 7 can be introduced in the clinic, if commercial interests or proprietary limitations would not interfere. Recombinant SCF, IL-6 and CH-296 are available. All experiments were performed in serum-free medium without the addition of animal serum. The preparation of SCM may be more difficult. But when SCM is prepared under the same clinical grade conditions as is the retroviral supernatant, this problem might also be avoided.

Vectors for gene transfer

A subject that is not discussed in this thesis but almost certainly may contribute to an improvement of gene therapy protocols is the choice of the vector system which introduces the gene expression cassette in the target cell. In the work for this thesis two murine leukemia viruses (MLV) have been used as retroviral vectors, namely the murine stem cell virus (MSCV) [32] and the MFG vector [33]. Both vectors have been shown in murine experiments to be able to transfect long-term repopulating HSC and express the gene *in vivo*. Several alternative vector systems have been proposed to improve gene delivery.

Virus envelope

In addition to the amphotropic MLV, the xenotropic MLV, the gibbon ape leukemia virus and feline leukemia virus are being studied for their relative transduction efficiency in several target cells [34]. Studies on receptor expression and modulation for the different MLV may and should reveal the possible applicability of the different leukemia virus envelopes for the transfection of HSC.

Retrovirus subfamilies or other virus families

The murine oncoviruses have been used most extensively for the transfection of HSC. More recently also foamy viruses and lentiviruses have been proposed as possible vectors for gene transfer into HSC [35]. Also other families of viruses have been used. The most extensively studied viruses are the adenoviruses and the adeno-associated viruses [36].

Gene targeting

Both viral and non-viral gene delivery systems can be targeted to specific elements of certain target cell types [37]. For example, the c-kit receptor on HSC can be used for this purpose [38].

Table 8.2 Comparison of Various Conditions of Gene Transfer into Peripheral Blood Stem Cells as Compared to the Current Clinical MDR1 Gene Therapy Protocol in Rotterdam*.

	Transfection Percentage		*Stem Cell Recovery*			*Total Number of Transfected Cells*	
Transfection Condition	CFC	CAFC wk 6	CFC	CAFC wk 6	LTC-CFC at wk 6	CFC	CAFC wk 6
2- to 4-day transfection with IL-3*	1	1	1	1	1	1	1
2-day transfection on CH-296 coated dishes with IL-3/SCF/IL-6	7.9	ND	1.3	ND	ND	13.5	ND
5-day culture + 2-day transfection on CH-296 coated dishes with IL-3/SCF/IL-6	8.2	12.4	4.1	0.2	0.1	90.2	2.8
5-day culture + 2-day transfection on CH-296 coated dishes with IL-3/SCF/IL-6 and FBMD-1 SCM	8.1	17.9	9.3	0.9	0.3	116	14.9
5-day culture + 2-day transfection on CH-296 coated dishes with IL-3/SCF/IL-6 and L88/5 SCM	5.1	17.6	15.8	1.4	0.3	141	21.3

*, Values indicate the ratio of improvement of gene transfer efficiency into CFC, CAFC week 6 and LTC-CFC week 6 as compared to 2- to 4- day control incubations with IL-3 without CH-296 (set at 1) (Hoogerbrugge et al., clinical protocol). CAFC, Cobblestone Area Forming Cell; CFC, Colony Forming Cell; IL, Interleukin; LTC-CFC, Long-Term Culture CFC Production; ND, Not Determined; SCF, Stem Cell Factor; SCM, Stroma-Conditioned Medium. Summary of conditions tested in chapter 7.

The fate of transduced hematopoietic stem cells *in vivo*

Theoretically, various circumstances may counteract the functional expression of the transfected gene following *in vivo* transplantation. The total number of transplanted and transfected stem cells could be too low to accomplish a therapeutic effect. But also a reduced seeding or engraftment of transfected HSC may limit the therapeutic effect of the genetically modified HSC and their descendants. A possible solution to the problem of the low number of transfected HSC might come from the *in vivo* selection of transfected cells using a co-transduced drug resistance gene. Murine studies have shown that this may in principle be feasible for the MDR1 gene [39]. However, there may still be the

problem of the high level of endogenous MDR1 expression of primitive HSC [40], a condition that may result in co-selection of non-transduced stem cells.

