Ex vivo expansion of human umbilical cord blood hematopoietic stem and progenitor cells

Nuray Kuşadası
Septet (1920) by Willem Pijper (1894-1947) in which he introduced ‘the germcell technique’ for the first time. The composition diverts from one rhythm such as a tree growing from one cell.

All parts of this thesis may be reproduced, stored, or transmitted in any form or by any means, electronical, mechanical, photocopying, recording or otherwise, provided that the source is mentioned.
Ex vivo expansion of human umbilical cord blood hematopoietic stem and progenitor cells

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus

Prof.dr.ir. J.H. van Bemmel

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 27 november 2002 om 9.45 uur

door

Nuray Kuşadası

geboren te Çayıralan (Turkije)
The work described in this thesis was performed at the Institute of Hematology, Erasmus Medical Center Rotterdam, The Netherlands. The experiments described in chapter 5 and 6 were performed in collaboration with the Department of Cell Biology and Genetics of the same center.

This thesis was financially sponsored by Amgen B.V., Sanquin bloedvoorziening (CLB) and SanverTECH B.V.
The idea of a duality such as air and water can be expressed in a picture by starting from a plane-filling design of birds and fish; the birds are “water” for the fish, and the fish are “air” for the birds. Heaven and Hell can be symbolized by an interplay of angels and devils. There are many other possible pairs of dynamic subjects, at least in theory, for in most cases, their realization meets with insuperable difficulties.

M.C. Escher
## Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>General introduction</td>
<td>11</td>
</tr>
</tbody>
</table>
| 2       | Successful short-term *ex vivo* expansion of NOD/SCID repopulating ability and CAFC<sub>ex vivo</sub> from umbilical cord blood.  
Published in Leukemia. 2001. | 47   |
| 3       | Stromal support augments extended long-term *ex vivo* expansion of hematopoietic progenitor cells.  
Published in Leukemia. 2001. | 73   |
| 4       | Gp130-Signaling synergizes with Flt3-L and Tpo for the long-term expansion of cord blood progenitors.  
Published in Leukemia. 2001. | 111  |
| 5       | Stromal cells from murine aorta-gonad-mesonephros region, liver and gut mesentery expand human umbilical cord blood-derived *in vitro* repopulating stem cells.  
Published in Leukemia. 2002. | 129  |
| 6       | Flt3-L and Tpo modulate the SDF-1 mediated chemotactic activities of marrow and embryonic stromal cells.  
Manuscript in preparation. 2002. | 153  |
<p>| 7       | General discussion                                                    | 177  |</p>
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary</td>
<td>199</td>
</tr>
<tr>
<td>Samenvatting</td>
<td>205</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>211</td>
</tr>
<tr>
<td>Curriculum vitae</td>
<td>213</td>
</tr>
<tr>
<td>Publications</td>
<td>214</td>
</tr>
<tr>
<td>Tot slot</td>
<td>216</td>
</tr>
</tbody>
</table>
Chapter 1

General introduction
Umbilical cord blood hematopoietic stem and progenitor cells

General introduction

1.1 Scope of the thesis

Hematopoietic stem cells (HSCs) and progenitors are a unique scientific and medical resource. Stem cell transplantation (SCT) has achieved significant therapeutic success over the last decades. While initially bone marrow (BM) HSCs were used for transplantation, mobilization of HSCs into the peripheral blood (PB) and their collection by leukapheresis and use as clinical grafts became feasible. Umbilical cord blood (UCB) is a most recent source of HSCs and progenitors, which has been used to treat patients with hematological diseases and other severely ill patients whose immune systems have been decimated by high dose chemotherapy. Among the various HSC sources for transplantation, UCB offers a number of potential advantages: easy access, ability to ensure ethnic diversity among donors, low risk of transmissible infectious diseases, no risk to donor and no contamination by residual tumor cells. However, reconstitution of hematopoiesis after intensive chemotherapy and grafting of UCB cells in children and even more in adults, is slower as compared to the use of BM or mobilized PB (MPB) grafts. This is most probably due to the low number of transplanted HSCs per kg bodyweight. Another problem regarding UCB transplantation is that the collection can only take place once, so that additional grafts are not available if so required. Combining blood from several umbilical cords has not been felt an option in the clinical transplantation setting due to the possible immunological consequences, and therefore research has focussed on the development of techniques to increase the number of UCB HSCs in laboratory incubators.

The ability to isolate and expand cells capable of reconstituting hematopoiesis keeps great promise to improve the outcomes of patients treated with autologous and allogeneic SCT. Ex vivo expansion may broaden the SCT modality in cases where limited HSCs and progenitors are available and where their quality are dramatically reduced, such as in UCB grafts or leukapheresis samples from heavily pretreated patients. The morbidity caused by prolonged neutropenia leaves patients at risk to develop serious infections. The potential for rapid recovery after myeloablative therapy may be accelerated by ex vivo generation of the required numbers of the necessary cells. Expansion studies may alternatively contribute to the development of culture conditions that permit efficient gene transfer into primitive HSCs without their concurrent differentiation-induced loss. These studies may also aid in purging stem cell transplants of contaminated tumor cells.
in an autologous setting for example by specific molecular targeting of tumor cells using immunotoxins directed specifically against cancer cell antigens. Finally, another clinical application could be the generation of immunologically active cells such as natural killer or dendritic cells with antitumor activity to be used in immunotherapeutic regimens. The main focus of this thesis is on the development of procedures that allow extensive numerical expansion of UCB HSCs and progenitors ex vivo without a concomitant loss of the ability for long-term engraftment.

Expansion in vivo occurs after conditioning and SCT in the BM of man, while it occurs in the yolk-sac, aorta-gonad-mesonephros (AGM) region and fetal liver during the embryonic development, showing that these regions can produce, maintain and/or amplify HSCs and progenitors in vivo. The mechanism(s) involved herein has(have) not been elucidated, but in part proposed as a complex interplay between hematopoietic cells and cytokines elaborated either locally by stromal cells or peripherally with stimulatory (interleukins (ILs), stem cell factor (SCF), fetal liver tyrosine kinase 3 ligand (Flt3-L) and thrombopoietin (Tpo)) or inhibitory (transforming growth factor-β (TGF-β), macrophage inflammatory protein-1α (MIP-1α)) effects on the one hand and/or the microenvironment through adhesion to matrix molecules or stromal cells on the other hand. Ex vivo culture systems have been based on mimicking the in vivo situation either by exogenous addition of cytokines and/or by the use of stromal cell layers. A major step toward extensive ex vivo amplification of human HSCs and progenitors has been reported after the introduction of the early acting cytokines fetal liver tyrosine kinase 3 ligand Flt3-L and Tpo [1-3]. Ample studies have demonstrated the supportive capacity of stromal cells on the HSC maintenance and ability to generate progenitors in co-cultures [4-9]. Despite intensive research, ex vivo expansion of HSCs with long-term repopulating (LTR) ability is still elusive because the studies either looked only at progenitor generation or was not able to obtain expansion of LTR-HSCs. Therefore, we aimed to expand LTR-HSCs from UCB in the experimental work presented in this thesis. The main topics herein include (1) the analysis of optimal conditions in order to numerically expand the HSCs, where in particular the effect of combination of cytokines either in the absence or presence of a BM-derived stromal layer has been evaluated; (2) the analysis of the HSC supportive capacity of several newly developed stromal clones derived from AGM region, where the first definitive adult HSCs appear and expand; and (3) studies aimed to identify factor(s) that are involved in stromal support of HSC expansion.
1.2 The hematopoietic system

Hematopoiesis

The production of mature blood cells, or hematopoiesis, is a multistep process and takes place in the BM. They have a limited life-span and have to be replenished on a daily basis so that a life-long production of billions of new blood cells is guaranteed. The replenishment takes place by transitory cells known as progenitors, which proliferate and differentiate under appropriate stimuli. These developmentally restricted progeny originate from a rare pool of BM cells, the hematopoietic stem cells (HSCs) [10]. The HSCs have been considered to be a quiescent population with only few clones contributing to hematopoiesis during adult life at any moment in time [11, 12]. HSCs are particularly confined to the BM [10], however, they are also present in other tissues such as spleen and PB. HSCs are characterized by their ability (a) to self-renew; (b) to generate all myeloid and lymphoid lineages, and thus to be pluripotent; (c) to long-term repopulate a myeloablated recipient; and (d) to display extensive proliferative potential [13, 14]. Their progeny are progressively less immature and less capable of proliferation and differentiation into more than one cell type. The decision of a HSC either to self-renew or differentiate, and the commitment to a specific lineage by a multipotent progenitor is believed to be a complex process under the control of stochastic and deterministic events [15-19]. The HSC compartment is heterogeneous in various aspects. From physical sorting of hematopoietic cells it has become apparent that the human HSC compartment, in analogy to the situation in the mouse, represents a hierarchy of primitive cells on the basis of increasing turn-over rate, increasing chemo- and radiosensitivity, decreasing ability to generate new HSCs, decreasing proliferative potential and pluripotentiality [20-27]. As a result, in a transplant setting this heterogeneity may be reflected in the different time periods that different HSC clones contribute to the reconstitution of a conditioned recipient. Murine transplantation studies have revealed a subdivision of HSCs in roughly two compartments, existing of cells with short-term repopulating ability that are transient and those with long-term repopulating ability which are responsible for sustained multilineage hematopoiesis both in vitro and in vivo [28-30].

Human hematopoietic stem and progenitor cell purification

The main problem in hematopoietic cell development studies is the isolation and identification of the pluripotent HSC. Many attempts to purify hematopoietic cells have used a combination of approaches based on physical and biological properties such as immunophenotype of the target cells. Density gradient separation is commonly used
as a pre-enrichment step in HSC purification [31]. As most primitive hematopoietic cells reside in the G₀ phase of the cell-cycle (having low metabolic activity and low levels of nucleic acids), the pharmacological cell-cycle specific agents can be used to enrich these cells. For this, the DNA stain Hoechst 33342 can be used to isolate G₀/G₁ cells, and the RNA stain PyroninY can be used to isolate G₀ cells specifically [32]. Rhodamine123 has also been used in enrichment strategies [33]. This stain passively diffuses into all viable cells, binds to mitochondria and can be efficiently removed by a multidrug resistance pump. Because this pump is more active in long-term repopulating hematopoietic cells, and resting cells have fewer mitochondria, HSCs are enriched in the Rhodamine123low cell population. Additionally, treatment with 5-fluorouracil or 4-hydroperoxy-cyclophosphamide results in elimination of proliferating cells and spared non-cycling stem cells [34]. This treatment might, however, be associated with potentially irreversible damage to primitive HSCs, which could lead to delayed engraftment. Another enrichment procedure uses the immunological characteristics of different hematopoietic cell populations. Initially, murine HSCs have been successfully characterized and phenotyped utilizing fluorescently labeled monoclonal antibodies to cell surface antigens that are present on different hematopoietic cell subsets [35]. These stained cells can subsequently be isolated using fluorescent activated cell sorting (FACS). The major advance in human HSC purification has been the identification of CD34 (a transmembrane glycoprotein as a marker of a heterogeneous cell population that is functionally characterized by its in vitro capability of generating colonies derived from early and late progenitors and by its in vivo ability to reconstitute the myelo-lymphopoietic system of an irradiated recipient [36-38]. The CD34 antigen is expressed on 1-4% of normal BM, 0.01-0.1% of PB and 0.1-0.4% of UCB cells [39]. In contrast, recent studies have demonstrated the presence of other candidate human HSCs within the CD34+ population that do not express lineage markers (Lin⁻) showing the ability of these cells to engraft both primary and secondary recipients [40-44]. The Lin⁻CD34+ cells were found with a frequency of 0.03%, 0.08% and 0.45% in normal BM, MPB and UCB, respectively [43]. Another glycoprotein antigen is AC133, which is selectively expressed on CD34+ HSCs and progenitors derived from fetal liver, BM and PB [45-47]. Studies have shown that CD34⁺AC133⁺ cells derived from UCB were highly enriched in long-term culture initiating cells (LTC-ICs), non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice repopulating cells (SRCs) and dendritic cell precursors [46, 48], providing evidence for the long-term repopulating potential of AC133⁺ cells. In summary, HSCs are rare and morphologically not readily distinguishable from other cell types within the hematopoietic compartment, contain multiple subsets,
and therefore present the investigators and clinicians with an identity problem. The most common approach for partial purification is the use of cell surface markers, especially the CD34 antigen. These isolated cells are heterogeneous and include cells that are long-term self-renewing stem cells, short-term progenitors and mature hematopoietic cells. To compare the \textit{in vitro} and \textit{in vivo} characteristics of different stem cell sources such as BM, PB and UCB, one should be aware of the differences in cell-cycle status, engrafting capability and cytokine response, which might potentially represent different subsets of pluripotent HSCs.

\textit{Human hematopoietic stem and progenitor cell transplantation}

HSC transplantation is increasingly used for the treatment of a variety of hematological and non-hematological diseases of neoplastic and non-neoplastic origin \cite{49}. The HSCs used in human clinical settings are derived from several sources. The classic source of HSC is the BM, which has been used for more than fifty years \cite{50, 51}. Other cells that are present in the BM include stromal and stromal progenitor cells, mature and maturing hematopoietic cells. Although the main source of hematopoietic cells for transplantation was the BM, limitations of this life saving procedure due to the frequent lack of an appropriate donor have led to search for alternative sources. As it was known for decades that a small number of HSCs with \textit{in vivo} repopulating ability circulated in the PB \cite{52, 53}. Additional studies revealed that administration of cytokines such as G-CSF, and/or chemotherapy to the donor dramatically increased the number of these cells in the systemic circulation, from which they can be collected by leukapheresis and successfully used in transplantations. The probable underlying drive of this mobilization process is a threatening depletion of HSC reserve due to infection, blood loss or the use of chemotherapy (so called stress hematopoiesis). After the first report of cytokine-MPB stem cell transplantation in 1989 \cite{54}, donor cells from PB were preferred to use in clinical transplantations. Several studies described a faster post-transplant recovery and higher survival rates after MPB transplantations as compared to BM \cite{55-57}. However, MPB transplants were likely to cause more chronic graft-versus-host disease related problems in allogeneic settings due to the fact that these transplants contained more T-lymphocytes than BM-derived ones, which has been shown to play an important role in transplant rejection \cite{58}. To overcome these problems, the transplants were additionally depleted for donor T-lymphocytes \cite{59, 60} and/or administered with immunosuppressive drugs \cite{61}. These latter procedures have also been performed in allogeneic BM transplantations to minimize the transplant rejection \cite{60, 62}. The presence of HSCs in UCB and placental blood \cite{63, 64} facilitated the potential use of these cells also for
HSC transplantation. These tissues support the developing fetus during pregnancy, are delivered along with the baby and usually discarded. Since the first successful UCB transplant in a child with Fanconi anemia [65], the collection and therapeutic interest in these cells has grown quickly [66-68]. Extensive banks of cryopreserved and HLA-typed UCB HSCs have been established in many institutions [69-71]. The clinical advantages of UCB transplants can be summarized as widespread availability, easy access, low risk of transmitting infections and possibly reduced graft-versus-host disease related problems [72-75]. The ability to form more colonies in culture, a higher cell-cycle rate and the presence of longer telomeres in UCB HSCs contributed to a distinctive proliferative advantage as compared to adult-derived transplants [76-82]. However, reconstitution of hematopoiesis after intensive chemotherapy and subsequent grafting of UCB cells in children, and even more in adults appeared to be slower as compared to the use of BM or MPB transplants, resulting in increased risk for infections and bleedings in patients. This limited potential for rapid hematological recovery after transplantation with UCB cells is probably due to a limited number of HSCs transplanted per kg body weight [83-85]. Therefore, a substantial amount of research, which has been conducted on UCB, includes the search for ways to expand the numbers of HSCs and compare the biological properties of UCB and adult transplants. An important alternative source of HSCs in research, but not in clinical use, is the blood producing tissues of embryonic and fetal animals [86-90]. The earliest hematopoietic activity, beginning at about day 7 of a mouse embryo (E7), is indicated by the appearance of blood islands in the yolk-sac. It has been suggested that the yolk-sac hematopoietic cells are transient [91]. Recent data strongly suggest that definitive adult HSCs derive from the intra-embryonic splanchnopleura, the so called aorta-gonad-mesonephros (AGM) region, rather than the extra-embryonic yolk-sac [92-94]. In the murine AGM region, hematopoietic cells which are capable of reconstituting definitive adult hematopoiesis, first appear autonomously at day 10.5 of gestation, expand in number rapidly in the following two to three days and migrate to the liver in subsequent couple of days. Here they continue to divide and migrate, spreading to the spleen, thymus, and near the time of birth to the BM. Ongoing efforts are devoted to identify and characterize the human embryonic and fetal HSCs [87, 88]. Recently, HSCs have been found in circulating blood of aborted human fetuses, and these circulating cells had different proliferative and differentiative abilities than cells from fetal liver, fetal BM and UCB [89, 90, 95].
Regulation of hematopoiesis

The complex process of hematopoiesis is regulated both positively and negatively by the BM microenvironment and/or cytokines, which pose their effects in synergistic, recruiting, or antagonistic ways. The fate of HSCs herein can be briefly hypothesized as (1) loss by differentiation; (2) maintenance or (3) expansion by self-renewal; (4) expansion by induction; or (5) expansion by dedifferentiation (figure 1). Steady-state hematopoiesis is believed to be a balance between these positive and negative stimuli, in which cytokines, cytokine receptors, direct cell-cell contact as well as indirect interactions mediated by matrix components constitute a complex network of growth modulators, regulating hematopoiesis within the marrow microenvironment [96-99]. In general, an important additional role of stochastic events in regulation of the HSC fate is also accepted. The heterogeneous stromal environment, itself a connective tissue, is composed of macrophages, endothelial cells, fibroblasts, adipocytes and smooth muscle cells. Fibroblasts, adipocytes and smooth muscle cells derive from a pluripotent cell located in the marrow [100, 101]. In conclusion, the hematopoietic microenvironment is defined largely by function as a complex of cells and factors critical for the maintenance and regulation of stem cells and their progeny in vivo. Initially, primary LTCs of marrow stroma have been used to approximate the microenvironment in vitro. A large amount of work has resulted in the identification of many gene products with in particular recombinant cytokines. Cytokines, membrane-bound and soluble glycoproteins, are produced by a variety of cells, such as stromal cells, mature blood cells (macrophages, T- and B-lymphocytes) and endothelial cells. Some common features of cytokines are their activity at picomolar concentrations, the wide range of activities that each displays (pleiotropy) and the similarity in activities that different cytokines share (redundancy). They are roughly subdivided into early and late acting groups according to the position of the target cells in the hierarchy of hematopoietic compartment [102]. Early acting cytokines tend to impact on proliferating multipotent HSCs, while late acting cytokines act on terminal differentiation of hematopoietic cells. Individual cytokines can preferentially act in one compartment, such as Flt3-L and SCF on early HSCs, and colony stimulating factors (CSFs) and erythropoietin (Epo) on more mature, lineage-committed progenitors, but they can also act on both compartments such as Tpo. Multipotent HSCs have been demonstrated to require activation by multiple cytokines for their proliferation and maintenance [103]. Cytokines mediate their biological activities by binding to specific receptors on the surface of the target cells which are coupled to intracellular signaling pathways [104].
Chapter 1

During steady-state hematopoiesis most stem cells are thought to be quiescent. This quiescence has been interpreted as an absence of sufficient positive stimulating signals and/or the presence of inhibitory signals. Considerable effort has been directed towards identifying signals that can inhibit hematopoiesis, and some have been identified. TGF-β, MIP-1α, interferon (INF) and tumor necrosis factor (TNF) have been shown to inhibit hematopoiesis in \textit{in vitro} assays, and in some cases when administered to mice they inhibited the cycling activity of progenitors \textit{in vivo} [105-109].

To study the activities of individual cells within the heterogeneous hematopoietic microenvironment stromal cell lines have been established from yolk-sac and embryonic, fetal and adult hematopoietic tissues. Stromal cells from the latter two tissues were able to maintain HSCs [110-112]. Stromal cell lines from yolk-sac have been demonstrated to expand yolk-sac and adult BM hematopoietic progenitors [110, 111]. The embryonic AGM region, which generates the first definitive HSCs, has additionally been shown

---

**Figure 1.** Scheme of the mechanisms involved in the regulation of HSC compartment. The decision of a HSC either to self-renew or differentiate is believed to be a complex process under the control of stochastic and deterministic events. As a result of these events the fate of HSC can be loss, maintenance or expansion.
to be an attractive alternative microenvironment to evaluate the HSC growth. To date, only two hematopoietic supportive embryonic stromal cell lines, day 11 embryo (E11)-derived DAS 104-4 and E10.5-derived S3, have been reported to maintain HSCs in vitro [112, 113]. Furthermore, Remy-Martin and colleagues demonstrated that most stromal cell lines express markers that are generally found on smooth muscle cells as they start to differentiate from mesenchymal populations [114]. Recently, it has been shown that a population of BM-derived mesenchymal cells can give rise to bone and cartilage, tendon, smooth and striated muscles, and stromal cells that support hematopoietic differentiation (reviewed in [115-117]). These newly identified non-hematopoietic stem cells have been referred to as mesenchymal stem cells.

1.3 Human hematopoietic stem and progenitor cell assays

A variety of experimental techniques, i.e. semisolid, suspension and stroma-based cultures investigating biological properties and the use of transplantation approaches, allowed a description of the heterogeneous and hierarchical developmental stages of pluripotent hematopoietic cells and their progeny. All these assays measure HSC activity from different perspectives and may therefore detect similar or overlapping (sub)populations (figure 2). From a practical point of view, hematopoietic cells that can generate active hematopoiesis for weeks in vitro or months in vivo are being considered to be LTR cells. This is a clinically adapted and useful criterion because it characterizes those cells which are important for sustained hematopoietic recovery after transplantation. As several of these assays have been used in this thesis to quantify the HSC and progenitor content in uncultured and cultured UCB CD34+ cell populations, a brief summary of commonly used in vitro and in vivo assays for human hematopoietic cells will be discussed in the following section.
1.3.1 Immunophenotypic analysis

To define the surface marker profile of HSCs, monoclonal antibodies against cell surface antigens were developed [118]. Based on the availability of such antibodies most of the mature and immature hematopoietic cell subsets can now be defined by their phenotype. For instance, cells expressing CD34 on their surface are hematopoietic cells with colony forming activity and long-term repopulating ability. The current acceptance of
experimental and clinical strategies for the enrichment of human HSCs and progenitors relies on the positive selection of cells expressing the CD34 antigen [119]. Additional enrichment strategies make use of the presence or absence of other cell surface markers on immature HSCs and progenitors and include: Thy-1⁺ [120], CD38low⁻ [121, 122], CD45RA⁻ [123], HLA-DRlow⁻ [124], c-kitlow⁻ [125, 126], AC133+ [46, 47], KDR1⁺ [127] and CXCR4⁺ [131,132]. These markers enable further characterization of the heterogeneous CD34 population into functionally distinct subsets. Moreover, recent studies define a new subset of cells characterized within the Lin⁻CD34⁻ population that can reconstitute hematopoiesis in xenogeneic transplantation systems [40-44]. Ex vivo manipulation of this new HSC subset has shown the generation of CD34⁺ cells, CFCS and SRCs, indicating that the CD34⁻ subset contain precursors of CD34⁺ cells [128]. Additional studies reveal that the CD34 expression is reversible and related to the activation state of HSCs, as assayed in a mouse transplantation model [129]. These findings have resulted in growing controversy on the status of CD34 expression as a marker for HSCs and increased the necessity of functional characterization of transplantable hematopoetic cells. Whether this new candidate HSC population with the CD34⁻ phenotype will find a place in clinical approaches requires the ultimate functional human HSC test, including genemarking and the transplantation of the long-term (several years) multilineage reconstituting HSCs in patients.

1.3.2 Semisolid colony forming cell (CFC) assays

CFU-C assays
The colony forming progenitors are hierarchically organized. Progenitor cells that are restricted in their lineage potential and have limited self-renewal capability can be detected in colony forming unit in culture (CFU-C) assays using semisolid media [130-132]. These media allow the clonal progeny of a single progenitor cell to stay concentrated. Upon culturing for 14 days, committed and multipotential progenitors can be distinguished as colonies that contain one or more myeloid and/or erythroid lineages. Using specific detection methods and exploiting the differences in size, color and morphological characteristics of progenitor subsets, committed progenitors, such as CFU-granulocyte, macrophage (CFU-GM) and burst forming unit-erythroid (BFU-E), as well as pluripotent progenitors, such as CFU-granulocyte, erythrocyte, megakaryocyte, macrophage (CFU-GEMM), can be assayed simultaneously in the same culture.
1.3.3 Stroma-supported long-term culture (LTC) assays

The hematopoietic microenvironment is a site of residence of quiescent stem cells and provides more than structural support for hematopoietic cells during proliferation and differentiation. Observations led to the theory that the microenvironment in which HSCs reside influences the commitment process, termed the hematopoietic inductive microenvironment. After the introduction of a culture system by Dexter and colleagues [133], in which the hematopoietic microenvironment is initiated in tissue culture flasks by establishment of a confluent stromal layer, the use of stromal layers has been adapted for quantitative and qualitative analysis of primitive HSCs. The commonly used stroma-dependent assay systems are briefly summarized below.

CAFC assay

The cobblestone area forming cell (CAFC) assay is a miniaturized stroma-dependent long-term BM culture in which hematopoietic cells are assayed. As an endpoint in the assay macroscopic, phase-dark clones formed beneath stromal layer, so called cobblestone areas, [134-136] are used as the readout (rather than replating in a progenitor cell assay as done in the LTC-IC assay). Initially, murine hematopoietic cells were overlaid on irradiated stromal layers in 96-well plates in a range of concentrations, and frequencies of cobblestone area-forming cells (CAFCs) were calculated by Poisson statistics. In these studies, the time-dependent cobblestone area formation reflected the renewal and primitiveness of CAFCs. The frequency of murine precursors forming cobblestone areas on day-28 after culture has been shown to be a measure for marrow repopulating ability and on day-35 for LTR ability, whereas day-10 CAFC frequency closely corresponded with day-12 CFU-S numbers [24, 29, 137]. In the following years this method has been adapted and used to analyze human HSC subsets. The late appearing human CAFCs have been shown to have overlapping characteristics to previously reported human LTC-ICs [138]. The frequency of more primitive progenitors, which are functionally closer to the pluripotent HSC in the hierarchical step, has been determined by prolonging the culture time of these two assay systems [138-140]. These so called extended LTC-ICs and CAFCs were exclusively found in the CD34+CD38- fraction of human BM cells, whereas standard LTC-ICs and CAFCs were heterogeneous for the expression of CD38 within the CD34+ subset.
**Umbilical cord blood hematopoietic stem and progenitor cells**

**LTC-IC assay**
The LTC-IC assay is a quantitative assay for primitive HSCs based on their capacity to generate clonogenic cells in long-term stromal co-cultures for at least five weeks [141-144]. Clonogenic cell output appeared to be linearly related to the input cell number over a wide range of cell concentrations. Using this limiting dilution analysis technique the frequency of LTC-ICs in normal human marrow has been established as 1 per $2 \times 10^4$ MNCs and in a CD34$^+$ purified subpopulation as 1 per 50-100 cells [141]. Although LTC-ICs are among the best available *in vitro* assay approximating the number of HSCs in humans, they represent a heterogeneous population in terms of proliferative capacity and sensitivity to different cytokines. A subpopulation of these cells has been shown to share phenotypic and functional properties with long-term *in vivo* repopulating cells [145].

**LTC-CFC assay**
The long-term culture colony forming cell (LTC-CFC) assay is routinely performed in 25T-flasks or large vessels. In this assay the ability of the transplant to generate committed progenitors for a period of several weeks to months can be determined. The number of progenitors produced at a certain time point in the LTC-CFC assay is accepted as a measure for the quality of the transplant [146-149]. The absolute number of these cells can be assayed by replating the hematopoietic cells from the *adherent* and *non-adherent* compartments of these stromal co-cultures into the CFU-C assay. Additionally, extension of the culture (referred to as extended LTC) made it possible to detect the ability of more primitive progenitors that are probably closer to long-term *in vivo* repopulating hematopoietic cells in the HSC hierarchy [139].