The HSC and their daughter cells might also fail to functionally express the introduced gene. For example, in chapter 6 it has been shown that the MDR1 gene transfer measured by PCR was greater than the functional vincristine resistance or the rhodamine-123 exclusion assays, another index of multidrug resistance.

Because ample evidence exists that during a given period of time only a limited number of primitive HSC are responsible for hematopoiesis in an organism [41], one may assume that transfected primitive HSC will not take part in hematopoiesis continuously. There are indications that transfected HSC contribute less to hematopoiesis *in vivo* than do non-transduced HSC. This might be concluded from the notable discrepancy between the number of retrovirally marked stem cells in the marrow and the number of marked cells present in the peripheral blood of beige/nude/xid mice [42]. In addition, this has also been observed in human gene therapy patients [43] and can be interpreted as possible evidence for an *in vivo* maturation block of transfected HSC.

Because gene therapy can lead to the expression of a foreign gene, this might elicit an immunological reaction of the organism against the transduced cells [44,45]. It is also possible that the gene product has direct toxicity to hematopoietic cells [46,47].

Some of the issues, which have been raised in this paragraph and are likely to influence the *in vivo* expression of transduced genes in transplanted HSC, can also be addressed using *in vitro* stem cell assays such as the ones presented in this thesis. This permits not only the analysis of the feasibility of transfection protocols with respect to the HSC recovery and the transfection percentage, but also the assessment of regulated and stable expression. In addition, these *in vitro* assays may assist in defining the contribution of transduced HSC to the total HSC compartment at various times after their transplantation.

8.4 References

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ABBREVIATIONS

5-FU	5-Fluorouracil
7-AAD	7-Aminoactinomycin D
α-	anti-
Ab	Antibody
ADA	Adenosine Deaminase
Adh	Adherent
BFU-E	Burst Forming Unit-Erythroid
BMC	Bone Marrow Cells
βME	β-Mercaptoethanol
BSA	Bovine Serum Albumin
CA	Cobblestone Area
CAFC	Cobblestone Area Forming Cell
CD	Cluster of Differentiation
CFC	Colony Forming Cell
CFU-GM	Colony Forming Unit-Granulocyte Macrophage
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ELTC-IC	Extended Long-Term Culture-Initiating Cell
EPO	Erythropoietin
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein-Isothiocyanate
FL	FLT3-Ligand
FN	Fibronectin
GAG	Glycosaminoglycan
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBSS	Hanks' Balanced Salt Solution
HC	Hydrocortisone 21-hemisuccinate
HLA	Human Lymphocyte Antigen
HS	Horse Serum
HSC	Hematopoietic Stem Cell
hu	Human
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
LIF	Leukemia Inhibitory Factor
LP	Leukaphersis Product
LTBMC	Long-Term Bone Marrow Culture
LTC	Long-Term Culture
LTC-CFC	Long-Term Culture-Colony Forming Cell
LTC-IC	Long-Term Culture-Initiating Cell
LTR	Long Terminal Repeat

MACS	Magnetic Cell Sorting
MDR1	Multidrug Resistance-1
MIP	Macrophage Inflammatory Protein
MLV	Murine Leukemia Virus
MoMuLV	Moloney Murine Leukemia Virus
MSCV	Murine Stem Cell Virus
mu	Murine
NA	Non-Adherent
NC	Nucleated Cell
ND	Not Determined
NEO	Neomycin phosphotransferase
No.	Number
NOD	Non-Obese Diabetic
PBS	Phosphate-Buffered Saline
PBSC	Peripheral Blood Stem Cell
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
Rh123	Rhodamine-123
RNA	Ribonucleic Acid
r_s	Spearman's rank correlation coefficient
SA	Stroma-Adherent
SCF	Stem Cell Factor
SCID	Severe Combined Immune Deficiency
SCM	Stroma-Conditioned Medium
SD	Standard Deviation
SEM	Standard Error of the Mean
SNT	Supernatant
SRC	Severe combined immune deficient mouse Repopulating Cell
TGF	Transforming Growth Factor
TPO	Thrombopoietin

SUMMARY

One of the main targets for gene therapy is the hematopoietic system because of well-developed procedures for bone marrow transplantation, the many types and wide distribution of hematopoietic cells in the body and the existence of many diseases and treatments that affect hematopoietic cells. The target cells for gene transfer are the most primitive hematopoietic stem cells (HSC), which are present at low frequency in the bone marrow, are responsible for long-term hematopoiesis and give rise to all hematological and lymphoid lineages. Up to now, most gene therapy studies of HSC have concentrated on stem cell marking, correction of genetic disorders and conferral of chemotherapy resistance.

The aim of this study was to develop protocols which improve gene transfer of human HSC. For this purpose suitable assays were developed to determine the frequency and proliferative capacity of different classes of human HSC. Subsequently, culture conditions for human HSC were studied. As a result, using optimized culture conditions and newly developed human HSC assays, improved human HSC gene transfer protocols could be proposed. In the presented studies we have opted for clinically relevant and acceptable protocols. This included the use of target cells which are most frequently used in clinical autologous stem cell transplantation, namely mobilized peripheral blood stem cells (PBSC).

The first goal of the project described in this thesis was the development of *in vitro* assays for human primitive HSC, enabling the *in vitro* analysis of human HSC before and after retroviral gene transfer. Stroma-dependent cultures were set up using a murine stromal cell line, the FBMD-1. As a result, the human cobblestone area forming cell (CAFC) assay was introduced as an assay for *in vitro* frequency analysis of different HSC subsets. In addition, using the same stromal cell line a flask long-term culture (LTC) assay was established which allows the determination of the production of colony forming cells (CFC) from a hematopoietic specimen. This parameter has been used as an *in vitro* parameter for stem cell ability. In combination these assays can be applied to calculate the average quality of individual stem cells (Chapter 2).

The validity of these stroma-based assays for primitive stem cells that are able to induce long-term chimerism *in vivo* has not formally been proven. However, various arguments support their validity. Firstly, the human CAFC assay has been extrapolated from the murine CAFC assay. Extensive correlation studies using physically separated stem cell subsets in four different murine transplantation models have shown that the established hierarchy of *in vivo* repopulating stem cell subsets is likewise apparent in the murine CAFC assay. This would suggest that in analogy with the murine CAFC assay, also in humans, early appearing CAFC represent transiently repopulating stem cells, while late appearing CAFC have *in vivo* long-term repopulating ability. Secondly, using sorted or 5-fluorouracil treated bone marrow cells it has been shown that the human CAFC assay can indeed discriminate between progenitor cells (CAFC

week 1 to 3) and more primitive stem cells (CAFC week 5 to 8) (Chapter 2). Thirdly, supportive evidence for the validity of these stroma-supported assays is also supplied by analysis of clinical stem cell transplants (Chapter 3). Mobilized PBSC harvests of intensively pretreated cancer patients contained low numbers of CAFC subsets and the individual stem cell quality of those stem cells was low. In contrast, stem cell grafts harvested from less heavily pretreated patients had higher CAFC yields. Attempts were made to correlate the number of CAFC week-types in grafts with the *in vivo* repopulation abilities of those transplants. The number of CAFC week 1 to 3 transplanted correlated better with early hematopoietic recovery than did the number of CAFC week 4 to 6. Thus, this would be consistent with the notion that CAFC week 1 to 3 are indicators of elatively mature progenitor cells.