**1.3.4 Human xenograft models**
The only functional measure of a long-term renewable HSC is the capacity to engraft myeloablated recipients and sustain long-term multilineage hematopoiesis *in vivo*. In order to strengthen the functional characterization of human HSCs, investigators have directed attention towards development of animal models for hematopoiesis. The most frequently used *in vivo* models for the evaluation of human HSC activities will be described briefly in this section.
Chapter 1

**SCID xenograft models**

Recent advances in mammalian genetics have provided a number of immunodeficient murine models for engraftment and quantitation of human HSCs. In initial experiments, mice triply homozygous for beige, nu, and X-linked immunodeficiency loci, the so-called bnx-mice, were used [150-152]. Other immunodeficient models include mice deficient in recombination activating gene-1 (Rag1) and Rag2 genes and mice homozygous for the Prkdc\({\text{scid}}\) (scid) locus [153-155]. The first successful engraftment of human cells into homozygous scid mice was reported in 1988 [156, 157]. In this model, human fetal liver or fetal BM, either with or without human fetal thymus tissue, was transplanted under the renal capsule of unirradiated scid mice (referred to as SCID-hu mice). Intravenous injection of irradiated scid mice with human BM, UCB, or G-CSF cytokine-MPB mononuclear cells resulted in the engraftment of a human hematopoietic system in the murine recipient [158-162]. Two major limitations of this SCID-hu model are the low engraftment levels and the need of human cytokines and/or microenvironment for proliferation and differentiation of transplanted HSCs. The use of NOD/SCID mice, which are characterized by extremely low NK cell activity, and defective myeloid development [163, 164], partly overcomes these problems by providing a niche for engraftment and little graft rejection. The cells responsible for the engraftment of the BM of NOD/SCID mice in both myeloid and lymphoid lineages have been termed “scid-repopulating cells” (SRCs) [38, 165-167]. These cells have been postulated to be more primitive than any of the HSC populations that have been identified using the currently available in vitro methods [158, 159, 168]. The cell surface phenotype of SRC was found to be CD34\(^+\)CD38\(^-\) [38, 122]. However, CD34\(^+\)CD38\(^-\) or CD34\(^+\)CD38\(^+\) cells were also reported to have limited engraftment capacities in transplanted NOD/SCID mice [43, 169]. Subsequent measurements revealed the frequencies of SRC in UCB, BM, and MPB as 1 in \(9.3 \times 10^5\), 1 in \(3 \times 10^6\) and 1 in \(6 \times 10^6\) mononuclear cells, respectively, as determined by limiting dilution experiments [167]. However, Van Hennik et al indicated that their frequencies might be much higher, as only very limited numbers of transplanted human HSCs (0.9%-4% depending on the source) home to the murine BM [170]. Recently, an alternative mouse model, the NOD/SCID \(\beta 2\)-microglobulin-deficient (\(\beta 2\ m^{\text{null}}\)) mouse, has been reported [171-173]. As few as \(8 \times 10^4\) human cord blood mononuclear cells transplanted into NOD/SCID/B2m\(^{\text{null}}\) mice gave multilineage differentiation in the murine BM, revealing a more than 11-fold higher SRC frequency than in NOD/SCID mice. However, the SRCs producing human hematopoietic progeny in these mice exhibited only short-term repopulating ability and contained cells also expressing the CD38 antigen [174]. While SRCs in the NOD/SCID model are exclusively
CD38\(^-\); these results indicate that human repopulating cells in NOD/SCID/B2m\(^{null}\) mice represent less primitive progenitors.

**Fetal sheep xenograft model**

Among the xenograft models to study the engraftment ability of human HSCs *in vivo*, the human/sheep xenograft model has several additional unique and advantageous aspects [175-178]. One advantage is the immunologic immaturity and developing spaces in fetal BM, which allows for donor engraftment in normal recipients without marrow conditioning. Transplanted human hematopoietic cells have been shown to colonize the BM, to remain there for several years contributing to multilineage differentiation, to respond to human cytokines, and to retain their ability to engraft secondary recipients. The large size of the recipient permits repeated evaluation of human cell activity in the same chimeric sheep over prolonged periods of time. All these features make this assay perhaps the most promising biological *in vivo* system for human HSCs. However, the high frequency of unsuccessful transplantations and abortions and the expensive costs limit the widespread use of this model.

1.3 Experimental/clinical history of *ex vivo* expanded human hematopoietic stem and progenitor cells

**Experimental studies**

*Effect of cytokines.* To date, most research on *ex vivo* expansion has explored different combinations of cytokines, culture medium ingredients and the role of stromal support. In the early 1990s our group started to evaluate the numerical expansion of murine HSCs and progenitors derived from the BM. In these initial studies many permutations of cytokines and their concentration have been analyzed [135, 179-182]. Although the combination of several cytokines (for example IL3 + IL11/IL12 + SCF) revealed a high increase in the numbers of progenitors and short-term repopulating murine HSCs (300 to 1000-fold the input in 5-7 days of culture), a moderate increase or even decrease was observed with LTR-HSCs (0.1 to 3-fold the input). In the following years, Breems *et al* attempted to expand human HSCs and progenitors derived from normal BM, MPB or UCB under the same conditions and within the same experiment. It was found that the frequency as well as the quality of HSCs and progenitors in primary samples of individual MPBs [136, 149] and UCBs (unpublished data) was extremely variable as compared to the BM samples. In these studies a moderate numerical expansion of
various HSC and progenitor subsets was observed. However, this was at the expense of their quality in functional assays [5]. The cytokine-supplemented cultures required stroma for optimal maintenance of graft quality [6]. Ample other studies, in which LTC-ICs or in vivo repopulating ability were used as endpoints, have reported that human HSCs could quantitatively be maintained or slightly increased (<5-fold the input values) in culture [183-187]. In addition, the culture time was found to be detrimental for the HSC survival. Primitive HSCs were reportedly lost after a culture period of 5-7 days, while the number of progenitors and CD34+ cells increased several hundred times as compared to the uncultured cells [188, 189]. All together, until the end of the 1990s the expansion of committed progenitors has been reported frequently, while the ability to expand more primitive HSCs has met with little success.

Based on our previous results and the results of others, IL3, IL6, IL11, G-CSF, SCF, Flt3-L and Tpo were selected as the candidate cytokines for use in HSC expansion studies. GM-CSF and Epo have also been used by many others but resulted only in extensive production of erythrocytes and granulocytes/monocytes with no significant effect on HSC and progenitor expansion. The most promising expansion results were obtained from the introduction of Flt3-L and Tpo. The receptors of both of these ligands are expressed on early and committed progenitors. Analysis of the effect of different cytokine combinations on stroma-free cultures of CD34+CD38- BM cells indicated that Flt3-L, SCF and IL3 gave the highest expansion of the input LTC-ICs [190, 191]. In another study the addition of Flt3-L, in conjunction with IL3, IL6 and SCF, in suspension culture during in vitro transductions maintained the ability of human CD34+ cells to sustain long-term hematopoiesis in vivo [192]. Tpo has also been reported to increase the multilineage growth of CD34+CD38- bone marrow cells under stroma-free conditions when added to the cytokine combination Flt3-L and SCF [193]. Others demonstrated that maximal expansion of progenitors by Flt3-L + IL11 required longer incubation than with SCF + IL11 indicating involvement of different kinetics in enhanced production of progenitors [194]. The evaluation of all these cytokines resulted in the conclusion that Flt3-L and Tpo were able to give a moderate increase of CD34+CD38- derived LTC-ICs. Other reports emphasize an important role for the multifunctional cytokine IL6 in the regulation of hematopoiesis. This pleiotropic molecule has been shown to act synergistically with IL3 and SCF to enhance the proliferation of human progenitors. Because the intracellular portion of its receptor (IL6R) is very short, an associated 130-kDa (gp130) molecule that lacks IL6 binding activity appears to be required for the signal transduction. Moreover, gp130-deficient mouse embryos show greatly reduced numbers of progenitors, thereby underlining the importance of this signal transduction.
pathway in the regulation of hematopoiesis. Gp130 is ubiquitously expressed on blood progenitors, whereas the expression of IL6R seems to be limited to approximately 30-50% of CD34+ UCB and 80% of CD34+ PB cells [195, 196]. These results support and extend the studies of several investigators [197-199] who tested a complex of the soluble IL6R and IL6 (IL6/sIL6R) in combination with SCF or Flt3-L in three-week stroma-free cultures and methylcellulose clonal assays, and demonstrated an increased expansion of multipotential and committed progenitors from UCB or BM-derived CD34+ cells. In addition, Zandstra et al reported a higher LTC-IC expansion in one-week serum-free cultures of CD34+CD38- UCB cells stimulated by Flt3-L, in combination with the IL6/sIL6R complex, when compared to the combinations of Flt3-L plus Tpo or Flt3-L, SCF, IL6, IL3 plus G-CSF [200]. Among the various early acting cytokine combinations tested for their ability to sustain long-term hematopoiesis in stroma-free cultures using CD34+ UCB cells, Flt3-L and Tpo were found to be necessary and sufficient to maintain early HSCs for several months [1, 2]. In these studies the number of LTC-ICs were reported to be 2 x 10^5-fold increased and the CFU-GM were 2 x 10^6-fold increased after 5 to 6 months of stroma-free cultures, respectively. These authors demonstrated that IL3 induced the production of committed progenitors and was unable to sustain maintenance of HSCs. Recently, these authors also demonstrated that the SRCs were markedly expanded using Flt3-L, Tpo, SCF and IL6 in combination under the same conditions after ten weeks of culture [3]. Also, the addition of IL6/sIL6R complex instead of IL6 to the cytokine combination Flt3-L, Tpo and SCF has been shown to expand the SRCs in a 7-day stroma-free cultures [201].

Another area where cytokines are involved is that of homing and migration of HSCs and progenitors. An important feature of intravenously infused HSCs and progenitors is the ability to migrate to sites that can sustain hematopoiesis, in order to guarantee a life-long regulated preservation and outgrowth of hematopoietic cells responding to the body's demand. This homing process can be considered to be multifactorial, similar to what has been found for trans-endothelial migration of leukocytes [202, 203]. The receptors involved in the regulation of homing of HSCs to the BM belongs to the so called G-protein-coupled seven transmembrane spanning receptor superfamily [204, 205]. The ligands for these receptors have been referred to as chemoattractant cytokines, or chemokines [206]. Progress in understanding the specific interactions of hematopoietic cells with chemokines has emerged from in vitro transwell migration studies [207-212]. The chemokine stromal cell-derived factor SDF-1 has been shown to strongly attract the CD34+ cells [207, 210, 211, 213-215]. It has also been shown to be a chemoattractant for monocytes and lymphocytes. In addition, this chemokine was found to be critical
for bone marrow engraftment. It binds to its receptor CXCR4, which is expressed on many cell types including CD34\(^+\)CD38\(^-\) cells, and attracts CD34\(^+\)CXCR4\(^+\) HSCs and progenitors. Its important role in homing is illustrated by the absence of hematopoiesis in the bone marrow of mice that lack SDF-1 or do not express CXCR4 [216-218]. CXCR4 expression on human CD34\(^+\) cells can be enhanced by SCF and IL6 [172]. This enhanced expression has been suggested to potentiate migration towards SDF-1 and engraftment in primary and secondary transplanted NOD/SCID mice. The CXCR4 antagonist 12G5 (Pharmingen) or MBA171 (R & D Systems) completely abrogate HSC engraftment in this model. Finally, the analysis of different HSC sources revealed that UCB have a significantly higher migratory potential than did BM or PB [210]. All together, the process of homing can also be of importance in ex vivo expansion studies, as it may change the expression of appropriate molecules on HSCs and progenitors, which in turn affects the homing of these cells in vitro or in vivo.

**Effect of stroma in the presence or absence of cytokines.** There is extensive data on the supportive capacity of stromal cells on the maintenance of primitive stem cells and their ability to generate progenitors in LTCs. However, the requirement for a stromal microenvironment in ex vivo amplification of HSCs and progenitors remains controversial. In the absence of exogenous cytokines, stromal support derived from adult animals fails to significantly expand in vitro and in vivo repopulating cells [135, 160, 188, 219]. In the presence of exogenous cytokines, lineage negative CD34\(^-\)Thy-1\(^+\) human BM cells expanded on a porcine microvascular endothelial cell layer were consistently capable of competitive marrow repopulation with multilineage progeny present eight weeks post-engraftment, while grafts composed of cells expanded in stroma-free cultures did not lead to multilineage repopulation in vivo [7]. We have previously reported that exogenous cytokines, in conjunction with stroma-conditioned medium (harvested without the use of cytokines), enhanced the expansion of primitive HSCs from CD34\(^+\) selected MPB in short-term stroma-free cultures, while stroma and cytokines were also required for optimal maintenance and graft quality [5, 6]. In particular, the inclusion of Tpo in Dexter-type cultures has been shown to dramatically increase the longevity of hematopoietic activity and maintenance of in vivo repopulating ability of stem cells, indicating the importance of this cytokine in stroma-supported cultures [220, 221]. In the presence of the cytokines Flt3-L, Tpo, SCF, IL6 and GM-CSF, endothelial-based culture of UCB CD34\(^+\)CD38\(^-\) cells has been reported to give a higher CAFC expansion (11-fold increase) as compared to a 4-fold increase under stroma-free conditions after three weeks of culturing [222]. Both myeloid and lymphoid progenitors from UCB and MPB could be expanded in the presence of the murine fetal liver cell
Umbilical cord blood hematopoietic stem and progenitor cells

line AFT024 under two to five-week stroma-contact and stroma-non-contact culture conditions supplemented with at least Flt3-L and Tpo [223]. A study of Xu et al. showed that the stromal cells without exogenous addition of cytokines can also maintain in vivo repopulating ability of CD34+CD38- UCB cells during a four-week culture period [112]. It should be noted that these cells were supported by a different stromal layer derived from the murine embryonic AGM region as compared to other studies. All these data together suggest that both stromal cells and exogenous cytokines, including at least Tpo, are required for maintenance and/or expansion of HSCs in ex vivo cultures. There is still insufficient data on which factors contribute to this support under stromal conditions. However, the combination of stromal support and cytokines may potentially lead to dramatic improvement of the longevity of repopulating characteristics of HSCs and progenitors in cultures exceeding a time period of about two weeks as is the case when Tpo is used in stroma-supported BM cultures.

Clinical studies

Preclinical studies in normal baboons have demonstrated the potential clinical benefit of ex vivo expanded cells [224]. Baboons that were transplanted with expanded CD34+ MPB cells and given G-CSF and Tpo, showed a significantly shorter duration of neutropenia. On the basis of experimental findings and the development of automated closed-system for cell growth [225, 226], human clinical trials began. Initially, ex vivo expanded cells derived from BM were infused in combination with conventional cells [227]. After infusion of these cells no toxicity was observed demonstrating the feasibility of the expansion procedures and the clinical safety of the approach. Most importantly, a potential benefit was observed as measured by shortening of hospital stays, time to platelet recovery and days of febrile neutropenia when ≥ 60% of a standard cell dose of 1 x 10^5 CFU-GM/kg were infused. Additionally, a successful engraftment with only expanded BM HSCs was achieved in patients after high dose chemotherapy [228, 229]. The expanded cells used for infusion were obtained using a stroma-based continuous perfusion method with addition of the cytokines GM-CSF/IL3 fusion protein, Flt3-L and Epo or Tpo. These studies verify the importance of the combination stromal cells and cytokines in order to ex vivo expand transplantable HSCs and extend our previous data [6] where we show the requirement of stromal support for optimal maintenance of the graft quality. Several other groups reported decreased time to neutrophil recovery with ex vivo expanded PB cells. These authors cultured either C34+ cells or mononuclear fraction under similar conditions in teflon bags containing Defined Media (Amgen) supplemented with SCF, G-CSF and Tpo at 100 ng/ml for ten days [230-232]. Another
application of expanded hematopoietic cells, particularly more mature cells, has been explored as a means to accelerate the rate of recovery after myeloablative therapy. Along this line, Paquette et al demonstrated that ex vivo expanded PB progenitors could ameliorate post-transplantation neutropenia, thrombocytopenia and anemia [232]. Also the successful clinical transplantation of ex vivo expanded UCB HSCs and progenitors has been described. In these studies transplantations have been performed with uncultured and ex vivo cultured cells together [233-236]. Therefore, the precise role of cultured UCB cells in post-transplant recovery could not be analyzed. To better evaluate the engraftment dynamics of cultured UCB cells in human, Fernandez et al used a model of simultaneously transplanting cells from two different donors to the same patient [237]. Preliminary results of patients that have received one uncultured UCB unit and one cultured UCB CD34+ cells derived from a secondary UCB unit showed no significant contribution of cultured cells to early engraftment. Interestingly, also no prohibitive unfavorable immunological problems have been observed. Most of these human trials indicate the potential clinical use and benefit of expanded cells in post-transplant rate of the neutrophil recovery, while the effect of expansion on long-term engraftment ability remains unanswered. These studies show no additional effect on platelet recovery after infusion of expanded cells independent of their source. Possible explanations therefore may be that the culture conditions do not support progenitors that differentiate into platelets and/or that expanded cells need cytokines such as Tpo for platelet development.

Although ex vivo expanded HSCs have been and are being used in clinical settings, it is still unknown how many cultured HSCs are needed to successfully and rapidly engraft a recipient. It is also unknown what the loss is in graft quality during cultures. Clinical analysis has emphasized that the factor in predicting a positive outcome for transplant is the number of UCB nucleated cells infused, and should be >3 x 10^7/kg body weight [70]. Others confirmed that reliable engraftment occurs after UCB transplantation with 3 to 4 x 10^6/kg CD34+ cells [238]. The median body weight in these studies ranged between 17-19 kg. In the experiments carried out in our laboratory we observed a mean absolute number of 2.2 x 10^4 CFU-GM and 4.1 x 10^3 CAFCwo' per 10^5 uncultured UCB CD34+ cells. The SRC frequency in UCB has been calculated as 1 in 1.5 x 10^4 uncultured UCB CD34+ cells [239]. According to these data we made an estimation of the expansion factor for CFU-GM, CAFCwo and SRC being around 4-fold the input values for an adult recipient of 70 kg. Another application of expanded HSCs is to reduce the recovery time of neutrophils and platelets after myeloablative therapy. The recovery time after SCT is three to four weeks depending on the stem cell source being used [68, 70, 75, 83, 84].
Several *in vitro* studies determined the doubling time of human HSCs and progenitors derived from UCB or MPB as being one to three days depending on the cytokines used in culture [240-242]. Assuming a one day doubling time, we estimated the required expansion factor of HSCs and progenitors range between $1.3 \times 10^2$ and $3 \times 10^4$ fold the input value if a reduction of one and two weeks, respectively, for neutrophil and platelet recovery would be needed. Therefore, we aimed to obtain these increases for HSCs and progenitors with maintenance of the individual stem cell quality in the grafts.

### 1.4 Outline of the thesis

The goal of this thesis research is to establish *ex vivo* expansion conditions for HSCs derived from UCB. To realize the expansion of HSCs, CD34+ or AC133+ UCB cells were cultured in the absence or presence of various cocktails of early acting cytokines including Flt3-L, Tpo, SCF or IL6 under stroma-free or stroma-supported conditions. The HSC and progenitor expansion was assessed using *in vitro* and *in vivo* long-term repopulating cells.

First, the experiments were designed to test whether HSC expansion would alter the *in vivo* long-term engraftment potential of CD34+ UCB cells in the presence of BM-derived stromal cells during two weeks. Also the cytokines required for expansion of HSCs and progenitors in either the presence or absence of stroma have been evaluated.

The experiments described in chapter 3 are closely linked to the work described in the previous chapter. They were designed to investigate whether HSC expansion could be improved when cultured for more than two weeks, and whether the presence of BM-derived stromal cells, and combinations of specific cytokines could affect the HSC and progenitor maintenance or expansion.

In chapter 4 the effect of a new fusion protein of IL6 and the soluble IL6R, H-IL6, has been evaluated on the long-term *ex vivo* expansion of HCSs derived from AC133+ UCB cells. To do this, we used stroma-free and stroma-supported LTCs and compared several cytokine combinations in the presence or absence of this chimeric protein, or IL6, and estimated the HSC and progenitor output by multiparameter FACS analysis and CAFC assays.

Following these experiments, nineteen newly established murine embryonic stromal clones have been investigated for their ability to sustain human HSCs and progenitors in extended LTCs of CD34+ UCB cells in the absence or presence of the cytokines Flt3-L and Tpo for periods as long as twelve weeks. A significant proportion of HSC and
progenitor subsets was found in the *non-adherent* compartment of these co-cultures. With an interest to elucidate the factors that determine the proportion of *adherent* and *non-adherent* compartments, we evaluated in chapter 6 the chemoattractive activity of different stromal cells and the effect of exogenously added cytokines herein.
References

4. Verfaillie CM. Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. Blood 1993; 82 (7): 2045.
24. Ploemacher RE, Brons NH. Cells with marrow and spleen repopulating ability and forming spleen colonies on day 16, 12, and 8 are sequentially ordered on the basis of increasing rhodamine 123 retention. J Cell Physiol 1988; 136 (3): 531.
Chapter 1


29. van der Loo JC, van den Bos C, Baert MR, Wagemaker G, Ploemacher RE. Stable multilineage hematopoietic chimerism in alpha-thalassemic mice induced by a bone marrow subpopulation that excludes the majority of day-12 spleen colony-forming units. Blood 1994; 83 (7): 1769.


Umbilical cord blood hematopoietic stem and progenitor cells

52. McCredie KB, Hersh EM, Freireich EJ. Cells capable of colony formation in the peripheral blood of man. Science 1971; 171 (968): 293.
Chapter 1


86. Lowenberg B. Fetal liver cell transplantation: role and nature of the fetal haemopoietic stem cell. Hematology, Erasmus University 1975: Rotterdam.


Umbilical cord blood hematopoietic stem and progenitor cells


Chapter 1


137. Ploemacher RE, van der Loo JC, van Beurden CA, Baert MR. Wheat germ agglutinin affinity of murine hemopoietic stem cell subpopulations is an inverse function of their long-term repopulating ability in vitro and in vivo. Leukemia 1993; 7 (1): 120.


Umbilical cord blood hematopoietic stem and progenitor cells

167. Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 1997; 89 (11): 3919.
182. van der Loo JC, Ploemacher RE. Marrow- and spleen-seeding efficiencies of all murine hematopoietic stem cell subsets are decreased by preincubation with hematopoietic growth factors. Blood 1995; 85 (9): 2598.
Chapter 1


Umbilical cord blood hematopoietic stem and progenitor cells


210. Veermans C, Gerritsen WR, van den Borre AE, van der Schoof CE. Increased migration of cord blood-derived CD34+ cells, as compared to bone marrow and mobilized peripheral blood CD34+ cells across uncoated or fibronectin-coated filters. Exp Hematol 1999; 27 (12): 1806.


222. Rosler E, Brandt J, Chute J, Hoffman R. Cocultivation of umbilical cord blood cells with endothelial cells leads to extensive amplification of competent CD34+CD38- cells [In Process Citation]. Exp Hematol 2000; 28 (7): 841.

223. Lewis ID, Verfaillie CM. Multi-lineage expansion potential of primitive hematopoietic progenitors. Superiority of umbilical cord blood compared to mobilized peripheral blood [In Process Citation]. Exp Hematol 2000; 28 (9): 1087.


Chapter 1


Successful short-term ex vivo expansion of NOD/SCID repopulating ability and CAFC from umbilical cord blood.

N. Kuşadası, P.L. van Soest, A.E. Mayen, J.L.M. Koevoet and R.E. Ploemacher
Institute of Hematology, Erasmus Medical Center Rotterdam

Published in Leukemia, 2001.
Umbilical cord blood hematopoietic stem and progenitor cells

Abstract

In view of the limited potential for rapid hematological recovery after transplantation of umbilical cord blood cells (UCB) in adults we have attempted to expand CD34+ selected hematopoietic stem cells (HSCs) and progenitors in two-week cultures of whole graft pools in the presence or absence of serum and stromal layers, and with various cytokine combinations including a) Flt3-L + Tpo, b) Flt3-L + Tpo plus SCF and/or IL6, or c) SCF + IL6. Both in the input material and cultured grafts we determined the number of colony-forming cells (CFC), cobblestone area forming cells (CAFC), the NOD/SCID repopulating ability (SRA), and CD34+CD38- subset by phenotyping. The highest fold-increase obtained for the number of nucleated cells (nc), CD34+, CD34+CD38- cell numbers and CFC content was, respectively, 102 ± 76, 24 ± 19, 190 ± 202 and 53 ± 37 for stroma-free and 315 ± 110, 25 ± 3, 346 ± 410 and 53 ± 43 for stroma-supported cultures. CAFC_wk 6 was maximally 11-fold expanded both under stroma-free and stroma-supported conditions. The FBMD-1 stromal cells supported a modest expansion of CD34+CD38- cells (27 ± 18-fold) and nc (6 ± 4-fold), while a loss of CFC and CAFC subsets was observed. The stromal cells synergized with Flt3-L + Tpo to give the highest expansion of hematopoietic progenitors. Stromal support could be fully replaced by complementing the Flt3-L + Tpo stimulated cultures with SCF + IL6. Flt3-L + Tpo were required and sufficient to give a 10- to 20-fold expansion of the ability of CD34+ UCB cells in two-week cultures to engraft the BM of NOD/SCID mice. Stromal support, or complementation of the medium with SCF + IL6, did not significantly improve the in vivo engraftment potential. If the SRA and CAFC_wk 6 assays are accepted as tentative estimates of in vivo engrafting stem cells in humans, our findings may assist in the preparation of UCB grafts to meet the requirements for improved repopulation in the clinical setting.

Introduction

The discovery that umbilical cord blood (UCB) HSCs could be used as an alternative source to rescue myeloablated pediatric patients broadened the bone marrow (BM) transplantation treatment modality. However, reconstitution of hematopoiesis after intensive chemotherapy and grafting of UCB cells in children and even more in adults is slower compared to the use of BM or mobilized peripheral blood grafts [1]. This prolonged period of aplasia results in increased risk for infections and bleedings in
patients. The limited potential for rapid hematological recovery after transplantation of UCB cells is probably due to a limited number of nucleated cells and HSCs transplanted per kg BW, and may therefore be abrogated by ex vivo generation of the required numbers of stem and progenitor cells. Expansion studies might also increase our current knowledge on the regulation of hematopoiesis, contribute to new treatment strategies of hematological diseases and aid in manipulating stem cells as vehicles for gene therapy.

Although ample studies on the culture of CD34+ UCB cells have been published in the last years, and expansion of committed progenitors has been reported, the ability to expand more primitive HSCs has met with little success [2]. The most promising results have been obtained after the introduction of Flt3-L, the ligand of the class III tyrosine kinase receptor Flt3, and Tpo, the ligand of c-mpl receptor, both receptors are expressed on early and committed hematopoietic progenitors. Analysis of the effect of 16 cytokines on stroma-free cultures of CD34+CD38− bone marrow cells indicated that Flt3-L, SCF and IL3 gave the highest (30-fold) expansion of the input long-term culture-initiating cells (LTC-IC) [3]. In another study addition of Flt3-L maintained the ability of human CD34+ cells to sustain long-term hematopoiesis [4]. Tpo has also been reported to increase the multilineage growth of CD34+CD38− bone marrow cells under stroma-free conditions when added to the cytokine combination Flt3-L and SCF [5]. The presence of additional cytokines e.g. IL3, IL6 and Epo did not significantly enhance the colony formation above that observed with Flt3-L, Tpo and SCF. Others demonstrated that maximal expansion of progenitors by Flt3-L + IL11 required a longer incubation than with SCF + IL11 indicating involvement of different kinetics in enhanced production of progenitors [6]. A major step toward extensive ex vivo amplification of early human progenitors has been reported by Piacibello et al. These authors demonstrated that IL3 induced production of committed progenitors and was unable to sustain maintenance of hematopoietic stem cells. Among the various early acting cytokine combinations tested for their ability to sustain long-term hematopoiesis in stroma-free cultures using CD34+ UCB cells, Flt3-L and Tpo were found to be necessary and sufficient to maintain early progenitors for several months [7-9].

Until recently, investigators have included CD34+ cells, or subsets, and LTC-IC or cobblestone area forming cell (CAFC) in vitro assays to measure the HSC activity after expansion studies, but few have evaluated the genetically immunodeficient mice repopulating ability of these expanded HSCs [10-12]. In HSC expansion studies, investigators also emphasized the role played by microenvironment, with stromal cells as essential components. Multiple mechanisms have been proposed to explain the regulatory effect of stromal cells on HSC functions such as survival of quiescent cells,
increased proliferation and differentiation, or, decreased cell proliferation mediated by contact with stromal elements [2, 13, 14]. However, there is incomplete evidence as to whether expansion of NOD/SCID repopulating cells (SRC) is increased in the presence of a BM-derived stromal layer. In the present study we examined if HSC expansion in the presence of a BM-derived stromal cells, in synergy with potent cytokines during two weeks, will alter the in vivo long-term engraftment potential of CD34+ UCB cells and which cytokines are required in the presence or absence of stroma. Both in vitro (CAFC) and in vivo (SRC) HSC assays that have been reported to correlate with (multilineage) engraftment potential have been used simultaneously in each experiment, to evaluate the changes of cultured cells from different perspectives. We demonstrate that stroma is not required for NOD/SCID repopulating ability in two-week cultures by a combination of cytokines when Flt3-L and Tpo are included.