The second goal of this thesis was to improve gene transfer into human primitive HSC from mobilized peripheral blood. The culture conditions for gene transfer into PBSC were studied in seven-day serum-free cultures (Chapters 4 and 5). It was shown that the addition of stroma-conditioned medium (SCM) to the seven-day cultures improved the recovery and expansion of both progenitor cells and primitive stem cells. The nature of the SCM effect could not be elucidated, although it was unlikely that the effect was mediated by known cytokines. In contrast, the LTC-CFC producing ability was reduced following these seven-day cultures. The only condition that could prevent this loss of stem cell quality was direct stroma-contact.

A possible strategy for protection of hematopoietic cells of cancer patients against chemotherapy is the conferral of the multidrug resistance-1 (MDR1) gene in PBSC destined for transplantation. HSC were studied after MDR1 gene transfer in CAFC and LTC-CFC assays. It was shown that the expression of the MDR1 gene in HSC resulted in resistance to vincristine (Chapter 6).

Using clinically relevant cell- and serum-free supernatant infection protocols, various parameters were studied that may contribute to an improvement of the observed poor transduction efficiency of PBSC (Chapter 7). Retroviral supernatant infection in combination with the carboxy-terminal fibronectin fragemnt CH-296 increased significantly the transfection efficiency as compared to supernatant alone and made the use of polycations redundant. Gene transfer of primitive HSC was specifically improved when this procedure was preceded by a five-day pre-culture period as compared to a two-day transfection procedure. The addition of SCM during the pre-culture period did not affect the individual CAFC quality or transduction efficiency, but increased greatly the recovery of the total number of transfected and untransfected HSC leading to larger grafts containing higher numbers of transfected stem cells.

These findings lead to an improved transfection protocol which can be clinically applied. As compared with the protocol of the clinical MDR1 gene therapy trial in Rotterdam, this improved protocol results in 141- and 21.3-fold higher number of transfected CFC and CAFC week 6, respectively, in the graft.

SAMENVATTING

Het hematopoiëtische (=bloedvormende) systeem is een van de voornaamste doelwitorganen voor gentherapie. Dit systeem is zo geschikt, omdat slechts een kleine groep primitieve hematopoiëtische stamcellen verantwoordelijk is voor een levenslange produktie van nieuwe stamcellen, alsmede de daarvan afstammende rijpere voorlopercellen en alle typen volledig uitgerijpte bloedcellen. Daarom kan de overdracht van een gen (=de informatie voor een bepaalde celfunctie) in primitieve stamcellen leiden tot de introductie van dit gen in alle voorloper- and bloedcellen die uit deze stamcel ontstaan. Een ander voordeel is dat hematopoiëtische stamcellen relatief eenvoudig te verkrijgen zijn uit beenmerg, navelstrengbloed en (na mobilisatie) uit perifeer bloed. Stamcellen kunnen betrekkelijk eenvoudig door middel van intraveneuze infusie worden getransplanteerd.

Tot nu toe hebben de meeste gentherapie studies met behulp van hematopoiëtische stamcellen zich gericht op drie mogelijke toepassingen. Ten eerste hebben studies van genetisch gemerkte stamcellen de uitvoerbaarheid van genoverdracht in hematopoiëtische stamcellen proberen aan te tonen. Ten tweede zouden genetische defecten kunnen worden gecorrigeerd door het relevante gen in de stamcellen van een patiënt te brengen. Ten derde zouden bloedcellen van oncologische patiënten beter kunnen worden beschermd tegen chemotherapie door het introduceren van resistentiegenen in hun stamcellen. Veelal worden deze genen in de cellen gebracht via retrovirale vectoren. Het succes van gentherapie wordt echter beperkt door de lage efficiëntie van genoverdracht in primitieve stamcellen.

Het doel van dit proefschrift was de verbetering van retrovirale genoverdracht in primitieve hematopoiëtische stamcellen van de mens. Hiertoe werden klinisch relevante kweek- en genoverdrachtcondities van stamcellen bestudeerd. Bovendien werden kweeksystemen ontwikkeld om het aantal en de kwaliteit van de hematopoiëtische stamcellen van de mens vooraf en na genoverdracht te bepalen.

Het eerste doel van het project beschreven in dit proefschrift was de ontwikkeling van *in vitro* kweeksystemen voor primitieve hematopoiëtische stamcellen van de mens (Hoofdstuk 2). Deze kweeksystemen maakten de *in vitro* analyse van stamcellen van de mens voor en na retrovirale genoverdracht mogelijk.

In deze kweeksystemen worden hematopoiëtische stamcellen van de mens gekweekt op een laag van stromale cellen, die werden opgegroeid uit het orgaanstroma van het beenmerg. De stamcellen kruipen onder de stromale laag en vormen voorlopercellen. Zo ontstaan groepjes bloedcellen onder het stroma, die onder een fase-contrast microscoop het aspect van donkere keienpaadjes (=cobblestone area) hebben. Deze voorlopercellen migreren tijdens hun verdere ontwikkeling omhoog naar het oppervlak van het stroma waar rijpe bloedcellen

ontstaan. Omdat elke cobblestone area wordt voortgebracht door een stamcel, kan dit cobblestone area vormende cel (=CAFC) kweeksysteem werd gebruikt om de frequentie van stamcellen te meten. Aangezien met het muizen CAFC systeem in transplantatiemodellen eerder was aangetoond dat de zogenaamde CAFC dag 10 verantwoordelijk zijn voor de bloedvorming kort na een beenmergtransplantatie en de CAFC dag 28 tot 42 voor de definitieve bloedvorming, werd de hypothese ontwikkeld dat de late CAFC in de menselijke kweken ook representatief zouden zijn voor de echte repopulerende stamcellen. Deze stamcellen kunnen worden beschouwd als de primitieve of "echte" stamcellen. Met behulp van gesorteerde of met 5-fluorouracil behandelde beenmergcellen werd aangetoond dat het CAFC kweeksysteem voor de mens daadwerkelijk verschillende stamceltypen kan onderscheiden. De cobblestone area's die na 1 tot 3 weken in kweek verschijnen ontstaan uit voorlopercellen met een beperkte groeicapaciteit, terwijl de cobblestone area's die veel later in kweek verschijnen (week 5 tot 8) daarentegen uit veel primitievere stamcellen voortkomen.

Daarnaast werden lange-termijn-kweken (=LTC) op stromale lagen in kweekflesje opgezet waarin het vermogen van een hematopoiëtisch monster om kolonievormende voorlopercellen (=CFC) te produceren werd bepaald (=LTC-CFC kweeksysteem). In combinatie kunnen de CAFC en LTC-CFC kweeksystemen worden toegepast om de gemiddelde stamcelkwaliteit te berekenen.

Vervolgens werden klinische, autologe stamceltransplantaten van oncologische patiënten met behulp van de CAFC en LTC-CFC kweeksystemen onderzocht (Hoofdstuk 3). Deze stamceltransplantaten werden verkregen uit perifeer bloed na een behandeling met chemotherapie en stimulatie met hematopoiëtische groeifactoren. Het bleek dat LTC-CFC producerende kwaliteit van deze perifeer bloedstamcellen gerelateerd was aan het aantal stamcellen (=CAFC week 6) dat voor transplantatie kon worden verkregen. Voorts werd waargenomen dat de transplantaten van patiënten die in het verleden reeds intensief waren behandeld met chemotherapie niet alleen een laag aantal stamcellen bevatten, maar bovendien stamcellen met een verminderde LTC-CFC producerende kwaliteit. Vervolgens werd getracht het aantal CAFC week-types in de transplantaten te correleren met het korte termijn hematologisch herstel na transplantatie. Het aantal CAFC week 1 tot 3 getransplanteerd kon het korte termijn hematologisch herstel beter voorspellen dan het aantal CAFC week 4 tot 6. Dit is in overeenstemming met de hypothese dat de CAFC week 1 tot 3 indicators zijn voor een relatief rijpe voorlopercel.