Materials and methods

*Human umbilical cord blood cells*. Human UCB samples were collected from umbilical cord vein after full-term delivery by the nursing staff of the Department of Obstetrics and Gynecology at the Sint Franciscus Gasthuis (Rotterdam, The Netherlands). Informed consent for taking samples for clinical study was obtained. UCB was collected in sterile flasks containing 10 ml citrate-glucose as anticoagulant, stored at room temperature and processed within 24 hours of collection.

*Isolation of CD34+ cells*. Low-density cells were isolated using Ficoll Hypaque density centrifugation (1.077g/cm², Lymphoprep, Nycomed Pharma, Oslo, Norway). After centrifugation at 600 g for 15 minutes, the mononuclear cell (MNC) band at the interface was removed, washed twice with Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) and resuspended in Iscove’s modified Dulbecco’s medium (IMDM, Gibco, Breda, The Netherlands). The MNCs were stored in liquid nitrogen until use. After thawing and before the CD34+ hematopoietic progenitor cells were isolated we pooled 5 to 20 different UCB samples. The CD34+ cells were harvested from the mononuclear cells using Variomacs Immunomagnetic Separation System (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD34+ cells were labeled indirectly using a hapten-conjugated primary monoclonal antibody and an anti-hapten antibody coupled to MACS microbeads. The magnetically labelled cells were enriched by passing them twice through a Variomacs positive selection column to reach a final purity of > 90% CD34+ cells. A nuclear cell count was performed and the progenitor and stem cell
numbers in the CD34+ selected UCB cells were assayed by CFC, CAFC and NOD/SCID repopulating cell assays. In addition, the immunophenotypic characteristics of the cells were assessed and part of the CD34+ cells were cultured for two weeks.

**Hematopoietic growth factors.** The following cytokines were used: recombinant human stem cell factor (SCF) and FLT3-ligand (Flt3-L), both gifts from Amgen (Thousand Oaks, CA, USA); recombinant human interleukin 6 (IL6), recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF), recombinant human granulocyte colony stimulating factor (G-CSF), murine stem cell factor (SCF), all gifts from Genetics Institute (Cambridge, MA, USA); recombinant human thrombopoietin (Tpo, a gift from Genentech, South San Francisco, CA, USA), recombinant human erythropoietin (Epo, Boehringer, Mannheim, Germany) and IL3 (Gist Brocades, Delft, The Netherlands).

**Ex vivo expansion cultures.** Various culture conditions were used in 10 consecutive experiments. In the first two experiments 5.0 x10^4 CD34+ UCB cells were cultured in tissue culture 6-well plates (Costar, Badhoevedorp, The Netherlands) in an 1 ml IMDM-cocktail containing 10% fetal calf serum (FCS, Summit, Fort Collins, CO), 1% bovine serum albumin (BSA, Sigma, Zwijndrecht, The Netherlands) supplemented with penicillin (100 U/ml, Gibco), streptomycin (0.1 mg/ml, Gibco), β-mercapto-ethanol (10⁻⁴ M, Merck, Darmstadt, Germany), bovine insulin (4 mg/ml, Gibco), cholesterol (15 μM, Sigma), linolic acid (15 μM Merck) iron-saturated human transferrin (0.62 g/l, Intergen, Uithoorn, The Netherlands), nucleic acids (cytidine, adenosine, uridine, guanosine, 2’-deoxycytidine, 2’-deoxyadenosine, thymidine, 2’-deoxyguanosine (all at 10⁻³ g/ml, Sigma)) and the cytokines Flt3-L (50 ng/ml), Tpo (10 ng/ml), SCF (100 ng/ml), IL6 (100 ng/ml). The cells were cultured with the following combination of cytokines: Flt3-L + Tpo, Flt3-L + Tpo + SCF, Flt3-L + Tpo + IL6, Flt3-L + Tpo + SCF + IL6 or SCF + IL6. Cultures were set up using six to eight replicates of each cytokine combination. Additionally, 5.0 x10^4 CD34+ cells were deposited in tissue culture 25T-flasks in 4 ml IMDM-cocktail containing 20% FCS with these same cytokine combinations. Cells were also cultures in the absence of cytokines. In the following part of the study, 6.45 x10^5 or 7.0 x10^5 CD34+ cells were cultured in tissue culture 175T-flasks (Falcon, Etten-Leur, The Netherlands) with the cytokine combination Flt3-L + Tpo + IL6 in 10 ml IMDM-cocktail without BSA containing 5, 10 or 20% FCS. Finally, 3.5 x10^4 or 4.0 x10^4 CD34+ cells were deposited in tissue culture 25T-flasks in 4 ml IMDM-cocktail without BSA containing 20% FCS supplemented with the following cytokines: Flt3-L + Tpo, Flt3-L + Tpo + SCF, Flt3-L + Tpo + SCF, Flt3-L + Tpo + SCF + IL6. The effect of the murine FBMD-1 stromal layer on the *in vitro* and *in vivo* repopulating ability was assessed.
for the cytokines Flt3-L + Tpo, Flt3-L + Tpo + IL6, Flt3-L + Tpo + SCF + IL6, and SCF + IL6. Insulin was not added in the stromal groups because it potently stimulated adipogenesis in FBMD-1 cells.

In the second part of the study, $3.5 \times 10^5$ and $7.75 \times 10^5$ CD34+ UCB cells were cultured in tissue culture 175T-flasks using 10 or 18 ml Cellgro® stem cell growth medium (SCGM, Boehringer Ingelheim, Heidelberg, Germany) with or without 10% FCS supplemented with the cytokine combinations Flt3-L + Tpo, Flt3-L + Tpo + SCF + IL6, Flt3-L + Tpo + IL6 or SCF + IL6. In the last set of experiments, $4.0 \times 10^4$ CD34+ cells were deposited in tissue culture 25T-flasks in 4 ml SCGM containing 20% FCS supplemented with the following cytokines: Flt3-L + Tpo, Flt3-L + Tpo + SCF, Flt3-L + Tpo + IL6 or Flt3-L + Tpo + SCF + IL6. The effect of the murine FBMD-1 stromal layer on the in vitro and in vivo repopulating ability was assessed for the cytokines Flt3-L + Tpo, Flt3-L + Tpo + IL6, Flt3-L + Tpo + SCF + IL6 and SCF + IL6. Cells were also deposited on a stromal layer without any cytokine supplements. SCGM without insulin was used in stromal groups to prevent adipocyte formation. In all experimental sets Flt3-L and Tpo were added twice a week to the expansion cultures.

The cultures were maintained at 37°C and 10% CO₂ in a humidified atmosphere and terminated at week 2 by collecting the supernate after scraping each well or flask with a cell scraper to include all the adherent cells. The stromal groups were first trypsinized with trypsin-EDTA (Life Technologies, Breda, The Netherlands). The content of the various dishes or flasks belonging to a single group were pooled, centrifugated at 250 g for 10 minutes and resuspended in IMDM. Suitable aliquots of the output suspensions were assayed in CFC, CAFC and SRC assays for determination of progenitor and stem cell numbers. In addition, the immunophenotypic characteristics of the cultured cells were analyzed.

**Clonogenic assay.** Granulocyte-macrophage colony forming unit (CFU-GM) and burst forming unit-erythroid (BFU-E) progenitor cells were assayed using a semisolid culture medium (1.2% methylcellulose), containing IMDM supplemented with 30% FCS, β-mercapto-ethanol (5 x 10⁻⁴ M), penicillin (100 U/ml), streptomycin (0.1 mg/ml), hu-Epo (1 U/ml), hu-IL3 (20 ng/ml), hu-GM-CSF (5 ng/ml), hu-G-CSF (50 ng/ml) and mu-SCF (100 ng/ml). Duplicate cultures were plated in 35 mm tissue culture dishes (Falcon) and incubated at 37°C and 10% CO₂ in a humidified atmosphere for 14 days. Colonies containing 50 cells or more were scored at day 14 using an inverted light microscope.

**Cobblestone area forming cell assay.** Confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates (Falcon) were overlaid with UCB cells in a limiting dilution
set up as described [15]. Briefly, twelve successive two-fold dilutions were used for each sample with 15 wells per dilution. The percentage of wells with at least one phase-dark hematopoietic clone (cobblestone area) of at least five cells beneath the stromal layer was determined at week 2, 4, and 6. At initiation of the cultures 250 CD34+ UCB cells per well were plated in the first dilution, while after two weeks of cultures 200-1000 CD34+ input equivalent cells per well were used in the first dilution. The CAFC frequencies were calculated using Poisson statistics.

**Immunophenotypic analysis.** At least 5.0 x 10^4 fresh or cultured CD34+ cells or femoral bone marrow cells of the NOD/SCID mice were stained with anti human CD45/CD33, CD34/CD38, CD34/CD19 (fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated) labeled monoclonal antibodies (Immunotech, Mijdrecht, The Netherlands). To analyse the lineage commitment the femoral bone marrow cells were also stained with anti human CD15/CD33, CD41/CD33, glycopherin A (GlyA), CD20/CD19, CD3/CD4 and CD3/CD8. After incubation of the cells in phosphate-buffered saline (PBS, Life Technologies, Breda, The Netherlands) containing 0.5% BSA and 2% normal human serum for 30 minutes on ice, the cells were washed in PBS with 0.5% BSA and resuspended in 0.35 ml PBS. Just before the acquisition 7-aminoactinomycin (7-AAD, Molecular Probes, Leiden, The Netherlands) was added to each sample to determine the viability of the cells. FITC and PE-conjugated mouse isotype antibodies were used as control for each group. At least 1.0 x 10^4 events were acquired using fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson).

**NOD/SCID repopulating cell (SRC) assay.** Specific pathogen free (SPF) NOD/LtSz-SCID/SCID (NOD/SCID) mice, six to fifteen weeks of age, were used as recipients of human cell grafts. The NOD/SCID mice were bred, maintained and used under SPF conditions in a laminar airflow unit, supplied with sterile food and acidified drinking water containing 100 mg/ml ciprofloxacin. All mice were sublethally irradiated with 3.5 Gy from a 137Cs source (Gammacell, Atomic Energy of Canada, Ottawa, Canada) 1 to 4 hours prior to intravenous transplantation. At day 0 of the experiments 5.0 x 10^4 or 1.0 x 10^5 CD34+ cells and on week 2 of the cultures 5.0 x 10^3 and 5.0 x 10^4 input equivalent cells were transplanted. The number of mice used for each group varied from 3 to 4. Six weeks after transplantation mice were killed by CO₂ inhalation and bone marrow cells were harvested by flushing all cells from two isolated femurs per animal. After counting, the cells were analyzed by FACS to determine the human cell engraftment in each mouse. Between 1.0 x 10^4 and 1.0 x 10^5 events were acquired for analysis. If the percentage of CD45+ cells was >0.1% the mice were considered positive, while all mice with a percentage >1.5% were analyzed for expression of lineage-specific markers.
**Statistical analysis.** The values are reported as mean ± SD. The significance levels were determined by two-tailed Mann-Whitney test analysis.

**Results**

*Characteristics of uncultured CD34*+ cells. Highly purified CD34+ UCB cells were cultured in an attempt to expand the graft content of primitive stem cells using serum-containing and serum-free conditions supplemented with two or more cytokines and in the presence or absence of a murine FBMD-1 stromal layer. After isolation of the MNC by low-density gradient from a pool of 5 to 20 UCB samples, immunomagnetic selection according to the CD34 antigen expression was performed. The purity of the selected cells, determined by FACS after staining with CD34-FITC conjugated monoclonal antibody, varied between 88% to 97%. The percentage of the cell fractions expressing the CD33+ (myeloid lineage), CD19+ (B-lymphocytes) and CD34+CD38− (primitive stem cells) from seven, eight and ten experiments were 21 ± 21%, 4 ± 2% and 3 ± 2% respectively. The absolute number of clonogenic cells was 30,861 ± 12,862 CFU-GM and 10,606 ± 6,474 BFU-E per 10^5 input cells. The CAFC_{week2} frequency as indicator of transiently repopulating stem cells ranged from 7,336 to 23,566, whereas the CAFC_{week6} as a measure of tentative long-term repopulating stem cells varied from 2,381 to 8,308 per 10^5 input cells. To analyze the ability of uncultured CD34+ cells to give multilineage engraftment in the BM of NOD/SCID mice 5.0 x 10^4 or 1.0 x 10^5 CD34+ UCB cells per mouse were injected i.v. into groups of sublethally irradiated mice. Six weeks after transplantation the mice were killed, BM cells harvested from both femurs and analyzed for the presence of different lineages of human hematopoietic cells using FACS. Fifty and hundred thousand CD34+ cells were able to engraft 100% of the mice. The percentage of CD45+ cells, as a marker for the presence of human hematopoietic cells in the murine BM, ranged from 5.3% to 19.5%. After two weeks of culture the numbers of ne, CFC, CAFC, immunophenotypic cell populations and the ability of the cultured cells to give multilineage engraftment in NOD/SCID mice were determined and compared with the corresponding input values. As the different medium conditions used in ten experiments did not significantly affect the output for the various parameters determined (data not shown), the results below represent pooled data for the groups with similar cytokine combination.

*Characteristics of cultured CD34*+ cells. **Stroma-free cultures.** Stroma-free culture of CD34+ UCB cells in the presence of different combinations of cytokines resulted in
expansion of NC, CD34+ and CD34+CD38- cells over the input values, while in absence of cytokines all cells died (figure 1). The fold-increase of the CD34+ phenotype as measure of hematopoietic progenitor cells, and that of the CD34+CD38- phenotype as indicator for a quiescent and functionally primitive population of progenitor cells, varied from 0.4 to 51 and 3.1 to 475, respectively. An increase of B-lymphoid and myeloid cells compared to the uncultured CD34+ UCB cells was also obtained (table 1). The maximum recovery of CFU-GM in stroma-free cultures was 42 ± 40 fold the input (figure 2), while the BFU-E were lost in all cytokine groups. In general, there was a higher fold increase of CD34+CD38- cells than of CD34+ cells. If this phenotype CD34+CD38- truly predicts the expansion of long term repopulating cells, then it would be likely to find similar dramatic expansion of the absolute number of CAFC subsets and SRC as well. However, the CAFC were maximally 4 and 11-fold expanded, respectively, while in some

Table 1. Expansion of B-lymphoid and myeloid cell populations following two weeks of ex vivo culture of CD34+ umbilical cord blood cells.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Fold-increase of total number of hematopoietic cells</th>
<th>B-lymphoid</th>
<th>Myeloid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD19+</td>
<td>CD34+CD19+</td>
</tr>
<tr>
<td>Stroma-free cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No cytokines; n=2</td>
<td></td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>Flt3-L + Tpo; n=3</td>
<td></td>
<td>3.4 ± 2.8</td>
<td>1.6 ± 1.5</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF; n=1</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL-6; n=6</td>
<td></td>
<td>2.9 ± 2.7</td>
<td>4.1 ± 4.5</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL-6; n=3</td>
<td></td>
<td>3.0 ± 1.2</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>SCF + IL-6; n=2</td>
<td></td>
<td>0.7 ± 0.8</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>Stroma-supported cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No cytokines; n=2</td>
<td></td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>Flt3-L + Tpo; n=3</td>
<td></td>
<td>3.4 ± 2.8</td>
<td>1.6 ± 1.5</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL-6; n=7</td>
<td></td>
<td>2.9 ± 2.7</td>
<td>4.1 ± 4.5</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL-6; n=2</td>
<td></td>
<td>3.0 ± 1.2</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>SCF + IL-6; n=2</td>
<td></td>
<td>0.7 ± 0.8</td>
<td>1.0 ± 1.0</td>
</tr>
</tbody>
</table>

The data represent the mean fold-increase ± SD of 2-7 experiments performed, in which CD34+ UCB cells were cultured for two weeks under stroma-free and stroma-supported conditions with or without cytokines. Abbreviations: ND, not determined.
Figure 1. Fold-expansion of absolute number of nucleated cells, CD34+ and CD34+CD38− cells following two weeks of culture of CD34+ UCB cells under stroma-free and stroma-supported conditions with or without cytokines. Data represent the mean (SD) of 2-11 experiments performed. Mann-Whitney test: * denotes significant difference (P<0.05) between stroma-supported cultures with cytokines and stroma-supported culture without cytokines, # denotes significant difference (P<0.05) between stroma-supported cultures and FT stroma-free culture. Abbreviations: FT, Flt3-L + Tpo; FTS, Flt3-L + Tpo + SCF; FT6, Flt3-L + Tpo + IL6; FTS6, Flt3-L + Tpo + SCF + IL6; S6, SCF + IL6. **, all cells died.
Figure 2. Fold-expansion of absolute number of colony forming unit-granulocyte-macrophage (CFU-GM) and cobble stone area forming cell (CAFC) subsets following two weeks of culture of CD34+ UCB cells under stroma-free and stroma-supported conditions with or without cytokines. Data represent the mean (SD) of 2-11 experiments performed. Mann-Whitney test: * denotes significant difference (P< 0.05) between stroma-supported cultures with cytokines and stroma-supported culture without cytokines, # denotes significant difference (P< 0.05) between stroma-supported cultures and FT stroma-free culture. Abbreviations: FT, Flt3-L + Tpo; FTS, Flt3-L + Tpo + SCF; FT6, Flt3-L + Tpo + IL6; FTS6, Flt3-L + Tpo + SCF + IL6; S6, SCF + IL6. **, all cells died.
cytokine combinations (i.e. Flt3-L + Tpo and SCF + IL6) even less CAFC\textsubscript{week2} and \textsubscript{6} than input were recovered (figure 2). The addition of SCF +/- IL6 to the cytokine combination Flt3-L + Tpo promoted the early and late progenitor amplification in terms of CFU-GM and CAFC subsets, indicating a synergistic effect of these cytokines in combination with Flt3-L + Tpo. As can be seen from figure 2, the optimal cytokine combination for expansion of hematopoietic progenitors (CFU-GM, CAFC) under stroma-free conditions was Flt3-L + Tpo + SCF + IL6.

Following two weeks of culture, a considerable expansion of the potential of CD34\textsuperscript{+} UCB cells to engraft the BM of sublethally irradiated NOD/SCID mice was also observed. The maximum levels of BM engraftment obtained for each group of mice infused with cultured cells is shown in figure 3. When cells had been cultured in the presence of Flt3-L + Tpo + SCF, upto 90% of the BM cells were of human origin, while culture with SCF + IL6 alone led to human chimerism levels below that of uncultured CD34\textsuperscript{+} UCB cells. In all other cytokine combination groups, higher levels of human chimerism were detected in mice injected with cultured cells than in mice infused with uncultured CD34\textsuperscript{+} UCB cells (figure 4). Addition of SCF+/- IL6 to the cytokine combination Flt3-L + Tpo elicited no significant effect on the estimated expansion of the SRA. The cultured cells also showed an increased ability to repopulate the mice with CD34\textsuperscript{+} and CD34\textsuperscript{+}CD38\textsuperscript{-} cells compared to the input cells. The highest absolute percentage of CD34\textsuperscript{+} cells (14% of all bone marrow cells) and CD34\textsuperscript{+}CD38\textsuperscript{-} (1.4% of all bone marrow cells) was found in mice infused with cells cultured in the presence of Flt3-L + Tpo + IL6 and Flt3-L + Tpo, respectively.

Figure 5 shows the level of human engraftment at six weeks after transplantation of the NOD/SCID mice with 5.0 \times 10^3 rather than 5.0 \times 10^4 input equivalent cells. Even these limited cell numbers were similarly able to engraft 100% of the mice. The percentage of human engraftment ranged between 0.1% and 47%, with the highest value was observed for the cytokine combination Flt3-L + Tpo + IL6. Although the engraftment capacity of the cells cultured with either Flt3-L + Tpo or SCF + IL6 was lower as compared to other cytokine combinations, these 5.0 \times 10^3 input equivalent cells gave the same (using Flt3-L + Tpo and Flt3-L + Tpo + SCF +/- IL6 under stroma-free and stroma-supported conditions, respectively) or an even higher (using Flt3-L + Tpo + IL6) human engraftment than did 1.0 \times 10^5 CD34\textsuperscript{+} uncultured cells. This strongly substantiates our contention that the ability of the cells to give engraftment in the bone marrow of NOD/SCID mice can be expanded in a two-week culture system. Assuming a linear relation between the number of input equivalent cells transplanted and the level of chimerism found in the BM of NOD/SCID mice we have estimated the expansion of \textit{in vivo} engraftment ability of cultured CD34\textsuperscript{+}
Figure 3. Flow cytometric analysis of femoral marrow cells from individual NOD/SCID mice six weeks after transplantation with two-week (a) stroma-free and (b) stroma-supported cultured CD34+ UCB cells. The FACS profiles represent the highest human engraftment obtained within different culture conditions tested. The percentage of human chimerism expressing CD45 is given in the upper-right quadrant. The cells expressing the myeloid fraction (CD33) ranged between 3-20% of the femoral marrow. Mice were infused with either $5.0 \times 10^4$ uncultured CD34+ cells, or $5.0 \times 10^4$ input equivalent cultured CD34+ cells. Abbreviations: FT, Flt3-L + Tpo; FTS, Flt3-L + Tpo + SCF; FT6, Flt3-L + Tpo + IL6; FTS6, Flt3-L + Tpo + SCF + IL6; S6, SCF + IL6.
Figure 4. Engraftment ability of stroma-free and stroma-supported uncultured and two-week cultured CD34+ UCB cells in bone marrow of NOD/SCID mice six weeks after transplantation. Mice were infused with either 5.0 x 10^4 (n=13) or 1.0 x 10^5 (n=4) CD34+ uncultured cells, or 5.0 x 10^4 (n=4-15) input equivalent cultured CD34+ cells. The values for each group represent the percentage of human cells (CD45+) in the femoral marrow. Data are mean (SD) of 3-20 NOD/SCID mice transplanted per experimental group. Abbreviations: ND, not determined; n, number of mice transplanted per group; FT, Flt3-L + Tpo; FTS, Flt3-L + Tpo + SCF; FT6, Flt3-L + Tpo + IL6; FTS6, Flt3-L + Tpo + SCF + IL6; S6, SCF + IL6.

UCB cells, as we realized that the expansion in mice with high chimerism percentages could be underestimated. As shown in table 3, all cytokine combinations except for SCF + IL6 led to a more than an average of 10-fold SRA expansion.

In order to evaluate whether the expanded cells maintained their ability for multilineage engraftment in the NOD/SCID mice we studied the expression of lineage markers on the human cells present in the femoral marrow of the mice at six weeks post-transplant. We did not observe a significant difference between the ability of the cultured and uncultured cells to produce various progenitors (CD34+, CD34+CD38, CD34+CD19+), B-lymphocytes (CD19+, CD20+CD19+, CD20+), myeloid cells (CD33+), granulocytes (CD15+) and monocytes (CD3+CD19+) (table 2). Human T-lymphocytes (CD3+CD4+, CD3+CD8+) were never detected in the bone marrow of the recipients. Also, no significant differences were found in the percentage of megakaryocytes (CD33+CD41+) formed by uncultured (3 ± 2%) and cultured cells (0.7 ± 0.6%) nor erythroid cells (GlyA+) (8 ± 8 versus 8 ± 8%) in 5-8 experiments.

Stroma-supported cultures. To investigate whether stromal support would improve the maintenance and/or expansion of primitive stem cells, we inoculated the CD34+ UCB cells on a murine stromal layer using the same liquid culture set up as used for stroma-
Figure 5. Enhanced engraftment ability of two-week stroma-free and stroma-supported cultured CD34+ UCB cells in the bone marrow of NOD/SCID mice six weeks after transplantation. Mice were infused with either 1.0 x 10^5 (n=4) uncultured CD34+ cells or with 5.0 x 10^5 (n=3-4) and 5.0 x 10^6 (n=3-4) input equivalent cultured CD34+ cells. The values for each group represent the mean (SD) percentage of human cells (CD45+) in the femoral marrow. Mann-Whitney test: * denotes significant difference (P< 0.005) between transplantation of 5.0 x 10^5 or 5.0 x 10^6 input equivalent cultured CD34+ cells. Abbreviations: ND, not determined; n, number of mice transplanted per group; FT, Flt3-L+Tpo; FTS, Flt3-L+Tpo+SCF; FT6, Flt3-L+Tpo+IL6; FTS6, Flt3-L+Tpo+SCF+IL6; S6, SCF+IL6; stroma-FT, stroma-supported culture in the presence of Flt3-L+Tpo.

Free cultures except for insulin. Cultures were either supplemented with Flt3-L+Tpo, SCF+IL6 or Flt3-L+Tpo+/-SCF+/-IL6. Stroma-supported cultures without additional cytokines were included as a control. From figures 1 and 2 it is clear that stroma support without additional cytokines did not lead to an expansion of CFU-GM, CAFC subsets and CD34+ cells, however, it gave a 20±17-fold increase of the CD34+CD38- subset. In the presence of Flt3-L+Tpo, stromal support synergized to give a significantly higher expansions of all parameters studied except of CD34+ cells as compared to stroma-free culture (P< 0.05). Stromal support could be fully replaced by complementation of the Flt3-L+Tpo stimulated cultures with SCF+IL6. In the presence of FBMD-1 stromal cells, other cytokine combinations could not significantly improve the total fold-expansion. Also in the presence of Flt3-L+Tpo+SCF, or Flt3-L+Tpo+IL6, stroma-support stimulated the average expansion of nc, CD34+, CD34+CD38- cells, although not always significantly. In the presence of stroma, SCF+IL6 stimulated remarkably few total nc, CD34+ cells and CFU-GM, and did not prevent loss of CAFC_{week 2} and <6, while it also did not give an improved maintenance of all parameters as compared to non-stroma supported cultures. Stromal support alone was not sufficient to maintain the SRA of
Table 2. Multilineage engraftment ability CD34+ UCB cells in the bone marrow of NOD/SCID mice before or after two-week culture period.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>% of human (CD45+) cells in the femoral marrow</th>
<th>(n= number of mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD34+</td>
<td>CD34+CD38+</td>
</tr>
<tr>
<td>Uncultured; n=17</td>
<td>14 ± 8</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Stroma-free cultures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No cytokines</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Flt3-L + Tpo; n=5</td>
<td>18 ± 12</td>
<td>5 ± 6</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF; n=4</td>
<td>7 ± 3</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL6; n=4</td>
<td>11 ± 3</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL6; n=7</td>
<td>8 ± 5</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>SCF + IL6; n=4</td>
<td>10 ± 6</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Stroma-supported cultures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No cytokines; n=1</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Flt3-L + Tpo; n=10</td>
<td>8 ± 5</td>
<td>2 ± 3</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL6; n=6</td>
<td>13 ± 10</td>
<td>3 ± 5</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL6; n=5</td>
<td>9 ± 9</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>SCF + IL6; n=4</td>
<td>15 ± 7</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

Cells infused into NOD/SCID mice were either uncultured, or propagated for two weeks in stroma-free or stroma-supported cultures. The inocula were either 50,000 (n=13) or 100,000 (n=4) CD34+ cells/mouse for uncultured cells or 50,000 input equivalent cells/mouse for cultured cells. Values represent the mean (± SD) percentage of human cells (CD45+) in the femoral marrow of 4-17 NOD/SCID mice transplanted per group. Abbreviations: N, not detectable; ND, not determined.
Table 3. Estimated fold-increase of the engraftment ability in NOD/SCID mice transplanted with cultured CD34+ UCB cells.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Fold-increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n= number of mice)</td>
<td></td>
</tr>
<tr>
<td><strong>Stroma-free cultures</strong></td>
<td></td>
</tr>
<tr>
<td>Flt3-L + Tpo; n=4</td>
<td>10 ± 8.5</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF; n=4</td>
<td>12.5 ± 6</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL6; n=4</td>
<td>13.8 ± 5.8</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL6; n=3</td>
<td>12.6 ± 5.5</td>
</tr>
<tr>
<td>SCF + IL6; n=3</td>
<td>2.6 ± 2.1</td>
</tr>
<tr>
<td><strong>Stroma-supported cultures</strong></td>
<td></td>
</tr>
<tr>
<td>Flt3-L + Tpo; n=4</td>
<td>12.7 ± 2.8</td>
</tr>
</tbody>
</table>

The fold-expansion of the engraftment ability, in the cell dose ranges as used, was calculated on the assumption that there is a linear relation between the number of SCID repopulating cells transplanted and the level of chimerism in the bone marrow of NOD/SCID mice. The expansion data show the mean ± SD calculated in one experiment, in which each mouse was transplanted with 100,000 CD34+ uncultured cells or with 50,000 input equivalent cells after two weeks of culture.

CD34+ UCB cells in two-week cultures. Flt3-L + Tpo were required and sufficient to give a comparable high SRA expansion under stroma-supported cultures as compared to stroma-free cultures. Stromal support, or complementation of the medium with SCF + IL6, did not significantly improve the NOD/SCID BM engraftment potential (figures 4 and 5).

Discussion

Our data show a more than 10-fold expansion of CAFCweek6 and of the potential of CD34+ UCB cells to multilineage engraft the BM of sublethally irradiated NOD/SCID mice after two weeks of both stroma-free and stroma-supported culture in the presence of at least Flt3-L + Tpo. Among the cytokine combinations tested, stroma significantly improved the maintenance of CAFCweek6 with the cytokine combination Flt3-L + Tpo. This stromal support could be replaced by adding additional SCF + IL6 to the Flt3-L + Tpo stimulated cultures. However, nor stromal support nor complementation of the medium with SCF + IL6 improved the in vivo engraftment potential of two-week cultured CD34+ UCB cells.

The four cytokines used in the present study were chosen due to their key involvement in previous expansion studies as well as being early acting cytokines.
Umbilical cord blood hematopoietic stem and progenitor cells

For example, while Tpo induces apoptosis with down regulation of CD44 in CD34+ UCB cells, Flt3-L does not [16]. In contrast, the continuous presence of Tpo in stromal cultures resulted in generation of both short- and long-term in vivo repopulating HSCs [17]. In the present study the results following two-week cultures have shown Flt3-L + Tpo + SCF + IL6 under stroma-free conditions to represent an optimum cytokine combination for expansion of early and late progenitors. The addition of SCF +/- IL6 to the combination Flt3-L + Tpo enhanced progenitor cell expansion in terms of CFU-GM and CAFC subsets. These data indicate a synergistic effect of SCF +/- IL6 with Flt3-L and Tpo in our two-week stroma-free culture system, which is in contrast with previous data published by Piacibello et al. indicating no difference in the extent of early progenitors to substantial addition of SCF +/- IL6. This may reflect differences in the sources of cytokines, the purification procedures of cell populations or the presence of uncharacterized factors in the sera.

One of the essential issues remaining to be clarified was the assessment of in vivo repopulating ability of expanded cells. In our two-week culture system we obtained a considerable expansion of cells capable of multilineage outgrowth in the BM of NOD/SCID mice. Luens et al. reported that human CD34⁺CD38⁻ bone marrow cells after 6 days stroma-free culture sustained, but not expanded, their in vivo repopulating ability in the qualitative SCID/hu bone assay [18]. A modest increase of competitive repopulating units was observed after 5-8 days stroma-free culture of CD34⁺CD38⁻ UCB cells in the presence of cytokines Flt3-L, SCF, IL3, IL6 and G-CSF [19]. Similarly, Bhatia et al. reported that under serum-free conditions a 2- to 4-fold increase of SRC could be obtained after 4 days culture of CD34⁺CD38⁻ UCB cells, while after 9 days of culture all SRC were lost [10]. All these data support the contention, that in contrast to CFU-GM, SRC are difficult to expand, while they are rapidly lost in culture. In the present study, the expansion of the ability of CD34⁺ UCB cells to multilineage engraft the BM of sublethally irradiated NOD/SCID mice following two-week stroma-free cultures was observed in the presence of various cytokine permutations including Flt3-L + Tpo and SCF + IL6. Remarkably, addition of SCF +/- IL6 to the cytokine combination Flt3-L + Tpo showed no significant effect on the estimated expansion of the engraftment ability. While preparing this manuscript Piacibello et al. showed that more than 70-fold SRC increase could be obtained after 9 to 10 weeks culture of CD34⁺ UCB cells under stroma-free conditions in the presence of the cytokines Flt3-L, Tpo, SCF and IL6 [11]. However, it is not clear from these studies whether SRC were expanded after two weeks of culture. The prolonged duration of the cultures would limit the usefulness of this expansion technique in a clinical setting.
There is ample evidence for the supportive role of stroma in maintenance of stem cells and progenitors [20-22]. Verfaillie et al. demonstrated that the absolute number of stem cells (LTC-IC) and the colony forming cell production in long-term cultures was increased after propagation of hematopoietic stem cells derived from normal BM in stroma-noncontact cultures [14, 23]. O-sulphated GAGs seemed to be responsible for this long-term stroma-supported maintenance of LTC-IC in culture [24]. Brandt et al. showed that three weeks stroma-free culture of CD34+Thy-I+ human BM cells with lineage-negative markers resulted in impairment of engraftment ability in NOD/SCID mice, which could be overcome by coculture with a layer of porcine microvascular endothelial cells [12]. Additionally, we have previously reported that stroma-conditioned medium enhanced the expansion of primitive hematopietic stem/progenitor cells from CD34+ selected mobilized peripheral blood in short-term cultures, while stroma-support was also required for optimal maintenance and graft quality [25, 26]. Chih et al. have suggested LIF as the cytokine that is responsible for maintaining and expanding transplantable CD34+Thy1+ cells in stroma-supported cultures through an action on the stroma [27]. Our present results indicate that the addition of Flt3-L + Tpo to the stroma-supported cultures significantly improved the expansion results in terms of nc, CD34+, CD34+CD38− cells, CFU-GM, CAFC subsets and estimated SRA. Surprisingly, in contrast to the stroma-free cultures, the addition of SCF +/- IL6 to Flt3-L + Tpo did not appear to substantially modify the extent of early progenitors, i.e. CAFC and estimated SRA. Although these data seem to contrast with several previous studies indicating that proliferation of hematopoietic stem cells in the presence of stroma is associated with loss of most in vivo repopulating ability [28, 29], it should be realised that Flt3-L and Tpo were not exogenously added to the cultures in the latter studies. Recently, a study by Xu et al. has shown that CD34+CD38− UCB cells after a four-week culture period could maintain their in vivo repopulating ability [30]. These cultures were supported by a stromal cell line derived from the aorta-gonad-mesonephros region of a 10.5 days postcoitum mouse embryo, a location currently considered to generate the first definitive hematopoietic stem cells. In our study, the expansion of CAFC and estimated SRA in Flt3-L + Tpo stroma-supported cultures was as high as in stroma-free cultures in the presence of Flt3-L + Tpo + SCF + IL6. These observations suggest that SCF and IL6, which is elaborated by the FBMD-1 stroma, was sufficient to compensate for the exogenously added SCF and IL6.

It may be likely, that the increased SRA of cultured CD34+ UCB cells is due to (a) an increase of the total SRC number, however, it is also possible that (b) quiescent SRC, or pre-SRC, are activated or recruited, respectively. Alternatively, it could be that (c)
cultured cells show an increased ability to home either to, or expand in the BM of NOD/SCID mice, or that (d) there is a numerical or functional gain of facilitating cells that is possibly required for engraftment of the NOD/SCID BM by SRC. A study by Peled et al. showed that the chemokine stromal cell-derived factor (SDF)-1 and its receptor CXCR4, which is expressed on some CD34+CD38- cells amongst others, may be important for engraftment of sublethally irradiated NOD/SCID mice [31]. The cytokines SCF and IL6 induced CXCR4 up-regulation on CD34+ cells, which subsequently potentiated migration of these precursor cells and their engraftment in primary and secondary transplanted mice. In addition, antibodies to CXCR4 completely prevented the engraftment in vivo.

According to these data, the use of at least SCF +/- IL6 in our culture system might have caused increased homing potential of the CD34+ UCB cells, which in turn contributed to the increased SRA. Recently, Yagi et al. demonstrated that continuous presence of Tpo in mouse long-term bone marrow cultures resulted in generation of short and long-term hematopoietic stem cells as detected by an in vivo competitive repopulation assay indicating that Tpo can mediate self-replication of HSCs ex vivo [17]. Our results on increased CAFC and SRA following two-week of culture suggest that the continuous presence of Tpo might also play an important role in ex vivo self-replication of human CD34+ cells.

The simultaneous assessment of in vivo repopulating ability, phenotypic and functional characteristics of ex vivo manipulated cells enabled us to quantify the changes of cultured cells from different perspectives. It is interesting to note that in the presence of Flt3-L + Tpo, expansion of CAFC numbers (0.04 to 6 and 0.2 to 11 fold the input for weektype 2 and 6, respectively) paralleled the estimated expansion of SRA (table 3), in contrast to the much more extensive increases in the total number of CD34+CD38- cells (5 to 1149 fold the input). The CAFC, SRA and CD34+CD38- cells are known to identify closely related populations in uncultured UCB, BM and MPB samples. The much more rapid increase of CD34+CD38- cells compared to CAFC and estimated SRA indicates that these cell types may not detect the same cell populations after the culture period used.

In conclusion, hematopoietic stem/progenitor cells can be expanded in short-term cultures and stroma does not seem to be required for this purpose. If assessment of SRA and CAFC subsets are measures of in vivo repopulating stem cells in humans these findings may contribute to a rapid preparation of UCB grafts to meet the requirements for improved repopulation in the clinical setting.
Acknowledgements

We would like to acknowledge the nursing staff and midwives of the St. Fransiscus Gasthuis for collection of UCB samples, and P.B. van Hennik, W.J.C. Rombouts, I. Blokland and J. Cancelas for technical assistance.
Umbilical cord blood hematopoietic stem and progenitor cells

References


Chapter 3

Stromal support augments extended long-term ex vivo expansion of hematopoietic progenitor cells.

N. Kusadası, J.L.M. Koëvoet, P.L. van Soest and R.E. Ploemacher
Institute of Hematology, Erasmus Medical Center Rotterdam.
Published in Leukemia, 2001.
Abstract

Current technology to numerically expand hematopoietic stem/progenitor cells (HSPC) ex vivo within one to two weeks is insufficient to warrant significant gain in reconstitution time following their transplantation. In order to more stringently test the parameters affecting HSPC expansion, we followed ex vivo cultures of CD34+ selected umbilical cord blood (UCB) HSPC for up to ten weeks and investigated the effects of stromal support and cytokine addition. The cytokine combinations included Flt3-L + Tpo, Flt3-L + Tpo plus SCF and/or IL6, or SCF + IL6. To identify the HSPC in uncultured and cultured material we determined the number of colony-forming cells (CFC), cobblestone area forming cells (CAFC), the NOD/SCID repopulating ability (SRA), and CD34+ subsets by phenotyping. The highest fold-increase obtained for CD34+ and CD34+CD38- cell numbers was, respectively, 1,197 and 30,937 for stroma-free and 4,066 and 117,235 for stroma-supported cultures. In general, CFC generation increased weekly in Flt3-L + Tpo containing groups up to week 5 with a 28 to 195-fold expansion whereafter the weekly CFC output stabilized. Stroma support enhanced the expansion of CAFC_{\text{week6}} maximally 11-fold to 89-fold expanded with Flt3-L + Tpo + IL6. Cultures stimulated with at least Flt3-L + Tpo gave an estimated 10 to 14-fold expansion of the ability of CD34+ UCB cells to multilineage engraft the BM of sublethally irradiated NOD/SCID mice at two weeks of stroma-free and stroma-supported cultures, while at week 5 and later the estimated SRA decreased to low or undetectable levels in all groups. Our results show that stroma and Flt3-L + Tpo but also inclusion of bovine serum albumin, greatly increase the long-term generation of HSPC as measured by in vitro assays and is indispensable for long-term expansion of CD34+CD38-CXCR4+ cells. However, the different surrogate methods to quantify the HSPC (CD34+CD38-, CFC, CAFC_{\text{week5}} and SRA) show increasing incongruency with increasing culture time, while especially the phenotypic analysis and the CFC generation greatly overestimate the CAFC and SRA expansion in ten-week cultures.

Introduction

Ex vivo expansion of repopulating HSPC may aid in abrogation of neutropenia in cancer patients receiving myeloablative high dose chemotherapy. It may also open new opportunities for stem cell transplantation in cases where limited HSPC are available, such as in UCB grafts and leukapheresis samples from heavily pretreated patients. In
addition, expansion may contribute in developing culture conditions that permit efficient gene transfer into primitive HSPC without their concurrent differentiation-induced loss. Although expansion of total nucleated cells and progenitors has been documented, infusion of these cells has not resulted in any improvement of the time to recovery of neutrophils or platelets. Upto date, most research on *ex vivo* expansion has explored different combinations of cytokines or chemokines, culture medium ingredients, and recently the role of stromal support [1-7]. Flt3-ligand (Flt3-L), thrombopoietin (Tpo), stem cell factor (SCF) and interleukin-6 (IL6) are known to be early acting cytokines and have been reported to support primitive HSPC proliferation with maintenance of long-term repopulating ability [8-11]. The important effects of these cytokines on early HSPC have led to the development of *in vitro* culture systems in order to expand these cells. Particularly, the long-term production of HSPC has been used as a stringent endpoint for HSPC maintenance or expansion [12-18]. Using a cytokine-supplemented stroma-free extended long-term culture (LTC) system Piacibello and colleagues have uniquely documented a dramatic expansion of primitive CD34+ UCB cells over periods as long as thirty weeks [19, 20]. Endpoints of primitivity in these studies included CD34+CD38- and long-term culture initiating cell (LTC-IC) subsets. Recently, these authors also demonstrated that the NOD/SCID repopulating cells were markedly expanded using Flt3-L, Tpo, SCF and IL6 under the same conditions after ten weeks of culture [21]. Additionally, we reported that the *in vivo* repopulating ability of CD34+ UCB cells could be expanded in two-week cultures containing at least Flt3-L and Tpo and that the stromal support was not required [22]. The requirement for a stromal microenvironment in *ex vivo* amplification of the HSPC remains controversial. Endothelial-based culture of UCB CD34+CD38- cells has been reported to obtain higher CAFC expansion (11-fold the input) as compared to a 4-fold in the stroma-free system after three weeks of culture in the presence of the cytokines Flt3-L, Tpo, SCF, IL6 and GM-CSF [23]. A recent study by Lewis *et al.* showed that both myeloid and lymphoid progenitors from UCB and mobilized peripheral blood can be expanded in the presence of the murine fetal liver cell line AFT024 under two- to five-week stroma-contact and stroma-non-contact culture conditions supplemented with at least Flt3-L and Tpo [24]. Although in the absence of exogenous cytokines stroma-support fails to significantly expand *in vitro* and *in vivo* repopulating cells in stroma-supported cultures [12, 25-28], the inclusion of Tpo in such cultures dramatically increased the longevity of hematopoietic activity and maintenance of repopulating ability of stem cells [29]. All of these data together suggest that both stromal cells and exogenous cytokines, including at least Tpo, are required for maintenance and/or expansion of HSPC in cultures exceeding a time period of about two weeks.
Although a shorter culture period is favourable in a clinical setting, the extend of HSPC expansion (3- to 15-fold increase of CAFC and SRA) obtained in one to two-week cultures is not sufficient for a successful clinical application [22]. Therefore, in the present study we further examined whether HSPC expansion could be improved when cultured for more than two weeks, and whether the presence of BM-derived stromal cells, and combinations of specific cytokines affected the HSPC maintenance or expansion. As we have previously documented that certain BSA batches were inhibitory for murine HSPC expansion due their TGFβ₁ content, we have also compared the effect of BSA on human HSPC cultures in the present study.

Materials and methods

Human umbilical cord blood cells. Human UCB samples were collected from the umbilical cord vein after full-term delivery by the nursing staff of the Department of Obstetrics and Gynaecology at the Sint Franciscus Gasthuis (Rotterdam, The Netherlands). Informed consent for taking samples for clinical study was obtained. UCB was collected in sterile flasks containing 10 ml citrate-glucose as anticoagulant, stored at room temperature and processed within 24 hours of collection.

Isolation of CD34+ cells. Low-density cells were isolated using Ficoll Hypaque density centrifugation (1.077g/cm², Lymphoprep, Nycomed Pharma, Oslo, Norway). After centrifugation at 600 g for 15 minutes, the mononuclear cell (MNC) band at the interface was removed, washed twice with Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) and resuspended in Iscove’s modified Dulbecco’s medium (IMDM, Gibco, Breda, The Netherlands). The MNCs were stored in liquid nitrogen until use. After thawing and before the CD34+ hematopoietic progenitor cells were isolated we pooled 3 to 16 different UCB samples. The CD34+ cells were harvested from the mononuclear cells using Variomacs Immunomagnetic Separation System (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD34+ cells were labeled indirectly using a hapten-conjugated primary monoclonal antibody and an anti-hapten antibody coupled to MACS microbeads. The magnetically labelled cells were enriched by passing them twice through a Variomacs positive selection column to reach a final purity of > 90% CD34+ cells. A nuclear cell count was performed and the progenitor and stem cell numbers in the CD34+ selected UCB cells were assayed by CFC, CAFC and NOD/SCID repopulating cell assays. In addition, the immunophenotypic characteristics of the cells were assessed and part of the CD34+ cells were cultured for ten weeks.
Hemopoietic growth factors. The following cytokines were used: recombinant human stem cell factor (SCF) and FLT3-ligand (Flt3-L), both gifts from Amgen (Thousand Oaks, CA, USA), recombinant human interleukin 6 (IL6), recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF), recombinant human granulocyte colony stimulating factor (G-CSF) and recombinant murine stem cell factor (SCF), all gifts from Genetic Institute (Cambridge, MA, USA); recombinant human thrombopoietin (Tpo, a gift from Genentech, South San Francisco, CA, USA), recombinant human erythropoietin (Epo, Boehringer, Mannheim, Germany) and recombinant human IL3 (Gist Brocades, Delft, The Netherlands).

Extended long-term expansion cultures. Various culture conditions were used in 5 consecutive experiments. In the first experiment 5.0 x10^4 CD34+ UCB cells were cultured in 6-well tissue culture plates (Costar, Badhoevedorp, The Netherlands) in an 1 ml IMDM-cocktail containing 10% fetal calf serum (FCS, Summit, Fort Collins, CO), 1% bovine serum albumin (BSA, Sigma, Zwijndrecht, The Netherlands) supplemented with penicillin (100 U/ml, Gibco), streptomycin (0.1 mg/ml, Gibco), β-mercapto-ethanol (10^{-4} M, Merck, Darmstadt, Germany), bovine insulin (4 mg/ml, Gibco), cholesterol (15 μM, Sigma), linolic acid (15 μM Merck) iron-saturated human transferrin (0.62 g/l, Intergen, Uithoorn, The Netherlands), cytidine (10^{-3} g/ml, Sigma), adenosine (10^{-3} g/ml, Sigma), uridine (10^{-3} g/ml, Sigma), guanosine (10^{-3} g/ml, Sigma), 2’-deoxycytidine (10^{-3} g/ml, Sigma), 2’-deoxyadenosine (10^{-3} g/ml, Sigma), thymidine (10^{-3} g/ml, Sigma), 2’-deoxyguanosine (10^{-3} g/ml, Sigma) and the cytokines Flt3-L (50 ng/ml), Tpo (10 ng/ml), SCF (100 ng/ml), IL6 (100 ng/ml). The cells were cultured with the following combination of cytokines: Flt3-L + Tpo, Flt3-L + Tpo + SCF, Flt3-L + Tpo + IL6, Flt3-L + Tpo + SCF + IL6, or SCF + IL6. Cultures were set up using six to eight replicates of each cytokine combination. In the subsequent two studies 5.0 x10^4 CD34+ cells were cultured in tissue culture 25T-flasks (Falcon, Etten-Leur, The Netherlands) in 4 ml IMDM-cocktail containing 20% FCS with the same cytokine combinations. The effect of the murine FBMD-1 stromal layer on the in vitro repopulating ability was assessed for the same cytokine combinations. Insulin was not added in the stromal groups because it potently stimulated adipogenesis in FBMD-1 cells. Finally, in the last two studies 3.5 x10^4 and 4.0 x10^4 CD34+ cells were deposited on FBMD-1 stromal layer in tissue culture 25T-flasks in 4 ml IMDM-cocktail without BSA containing 20% FCS supplemented with the following cytokines: Flt3-L + Tpo, Flt3-L + Tpo + SCF, Flt3-L + Tpo + IL6 and Flt3-L + Tpo + SCF + IL6. The cytokines Flt3-L and Tpo were added twice a week during the culture period.
Umbilical cord blood hematopoietic stem and progenitor cells

The cultures were maintained with splitting of the cultured cells every one to two weeks starting from the second week of culture. When stroma-supported cultures had to be splitted, the adherent and non-adherent compartments of all corresponding dishes or flasks were pooled, and cells were reinoculated into culture on a newly preformed stroma. If at splitting a part of the cells were removed for analysis (on week 2 and 5), this was compensated in the calculations for total support. The cells were incubated at 37°C and 10% CO₂ in a humidified atmosphere and harvested at different time points by collecting the supernate of each well or flask, whereafter all groups were trypsinized with trypsin-EDTA (Life Technologies, Breda, The Netherlands) to include all the adherent cells. The content of the various wells or flasks belonging to a single group were pooled, centrifugated at 250 g for 10 minutes and resuspended in IMDM. Suitable aliquots of the output suspensions were assayed in CFC, CAFC and SRC assays for determination of progenitor and stem cell numbers. The immunophenotypic characteristics of the cultured cells were also assessed. Remaining cell numbers of the output suspensions were cultured further for analysis at later time points.

Clonogenic assay. Granulocyte-macrophage colony forming unit (CFU-GM) and burst forming unit-erythroid (BFU-E) progenitor cells were assayed using a semisolid culture medium (1.2% methylcellulose), containing IMDM supplemented with 10% FCS, β-mercapto-ethanol (5 x 10⁻⁵ M), penicillin (100 U/ml), streptomycin (0.1 mg/ml), human(hu)-Epo (1 U/ml), hu-IL3 (20 ng/ml), hu-GM-CSF (5 ng/ml), hu-G-CSF (50 ng/ml) and murine-SCF (100 ng/ml). Duplicate cultures were plated in 35 mm tissue culture dishes (Falcon) and incubated at 37°C and 10% CO₂ in a humidified atmosphere for 14 days. Colonies containing 50 cells or more were scored at day 14 using an inverted light microscope.

Cobblestone area forming cell assay. Confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates (Falcon) were overlaid with UCB cells in a limiting dilution set up as described [30]. Briefly, twelve successive two-fold dilutions were used for each sample with 15 wells per dilution. The percentage of wells with at least one phase-dark hematopoietic clone (cobblestone area) of at least five cells beneath the stromal layer was determined at week 2, and 6. At initiation of the cultures 250 CD34⁺ UCB cells per well were plated in the first dilution, while after cultures 200-1000 CD34⁺ input equivalent cells per well were used in the first dilution. The CAFC frequencies were calculated using Poisson statistics.

Immunophenotypic analysis. At least 5.0 x 10⁴ fresh or cultured CD34⁺ cells or femoral bone marrow cells of the NOD/SCID mice were stained with anti human CD45/CD33, CD34/CD38 or CD34/CD19 (fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-
conjugated) labeled monoclonal antibodies (Immunotech, Mijdrecht, The Netherlands). The cultured cells were also analyzed with anti human CD34, CD38 and CXCR4 (Becton Dickinson, Woerden, The Netherlands) using allophycocyanin (APC), FITC and PE-conjugated monoclonal antibodies, respectively. Human cells from the femoral bone marrow of the NOD/SCID mice were also stained with anti human CD15/CD33, CD20/CD19, CD3/CD4 and CD3/CD8 to analyse the multilineage differentiation. After incubation of the cells in phosphate-buffered saline (PBS, Life Technologies, Breda, The Netherlands) containing 0.5% BSA and 2% normal human serum for 30 minutes on ice, the cells were washed in PBS with 0.5% BSA and resuspended in 0.35 ml PBS. Just before the acquisition 7-aminoactinomycin (7-AAD, Molecular Probes, Leiden, The Netherlands) was added to each sample to determine the viability of the cells. APC, FITC and PE-conjugated mouse isotype antibodies were used as control for each group. At least 1.0 x 10⁴ events were acquired using fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson).

**NOD/SCID repopulating cell (SRC) assay.** The NOD/LtSz-SCID/SCID (NOD/SCID) mice, 8 to 13 weeks of age, were used as recipients of human cell grafts. The NOD/SCID mice were bred, maintained and used under specific pathogen free conditions in a laminar airflow unit, supplied with sterile food and acidified drinking water containing 100 mg/ml ciprofloxacin. All mice were sublethally irradiated with 3.5 Gy from a ¹³⁷Cs source (Gammacell, Atomic Energy of Canada, Ottawa, Canada) 1 to 4 hours prior to intravenous transplantation. At initiation of the experiment the mice were transplanted with 1.0 x 10⁵ CD34+ uncultured cells and at week 2 of cultures with 5.0 x 10⁴ input equivalent cells. At week 5 of culture each mouse was infused with 391, 195, 391, 195, 195 or 195 input equivalent cells for the groups Flt3-L + Tpo, Flt3-L + Tpo + SCF, Flt3-L + Tpo + IL6, Flt3-L + Tpo + SCF + IL6, SCF + IL6 or stroma-supported Flt3-L + Tpo, respectively, and at week 10 of culture with 73, 37, 73, 37, 37 or 37 input equivalent cells for the same groups, respectively. The number of mice used for each group varied from three to four. Six weeks after transplantation mice were killed by CO₂ inhalation and bone marrow cells were harvested by flushing all cells from two isolated femurs per animal. After counting, the cells were analyzed by FACS to determine the human cell engraftment in each mouse. Between 1.0 x 10⁴ and 1.0 x 10⁵ events were acquired for analysis. If the percentage of CD45+ cells was > 0.1% the mice were considered positive, while all mice with a percentage > 5% were analyzed for expression of lineage-specific markers.

**Statistical analysis.** The values are reported as mean ± 1 SD. The significance levels were determined by two-tailed Mann-Whitney test analysis.
Umbilical cord blood hematopoietic stem and progenitor cells

Results

Uncultured CD34+ UCB cells. The purity of the immunomagnetic selected cells, determined by FACS after staining with CD34-FITC conjugated monoclonal antibody, varied between 92% to 96%. The percentage of the primitive stem cells with the CD34+CD38- and CD34+CD38-CXCR4+ phenotypes, which are assumed to contain all BM repopulating ability, was 3 ± 1% (as percentage of total nc in five experiments) and 4% (as percentage of total CD34+ cells determined in one experiment), respectively. The mean number of clonogenic cells was 29,688 ± 9,781 CFU-GM and 10,500 ± 4,093 BFU-E per 10^5 input cells. The CAFC frequencies as a measure of transiently and tentative long-term repopulating stem cells, respectively, ranged from 6,230 to 23,566 and 3,988 to 9,997 per 10^5 input cells, respectively. To analyze the ability of uncultured CD34+ cells to give multilineage engraftment in the BM of NOD/SCID mice, 1.0 x 10^5 CD34+ UCB cells per mouse were injected i.v. into groups of sublethally irradiated mice. Six weeks after transplantation the mice were killed, BM cells harvested from both femurs and analyzed for the presence of different lineages of human hematopoietic cells using FACS. Hundred thousand CD34+ cells were able to multilineage engraft 100% of the mice. The percentage of CD45+ cells, as a marker for the presence of human hematopoietic cells in the murine BM, ranged from 1% to 19%.

Cultured CD34+ UCB cells. Stroma-free extended LTC. To assess the ability of serum-containing stroma-free cultures to support the growth of primitive hematopoietic progenitors including CD34+ subsets, CFC, CAFC and NOD/SCID repopulating ability (SRA), their content was determined in each sample before and during ten-week cultures in the presence or absence of cytokines. First we evaluated the influence of medium conditions for expansion of CD34+ cells. The addition of BSA gave a significant increase of the absolute number of nc, CD34+ subsets and CFU-GM (P< 0.05). While the absence of BSA resulted in loss of early progenitors, the presence of BSA was able to give maintenance or moderate expansion of these cells during the culture period (table 1). For this reason, data below represent the results obtained under BSA-containing conditions. Stroma-free culture of CD34+ UCB cells in the presence of different combinations of cytokines resulted in a dramatic expansion of nc and CD34+ subsets (figure 1). The fold-increase of the CD34+ phenotype as a measure of HSPC, and that of the CD34+CD38- phenotype as an indicator of quiescent and functionally primitive population of HSPC, varied from 14 to 1197 and 54 to 30,937, respectively, at week 10 of the cytokine-supplemented cultures. In general, there was a rapid increase in the fold-expansion of these parameters during the first few weeks of culture whereafter the rate of increase
Table 1. Expansion of various parameters during culture with Flt3-L + Tpo + IL6 under different medium conditions.