Het tweede doel van het onderzoek was het ontwikkelen van verbeterde protocollen voor genoverdracht in hematopoiëtische stamcellen. De kweekcondities van stamcellen in genoverdracht procedures werden onderzocht in zevendaagse serum-vrije kweken (Hoofdstukken 4 en 5). In deze kweken werd het effect van medium onttrokken aan stromale cellijnkweken (=stroma geconditioneerd medium) getest. Het kon worden aangetoond, dat de toevoeging

van dit stroma geconditioneerd medium aan kweken de overleving en expansie van zowel voorlopercellen als primitieve stamcellen verbeterde. De aard van het stroma geconditioneerd medium effect kon echter niet worden opgehelderd. Het is echter onwaarschijnlijk dat het effect wordt veroorzaakt door reeds bekende hematopoiëtische groeifactoren. Echter deze kweekexperimenten toonden ook een tekort aan. De produktie van LTC-CFC in deze zevendaagse kweken was afgenomen. De enige conditie, die de afname van stamcelkwaliteit in deze zevendaagse kweken kon compenseren, was een direct contact met een stromale laag. Uit deze bevindingen werd de conclusie getrokken dat voor de expansie van stamcellen oplosbare stromale factoren gunstig zijn, maar dat voor een behoud van stamcelkwaliteit contact met stroma nodig is.

Een mogelijke strategie voor het beter beschermen van bloedcellen van oncologische patiënten tegen chemotherapie is de overdracht van het multidrug resistentie-1 (=MDR1) gen in perifeer bloedstamcellen. Wanneer dit gen actief is in een cel leidt dit tot het verwijderen van een reeks stoffen, waaronder cytostatica, uit deze cel. Ten behoeve van de analyse van MDR1 genoverdracht naar stamcellen werden de CAFC en LTC-CFC kweeksystemen gebruikt (Hoofdstuk 6). Het MDR1 gen werd in perifeer bloedstamcellen gebracht en vervolgens werden deze cellen in de kweeksystemen geanalyseerd. Expressie van het MDR1 gen in hematopoiëtische stamcellen leidde tot resistentie tegen cytostatica. Dit kon worden aangetoond door deze kweeksystemen in de aan- en afwezigheid van het cytostaticum vincristine uit te voeren.

Om genoverdracht in perifeer bloedstamcellen te verbeteren werden verschillende klinisch relevante genoverdrachtprotocollen onderzocht (Hoofdstuk 7). Hierbij werd gebruik gemaakt van een fibronectine-fragment (=CH-296). De bodem van plastic kweekschaaltjes werd voorbehandeld met CH-296 waardoor zowel stamcellen als retrovirussen op de bodem werden gefixeerd. Hierdoor kwamen, naar verwachting, stamcellen en retrovirussen dichter bij elkaar te liggen, waardoor de efficiëntie van genoverdracht werd verhoogd. Voorts kon worden aangetoond dat een voorafgaande vijfdaagse kweekperiode de retrovirale genoverdracht naar primitieve stamcellen verhoogde. Daarnaast verbeterde de toevoeging van het bovengenoemde stroma geconditioneerd medium tijdens deze vijfdaagse kweekperiode de overleving en expansie van alle stamceltypen. Deze gegevens leidden tot een verbeterd genoverdrachtprotocol, dat klinisch zou kunnen worden toegepast. Wanneer men dit verbeterde genoverdrachtprotocol vergelijkt met het klinische genoverdrachtprotocol, dat in het MDR1 gentherapie-onderzoek in het Academisch Ziekenhuis te Rotterdam wordt uitgevoerd, dan zou dit kunnen leiden tot een 141- en 21-voudig efficiëntere genoverdracht in respectievelijk voorlopercellen (=CFC) en primitieve stamcellen (=CAFC week 6).

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