<table>
<thead>
<tr>
<th>Cultures in the presence (+) or absence (-) of BSA</th>
<th>2-week cultured</th>
<th>5-week cultured</th>
<th>10-week cultured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nucleated cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>9 ± 6</td>
<td>30 ± 18</td>
<td>43 ± 43</td>
</tr>
<tr>
<td>+</td>
<td>96 ± 14</td>
<td>113*</td>
<td>186 ± 70</td>
</tr>
<tr>
<td>CD34+ cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>3 ± 3</td>
<td>6 ± 4</td>
<td>4 ± 4</td>
</tr>
<tr>
<td>+</td>
<td>54 ± 8</td>
<td>37*</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>CD34+CD38- cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>69 ± 60</td>
<td>7 ± 7</td>
</tr>
<tr>
<td>+</td>
<td>54 ± 4</td>
<td>285*</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>CFU-GM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>1 ± 1</td>
<td>15 ± 7</td>
<td>9 ± 9</td>
</tr>
<tr>
<td>+</td>
<td>13 ± 3</td>
<td>117 ± 83</td>
<td>39 ± 13</td>
</tr>
<tr>
<td>CAFC week 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>3 ± 2</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>5 ± 1</td>
<td>9 ± 11</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

UCB cells were either uncultured, or propagated for two, five and ten weeks in the presence of absence of stroma and/or BSA. Values represent the mean fold-increase (± 1 SD) of 2-3 groups determined in four independent experiments (data from 10% and 20% serum containing cultures were pooled for each group). Abbreviations: * measured in 1 experiment.
Figure 1. Fold-expansion of absolute number of nucleated cells, CD34⁺ and CD34⁺CD38⁻ cells following ten weeks culture of CD34⁺ UCB cells under stroma-free and stroma-supported conditions with or without cytokines. Data represent the mean of 1-2 experiments performed. Mann-Whitney test: **denotes significant difference (\(P<0.005\) for CD34⁺ cells, \(P<0.05\) for CD34⁺CD38⁻ or CD34⁺CD38⁻CXCR4⁺ cells) between stroma-free and stroma-supported cultures in the presence of at least Flt3-L + Tpo.
Figure 2. Fold-expansion of absolute number of colony forming unit-granulocyte-macrophage (CFU-GM) and cobble stone area forming cell (CAFC) subsets following ten weeks of culture of CD34+ UCB cells under stroma-free and stroma-supported conditions with or without cytokines. Data represent the mean of 1-2 experiments performed. Mann-Whitney test: ** denotes significant difference (P<0.05 for CFU-GM, P<0.005 for CAFCweek2) between stroma-free and stroma-supported cultures in the presence of at least Flt3-L + Tpo.

became slower. Interestingly, the CD34+CD38+CXCR4+ cells as an estimation of the NOD/SCID marrow repopulating fraction of HSPC [31] showed a rapid increase until week 2 of stroma-free cultures, a plateau between week 2 and 5 and a very rapid decline to undetectable levels at week 10. The CFU-GM (figure 2) showed a continuous increase during the first five week of stroma-free culture whereafter their levels remained more or less constant, while the BFU-E were undetectable in all cytokine groups at all time
Umbilical cord blood hematopoietic stem and progenitor cells

points analyzed. Among the different in vitro parameters determined in extended LTC, the CAFC subsets showed the lowest levels and fastest disappearance in all cytokine groups under stroma-free conditions except in the Flt3-L + Tpo + IL6 group, where there was a continuous increase during stroma-free culture reaching almost 10-fold expansion at week 10. Interleukin 6 +/- SCF synergized with Flt3-L + Tpo to promote the early and late progenitor amplification in terms of CAFC subsets and CFU-GM. The addition of SCF to the cytokine combination Flt3-L + Tpo in stroma-free cultures resulted in loss of CFU-GM and CAFC subsets at week 10 of culture which was abrogated by the addition of IL6.

![Figure 3](image-url)

**Figure 3.** The percentage of human chimerism of uncultured CD34+ UCB cells and stroma-free and stroma-supported cultured CD34+ UCB cells in bone marrow of NOD/SCID mice six weeks after transplantation. Mice were infused with either 1.0 x 10^5 CD34+ uncultured cells or with 5.0 x 10^6. 195 to 391 and 37 to 93 1 input equivalent cultured CD34+ cells at week 2, 5 and 10 of culture, respectively. The values for each group represent the percentage of human cells (CD45+) in the femoral marrow. Data are mean (± 1 SD) of 3-4 NOD/SCID mice transplanted per experimental group. Mann-Whitney test: **denotes significant difference (P< 0.05) between SCF + IL6 and at least Flt3-L + Tpo containing groups.

In order to evaluate whether the expanded cells maintained their ability for multilineage engraftment in the NOD/SCID mice we studied the SRA of the cytokine-supplemented stroma-free cultured cells. In agreement with our previous observations, a considerable expansion of the ability of CD34+ UCB cells to engraft the BM of sublethally irradiated NOD/SCID mice was observed after two weeks of culture (figure 3). The percentage of human chimerism was 49 ± 42, 62 ± 29, 68 ± 29, 62 ± 27, 13 ± 10
Chapter 3

Table 2. Multilineage engraftment ability of CD34+ UCB cells in the bone marrow of NOD/SCID mice.

<table>
<thead>
<tr>
<th>Cell populations</th>
<th>Uncultured</th>
<th>Stroma-free</th>
<th>Stroma-supported</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flt3-L + Tpo</td>
<td>Flt3-L + Tpo + SCF</td>
<td>Flt3-L + Tpo + IL6</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=2)</td>
<td>(n=4)</td>
</tr>
<tr>
<td>CD34+</td>
<td>7 ± 3</td>
<td>7 ± 3</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>CD34+CD38+</td>
<td>0.6 ± 0.6</td>
<td>0.6 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>CD34+CD19+</td>
<td>5 ± 2</td>
<td>10 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>CD19+</td>
<td>63 ± 10</td>
<td>63 ± 10</td>
<td>73 ± 15</td>
</tr>
<tr>
<td>CD20+</td>
<td>8 ± 4</td>
<td>8 ± 4</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>CD33+</td>
<td>13 ± 2</td>
<td>13 ± 2</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>CD15+</td>
<td>13 ± 7</td>
<td>10 ± 5</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>CD3+CD4+</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>CD3+CD4+</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>CD3+CD8+</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

Cells infused into NOD/SCID mice were either uncultured, or propagated under stroma-free or stroma-supported conditions. The inocula were either 100,000 CD34+ cells/mouse for uncultured cells or 37 to 50,000 input equivalent cells/mouse for cultured cells. Values represent the mean (± 1 SD) percentage of human cells (CD45+) in the femoral marrow of 23 NOD/SCID mice with more than 5% human chimerism. Abbreviations: N, not detectable; n, number of mice per group.

for Flt3-L + Tpo, Flt3-L + Tpo + SCF, Flt3-L + Tpo + IL6, Flt3-L + Tpo + SCF + IL6 and SCF + IL6, respectively, at week 2 of culture. The percentage of human chimerism in the BM of mice infused with cultured cells ranged between 1 % and 90 % with no significant difference between the cytokine combinations when at least Flt3-L + Tpo was included. In all Flt3-L + Tpo containing groups, higher levels of human chimerism on average were detected in mice infused with cultured cells equivalent to 5.0 x10^4 input cells than in mice infused with 1.0 x10^5 uncultured CD34+ UCB cells. The cultures with SCF + IL6 alone led to human chimerism levels as high as that of uncultured CD34+ UCB cells and ranged between 1% and 20%. Addition of SCF +/- IL6 to the cytokine combination Flt3-L + Tpo did not significantly affect the expansion of the SRA after two weeks of culture.

The expression of lineage markers on human cells present in the femoral marrow of the mice at six weeks post-transplant showed no significant difference between the cultured and uncultured cells (table 2). Although we were able to expand the ability of UCB CD34+ cells to multilineage engraft the BM of NOD/SCID mice in two-week cultures, hardly or no detectable levels of human engraftment were observed in the mice transplanted when the CD34+ UCB cells had been cultured for five weeks or longer.

Stroma-supported extended LTC. To investigate whether stroma would improve the support of primitive HSPC, the CD34+ UCB cells were cultured on the murine BM-derived stromal cell line FBMD-1 using the same cytokine combinations and liquid
Umbilical cord blood hematopoietic stem and progenitor cells

culture conditions as used for stroma-free cultures except for insulin. Insulin was omitted from these cultures as it induced extensive adipogenesis and consequently affected the rigidity of the stromal layers. Under suboptimal culture conditions, e.g. using medium lacking BSA, stroma was required to increase the absolute number of CFC, CAFC and CD34+CD38− cells as compared to stroma-free culture (table 1). The nc, CD34+ subsets and CFU-GM expansions were significantly improved with the addition of BSA into the medium both under stroma-free and stroma-supported conditions (P< 0.05), as were the CAFCweek6. We have therefore included BSA, except otherwise specified, into the medium to improve the growth of the inoculated CD34+ cells in the remainder of the experiments. The stroma support without additional cytokines gave a maximum of 26, 161 and 74-fold increase of the CFU-GM, CD34+ and CD34+CD38− subsets respectively during the culture period of ten weeks, however, it did not lead to an expansion of CAFC subsets (figures 1 and 2). At weeks two and five of culture no statistically significant difference in HSPC parameters was observed between stroma-free and stroma-supported cultures in the presence of at least Flt3-L + Tpo. Interestingly, stroma support with or without cytokine addition improved the support of nc, CD34+, CD34+CD38− and CAFC subsets with at least one-log increase as compared to the corresponding stroma-free parameters at week 10 of culture, with the exception of the cytokine combination SCF + IL6 which showed similar (nc, CD34+) or decreased (CD34+CD38−, CFU-GM and CAFC subsets) levels of the HSPC parameters (figures 1 and 2). More interestingly was the observation that the CD34+CD38−CXCR4+ cells, which have been shown to represent the BM repopulating progenitors in uncultured grafts [31], required both stromal support and cytokines to expand for a time period of more than five weeks (figure 1). The stroma support gave a similar increase of CFU-GM as the corresponding stroma-free groups under Flt3-L + Tpo containing conditions (figure 2). In contrast to the stroma-free cultures, stroma support induced a continuous increase of CAFC subsets during the culture period when at least Flt3-L + Tpo was included, reaching a maximum of 89-fold (mean 72-fold) expansion with Flt3-L + Tpo + IL6 at week 10 of culture (figure 2). Additionally, the inclusion of SCF to the cytokine combination Flt3-L + Tpo under stroma-supported conditions showed a trend of diminished generation of CFU-GM and CAFC subsets as compared to the Flt3-L + Tpo group at week 10 of the culture (CFU-GM, 141 versus 977-fold; CAFCweek6, 10 versus 54-fold). This effect of SCF was abrogated by the addition of IL6. In conclusion, in Flt3-L + Tpo containing cultures stroma support dramatically increased the expansion of HSPC as assessed by CAFC and CD34+CD38−CXCR4+ cells. We previously reported that Flt3-L + Tpo were required and sufficient to give a comparable high SRA expansion in two-week cultures. We therefore
Figure 4. Comparative expansion of in vitro and in vivo HSPC parameters during ten weeks stroma-free and stroma-supported culture of CD34+ UCB cells. Data represent the mean of 2 independent experiments for CD34+CD38− and CAFCweek 6 and the mean of 4 mice at each time point analyzed within one experiment for estimated SRA. The calculation of SRA expansion has been performed as previously published [22].

evaluated only the stroma-supported Flt3-L + Tpo group using the NOD/SCID assay in this study (figure 3). The percentage of human chimerism was 62 ± 26% when 5.0 x10⁴ input equivalent cells were inoculated from Flt3-L + Tpo supplemented stroma-supported two-week culture, which was as high as under stroma-free conditions in the presence of at least Flt3-L + Tpo. A similar pattern of multilineage SRA expansion as compared to stroma-free cultures was observed with an increase at two weeks, whereafter hardly or no detectable levels of human engraftment with Flt3-L + Tpo propagated cells was observed.
Table 3. Lineage restriction within CD34\(^+\) subset before and after stroma-supported culture of CD34\(^+\) UCB cells.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Lineage restricted cells as % of CD34(^+) subset</th>
<th>CD2(^+)</th>
<th>CD19(^+)</th>
<th>CD33(^+)</th>
<th>CD14(^+)</th>
<th>CD15(^+)</th>
<th>CD41(^+)</th>
<th>GlyA(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncultured</td>
<td></td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>94 ± 5</td>
<td>N</td>
<td>3 ± 0</td>
<td>5 ± 3</td>
<td>12* N</td>
</tr>
<tr>
<td>2-week cultured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flt3-L + Tpo</td>
<td></td>
<td>9 ± 9</td>
<td>1 ± 1</td>
<td>92 ± 1</td>
<td>N</td>
<td>13 ± 0</td>
<td>3 ± 2</td>
<td>4 ± 0</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF</td>
<td></td>
<td>6 ± 6</td>
<td>N</td>
<td>92 ± 1</td>
<td>1 ± 1</td>
<td>9 ± 2</td>
<td>2 ± 1</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL6</td>
<td></td>
<td>10 ± 10</td>
<td>N</td>
<td>94 ± 0</td>
<td>4 ± 2</td>
<td>15 ± 0</td>
<td>N</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL6</td>
<td></td>
<td>8 ± 8</td>
<td>N</td>
<td>95 ± 0</td>
<td>3 ± 2</td>
<td>17 ± 4</td>
<td>N</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>5-week cultured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flt3-L + Tpo</td>
<td></td>
<td>N</td>
<td>N</td>
<td>81 ± 19</td>
<td>N</td>
<td>2 ± 1</td>
<td>N</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF</td>
<td></td>
<td>N</td>
<td>N</td>
<td>74 ± 25</td>
<td>1 ± 1</td>
<td>5 ± 1</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL6</td>
<td></td>
<td>N</td>
<td>N</td>
<td>74 ± 26</td>
<td>1 ± 1</td>
<td>11 ± 6</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL6</td>
<td></td>
<td>N</td>
<td>N</td>
<td>78 ± 22</td>
<td>4 ± 4</td>
<td>9 ± 2</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>10-week cultured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flt3-L + Tpo</td>
<td></td>
<td>N</td>
<td>N</td>
<td>83 ± 16</td>
<td>2 ± 1</td>
<td>4 ± 1</td>
<td>N</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF</td>
<td></td>
<td>N</td>
<td>N</td>
<td>60 ± 10</td>
<td>N</td>
<td>4 ± 2</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL6</td>
<td></td>
<td>N</td>
<td>N</td>
<td>43 ± 1</td>
<td>1 ± 1</td>
<td>3 ± 0</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL6</td>
<td></td>
<td>N</td>
<td>N</td>
<td>76 ± 23</td>
<td>2 ± 2</td>
<td>6 ± 3</td>
<td>N</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

UCB cells were either uncultured, or propagated for two, five and ten weeks under stroma-supported conditions with cytokines. Values represent the mean (± 1 SD) percentage of lineage restricted markers of two independent experiments within the CD34\(^+\) window. Abbreviations: N, not detectable; * measured in 1 experiment.

Comparative expansion of various parameters after extended LTC. In the present study, the presence of HSPC in uncultured and cultured CD34\(^+\) UCB cells was evaluated by different surrogate methods. Although the CD34\(^+\)CD38\(^-\) phenotype in uncultured material defines an immature population of progenitors and contains most long-term in vivo repopulating cells, the expansion of CD34\(^+\)CD38\(^-\) cells was not related to the CFC, CAFC and SRA present in the inoculum of cultured cells, both in stroma-free and stroma-supported conditions (figure 4). There was also no direct correlation between the number of CFC present in the inoculum of cultured cells and repopulation of sublethally irradiated recipients or CAFC\(_{w6}^\)\. This is not surprising, as it is known that CFC define a relatively mature population of progenitors lacking long-term repopulating ability. Also the CAFC\(_{w6}^\)\ which is considered to include long-term in vitro repopulating stem cell subsets, was not indicative for the in vivo repopulating ability. The expansion of CAFC\(_{w6}^\) estimated in the present study was maximally 17-fold at week 2 and 89-fold at week 10 of cultures. However, the estimated SRA expansion, as calculated previously [22], was approximately 14-fold at week 2, whereafter SRA was lost. In summary, our data show increasing incongruity with increasing culture time between
The expression of CD38 antigen after extended LTC. The incongruity of the dramatic expansion of CD34+CD38- cells and the limited increase in SRA and CAFC numbers during ex vivo propagation made us question whether the generated CD34+CD38- cells arose from the input CD34+CD38- cells or by loss of CD38 antigen expression on more lineage-restricted cell populations. Therefore, we assessed immunophenotypically the expression of lineage markers within the CD34+CD38- cell population during the ten-week culture period and determined the corresponding progenitor content of UCB CD34+ cells under BSA-lacking stroma-supported conditions in the presence of at least Flt3-L + Tpo. The UCB CD34+ cells maintained in vitro their ability to differentiate into myeloid cells (CD33+), monocytes (CD14+) and granulocytes (CD15+) for ten weeks (table 3). The CD34+ cells co-expressing CD2 (T-lymphocytes, natural killer cells), CD19 (B-lymphocytes), CD41 (megakaryocytes) and CD71 (transferrin receptor) were lost with increasing culture time and erythroid cells (GlyA+) were never detected at the
Umbilical cord blood hematopoietic stem and progenitor cells

time points analyzed. The CD34+CD38- cells were considered lineage positive (Lin+) if they additionally expressed at least one of the following markers CD2, CD14, CD15, CD33, CD41, CD71 or GlyA. Before culture the CD34+CD38- cells did not express any of the above mentioned lineage markers. However, during culture the majority of the CD34+CD38- cells co-expressed the lineage marker CD33. At two, four and ten weeks of culture the CD34+CD38- cells expressing CD33 antigen ranged between 15 to 27%, 2 to 40% and 17 to 72%, respectively (table 4). No significant difference in expression of the detected markers was observed between the different cytokine combinations used. These observations clearly indicate that many CD34+CD38- cells were in fact lineage marker expressing cells that failed to express the CD38 epitope.

Discussion

In the present study we have evaluated the ability of a murine BM-derived stromal cell line to increase maintenance and/or undifferentiated expansion of UCB CD34+ cells in extended cultures. We show that both stromal support and the inclusion of BSA dramatically improved the expansion of CAFC subsets in cultures exceeding a time period of two weeks and that important differences exists between the used stem and progenitor cell assays. Especially, the enumeration of CD34+ subsets and CFC generation greatly overestimated the expansion of CAFC or SRA at the time points analyzed, confirming that the different surrogate methods to quantitate HSPC are unlikely to detect identical populations after culture.

There is extensive data on the supportive capacity of stromal cells on the maintenance of primitive stem cells and their ability to generate progenitor cells in long-term cultures [32]. The plethora of cytokines that have become available in the last decennium has offered an attractive alternative for the use of stroma in ex vivo expansion of stem cells for clinical applications. However, the combination of stroma support and cytokines has been insufficiently explored, but potentially may lead to dramatic improvement of the longevity of repopulating characteristics of HSPC as is the case when Tpo is induced in stroma-supported BM cultures [29]. In the absence of exogenous cytokines, proliferation of HSPC in the presence of BM-derived stroma is associated with loss of most in vivo repopulating ability of hematopoietic progenitors [25, 33, 34]. Brandt et al. showed that three weeks stroma-free culture of CD34+Thy-1+ human BM cells with lineage-negative markers resulted in impairment of engraftment ability in NOD/SCID mice, which could be overcome by coculture with a layer of porcine microvascular endothelial cells [28].
We and others have previously reported that stroma-conditioned medium enhanced the expansion of primitive HSPC from CD34+ selected mobilized peripheral blood in short-term cultures, while stroma-support was also required for optimal maintenance and graft quality [12, 35, 36]. In addition, Xu et al. have shown that CD34+CD38- UCB cells could maintain their in vivo repopulating ability during a four-week culture period [37] when supported by an embryonic stromal cell line derived from the murine aorta-gonad-mesonephros region, a location currently considered to generate the first definitive HSPC. There is still insufficient data on which factors contribute to maintenance and/or expansion under stromal conditions. O-sulphated GAGs have been shown to aid the long-term maintenance of LTC-IC under stroma noncontact conditions [38], while LIF have been suggested to be responsible for maintaining and expanding transplantable CD34+Thy1+ cells in stroma-supported cultures through an action on the stroma [39]. It is likely, that additionally cell adhesion molecules and extracellular matrix molecules contribute to the specific contextual interaction between stem cells and the stromal environment, and that chemotactic factors as SDF-1 and specific lipids help to guide and maintain HSPC in their niches [40-44]. The present study indicate that stroma and cytokines are required to obtain expansion of CAFC when cultured for more than two weeks. Also the CD34+CD38-CXCR4+ cells representing the NOD/SCID repopulating cells [31] required both stromal support and cytokines to expand over a time period of five weeks. Our data contrast with the previous data published by Piacibello and colleagues indicating no stromal requirement in expansion of LTC-IC for more than six months and of SRC for ten weeks. In initial studies these authors used FCS in stroma-free long-term cultures for evaluation of optimal conditions which resulted in massive and prolonged expansion of in vitro hematopoietic progenitors (CFC) and more primitive hematopoietic stem cells (LTC-IC) from UCB CD34+ cells [19, 20]. Secondly, they switched to pooled human serum as a substitute for FCS in order to make a step further toward a clinical application and assessed whether the combination of early acting cytokines with best results could maintain and amplify SRC [21]. The latter resulted in an increase of more than 70-fold the input SRC after a culture period of nine to ten weeks. So, the differences observed between these and our extended long-term data may partly be attributable to the culture conditions used with special reference to serum batches or the source of serum used. Also the sources of cytokines and the cell purification procedures may have contributed to the contradictory results.

Previous attempts to expand stem cells in vitro have also emphasized the role of the early acting cytokines Flt3-L, Tpo, SCF and IL6 in regulation of HSPC growth and survival [8-11, 45-47]. Using combinations of these cytokines we observed synergistic
Umbilical cord blood hematopoietic stem and progenitor cells

effect in support of early progenitors for extended periods of ten weeks if at least Flt3-L + Tpo were included. While we observed that the use of cytokines alone expanded early progenitors, and the use of stroma alone decreased their numbers, the combination of stroma and cytokines greatly increased the maintenance and production of HSPC in our extended LTC. The effect of cytokines could be partly explained by a direct effect on HSPC, however, they are also likely to act indirectly by an effect on stroma. Human Flt3-L, Tpo and IL6 are known to exhibit cross-species activity on mouse cells. Unpublished observations in our lab indicate that the FBMD-1 cell line does not express murine c-kit (receptor for SCF) and FLT3 (receptor for Flt3-L), however, expresses the murine IL6-receptor-α and c-mpl (Tpo receptor), while human Tpo down modulates IL6-receptor-α expression on these cells. These data substantiate our contention that at least the cytokines Tpo and IL6 used in the present study might have reacted on both HSPC and stroma.

Interestingly, in SCF + IL6 stimulated cultures, stroma decreased the expansion magnitude of CD34+ and CD34+CD38− cells, CFU-GM and CAFC subsets as compared to the stroma-free conditions. A possible explanation for this observation is that the exogenous soluble ligand SCF may compete with the stroma membrane-bound SCF for c-kit receptors on HSPC, assuming that the interaction with the membrane-bound SCF results in the modification of the most effective signaling pathways in maintaining HSPC. The importance of membrane-bound SCF in hematopoiesis, fertility and skin pigmentation has been documented in studies on the anemic mouse traits W/Wv and Sl/Sl [48, 49]. An alternative explanation for this SCF effect on primitiveness of HSPC observed in the presence of stroma is that SCF induces differential expression of adhesion molecules on HSPC and/or stromal cells, which in turn could cause different stem cell subsets to contact different adherent cell compartments capable of differently affecting the HSPC self-renewal.

In agreement with our previous observation [22], human cells were found in the BM of NOD/SCID mice following transplantation with two-week cultured cells. These cells showed multilineage engraftment in vivo, and no significant difference between the cytokine combinations used was observed if at least Flt3-L + Tpo was included. The extended cultivation time and consequent dilution of input equivalent stem cells over time prohibited the use of a limiting dilution setup for calculating the numbers of SRC present in our samples. Instead, we have used the remaining cells to infuse limited numbers of NOD/SCID recipients to arrive at meaningful repopulation percentages. For this reason we were not able to precisely determine the fold expansion of SRC, but rather calculated an estimated increase in the ability of the infused cells to repopulate the BM.
of the recipient mice with human hematopoietic cells as previously published [22]. The very high percentages of human cells (over 90 percent) in some groups having received cultivated cells indicates, that we might have reached the maximal human chimerism percentage in the NOD/SCID assay, which in turn suggests that our SRA calculations are conservative. While at week 2 the expansion of SRA was similar under both stroma-free and stroma-supported conditions, after five weeks of culture cells were hardly (Flt3-L + Tpo + IL6 group) or not at all able to give detectable levels of human engraftment in the mice. Possibly, this low SRA is due to a loss of NOD/SCID repopulating cells (SRC), or due to their differentiation. It is not yet clear whether inclusion of FCS has an inhibitory or beneficial effect on the maintenance or expansion of SRC [50, 51]. However, using 10% or 20% FCS we recently obtained expansion of in vivo repopulating ability of UCB CD34+ cells [22] and present study). It is very likely that the concentration or batch of FCS used may be responsible for different results. Alternatively, SRC in the current study may have been diluted out as limited numbers of input equivalent cells were infused at weeks 5 and 10 of the culture with 37 to 391 input equivalent cells infused per mouse, when compared with the two-week time point where we transplanted 5.0 x10^4 input equivalent cells per mouse. As a fourth alternative, it could be that more than two-week cultured cells show a decreased ability to home to and to expand in the BM of NOD/SCID mice. A study by Peled et al. showed that the chemokine stromal cell-derived factor (SDF)-1 and its receptor CXCR4, which is expressed on some CD34+CD38- cells amongst others, may be important for engraftment of sublethally irradiated NOD/SCID mice, although there is evidence that CXCR4 is heterogeneously expressed on SRC [31, 52]. The cytokines SCF and IL6 induced CXCR4 up-regulation on CD34+ cells, which subsequently potentiated migration of these precursor cells and their engraftment in primary and secondary transplanted mice. Thus, the use of at least SCF +/- IL6 in the present study might have caused increased homing potential of the CD34+ UCB cells in two-week cultures, which in turn contributed to the increased SRA.

It is generally accepted that the CD34+CD38- phenotype in uncultured material defines an immature population of progenitors and includes many CAFC\_week6/LTC-IC and all long-term in vivo repopulating cells. However, in the present study the expansion of CD34+CD38+(CXCR4+) cells was not related to the CAFC and SRA after culture. These results are in agreement with previously published data indicating a differential maintenance of LTC-IC and SRC during stromal or endothelial-based cultures in three-week cultures of UCB or BM cells [23, 34]. Recently, also others reported a large expansion of CD34+CD38- cells (166-fold) during retroviral transduction period of 2 to 6 days without an increase in SRC numbers indicating a dissociation between the
primitive CD34⁺CD38⁻ phenotype and SRC function [51]. Overall, our data indicate
that the different surrogate methods to quantitate HSPC show great incongruency
with increasing culture time, in which especially phenotypic characterization and the
CFC assay seem to greatly overestimate the *in vivo* and *in vitro* repopulating ability of
cultured cells at the time points analyzed. One of the essential issues that we wished
to clarify was whether the dramatic CD34⁺CD38⁻ expansion during culture arose from
the input CD34⁺CD38⁻ cells or by downregulation of CD38 antigen expression within
a population of cultured CD34⁺CD38⁺ cells that no longer contained any long-term
repopulating ability, both *in vitro* and *in vivo*. While preparing this manuscript, Von
Lear *et al.* demonstrated for the first time that during four days of stroma-free culture
the CD34⁺CD38⁺ cells lost the CD38 antigen while remaining CD34⁺ resulting in an
overestimation of the expansion of the CD34⁺CD38⁻ population [53]. In our study we
evaluated immunophenotypically the expression of lineage-restricted markers within
the CD34⁺CD38⁻ cell population under BSA-lacking stroma-supported conditions
during ten weeks of culture. Remarkably, although before culture the CD34⁺CD38⁻ cells
were all Lin⁻, after culture the majority of the CD34⁺CD38⁻ (> 70%) cells co-expressed
lineage restricted markers. These observations clearly indicate that this phenotype is not
a reliable indicator for HSPC assessment after culture.

**Acknowledgements**

We would like to acknowledge the nursing staff and midwives of the St. Fransiscus
Gasthuis for collection of UCB samples, and P.B. van Hennik, W.J.C. Rombouts and I.
Blokland for technical assistance.
Chapter 3

References


6. Verfaillie CM. Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. Blood 1993; 82 (7): 2045.


Umbilical cord blood hematopoietic stem and progenitor cells


Chapter 4

Gp130-Signaling synergizes with Flt3-L and Tpo for the long-term expansion of cord blood progenitors.
Umbilical cord blood hematopoietic stem and progenitor cells

Abstract

We investigated the effect of a new fusion protein of IL6 and the soluble IL6R, H-IL6, on the long-term ex vivo expansion of hematopoietic progenitors derived from AC133+ cord blood cells. H-IL6, which acts on both IL6Rα-positive and IL6Rα-negative cells, effectively synergized with Flt3-L and Tpo with or without SCF for the propagation of primitive progenitors. However, IL6 showed a greater synergistic effect with Flt3-L and Tpo than H-IL6 for long-term progenitor propagation. During the first six weeks of culture under stroma-free serum-containing conditions, IL6 induced a 1.96 ± 0.64-fold higher expansion of nucleated cells, a 2.28 ± 0.33-fold higher expansion of CD34+ cells and a 2.74 ± 0.28-fold higher expansion of CD34+AC133+ cells than H-IL6 in combination with Flt3-L and Tpo. The propagation of CAFC<sub>week</sub> was up to four-fold higher in the presence of IL6 than with H-IL6. While the expansion of CD34+ and CD34+AC133+ cells dropped after five-seven weeks in the stroma-free cultures with Flt3-L, Tpo and H-IL6, a sustained expansion for twelve weeks was obtained in the presence of Flt3-L, Tpo and IL6. Stroma-contact greatly enhanced the progenitor expansion induced by Flt3-L and Tpo or Flt3-L, Tpo and H-IL6 although the highest proliferation was again obtained in the presence of IL6. In contrast, the presence of SCF resulted in increased differentiation. Since the majority of primitive progenitors are proposed to be IL6Rα negative, the results suggest that the synergistic effect of IL6 is mediated by accessory cells, which have been more effectively stimulated by IL6 than by the fusion peptide, H-IL6, in this culture system.

Introduction

Over the past 5 years, umbilical cord blood (UCB) has become an important alternative source of hematopoietic stem cells for transplantation and gene transduction protocols [1], since it is highly enriched for hematopoietic progenitors compared to bone marrow (BM) and adult peripheral blood (PB) [2, 3]. However, due to the small volume and the high variability in stem cell numbers, a single UCB collection is often considered to be insufficient to secure marrow engraftment in adults, and so far, UCB transplantations have been limited to pediatric patients. Since UCB is available without donor risk, and has the advantage of reduced risks for graft-versus-host disease and infections [3], it would be of great benefit if UCB-derived long-term repopulating cells could be expanded in vitro. Such expansion studies might also improve our current understanding
Chapter 4

of the regulation of hematopoiesis and open the way for new strategies in the treatment of hematological disorders and for the optimization of PB mobilization protocols.

High proliferation rates accompanied by a rapid differentiation of primitive progenitor cells have been obtained with previous \textit{ex vivo} expansion systems [4-6]. More recently, several groups have reported the qualitative and quantitative maintenance \textit{in vitro} of engrafting stem cells [7-9], and there is now evidence for a slight, but net increase in SCID repopulating cells during \textit{ex vivo} culture [10-12]. However, the culture conditions and growth factor combinations used to promote stem cell maintenance and proliferation differ substantially, although many of the cytokine cocktails include Flt3 ligand (Flt3-L), KIT ligand (SCF) and IL3 [11, 13-18]. Recent studies indicate that the c-mpl ligand, Tpo, may also be a potent agent to support the survival and expansion of primitive hematopoietic progenitors [19-23]. In fact, Piacibello \textit{et al.} [24, 25] have described the extensive amplification of primitive human UCB-derived progenitor cells, using the single growth factor combination of Flt3-L and Tpo.

Other reports emphasize an important role for the multi-functional cytokine IL6 in the regulation of hematopoiesis [11, 22, 26, 27]. This pleiotropic molecule has been shown to act synergistically with IL3 and SCF to enhance the proliferation of human hematopoietic progenitor cells [28, 29]. The intracellular portion of the IL6Rα is very short and signal transduction requires an associated 130-kDa (gp130) molecule that lacks IL6 binding activity [30]. This transducer unit is also shared by receptors for IL11, leukemia inhibitory factor (LIF), oncostatin M (OM), ciliary neurotrophic factor (CNTF), and cardiotrophin 1 (CT-1), and therefore explains the overlapping activities of these cytokines. Binding of IL6 or IL11 to their respective receptors leads to the homodimerization and activation of gp130, while LIF, OM, CNTF, and CT-1 induce a gp130 heterodimerization [30]. Interestingly, gp130-deficient mouse embryos show greatly diminished numbers of hematopoietic progenitors, thereby underlining the importance of this signal transduction pathway in the regulation of hematopoiesis [31]. Gp130 is ubiquitously expressed on blood progenitors, whereas the expression of IL6Rα seems to be limited to approximately 30-50\% of CD34+ UCB and 80\% of CD34+ PB cells [32, 33]. These CD34+IL6Rα+ cells are predominantly myeloid precursors. In contrast, erythroid and megakaryocytic progenitors as well as long-term culture initiating cells (LTC-IC) have been found to be enriched in the CD34+IL6Rα+ UCB fraction and to lack responsiveness to IL6 [32]. However, a soluble form of IL6Rα (sIL6R) has been shown to complex with IL6 and to act agonistically on gp130+IL6Rα+ cells [34]. In vitro short-term culture studies, using a combination of IL6 and sIL6R, report a dramatic expansion of primitive hematopoietic cells as well as an erythropoietin (Epo)-independent
Umbilical cord blood hematopoietic stem and progenitor cells

erthrocyte production, and a trilineage blood cell generation in the presence of SCF and IL3 [32, 35, 36]. Similar results have been obtained using a combination of IL6, sIL6R and Flt3-L although the synergistic action was weaker than with SCF [37]. Recently, the complex of IL6 and sIL6R has also been shown to stimulate megakaryopoiesis in the presence of SCF or IL3 and Tpo [38]. The importance of this IL6/sIL6R complex is further demonstrated in IL6/sIL6R double transgenic mice, which develop a marked extramedullary hematopoiesis in the spleen and liver. It has been hypothesized that the IL6/sIL6R complex is able to expand primitive resident stem cells in these primary hematopoietic organs in vivo [34, 39, 40].

An important objective of the present study was to investigate the potential benefit of the new designer recombinant IL6 and sIL6R fusion protein, H-IL6, for the ex vivo expansion of hematopoietic UCB progenitors. Using stroma-free and stroma-supported long-term cultures, we compared several cytokine combinations in the presence or absence of this chimeric protein or with IL6 and estimated the progenitor output by multiparameter FACS analyses and CAFC (cobblestone area forming cell) assays. Our results indicate that H-IL6 effectively synergizes with Flt3-L and Tpo with or without SCF for the expansion of CD34+ and CD34+AC133+ progenitors including primitive CAFCwock6. However, in this respect, the action of the H-IL6 fusion peptide was less potent than that of the full-length recombinant human IL6 under our culture conditions.

Materials and methods

Cells. UCB samples were obtained from the placenta of full-term deliveries at the St Franciscus Hospital, Rotterdam, The Netherlands, after informed consent of donors and with the permission of the local ethical committee. The cells were separated over a Ficoll-Hypaque density gradient (1.077 g/ml; Nycomed Pharma, Oslo, Norway) and mononuclear cells (MNC) were either processed directly or cryopreserved at -196°C in 10% (vol/vol) dimethylsulphoxide (DMSO), 20% fetal calf serum (FCS; Summit, Beunde Ronde), and 70% Hank’s balanced salt solution (HBSS; Gibco-BRL, Breda, The Netherlands).

Cell purification. Fresh or thawed UCB MNCs were purified for AC133+ or CD34+ cells using the VarioMACS Immunomagnetic Separation System (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s protocol. The purity assessed by FACS analysis ranged between 75% (single-selected) and 99% (double-selected). The recovered cells were either directly cultured or further purified for
Chapter 4

CD34+CD38-, CD34+AC133+, CD34+AC133· and CD34-AC133+ cells by FACS sorting using standard protocols (see below).

**Antibodies.** The following monoclonal antibodies (MoAbs) were used in this study: AC133-PE (clone AC133; Miltenyi Biotec), CD34-APC, -PE, -FITC (clone 581), CD38-PE, -FITC (clone T16), CD14-PE (clone RMO52), CD15-FITC (clone D3HL60.251), CD41-FITC (clone P2), CD45-FITC, -PE, -FITC (clone J4.119), CD41-FITC (clone J33), CD71-FITC (clone YDJL2.2), GlycA-PE (clone 11E4B7.6; all from Coulter Immunotech, Mijdrecht, The Netherlands), CD90-PE (clone 5E10; PharMingen, San Diego, CA, USA), HLA-DR-PE, -FITC (clone L243), IgG1-FITC, -PE, -APC (all from Becton Dickinson, San Jose, CA, USA).

**FACS analysis and FACS sorting.** FACS analyses and FACS sorting were performed on a FACS-Calibur flow cytometer (Becton Dickinson) equipped with an argon laser tuned to 488 nm and a helium neon laser tuned to 630 nm using Cellquest software (Becton Dickinson). Cell labeling was carried out either in 96-well round-bottom plates (non-sterile staining) or in sterile polystyrene tubes. Immunoglobulin binding sites were blocked with 10% (vol/vol) human serum and the cells were labeled on ice with the respective antibodies for 25 min in HBSS containing 0.5% (wt/vol) BSA ± 0.1% (wt/vol) sodium azide. The stained cells were washed twice, resuspended in HBSS, and 1 mg/ml 7-amino-actinomycin D (7-AAD; Molecular Probes, Leiden, The Netherlands) was added for viable cell selection prior to analysis and sorting. Non-specific background staining was determined using cells labeled with isotype-matched control antibody conjugates. Sorted cells were collected in 50 ml tubes pre-filled with 5 ml FCS and one sorted sample per sorting gate was re-analyzed to verify cell purity (85-97%).

**Stroma-free cultures.** Cells were cultured in Cell Gro SCGM (Cell Gro; Boehringer Ingelheim Bioproducts, Verviers, Belgium) supplemented with 10% (vol/vol) FCS or 10% (vol/vol) human AB serum. The medium was supplemented with 10^{-4} M β-mercaptoethanol (β-ME; Merck, Darmstadt, Germany), 100 U/ml penicillin, 50 mg/ml streptomycin (Gibco-BRL), and various recombinant human growth factor combinations at final concentrations of 100 ng/ml rhFlt3-L (Amgen, Thousand Oaks, CA, USA), 10 ng/ml huTpo (Genetech, San Francisco, CA, USA), 1-20 ng/ml H-IL6 [41], 100 ng/ml rhIL6 (Genetics Institute, Cambridge, MA, USA), 100 ng/ml huSCF (Amgen) and 50 U/ml rhIL11 (Genetics Institute). The cytokines were added weekly, except for Flt3-L and Tpo, which were added twice per week. 5 x 10^4 AC133+ or CD34+ cells were plated in 6-well plates (Corning Costar, NY, USA) in duplicate or triplicate in 1 ml medium with the respective cytokine combinations to initiate the cultures. At weekly intervals, the cells were harvested by rinsing the wells twice with HBSS. All cells from each group...
Umbilical cord blood hematopoietic stem and progenitor cells

were pooled, demidepopulated by reducing the volume by 50% (or 33% at week 1), and replated in fresh medium. The remaining cells were analyzed for viability (using trypan blue), cell number immunophenotype, and for CAFC and CFC content. Cell samples were also cytocentrifuged at various time points and stained with MayGrunwald-Giemsa for morphological analysis. Single-cell cultures were performed in round-bottom 96-well plates (Falcon, Becton Dickinson). Sorted CD34⁺CD38⁻ cells were diluted to a concentration of 0.7 cells/100 ml in CellGro SCGM with 10% (vol/vol) FCS. The cell suspension was mixed well, aliquoted, supplemented with the various cytokine combinations and seeded into microtiter plates at a volume of 100 ml per well (60 wells per plate). Fresh medium and cytokines were added every 10 days. The number of cells in the single wells was scored weekly using an inverted microscope. Cluster and clones up to 100 cells were counted, larger clones were estimated according to their surface occupancy. All cultures were maintained in a humidified atmosphere of 10% CO₂ at 37°C.

Stroma-contact cultures. AC133⁺ cells were seeded in duplicate at a concentration of 5 x 10⁴ cells per ml in 6-well plates previously coated with murine FBMD-1 stromal cells. Every week the cells were harvested by collecting first the non-adherent fraction and then carefully trypsinizing the adherent cells. Both fractions were pooled and suspended in fresh CellGro SCGM medium without insulin (supplemented with penicillin/streptomycin and β-ME). Half of the volume was replated on a new confluent FBMD-1 layer, and supplemented with the respective cytokines, while the other half was used for analyses.

CAFC and CFC assays. CAFC assays were performed as follows: confluent stromal layers of the mouse FBMD-1 cell line in 96-well plates were overlaid with primary MACS-separated or ex vivo cultured cells in a limiting dilution assay. Twelve dilutions, two-fold apart, with 8-15 replicate wells were prepared in IMDM (Iscove's modified Dulbecco's medium; BioWhittaker, Boehringer Ingelheim, Verviers, Belgium) supplemented with 1% (vol/vol) Glutamax (GibcoBRL), 10⁴ M β-ME, 100 U/ml penicillin, 50 mg/ml streptomycin, 10% FCS (Summit), 5% horse serum (Integro, Zaandam), 10⁻⁵ M hydrocortisone 21-hemisucc-cinate (Sigma), 10 ng/ml rhIL3 (Gist Brocades, Delft, The Netherlands) and 20 ng/ml rhG-CSF (Amgen). Half of the medium was replaced weekly with fresh medium supplemented with cytokines as described above. The cell input values ranged between 1.0 x10² and 2.0 x10⁴ cells per well. The percentage of wells with at least one cobblestone area (minimum of five cells) beneath the stroma layer was determined after two, four and six weeks, and CAFC frequencies were calculated using Poisson statistics. The clonal cell cultures were plated in duplicate.
in 35-mm petri dishes (Becton Dickinson) in 1 ml methylcellulose-based medium consisting of: 1.2% methylcellulose (Methocel, Stade, Germany), 30% (vol/vol) FCS, 10^{-4} M β-ME, 1 U/ml recombinant human (rh) Epo (Boehringer Mannheim, Mannheim, Germany), 10 ng/ml rhIL3 (Gist Brocades), 5 ng/ml rhGM-CSF (Genetics Institute), 20 ng/ml rhG-CSF (Amgen), 100 ng/ml murine rSCF (Genetics Institute) in IMDM. AC133+ and CD34+ cells were plated at a concentration of 50 cells per dish and cultured cells at a concentration of $1.0 \times 10^2$ to $2.0 \times 10^4$ cells per dish. Granulocyte/macrophage colony-forming (CFU-G/M) pro-genitor numbers were scored after 14 days at 37°C and 5% CO₂.

Statistical analysis. Results were expressed as means (± SD) of data obtained from three or more experiments, unless otherwise specified. Significance levels were determined using the Student’s t-test.

Results

The H-IL6 fusion protein increases the expansion of CD34+ and CD34+AC133+ progenitors in the presence of Flt3-L and Tpo. The combination of Flt3-L and Tpo has recently been described as inducing an extensive amplification and ’self-renewal’ of UCB-derived ‘stem cells’ under serum-containing conditions [24, 25]. We therefore analyzed various concentrations of the H-IL6 fusion protein for its ability to enhance progenitor output in liquid cultures supplemented with Flt3-L and Tpo in the presence of 10% serum. As shown in figure 1, the expansion of CD34+ progenitor cells over a four-week period increased in a H-IL6 dose-dependent manner, reaching maximal values at 3 ng/ml H-IL6, when assayed using FACS analyses. As a single agent H-IL6 failed to induce an expansion of CD34+ cells (data not shown). In order to evaluate the effect of the fusion protein in more detail, AC133+ and CD34+ UCB cells, separated using MiniMACS columns, were grown in Flt3-L and Tpo containing suspension cultures in the presence or absence of H-IL6 in a defined serum-free medium (CellGro SCGM) supplemented with FCS concentrations varying from 0% to 20%. The progenitor output over a period of five-six weeks was estimated using CFC and CAFC assays and FACS analyses. The proliferation of nucleated cells and CD34+ cells increased in a FCS concentration-dependent manner reaching plateau values at 10% FCS. Figure 2 shows the results of a representative experiment analyzing the expansion of nucleated cells, CD34+ cells and CD34+AC133+ cells from AC133-separated or CD34-separated UCB cells during five-six weeks of culture in the presence of 10% FCS and Flt3-L plus
Tpo with or without 3 ng/ml H-IL6. In three independent experiments, Flt3-L and Tpo induced an 86.04 ± 45.10-fold increase in nucleated cell numbers, a 10.58 ± 5.19-fold increase in CD34+ cells, and a 6.29 ± 2.29-fold increase in CD34+ AC133+ cells, all derived from AC133-separated UCB cells. In the presence of the fusion protein, a 384.0 ± 170.54-fold expansion of nucleated cells, a 68.41 ± 35.91-fold expansion of CD34+ cells, and a 37.69 ± 25.65-fold expansion of CD34+AC133+ cells was obtained from AC133-separated UCB cells (i.e. a respective 4.5-fold, 6.5-fold and six-fold higher expansion than for Flt3-L plus Tpo alone, P< 0.01 in all cases). The expansion potential of the CD34-separated UCB cells, derived from the same donor pools, was lower than that of the AC133-separated cells, and the addition of H-IL6 to the combination of Flt3-L and Tpo enhanced the CD34+ and CD34+AC133+ output only three- and four-fold, respectively. Flt3-L and Tpo, in the presence or absence of H-IL6, were not sufficient to increase total cell numbers for longer than two weeks at serum concentrations lower than 5% (data not shown). Furthermore, no maintenance or expansion of primitive CAFC_{week6} could be obtained using this medium without serum addition (data not shown). The growth-promoting effect of H-IL6 on progenitor cells is also illustrated in a typical
Figure 2. Expansion of nucleated cells (nc), CD34+ cells and CD34+AC133+ cells from AC133-separated (—) and CD34-separated (---) pooled UCB cells in the presence of Flt3-L (100 ng/ml) and Tpo (10 ng/ml) with or without H-IL6 (3 ng/ml).

FACS analysis in figure 3. While the percentage of CD34+AC133+ and CD34+AC133- cells rapidly decreased after one-week of culture if only Flt3-L and Tpo were present, a slower decrease was found in cultures supplemented with the H-IL6 fusion protein. In three independent experiments, the percentage of CD34+ cells after six weeks of culture was 7.2 ± 4.2-fold higher in the presence of H-IL6 than in its absence (P= 0.001).
Figure 3. Flow cytometric analyses of AC133-separated UCB cells before and during ex vivo culture with Flt3-L and Tpo in the presence or absence of H-IL6. Cells were stained with CD34-APC and AC133-PE. The quadrants were set according to negative control samples labeled with IgG1-PerCP and IgG1-PE.
Table 1. Ex vivo expansion of CFU-GM and CAFC in liquid cultures with Flt3-L and Tpo in the presence or absence of H-IL6.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Week</th>
<th>Fold-expansion of progenitor cells compared with input numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>wk2 CFU-GM</td>
</tr>
<tr>
<td>Flt3-L + Tpo</td>
<td>wk3</td>
<td>14.70 ± 5.43</td>
</tr>
<tr>
<td></td>
<td>wk6</td>
<td>3.10 ± 1.19</td>
</tr>
<tr>
<td>Flt3-L + Tpo + H-IL6</td>
<td>wk3</td>
<td>36.90 ± 4.78</td>
</tr>
<tr>
<td></td>
<td>wk6</td>
<td>50.0 ± 7.01</td>
</tr>
</tbody>
</table>

AC133⁺ UCB cells were grown in serum-containing cultures supplemented with Flt3-L and Tpo in the presence or absence of H-IL6. After three and six weeks of culture fractions of cells were plated in methylcellulose for CFU assays and stroma-coated 96-well plates for CAFC assays. The colony assays were initiated with 400-1000 cells per ml, the CAFC assays with 750-5000 cells in the first dilutions, and analyzed as described in Material and methods. Results are means ± SD of n=3 experiments.

H-IL6 improves the CFU-G/M and CAFC expansion in the presence of Flt3-L and Tpo. The CAFC assay allows the frequency analysis of very primitive (CAFC week 5-8) as well as more mature progenitor cells (CAFC within the first four weeks), while the CFC assay determines the frequency and lineage commitment of colony-forming cells [42]. As shown in table 1, the combination of Flt3-L and Tpo induced only a small expansion of CAFC<sub>week6</sub> (two- to four-fold) from AC133⁺ UCB cells during three weeks of ex vivo culture, but was insufficient to maintain week 4 and CAFC<sub>week6</sub> over six weeks of culture. In contrast, the addition of H-IL6 resulted in a 6.92 ± 2.61-fold expansion of CAFC<sub>week6</sub> during six weeks of culture (P= 0.001). The highest increase was obtained for the more mature progenitor fractions, with the total expansion of CAFC<sub>week6</sub> being 8.1-fold higher (P= 0.001) and the expansion of CFU-G/M being three- to four-fold higher in the presence of H-IL6 (P= 0.001).

Gp130-signaling and long-term expansion. To investigate the effect of H-IL6 in long-term cultures, AC133⁺ UCB cells were grown under stroma-free, serum-containing or stroma-supported, serum-free conditions for up to twelve weeks in the presence or absence of the H-IL6 fusion protein or IL6 and various combinations of Flt3-L, Tpo and SCF. A representative experiment with a single pool of AC133-separ-ated UCB cells is shown in figure 4.

Under stroma-free, serum-containing conditions, the two-factor combination Flt3-L and SCF induced a 190 ± 32.5-fold expansion of nucleated cells, a 15.5 ± 3.5-fold expansion of CD34⁺ cells, and a 5.5 ± 0.1-fold expansion of CD34⁺AC133⁺ cells during
Umbilical cord blood hematopoietic stem and progenitor cells

Figure 4. Expansion of nucleated (a), CD34⁺ (b) and CD34⁺AC133⁺ (c) UCB cells in the presence or absence of H-IL6 or IL6. AC133-separated cells were cultured with the indicated cytokine combinations under either stroma-free serum-containing conditions or on the FBMD-1 stromal cell line without serum. The cells were demidepleted weekly and re-plated as described.
the first two to four weeks of culture before declining. The addition of H-IL6 to Flt3-L and SCF increased the number of total nucleated cells a further two-fold (1.85 ± 0.92-fold, P = 0.1; figure 4a). Interestingly, the highest propagation was obtained for CD34+ cells with the expansion of these being 4.7 ± 1.9-fold higher (P = 0.049; figure 4b), and the expansion of CD34+AC133+ cells being 4.0 ± 1.8-fold higher (P = 0.03; figure 4c) in the presence of H-IL6 than with Flt3-L and SCF alone. These progenitors also persisted for a longer time in culture in the presence of H-IL6 than in its absence. The addition of Tpo to Flt3-L plus SCF induced similar levels of expansion of nucleated cells, CD34+ cells and CD34+AC133+ cells during the first two to four weeks of culture when compared to Flt3-L and SCF plus the H-IL6 fusion protein. Over twelve weeks of culture, the highest proliferation was obtained with a combination of all four factors. The number of nucleated cells increased up to 12705-fold, and the number of CD34+ and CD34+AC133+ cells up to 1477-fold and 1455-fold, respectively, over input values during twelve weeks of culture in Flt3-L, Tpo, SCF and H-IL6.

The two-factor combination Flt3-L and Tpo induced a two-fold lower expansion of nucleated cells and CD34+ cells than Flt3-L plus SCF under stroma-free conditions during the early phases of the culture. The addition of the H-IL6 fusion protein to Flt3-L and Tpo increased the number of CD34+AC133+ cells to the same level as Flt3-L, SCF plus H-IL6 but resulted in a three-fold lower expansion of CD34+ cells, which were negative for AC133 (data not shown). Interestingly, IL6 showed a greater synergistic effect with Flt3-L and Tpo for the long-term expansion of UCB progenitors than the H-IL6 fusion protein. In four different experiments, IL6 induced, during the first six weeks of culture, a 1.96 ± 0.64-fold higher expansion of nucleated cells (P = 0.039), a 2.28 ± 0.33-fold higher expansion of CD34+ cells (P = 0.037) and a 2.74 ± 0.28-fold higher expansion of CD34+AC133+ cells (P = 0.022) than the H-IL6 fusion protein in combination with Flt3-L and Tpo. While the progenitor expansion dropped after five-seven weeks in the H-IL6 supported cultures, a long-term expansion could be sustained in the presence of Flt3-L, Tpo and IL6 resulting in an up to 10-fold higher propagation of CD34+ cells over twelve weeks of culture.

Co-culture with the murine FBMD-1 stromal cell line in the absence of serum greatly increased the progenitor output in cultures containing Flt3-L and Tpo. Although the addition of H-IL6 to Flt3-L and Tpo induced a further 10-fold increase in CD34+ numbers, the synergistic effect of the fusion protein for the propagation of CD34+ and CD34+AC133+ cells was approximately four-fold lower than that of IL6 under the same conditions, while the increase in nucleated cells differed only by a factor of 2.5-fold between IL6 and the H-IL6 fusion protein (n = 2). The benefit of the full-length
Umbilical cord blood hematopoietic stem and progenitor cells

recombinant IL6 compared to the designer protein was also exemplified by the finding that IL6 was more potent in enhancing the expansion of CAFC<sub>week6</sub> in the presence of Flt3-L and Tpo. A preliminary but direct comparison showed an 18.5 ± 3.9-fold (mean ± SD of Poisson analysis) increase in primitive CAFC<sub>week6</sub> after six weeks of culture with Flt3-L, Tpo and IL6 in the absence of stroma, while in the presence of Flt3-L, Tpo and

![Graph](image)

**Figure 5.** Lineage commitment of AC133-separated UCB cells after two weeks of culture in serum-containing medium supplemented with the indicated cytokine combinations. The cells were labeled with different lineage-specific antibodies as described in Materials and methods, and analyzed on a FACS-Calibur flow cytometer.
H-IL6 were only 4.6 ± 1.8-fold higher than the input. Stroma-contact greatly increased the expansion of CAFCw in the presence of Flt3-L and Tpo, and the addition of IL6, or to a lesser extent H-IL6, further enhanced the number of these primitive progenitors. The effect of stroma on the propagation of primitive progenitor cells is described in more detail in Kuşadası et al. (manuscript in preparation).

Lineage commitment of ex vivo expanded cells in the presence of gp130 signaling.
Flt3-L is known to stimulate the growth/differentiation of myelo/monocytic cells [18] while Tpo has been shown to regulate megakaryocyte and platelet development [20]. Accordingly, approximately 25% of the in vitro expanded cells expressed the monocytic marker CD14, and up to 15% (9.6-14.8%) showed a CD33·CD41+ megakaryocytic phenotype after two weeks of culture in serum-containing medium supplemented with Flt3-L and Tpo (figure 5). The addition of H-IL6 increased the percentage of CD33·CD41+ cells 2.5-fold (26-33% CD33·CD41+ cells) reducing the fraction of myelo/monocytic cells by about the same amount. IL6 also synergized with Flt3-L and Tpo for the differentiation/proliferation of megakaryocytic cells although to a lesser extent than the fusion protein (15-20% less CD33·CD41+ cells).

The presence of SCF supported the differentiation to CD14·CD15+ granulocytic cells but decreased the megakaryocytic differentiation. No CD33·CD41+ cells could be detected when only Flt3-L and SCF (without Tpo) were present, and the addition of the fusion protein to this combination was also insufficient to increase the percentage of CD33·CD41+ megakaryocytic cells over 1%. Interestingly, no CD33·CD41+ cells were detected in the FBMD-1-contact cultures, even in the presence of Flt3-L, Tpo and gp130-signaling. The addition of serum also failed to increase the number of CD33·CD41+ cells in the presence of stroma (data not shown). In contrast, the percentage of CD14·CD15+ monocytic cells was more than two-fold higher in the stroma-supported than in the stroma-free cultures. A small fraction of CD71·GlycA+ erythroid progenitors (0.9-2.1%) could be detected after two weeks in the stroma-free cultures with Flt3-L and Tpo ± IL6 or H-IL6. 0-0.5% erythroid cells were found in the other cultures (data not shown).

Phenotype of long-term expanded cells in the presence or absence of stroma.
The immunofluorescence analysis of long-term expanded cells showed considerable differences between the stroma-free and stroma-supported cultures. As shown in figure 6 for the cytokine combination Flt3-L, Tpo and IL6, the percentage of CD34·CD38low and CD34·CD90+ (hu Thy-1 antigen) cells after twelve weeks of culture was more than five times higher in the presence of stroma than in its absence. The percentage of CD34·AC133+ cells was similar under both types of culture conditions. However, the majority of AC133+ cells were negative for CD34 under stroma-free conditions, while the expression of this
Figure 6. Phenotype of cells cultured for twelve weeks in the presence of Flt3-L, Tpo and IL6 with or without stromal support. The cells were labeled with CD34-APC, CD38-FITC, and either AC133-PE or CD90-PE as well as 7-ADD to exclude dead cells. The cells were analyzed on a FACS-Calibur flow cytometer, and the quadrants were set according to control samples stained with isotype-matched control antibodies.

antigen was, as on primary cells, restricted to the CD34+ fraction under stroma-supported conditions. Similar percentages (i.e. 5-7% CD34+CD38low, 1-1.5% CD34+CD90+, and 4.5-10% CD34+AC133+ cells) were obtained in the stroma-supported cultures containing Flt3-L, Tpo plus H-IL6 with or without serum after twelve weeks of culture. The proportion of CD34+AC133+ cells increased with time of culture under stroma-free conditions and showed a transition from CD34low to CD34+AC133+ cells. CD34+AC133+ cells could also be detected in the stroma-free cultures with Flt3-L, Tpo plus SCF but the frequency was higher in the presence of H-IL6 or IL6. The majority of these cells co-expressed CD15, and preliminary cell sorting results indicate that CD34+AC133+ cells are enriched in CAFCweek2 but do not comprise the more primitive CAFCweek6+.
Figure 7. Proliferation of single CD34⁺CD38⁻ UCB cells during four weeks of culture in the indicated cytokine combination with or without IL6. Sorted CD34⁺CD38⁻ cells were plated by limiting dilution to 0.7 cells per well into 96-well plates. The number of cells per well was counted weekly or estimated (clones >100 cells) by surface occupancy, and fresh medium with cytokines was added every 10 days.

Growth promoting effect of gp130-signaling on single CD34⁺CD38⁻ cells. To further analyze the effect of gp130-signaling on the proliferation of primitive progenitor cells, we FACS-sorted CD34⁺CD38⁻ UCB cells and plated single cells in 96-well plates in the presence of various cytokine combinations. Figure 7 shows the number and size of individual clones after one to four weeks of culture. IL6 increased the number of clones (>5 cells) seven- and five-fold when added to Flt3-L plus SCF and Flt3-L plus Tpo, respectively. Notably, H-IL6 was less potent than IL6 in recruiting CD34⁺CD38⁻ cells into cell cycle in synergism with Flt3-L and Tpo. The highest cloning frequency (52%) was found in the presence of Flt3-L, Tpo plus SCF, which could not be further enhanced by IL6 (50%). However, the number of viable clones decreased after one week of culture in the presence of Flt3-L, Tpo and SCF, while a further recruitment of quiescent cells into cell cycle was observed with Flt3-L, Tpo and IL6. In addition, clones containing more than 5000 cells were found exclusively in the presence of IL6, and were most frequent in the wells supplemented with the four-factor combination.
Discussion

In this study, we have examined the effect of a designer fusion protein of IL6 and the soluble IL6R, H-IL6, on the ex vivo expansion of cord blood progenitors. As a single agent, the fusion protein failed to induce the propagation of UCB-derived CD34+ cells and CAFC, but synergized effectively and in a dose-dependent fashion with Flt3-L and Tpo for the expansion of hematopoietic progenitor cells under both stroma-free and stroma-supported conditions. In addition, we have shown that IL6 is more potent than the fusion protein in supporting the long-term propagation of UCB CD34+AC133+ or CD34+ cells and CAFC in the presence of Flt3-L and Tpo.

Most data indicate that the proliferation of primitive hematopoietic progenitor cells requires co-stimulation by different regulatory factors [43]. Among the cytokines that are considered primary mediators in early hematopoiesis are Flt3-L and Tpo. Primitive CD34+CD38- rhodamine-123 low 4-hydroperoxycyclophosphamide (4-HC)-resistant cells respond to Flt3-L [16], and several studies report a higher expansion of primitive progenitors in the presence of this cytokine [15, 44]. Tpo has also been shown to enhance the recruitment of early hematopoietic progenitors into cell cycle and to increase the clonal growth and the viability of single CD34+CD38+ cells [19, 21-23]. Studies by Piacibello et al. [24] have described a 1000-fold amplification of CD34+ cells and a 300-fold amplification of primitive LTC-IC after three to six weeks in serum-containing cultures supplemented with the single combination of Flt3-L and Tpo. Using AC133+ or CD34+ UCB cells, we were unable to reproduce such levels of expansion for CD34+, CD34+AC133+, CFC or CAFC subsets with Flt3-L and Tpo under serum-containing conditions in our culture system. This may reflect differences in the sources of cytokines or the presence of uncharacterized factors in the sera. The addition of H-IL6 increased the propagation of CD34+ cells more than six-fold over Flt3-L and Tpo and further enhanced the proliferation of both CFC by three- to four-fold and primitive CAFC week 6 by two-fold during six weeks of culture. The fusion protein also acted synergistically with Flt3-L plus SCF and with Flt3-L, Tpo plus SCF for the expansion of CD34+ cells and CAFC week 6 both under serum-containing stroma-free and serum-free stroma-supported conditions. These results support and extend the studies of Sui et al. [35] and Ebihara et al. [37], who tested a complex of the soluble IL6R and IL6 (IL6/sIL6R) in combination with SCF or Flt3-L in three-week suspension cultures and methylcellulose clonal assays and demonstrated an increased expansion of multipotential and committed progenitors from UCB or BM-derived CD34+ cells. Kimura et al. [33] did not find a significant colony formation from FACS-sorted CD34+IL6R+ or CD34+IL6R- PB cells in the presence of
SCF and IL6/sIL6R alone, but a high proliferation of BFU-E and CFU-Mix colonies was induced in the IL6R-negative fraction when IL3 was added to this cytokine combination. Recently, Zandstra et al. [45] reported a higher LTC-IC expansion in one-week serum-free cultures of CD34+CD38− UCB cells stimulated by Flt3-L in combination with the IL6/sIL6R complex when compared to the combinations of Flt3-L plus Tpo or Flt3-L, SCF, IL6, IL3 plus G-CSF. Since the half-life of this IL6/sIL6R complex is very short, high concentrations of the soluble IL6R were needed to obtain effective stimulation [34, 35, 45]. In contrast, the H-IL6 fusion protein has proven to be fully active at 100- to 1000-fold lower concentrations than the combination of IL6 and IL6R [41], and in the present study 3 ng/ml H-IL6 were found to be sufficient to induce maximal expansion of CD34+ cells in the presence of Flt3-L and Tpo.

Fischer et al. [41] were the first to test the effect of the H-IL6 fusion protein on hematopoietic progenitors in short-term cultures. They found an increased expansion of CFU-GM when H-IL6 or the IL6/sIL6R complex was added to the combination of SCF and IL3 in two-week cultures of CD34+ mobilized PB cells. Notably, under the same conditions, stimulation with IL6 in place of the IL6/sIL6R complex resulted in a lower expansion of these committed progenitors. Sui et al. [35, 36] also described an increased number of clonogenic progenitors generated from CD34+ UCB cells in the presence of SCF and IL6/sIL6R compared to SCF and IL6. In their study, the IL6/sIL6R complex stimulated in particular the expansion of erythroid and mixed colonies, while the number of CFU-GM was similar in the cultures supplemented with either IL6 or the IL6/sIL6R complex. This is in line with the findings of Tajima et al. [32], who showed that erythroid progenitors and CFU-GEMM segregate with the CD34+gp130+IL6Rα− fraction of FACS-sorted UCB cells, whereas the CFU-GM are concentrated in the CD34+gp130+IL6Rα+ fraction. In addition, a six-fold higher LTC-IC frequency was found in the CD34+gp130+IL6Rα− subset compared to the CD34+gp130+ IL6Rα+ fraction, leading to the conclusion, that 'stem cells' or at least LTC-IC are IL6Rα negative, and cannot be stimulated by IL6 alone. However, due to the low IL6Rα receptor expression, positive and negative fractions are not clearly distinguishable, and LTC-IC were not found to be restricted solely to the IL6R-negative fraction. McKinstry et al. [46], using radiolabeled IL6, also detected IL6Rα expression on 13% of primitive murine Rh low Lin-Sca1+Kit+ progenitors. Furthermore, murine 5FU-treated BM cells could be shown to proliferate in response to IL6, SCF and IL3 [47]. Evidence that IL6 complexed to the soluble IL6R, but not IL6 alone, may be able to stimulate early murine progenitors, comes from phenotypic analyses of mice single transgenic for either IL6 or sIL6R or double transgenic for both IL6 and sIL6R [34, 39, 40]. Interestingly, double transgenic
Umbilical cord blood hematopoietic stem and progenitor cells

mice developed a marked extramedullary hematopoiesis in the spleen and to a lesser extent the liver while the bone marrow cellularity was not significantly changed. The liver showed distinct foci with predominantly granulopoietic cells, while large numbers of erythrocytes, granulocytes and megakaryocytes were found equally distributed in the spleen. In addition, the number of primitive Lin\(^{-}\)Sca1\(^{+}\)kit\(^{+}\) cells in the liver of double transgenic mice was increased from about 0.03% to 0.3% leading to the hypothesis that persisting fetal progenitor cells in spleen and liver can be recruited into proliferation by IL6 in combination with the soluble IL6R. However, as mentioned above, high concentrations of IL6R seemed to be necessary to generate this effect, as Hirota et al. [48] did not find hematological abnormalities in their IL6 and sIL6R transgenic mice with lower shedding levels of the membrane-bound IL6R.

Interestingly, although the H-IL6 fusion protein enhanced the expansion of AC133-separated hematopoietic progenitors with a variety of cytokine combinations in our culture system, IL6 synergized more potently than the fusion protein with Flt3-L and Tpo for the expansion of CD34\(^{+}\) and CD34\(^{+}\)AC133\(^{+}\) cells as well as of CAFC. This became more pronounced after prolonged ex vivo culture and may in part be due to the use of MACS-separated AC133\(^{+}\) UCB cells in preference to CD34-separated cells, which have been found to possess a lower expansion potential in our culture system. In this respect, the AC133 antigen is expressed on a subfraction of CD34\(^{+}\) cells, which comprises committed progenitors as well as LTC-IC and long-term repopulating cells. Our own unpublished data confirm that approximately 90% of the CAFC\(_{\text{week6}}\) and approximately 80% of the CFU-GM can be recovered in the CD34\(^{+}\)AC133\(^{+}\) fraction of sorted primary UCB cells. In contrast, CD34\(^{+}\)AC133\(^{-}\) cells have no long-term repopulating ability and are enriched for erythroid progenitors [49, 50]. Since erythroid progenitors have been shown to be IL6R\(\alpha\) negative [32], we would therefore expect that the separation of AC133\(^{+}\) cells results in an enrichment of IL6R\(\alpha\)\(^{+}\) cells and an enhanced response to IL6. Of further interest was the observation that the expansion of UCB-derived CD34\(^{+}\) and CD34\(^{+}\)AC133\(^{+}\) cells in serum-containing liquid cultures with Flt3-L, Tpo and the H-IL6 fusion protein resembled more the expansion induced by IL11 than by IL6 under the same conditions (unpublished data). Since both IL11 and IL6 induce a homodimerization of gp130, it can also be speculated that the activation of gp130 by the H-IL6 fusion protein may differ from the stimulation induced by an interaction of IL6 with the IL6R\(\alpha\) chain. Recently, Rakemann et al. [51] have reported, that STAT3-dependent gene transcription in HepG2 cells is about 10-fold higher after stimulation with H-IL6 than after activation with IL6. However, with increasing amounts of IL6R\(\alpha\) expression transgenic HepG2 cells became less sensitive to H-IL6 while the response to
Chapter 4

IL6 increased. Upregulation of IL6Rα expression during differentiation could therefore favor stimulation by IL6 compared with H-IL6 and indirectly mediate an as yet unknown growth promoting signal to more primitive progenitor cells.

While it has not as yet been shown that the direct stimulation of primitive CD34*gp130*IL6Rα· cells by the IL6/sIL6R complex or the H-IL6 fusion protein results in an expansion of long-term repopulating cells, IL6 and IL11 have been shown to synergize with other cytokines for the expansion of SCID repopulating cells (SRC). For instance, our recent studies indicate that IL6 in combination with Flt3-L and Tpo induces a 10- to 20-fold expansion of the ability of UCB-derived SRC in serum-containing liquid cultures to repopulate the murine bone marrow (Kuşadası, manuscript in preparation), and also causes a higher expansion of CAFC<sub>weeks</sub> than the combination of Flt3-L, Tpo and SCF. In addition, a cytokine cocktail of IL3, IL6, G-CSF, SCF and Flt3-L is reported to support a two-fold expansion of NOD/SCID competitive repopulation units (CRU) from CD34*CD38- UCB cells in serum-free suspension cultures [11]. Bhatia et al. [12] have also described a two- to four-fold increase in SRC after short-term culture of CD34*CD38- UCB cells with an IL6 containing cytokine cocktail, while murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability could be expanded three-fold in serum-free medium containing IL11, Flt3-L and SCF [10]. Notably, Peled et al. [52] recently reported an upregulation of CXCR4 expression on CD34+ cells by SCF and IL6. This receptor for the chemokine stromal cell-derived factor-1 (SDF-1) has been found to be critical for human stem cell engraftment and repopulation of NOD/SCID mice. Very recently, Piacibello et al. [53] reported that CD34+ UCB cells could be expanded for up to ten weeks in stroma-free cultures in the presence of Flt3-L, Tpo, SCF and IL6 without losing their <i>in vivo</i> repopulating potential.

In addition to the effects on primitive progenitor amplification, we observed that both IL6 and H-IL6 further enhanced the <i>ex vivo</i> expansion of megakaryocytic cells when combined with Flt3-L and Tpo. This observation is important in a clinical setting where it is necessary to shorten the period of chemotherapy-induced thrombocytopenia, which is prolonged after UCB transplantation when compared to BM transplantation [3]. Contact with the murine stromal line FBMD-1 reduced the percentage of CD33-CD41+ cells almost to background levels but, on the other hand, greatly enhanced the amplification of phenotypically and functionally primitive progenitor cells in the presence of Flt3-L and Tpo with or without H-IL6 or IL6. Again, the highest progenitor expansion was found in the presence of IL6. Interestingly, the phenotype of UCB progenitors expanded in coculture with FBMD-1 stromal cells (in the presence or absence of serum) corresponded to the phenotype of primary progenitors with the expression of the AC133 antigen.
being restricted to the CD34\textsuperscript{high} subset. In contrast, a fraction of CD34\textsuperscript{low}/AC133\textsuperscript{+} cells, which most likely represents more mature myeloid progenitors, appeared and increased during liquid culture in the absence of stroma. In conclusion, although the H-IL6 fusion protein supported the \textit{ex vivo} expansion of different progenitor populations in synergy with various cytokine combinations, no advantage over IL6 could be observed in our culture system. Further studies are necessary to investigate whether this fusion protein can directly stimulate primitive marrow engrafting cells or if, like IL6, it acts indirectly via accessory cells.

**Acknowledgements**

We would like to thank Prof Sir David Weatherall for his support. This research was supported by a 4th Framework EU Biotech Grant (IR, SMW), the Leukemia Research Fund (London, UK) (IR, SMW), the Deutsche Forschungsgemeinschaft (Bonn, Germany) (SR-J) and the Stiftung RheinlandPfalz fur Innovation (Mainz, Germany) (SR-J).
References

2. Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 1997: 89 (11): 3919.
15. Dooley DC, Xiao M, Oppenlander BK, Plunkett JM, Lyman SD. Flt3 ligand enhances the yield of primitive cells after Ex vivo cultivation of CD34+ CD38dim cells and CD34+ CD38dim CD33dim CD34dim HLA-DR+ cells. Blood 1997; 90 (10): 3903.
Umbilical cord blood hematopoietic stem and progenitor cells


Chapter 5

Stromal cells from murine aorta-gonad-mesonephros region, liver and gut mesentary expand human umbilical cord blood-derived in vitro repopulating stem cells.

Institute of Hematology and Cell Biology and Genetics, Erasmus Medical Center Rotterdam.
Published in Leukemia, 2002.
Abstract

The first definitive long-term repopulating hematopoietic stem cells (HSCs) emerge from and undergo rapid expansion in the embryonic aorta-gonad-mesonephros (AGM) region. To investigate the presumptive unique characteristics of the embryonic hematopoietic microenvironment and its surrounding tissues, we have generated stromal clones from subdissected day 10 and day 11 AGMs, embryonic livers (ELs) and gut mesentery. We here examine the ability of nineteen of these clones to sustain extended long-term cultures (LTCs) of human CD34+ umbilical cord blood (UCB) cells in vitro. The presence of in vitro repopulating cells was assessed by sustained production of progenitor cells (extended LTC-CFC) and cobblestone area forming cells (CAFC). The embryonic stromal clones differed greatly in their support for human HSCs. Out of eight clones tested in the absence of exogenous cytokines, only one (EL-derived) clone was able to provide maintenance of HSCs. Addition of either Tpo or Flt3-L + Tpo improved the long-term support of about 50% of the tested clones. Cultures on four out of nineteen clones, i.e. the EL-derived clone mentioned, two urogenital-ridge (UG)-derived clones and one gastrointestinal (GI)-derived clone, allowed a continuous expansion of primitive CAFC and CFU-GM with over several hundred-fold more CAFC produced in the twelfth week of culture. This expansion was considerably higher than that found with the FBMD-1 cell line, which is appreciated by many investigators for its support of human HSCs, under comparable conditions. This stromal cell panel derived from the embryonic regions may be a powerful tool in dissecting the factors mediating stromal support for maintenance and expansion of HSCs.

Introduction

A major clinical challenge in HSC transplantation is the establishment of culture systems that mediate extensive numerical expansion of HSCs in vitro with maintenance of HSC potential for proliferation and multilineage differentiation. Using well-defined early-acting cytokines, we and others have reported an increased ability of UCB cells for multilineage repopulation of NOD/SCID mice following ex vivo culture [1-4]. In addition, several stromal cell lines derived from adult, fetal or embryonic origin have been shown to support HSCs and/or progenitors, emphasizing the role of microenvironment in promoting the survival, proliferation and differentiation of hematopoietic cells in vitro [4-9]. However, the use of cytokines or stromal monolayers alone, or in
combination, has met with limited success in expanding long-term repopulating HSCs for clinical applications, or improving rapid engraftment and early recovery of platelet and neutrophil numbers.

In the past few years, we have demonstrated that definitive murine HSCs and progenitors first emerge from the intra-embryonic region, which forms the aorta-, gonads-, and mesonephros (AGM) region [10]. These hematopoietic cells capable of reconstituting definitive adult hematopoiesis first appear at day 10.5 after gestation (E10.5) and expand rapidly in the subsequent two to three days. Furthermore, from AGM explant cultures we learned that HSC activity is autonomously generated and amplified in the AGM [10]. In addition, we demonstrated that functional definitive HSCs developed particularly from the major arterial regions, i.e. the dorsal aorta, vitelline and umbilical vessels, indicating a close relationship between the developing hematopoietic and vascular systems [11]. The fetal liver, which is an important hematopoiesis-supportive microenvironment, does not become a major site for HSC expansion until E12 [12-14].

In subsequent years, a few stromal cell lines from the E11 AGM region have been reported to maintain HSCs with competitive repopulating activity in vitro [6, 9]. However, this support did not seem to be better than that of earlier established stromal cell lines from E14.5 fetal liver or adult bone marrow [7, 8]. As the factors responsible for the induction and expansion of HSCs are still incompletely defined, we hypothesized that the E10 and E11 AGM region with surrounding tissues and the embryonic E11 liver (EL), display unique characteristics with regard to support of HSC activity and that the cloning of stromal cells from these regions would aid in identifying mechanisms involved in HSC expansion.

We have generated more than hundred stromal clones from subdissected day 10 or 11 AGMs, ELs and gut mesentery of murine embryos transgenic for a temperature-sensitive mutant of the SV40 large T antigen (SV40 Tag) gene or the LacZ marker gene [15]. In the study presented here, we investigated the ability of nineteen embryonic stromal clones to sustain extended Dexter-type LTCs of CD34+ umbilical cord blood (UCB) cells for periods as long as twelve weeks. Our previous experiments indicated that addition of the cytokines Flt3-L and Tpo results in superior maintenance of immature cobblestone area forming cells (CAFCw) [4, 16]. Moreover, UCB cells cultured in the presence of at least these cytokines maintained their ability to undergo multilineage differentiation in vitro along myeloid and lymphoid pathways [17, 18]. The production of CAFCw or LTC-initiating cells(IC) in ex vivo expansion cultures has been shown to be indicative for the presence and quantity of in vivo repopulating HSC as measured.
Umbilical cord blood hematopoietic stem and progenitor cells

using the quantitative NOD/SCID xenotransplant model [3, 4, 19-21]. However, behaviour of the LTC-IC populations may only partly overlap with that of the HSC and show distinct characteristics under specific experimental conditions [16, 22]. Screening a large number of stromal cell lines using this in vivo model is not practicable. Thus, we have used the in vitro production of CFC and CAFC_{week6} in extended LTC as a pseudo-assay for repopulating HSC instead. Using this assay we were able to identify several embryonic stromal co-cultures (one EL-, two UG- and one GI-derived) that supported an expansion of CAFC_{week6} largely exceeding that of the reference co-cultures under comparable conditions. All co-cultures required at least human Tpo for their prolonged expansion of human HSCs and progenitors.

Material and methods

*Human umbilical cord blood cells.* Human UCB samples were collected in sterile flasks containing 10 ml citrate-glucose as anticoagulant from umbilical cord vein after full-term delivery by the nursing staff of the Department of Obstetrics and Gynecology at the Sint Franciscus Gasthuis (Rotterdam, The Netherlands). UCB was stored at room temperature and processed within 24 hours of collection. Low-density cells were isolated using Ficoll Hypaque density centrifugation (1.077g/cm², Lymphoprep, Nycomed Pharma, Oslo, Norway) by centrifugation at 600 g for 15 minutes. The mononuclear cell (MNC) band at the interface was removed, washed twice with Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) and resuspended in Iscove's modified Dulbecco's medium (IMDM, Gibco, Breda, The Netherlands). The MNCs were stored in 10% FCS, 20% DMSO in IMDM in liquid nitrogen until use.

*Isolation of CD34+ cells.* In all experiments human UCB were positively selected for the expression of CD34 using either Variomacs or Automacs Immunomagnetic Separation System (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers instructions. After thawing and before the CD34+ hematopoietic progenitor cells were isolated we pooled 3 to 16 different UCB samples. The purity of the CD34 selected cells from UCB, as determined by FACS after staining with a CD34 antibody, was 90% ± 3%, and the percentage of cells with a CD34+CD38- phenotype was 8% ± 7%. The progenitor and stem cell numbers in the input and output suspensions were determined by CFC and CAFC assays. The absolute number of progenitors present in uncultured material was 1.8 ± 1.0 x10⁴ and 2.7 ± 1.6 x10³ per 10⁵ CD34+ cells for CFU-GM and CAFC_{week6}, respectively.
Hematopoietic growth factors. The following cytokines were used: recombinant human Flt3-L, a gift from Amgen (Thousand Oaks, CA, USA); recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF), recombinant human granulocyte colony stimulating factor (G-CSF) and recombinant murine stem cell factor (SCF), all gifts from Genetic Institute (Cambridge, MA, USA); recombinant human Tpo, a gift from Genentech (South San Francisco, CA, USA), recombinant human erythropoietin (Epo, Boehringer, Mannheim, Germany) and recombinant human interleukin-3 (IL3) (Gist Brocades, Delft, The Netherlands).

Stromal cells and clones. The FBMD-1 cells were cultured as previously described [23]. The embryonic stromal clones were generated from subdissected AGM regions, gut and stomach tissues and livers from E10 and E11 embryos of BL1b, Tag05 and Tag11 mice with BL1b transgenic for LacZ inserted into the first intron and Tag 05/11 transgenic for temperature-sensitive mutant of the SV40 large T antigen. Details about the establishment of these stromal clones have been described elsewhere [15]. The embryonic stromal clones were cultured in 25 cm² flasks (Costar, Cambridge, MA, USA) with 5 ml IMDM containing 10% fetal calf serum (FCS, Summit, Fort Collins, CO) and 10% horse serum (HS, Gibco), supplemented with penicillin (100 U/ml, Gibco), streptomycin (0.1 mg/ml, Gibco), β-mercapto-ethanol (10⁻⁴ M, Merck), Darmstadt, Germany). The stromal cells were grown to confluence at 33°C and 10% CO₂ and irradiated with 40 Gy prior to co-cultures.

Extended LTCs. Confluent 25 cm² flasks of different stromal cells were overlaid with 1 or 2 x10³ CD34⁺ UCB cells. These cells were cultured in 5 ml LTC-medium (IMDM containing 20% FCS, further supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), β-mercapto-ethanol (10⁻⁴ M, Merck), cholesterol (15 μM, Sigma), linolic acid (15 μM Merck), iron-saturated human transferrin (0.62 g/l, Intergen, Uithoom, The Netherlands), nucleic acids (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'-deoxyadenosine, thymidine, 2'-deoxyguanosine (all at 10⁻⁲ g/ml, Sigma)). The cultures were either supplemented with Tpo (10 ng/ml) alone or a combination of Flt3-L (50 ng/ml) and Tpo (10 ng/ml). In some experiments cells were also deposited on stromal layers without any cytokine supplements.

Depleted extended LTCs. Cultures were set up in quadruplicate and maintained at 33°C and 10% CO₂ for twelve weeks with weekly half medium change and consequently depletion of half of the non-adherent cells. Every two weeks the CFC output in non-adherent supernatant was determined without correcting for weekly demidepopulations. At weeks seven/eight or twelve the adherent layers were assayed for the CFC and CAFC numbers as well. Thus, the total CFC and CAFC production (in adherent plus
Umbilical cord blood hematopoietic stem and progenitor cells

non-adherent cells) could be determined at these timepoints. In the subsequent two experiments four stromal clones and one control line were set up in six-fold in flask cultures and the adherent and non-adherent compartments were analyzed separately for their progenitor and stem cell contents at week 2, 4 and 6 of culture. To harvest the adherent layer, the non-adherent cells were first collected and the layer washed with PBS and subsequently incubated with trypsin-EDTA (Life Technologies, Breda, The Netherlands) at 37°C for 5-10 minutes. The digestion was stopped by adding 1 ml FCS.

Non-depleted extended LTCs. In the last experiment, confluent layers of FBMD-1, UG26-1B6, EL08-1D2 and Gl29-2B4 were irradiated with 40 Gy and seeded with 2 x 10⁴ UCB CD34+ cells in LTC-medium additionally containing 1% bovine serum albumin (BSA, Sigma, Zwijndrecht, The Netherlands). In stead of weekly demidepopulating the non-adherent cells, these cultures were maintained by splitting the cultures every four weeks and seeding suitable aliquots of 5 x 10³ and 1,25 x 10³ input equivalents cells (both adherent and non-adherent cell fractions harvested and pooled) on two newly established corresponding stromal layers at weeks 4 and 8, respectively. The remaining number of cultured cells was analyzed for their progenitor and stem cell numbers using the CFC and CAFC assays every fourth week. Every two weeks the non-adherent fraction of the flask cultures was removed, centrifuged to collect the non-adherent cells, which were returned to the cultures, and replaced with fresh medium. The cytokines Flt3-L (50 ng/ml) and Tpo (10 ng/ml) were added twice weekly. The other culture conditions were the same as mentioned above.

Clonony forming cell assay. Granulocyte-macrophage colony forming unit (CFU-GM) and burst forming unit-erythroid (BFU-E) progenitor cells were assayed using a semisolid culture medium (1.2% methylcellulose), in IMDM supplemented with 30% FCS, β-mercaptopo-ethanol (5 x 10⁻⁵ M), penicillin (100 U/ml), streptomycin (0.1 mg/ml), hu-Epo (1 U/ml), hu-IL3 (20 ng/ml), hu-GM-CSF (5 ng/ml), hu-G-CSF (50 ng/ml) and mu-SCF (100 ng/ml). Duplicate cultures were plated in 35 mm tissue culture dishes (Falcon) and incubated at 37°C and 10% CO₂ in a humidified atmosphere. Colonies containing 50 cells or more were scored at day 14 using an inverted light microscope.

Cobblestone area forming cell assay. Confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates (Falcon) were overlaid with UCB cells in a limiting dilution set up as described [24]. Briefly, twelve successive two-fold dilutions were used for each sample with 15 wells per dilution. The percentage of wells with at least one phase-dark hematopoietic clone (cobblestone area) of at least five cells beneath the stromal layer...
Figure 1. Flowcytometric analysis of uncultured and cultured CD34+ UCB cells in a representative experiment. The FACS profiles represent the percentage of CD34+ UCB cells within the life-gate before and after ten weeks of ex vivo culture on EL08-1D2 stromal cells under different conditions. The total CD34+ percentage is given in the corner of the right quadrant for each sample. Cells were stained with CD34-FITC or CD34-APC and CD38-PE. The quadrants were set according to negative control samples labeled with IgG1-FITC or IgG1-APC and IgG1-PE.

was determined at week 6 (CAFC_{week6}). The CAFC frequencies were calculated using Poisson statistics.

**Immunophenotypic analysis.** At least 50,000 fresh or cultured CD34+ cells were stained with anti human CD45/CD34/CD38 (allophycocyanin (APC)-, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated) labeled monoclonal antibodies (Immunotech, Mijdrecht, The Netherlands). After incubation of the cells in phosphate-buffered saline (PBS, Life Technologies, Breda, The Netherlands) containing 0.5% BSA and 2% normal human serum for 30 minutes on ice, the cells were washed
in PBS with 0.5% BSA and resuspended in 0.35 ml PBS. Just before the acquisition 7-
aminoactinomycin (7-AAD, Molecular Probes, Leiden, The Netherlands) was added to
each sample to determine the viability of the cells. APC, FITC and PE-conjugated mouse
isotype antibodies were used as control for each group. At least 10,000 events were
acquired using fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson).
The flow cytometric analysis of a representative experiment is given in figure 1.

Statistical analysis. The values are reported as mean ± 1 SD. The significance levels
were determined by two-tailed Mann-Whitney U-test analysis.

Results

The strategy for selecting functionally supportive embryonic stromal cell clones is
shown in figure 2. In initial studies more than hundred stromal clones from different
embryonic tissues and subregions of the AGM, i.e. aorta-mesenchyme (AM), UG,
EL and GI, were established from E10 and E11 mouse embryos transgenic for the
temperature sensitive tsA58 mutant of the SV40 large T antigen gene or a control LacZ
marker gene [15]. The stromal clones have been tested for their support of sustained
Figure 3. Extended generation of non-adherent CFU-GMs in three representative stromal co-cultures. Data represent one experiment with duplicate cultures. The cultures were maintained by weekly half medium change with removal of half of the non-adherent cells. The cells were assayed after pooling the corresponding cultures prior to CFC. The CFU-GM generation of the reference line FBMD-1 and two embryonic clones with distinct patterns are included for comparison. The CFU-GM and CAFCweek6 numbers present in uncultured material were 1.8 ± 1.0 x10^4 and 2.7 ± 1.6 x10^3 per 10^5 CD34+ UCB cells, respectively. Abbreviations: AM, aorta mesenchyme; UG, urogenital ridge; EL, embryonic liver; GI, gastrointestinal tract.
Figure 4. Non-adherent CFU-GM generation with stromal cells from embryonic subregions in extended co-cultures. Data represent the mean non-adherent CFU-GM production in different stromal co-cultures, averaged per subdissected region. The number of stromal clones averaged per region were 4-8, 2-9, 1-2, 1-2 and 2-3 for UG, AM, EL, GI regions and FBMD-1, respectively. The cultures were maintained by weekly half medium change with removal of half of the non-adherent cells. Mann-Whitney U-test: P< 0.05 or P< 0.005 between cultures without and with supplementation of Tpo or Flt3-L + Tpo at all time points; P< 0.05 between Tpo and Flt3-L + Tpo supplemented cultures on weeks 2 and 4. Because there was no significant difference in non-adherent CFU-GM generation between the stromal cells with respect to their origin we pooled these groups for statistical analysis. Abbreviations: AM, aorta mesenchyme; UG, urogenital ridge; EL, embryonic liver; GI, gastrointestinal tract.
generation of murine CFC in LTC. Twenty-five clones supported the growth of murine CFC better than several reference stromal lines [25]. Seven of these clones were able to maintain transplantable murine HSCs in a short-term co-culture. Though prolonged culture decreased the maintenance of HSCs on most stromal clones, the urogenital ridge-derived clone, UG26-1B6, maintained in vivo repopulating murine HSCs for at least four weeks. For the evaluation of the functional support for human HSCs and progenitors nineteen best supportive embryonic stromal clones, including the seven which supported murine transplantable HSCs, were selected.

Generation of CFC and CD34+ subsets in extended long-term co-cultures with human CD34+ UCB cells. Highly purified CD34+ cells from UCB were cultured on twenty-one different stromal layers, including the reference line FBMD-1, under serum-containing conditions for up to twelve weeks with medium containing either no cytokines, Tpo or Flt3-L + Tpo. We have previously shown that addition of these cytokines improves the maintenance of primitive CAFCw'k' on FBMD-1 stromal cells dramatically [4]. The stromal cells were grown to confluency and irradiated with 40 Gy prior to culture. In the first set of experiments we studied the ability of these stromal cells to sustain production of human progenitors in extended LTCs either in the absence or presence of cytokines. The extended LTCs were maintained by weekly half medium change resulting in removal of half of the non-adherent cells. The corresponding supernatants of the same cultures were pooled every two weeks and used to determine the CFU-GM production in the non-adherent compartment.

The non-adherent CFU-GM kinetics of the reference cell line and two representative clones are presented in figure 3. We observed a gradual decline in non-adherent CFU-GM production in the absence of cytokines with the majority of the stromal clones similar to the FBMD-1 reference line. In contrast, co-culture with the EL08-1D2 clone sustained human CFU-GM production under these conditions for more than eight weeks. Moreover, the same clone supported the generation of more CFC than observed for the reference cell line FBMD-1 when either Tpo alone or a combination of Flt3-L and Tpo was added. In figure 4 all stromal cell lines tested are plotted per embryonic region. Long-term non-adherent CFU-GM production was observed in cultures supported by stromal clones derived from all of the different embryonic regions. The limited number of clones did not allow statistical analyses of possible region-related differences in support. Cytokine supplementation dramatically improved the long-term non-adherent CFU-GM generation supported by stromal cell layers derived from all regions. This resulted in a 30-700 fold significant increase in CFU-GM retrieved from Tpo-supplemented cultures as compared to control cultures on week 12 (figure 4, P<
Figure 5. Extended long-term production of non-adherent CD34+ cells in three representative stromal co-cultures. Data represent one experiment with duplicate cultures. The cultures were maintained by weekly half medium change with removal of half of the non-adherent cells. The cells were assayed after pooling the corresponding cultures prior to phenotyping. The production of cells by the reference line FBMD-1 and two embryonic clones with distinct patterns are included for comparison. Abbreviations: AM, aorta mesenchyme; UG, urogenital ridge; EL, embryonic liver; GI, gastrointestinal tract.
0.05, Mann-Whitney U-test). The addition of Flt3-L to Tpo-complemented co-cultures showed no substantial improvement of the CFU-GM generation over that seen with TPO alone, except for weeks 2 and 4.

In addition to the functional screening, we also looked at the expression of CD34 and the CD34⁺CD38⁻ cells as an indicator of progenitors and stem cells within the non-adherent compartment. The results of CD34 assessment after co-culture experiments with the reference cell line and two representative clones are shown in figure 5. The non-adherent compartment of different embryonic stromal co-cultures showed a large variability in the generation of CD34⁺ and CD34⁺CD38⁻ cells. Several clones maintained or slightly increased their numbers in the presence of cytokines as compared to the reference line. In the presence of Flt3-L + Tpo the CD34⁺CD38⁻ cell production reached a maximum of 2330-fold the input with UG26-1B6 stromal cells at week 12 of co-culture (data not shown).

**Stromal support for primitive stem and progenitor cells in human extended long-term co-cultures.** To test whether the stromal clones supported the generation of primitive human progenitors, we determined the total (combined adherent and non-adherent cells) CFU-GM and CAFC fractions of co-cultures after 7/8 and twelve weeks in the presence of Tpo alone or a combination of Flt3-L and Tpo. The embryonic stromal clones showed substantial variation in their ability to support primitive progenitors (table 1). Seven stromal co-cultures that maintained in vivo repopulating ability of murine HSCs are highlighted (bold typeface) in table 1. Two of these clones, UG15-1B7 and UG26-2D3, did not long-term support human HSCs indicating different requirements of human HSCs for in vitro maintenance, or the lack of cross-specificity of one or more factors involved in the cross-talk between murine stromal cells and human progenitors. The clones GI29-2B4, UG26-1B6, UG26-3D4 and EL08-1D2, which most optimally support murine progenitors, also gave the best support for human HSCs and progenitors.

The distribution of stem and progenitor cells among adherent and non-adherent compartments in stromal co-cultures. The extended LTCs were maintained with half-medium changes each week and removal of half of the non-adherent cells. We observed that with some stromal clones only few cells adhered and realized therefore that the weekly depletion of half of all non-adherent cells would lead to a different depletion of HSCs and progenitors on different stromal cells. Therefore, we determined the percentage of CFU-GM in both adherent and non-adherent fractions of several stromal co-cultures of CD34⁺ UCB cells in the presence of Tpo or a combination of Flt3-L and Tpo. Remarkably, the different stromal clones showed different non-adherent proportions of CFU-GM at week 7/8 of the cultures for Tpo (range 7-95%) and Flt3-L +
### Table 1. Functional characteristics of stromal clones in extended long-term co-cultures with CD34+ UCB cells.

<table>
<thead>
<tr>
<th>Stromal co-cultures</th>
<th>Total stem and progenitor cell subsets</th>
<th>CFU-GM</th>
<th>CAFC&lt;sub&gt;weeks&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tpo</td>
<td>Flt3-L + Tpo</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/8</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/8</td>
<td>12</td>
</tr>
<tr>
<td>FBMD-1</td>
<td>750</td>
<td>275</td>
<td>648</td>
</tr>
<tr>
<td>AM06-1C4</td>
<td>7</td>
<td>Nd</td>
<td>77</td>
</tr>
<tr>
<td>AM06-2C4</td>
<td>35</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>AM20-1A4</td>
<td>395</td>
<td>Nd</td>
<td>245</td>
</tr>
<tr>
<td>AM20-1B4</td>
<td>4</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>AM30-2A4</td>
<td>290</td>
<td>80</td>
<td>82</td>
</tr>
<tr>
<td>AM30-3A3</td>
<td>158</td>
<td>175</td>
<td>96</td>
</tr>
<tr>
<td>AM30-1C6</td>
<td>443</td>
<td>312</td>
<td>415</td>
</tr>
<tr>
<td>AM30-3F4</td>
<td>292</td>
<td>133</td>
<td>383</td>
</tr>
<tr>
<td>AM30-3F5</td>
<td>270</td>
<td>Nd</td>
<td>158</td>
</tr>
<tr>
<td>UG07-1C6</td>
<td>75</td>
<td>Nd</td>
<td>347</td>
</tr>
<tr>
<td>UG15-1B7</td>
<td>33</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>UG26-1B4</td>
<td>258</td>
<td>133</td>
<td>500</td>
</tr>
<tr>
<td>UG26-1B6</td>
<td>327</td>
<td>360</td>
<td>732</td>
</tr>
<tr>
<td>UG26-2D3</td>
<td>31</td>
<td>2</td>
<td>Nd</td>
</tr>
<tr>
<td>UG26-3D4</td>
<td>254</td>
<td>167</td>
<td>Nd</td>
</tr>
<tr>
<td>EL08-1D2</td>
<td>2538</td>
<td>12544</td>
<td>3163</td>
</tr>
<tr>
<td>EL23-1C2</td>
<td>168</td>
<td>4</td>
<td>145</td>
</tr>
<tr>
<td>GI09-2E6</td>
<td>260</td>
<td>375</td>
<td>310</td>
</tr>
<tr>
<td>GI29-2B4</td>
<td>506</td>
<td>336</td>
<td>650</td>
</tr>
</tbody>
</table>

Data represent one experiment with duplicate cultures. Values are given as percentage of input numbers generated in total (adherent + non-adherent) flask cultures at 7/8 or 12 weeks of co-cultures. The extended LTCs were maintained by weekly half medium change with removal of half of the non-adherent cells in the presence of cytokines. The cultured cells were assayed after pooling the corresponding cultures prior to CFC and CAFC assays. The CFU-GM and CAFC<sub>weeks</sub> numbers present in uncultured material were $1.8 \pm 1.0 \times 10^4$ and $2.7 \pm 1.6 \times 10^3$ per $10^5$ CD34+ UCB cells. Co-cultures with seven clones that maintained in vivo repopulating ability of murine HSC are highlighted (bold face). Abbreviations: AM, aorta-mesenchyme; UG, urogenital ridge; GI, gastrointestinal tract; EL, embryonic liver; Nd, not determined.
Chapter 5

Table 2. The number of progenitors present in the non-adherent compartment of extended long-term stromal co-cultures in the presence of cytokines.

<table>
<thead>
<tr>
<th>Stromal co-cultures</th>
<th>Non-adherent CFU-GM proportion (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tpo</td>
</tr>
<tr>
<td>FBMD-1</td>
<td>50</td>
</tr>
<tr>
<td>AM06-1C4</td>
<td>7</td>
</tr>
<tr>
<td>AM06-2C4</td>
<td>24</td>
</tr>
<tr>
<td>AM20-1A4</td>
<td>27</td>
</tr>
<tr>
<td>AM20-1B4</td>
<td>9</td>
</tr>
<tr>
<td>AM30-2A4</td>
<td>36</td>
</tr>
<tr>
<td>AM30-3A3</td>
<td>44</td>
</tr>
<tr>
<td>AM30-1C6</td>
<td>19</td>
</tr>
<tr>
<td>AM30-3F4</td>
<td>66</td>
</tr>
<tr>
<td>AM30-3F5</td>
<td>31</td>
</tr>
<tr>
<td>UG07-1C6</td>
<td>24</td>
</tr>
<tr>
<td>UG15-1B7</td>
<td>95</td>
</tr>
<tr>
<td>UG26-1B4</td>
<td>51</td>
</tr>
<tr>
<td>UG26-1B6</td>
<td>32</td>
</tr>
<tr>
<td>UG26-2D3</td>
<td>82</td>
</tr>
<tr>
<td>UG26-3D4</td>
<td>40</td>
</tr>
<tr>
<td>EL08-1D2</td>
<td>64</td>
</tr>
<tr>
<td>EL23-1C2</td>
<td>47</td>
</tr>
<tr>
<td>GI09-2E6</td>
<td>31</td>
</tr>
<tr>
<td>GI29-2B4</td>
<td>55</td>
</tr>
</tbody>
</table>

Data represent one experiment with duplicate cultures. Values are given as percentage of total (adherent + non-adherent) numbers generated at 7/8 weeks of co-culture. The extended LTCs were maintained by weekly half medium change and thus removal of half of the non-adherent cells, in the presence of Flt3-L +/- Tpo. The cultured cells were assayed after pooling the corresponding cultures prior to CFC assay. The adherent and non-adherent fractions of the corresponding stromal co-cultures were analyzed separately. Abbreviations: AM, aorta-mesenchyme; UG, urogenital ridge; GI, gastrointestinal tract; EL, embryonic liver; Nd, not determined.
**Table 3.** Variable proportions of human UCB progenitor and stem cells are found in the non-adherent compartment of extended long-term stromal co-cultures.

<table>
<thead>
<tr>
<th>Stromal co-cultures</th>
<th>Non-adherent stem and progenitor cell subsets (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU-GM</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>FBMD-1</td>
<td>62±22</td>
</tr>
<tr>
<td>UG26-1B6</td>
<td>55±10</td>
</tr>
<tr>
<td>EL08-1D2</td>
<td>50±2</td>
</tr>
<tr>
<td>GI29-2B4</td>
<td>56±0.2</td>
</tr>
</tbody>
</table>

Data represent the mean of two independent experiments (± 1 SD) with duplicate cultures. Values are given as percentage of total (adherent + non-adherent) numbers generated at weeks 2, 4 or 6 in whole flask co-cultures. The extended LTCs were contained supplemental with Flt3-L + Tpo, and maintained by weekly half medium change with removal of half of the non-adherent cells. The cultured cells were assayed after pooling the corresponding cultures prior to CFC and CAFC assays. The adherent and non-adherent fractions of the corresponding stromal co-cultures were analyzed separately. Abbreviations: UG, urogenital ridge; GI, gastrointestinal tract; EL, embryonic liver.

**Table 4.** The expansion of CFU-GM and CAFC_{week6} subsets after extended long-term stromal co-cultures without weekly depletion.

<table>
<thead>
<tr>
<th>Stromal co-cultures</th>
<th>Total stem and progenitor cell subsets (fold the input)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU-GM</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>FBMD-1</td>
<td>211</td>
</tr>
<tr>
<td>UG26-1B6</td>
<td>49</td>
</tr>
<tr>
<td>EL08-1D2</td>
<td>59</td>
</tr>
<tr>
<td>GI29-2B4</td>
<td>144</td>
</tr>
</tbody>
</table>

Data represent one experiment with duplicate to quadruple cultures. Values are given as fold of input numbers generated in total (adherent + non-adherent) total cultures at 4, 8 or 12 weeks of co-cultures. The extended LTCs were maintained in the presence of Flt3-L + Tpo and splitted when necessary without weekly depletion of half of the non-adherent cells. The cultured cells were assayed after pooling the corresponding cultures prior to CFC and CAFC assays. The CFU-GM and CAFC_{week6} numbers present in uncultured material were 2.2 ± 0.6 x 10^4 and 2.9 ± 1.5 x 10^5 per 10^5 CD34^+ UCB cells. Abbreviations: BM, bone marrow; AM, aorta-mesenchyme; UG, urogenital ridge; GI, gastrointestinal tract; EL, embryonic liver; Nd, not determined.
Tpo (range 8-100%) groups (table 2). The median non-adherent CFU-GM fraction was 66% (range 37-91%) and 60% (range 0-86%) at week 12 for the same groups (data not presented). We subsequently determined the stem cell and progenitor fractions in the adherent and non-adherent compartments with the best supportive stromal clones during the first six weeks of co-cultures in the presence of Flt3-L and Tpo. The percentage non-adherent CFU-GM fraction in these co-cultures ranged from 40-90% (table 3). All together, the majority (> 80%) of the stromal clones contained most (30-90%) of the human progenitors in the non-adherent fraction during the two-twelve weeks of culture. Surprisingly, also high CAFC_{weeks} numbers were contained in the non-adherent compartment of stromal co-cultures (table 3). This observation is at variance with those of other investigators, who found that most primitive progenitors are located in the adherent compartment of primary BM stromal co-cultures [26-30]. Our data indicate that the weekly half-medium change in our culture system may have strongly affected the CFC and CAFC numbers in the total cultures leading to an underestimation of the HSC and progenitor expansion.

In order to assess the full potential of stromal co-cultures to expand human HSCs and progenitors, we measured the expansion factor of HSCs and progenitors under conditions where no weekly depletion of non-adherent cells was applied. For this purpose we performed extended LTCs of CD34+ UCB cells on the three best supporting stromal clones and the reference line FBMD-1 in the presence of the cytokine combination Flt3-L + Tpo, because the addition of these cytokines resulted in superior maintenance of CAFC_{weeks} in our previous FBMD-1 co-culture studies [16]. These cultures were maintained by splitting the flask cultures at weeks 4 and 8 and seeding the harvested cells on newly established corresponding stromal layers. Indeed, under these conditions a dramatic expansion of both CFU-GM and CAFC_{weeks} were observed (table 4). The AGM region derived stromal clone UG26-1B6 was superior in the expansion of CAFC_{weeks} as compared to other clones tested and contained 825-fold more CAFC per culture as had been inoculated at the start of the experiment. However, the superior support of this stromal clone became only apparent after more than eight weeks of in vitro propagation. During the whole culture time cobblestone areas and single phase dark cells were observed in high frequencies in the stromal layers.
Discussion

During murine development HSC activity has been found in the AGM region, vitelline and umbilical arteries, yolk-sac and liver, suggesting that these sites provide a supportive microenvironment for maintenance and probably numerical expansion of these HSCs. However, the precise role of the microenvironment in these midgestational sites in maintenance and growth of human HSCs is largely unexplored. In the study presented here we have identified four embryonic stromal clones, two UG-, one GI- and one EL-derived, that support a dramatic expansion of CFU-GM and CAFC<sub>week6</sub> during a twelve week co-culture with human CD34<sup>+</sup> UCB cells in the presence of at least Tpo. This expansion was higher than that obtainable with the FBMD-1 stromal line known for its capability to maintain or expand HSC activity. Without addition of cytokines only one EL-derived cell clone maintained the CFU-GM generation for up to twelve weeks, while in the presence of at least Tpo to the stromal co-cultures a continuous HSC and progenitor production was supported by twelve out of nineteen stromal clones. The limited number of stromal clones analyzed does not allow identification of the embryonic region that best supports the maintenance and expansion of human HSC subsets.

Five out of seven stromal clones that supported murine in vivo repopulating HSCs also supported the generation of human newborn HSCs and progenitors in vitro. This strongly suggests that the factors responsible for in vivo expansion of murine HSCs in E11 AGM, GI and EL subregions also play an important role in the maintenance of newborn human HSCs in vitro and can be used to amplify the CFU-GM and CAFC<sub>week6</sub> from CD34<sup>+</sup> UCB cells. In contrast, two stromal clones, UG15-1B7 and UG26-2D3, supported murine but not human progenitors in long-term co-cultures. It might therefore be speculated that one or more UG stromal factors involved in maintenance or expansion of human CAFC<sub>week6</sub> in extended LTC-CFC do(es) not have cross-species restrictions, or is(are) produced in sub-effective concentrations. Such factors could be derived from the murine stromal cells, but they could also be elaborated by the human progenitors and function to communicate with the stromal cells. In this context, it is of relevance to note that cytokine addition improved LTC-IC maintenance in part through interaction with progenitors and/or in part through interaction with stromal cells [31, 32]. Chemokines, such as the AGM-expressed WECHE, have been reported to inhibit the growth of progenitors [33, 34]. These data suggest that the ability of different AGM-derived stromal clones to support human HSCs and progenitors may be explained in part by proportionate differences in expression of stimulatory and inhibitory factors for HSC and progenitor expansion.
Although most of the embryonic clones used here were derived from different embryonic regions, we observed large variability in support of human HSCs and progenitors even between the clones derived from the same region. Such variability is most likely representative of the complex interplay between stroma and hematopoietic cells, regulated by adhesion molecules, extra-cellular matrix molecules, membrane-bound cytokines and cytokine receptors. Chemotactic factors such as SDF-1α may additionally help to guide and maintain HSCs in their niches [35-37]. Previously, we showed that the presence of Tpo in long-term stromal co-cultures prolonged the hematopoietic activity and stimulated the generation of human progenitors for up to twentyfive weeks [38]. Recently, we demonstrated the synergistic role of stroma-cytokine combinations in expansion of human primitive progenitors [16]. In the present study, the addition of Tpo to different stromal co-cultures often dramatically prolonged the hematopoietic activity, indicating the importance of this cytokine in long-term stromal survival of human HSCs. These data support and extend the requirement of Tpo to maintain HSCs in stromal co-cultures [39].

We show that a significant proportion of human CAFC and CFU-GM in co-cultures with a majority of murine stromal clones is found in the non-adherent compartment. We have also shown that both the adherent and non-adherent HSCs and progenitors can be gradually lost during extended cultures due to medium changes and that consequently the support of stromal clones for maintenance of HSCs and progenitors can be dramatically underestimated. In general, different methods are applied for maintenance of long-term stromal co-cultures. These include (a) half-medium changes each week and removal of half of the non-adherent cells as is the case in a part of our experiments, (b) medium changes without discarding cells, or (c) maintenance by splitting and refeeding the cultures with seeding the total number of harvested adherent and non-adherent cells on newly established stromal layers without discarding cells. All these methods will affect the number of HSCs and progenitors obtained after extended LTCs. It is generally assumed that in primary long-term co-cultures the majority of the most primitive stem cells adheres to the stromal layer. For example, Mauch et al. showed that about one third of murine spleen colony forming cells (CFU-S) was contained in the non-adherent compartment of stromal co-cultures, however, the adherent CFU-S had greater ‘self-renewal’ capacity than did non-adherent CFU-S [40]. Whether our stromal cell lines differentially affect the extent of self-renewal in our extended LTC is presently under investigation. Our findings that a large proportion of HSCs and progenitors was present in the non-adherent fraction of extended LTCs demonstrate that the ability of stromal cell lines to sustain HSC and progenitor production cannot be evaluated on the basis of the adherent or non-adherent fraction alone.
In the present study, we describe a set of embryo-derived stromal cell lines capable of sustaining human hematopoiesis in extended cultures. These stromal cells provide us with tools to investigate factors required for \textit{in vitro} maintenance of human hematopoiesis as well may assist in establishing of culture systems that may mediate numerical expansion of HSCs \textit{in vitro}.

\textbf{Acknowledgements}

We would like to acknowledge the nursing staff and midwives of the St. Fransiscus Gasthuis for collection of UCB samples, and P.B. van Hennik, W.J.C. Rombouts and I. Blokland for technical assistance.
Chapter 5

References

17. Lewis ID, Verfaillie CM. Multi-lineage expansion potential of primitive hematopoietic progenitors. Superiority of umbilical cord blood compared to mobilized peripheral blood [In Process Citation]. Exp Hematol 2000; 28 (9): 1087.
Umbilical cord blood hematopoietic stem and progenitor cells

31. Punzel M, Gupta P, Roodell M, Mortari F, Verfaillie CM. Factor(s) secreted by AFT024 fetal liver cells following stimulation with human cytokines are important for human LTC-IC growth. Leukemia 1999; 13 (7): 1079.
Chapter 6

Flt3-L and Tpo modulate the SDF-1 mediated chemotactic activities of marrow and embryonic stromal cells.


1Institute of Hematology and 2Cell Biology and Genetics, Erasmus Medical Center Rotterdam

In preparation, 2002.
Abstract

Studies of hematopoietic cell interactions with their microenvironment have resulted in establishment of many stromal cell lines from adult bone marrow (BM), fetal liver, aorta-gonad-mesonephros (AGM) region and yolk-sac. In the studies presented here we examine chemoattractive activities of stromal cell lines derived from several of these tissues using human hematopoietic cells as targets in a transwell migration assay. In addition, we examine the effect of exogenous cytokine addition on the chemotactic activity elaborated by these stromal cells, as well as on the proportion of adherent or non-adherent human umbilical cord blood (UCB)-derived colony forming cells (CFCs) in stromal co-cultures. Our results demonstrate that varying chemotactic activities are present in media conditioned by different stromal cell lines (SCM). The presence of Flt3-L and/or Tpo during the conditioning phase modulated this activity in a stromal cell specific way. Flt3-L or Tpo increased the chemotactic activity of marrow-derived MS-5 and FBMD-1 stromal cells, whilst they inhibited the release of chemotactic activity by AGM-derived stromal cells. Furthermore, the cytokine combination increased the chemotactic activity of embryonic dorsal aorta/mesenchyme(AM)-derived stromal cells. A strong decrease in the migration of HL60 cells towards SCM was observed in the presence of a CXCR4 antagonist, indicating that CXCR4/SDF-1 interaction is involved in the migration of HL-60 and CD34+ UCB cells towards SCM produced by various stromal cells. Since non-adherent CFU-GM in stromal co-cultures could be modulated by addition of cytokines, these results suggest that chemotactic activities are regulated through the interactions of cytokines with stromal cells.

Introduction

The microenvironment of hematopoietic tissues regulates embryonic and adult hematopoiesis in vitro and in vivo via direct cell-cell contact, membrane-bound presentation of cytokines and extracellular matrix components. The use of Dexter-type long-term cultures closely mimicking the in vivo hematopoietic microenvironment allowed in vitro maintenance of progenitors for extended periods and analysis of stroma-dependent hematopoiesis [1, 2]. In these stromal co-cultures the primitive progenitors present in the inoculum are assumed to adhere to and migrate into the stromal layer, i.e. the adherent compartment, where they may quiescently survive or proliferate and release colony-forming cells (CFC) in the supernatant, i.e. the non-adherent
compartment. Although the non-adherent CFC are routinely assayed as an indicator of the hematopoietic activity in stromal co-cultures, there are indications that the majority of primitive progenitors are located in the adherent compartment [1-5]. Thus, although spleen colony forming cells (CFU-S) and CFC have been described to localize in both the adherent and non-adherent compartments [6-8], the majority of primitive progenitors can be recovered from the stromal layers [9, 10]. Although there is a close association between progenitors and stromal cells, their direct interaction appears not to be required for the maintenance of long-term in vitro hematopoiesis [11].

To examine the interactions of hematopoietic cells with their microenvironments, many stromal cell lines and clones from adult, fetal and embryonic hematopoietic sites have been established and demonstrated to maintain in vitro and in vivo repopulating hematopoietic cells [12-19]. On the other hand, stromal cells have been shown to increase expansion of long-term culture-initiating cells (LTC-ICs) [20] and lineage(Lin)·CD34+ [21] cells and extend maintenance of graft quality [22]. Our preliminary data indicate that the majority of primitive human progenitors (CAFC wk6) localizes in the non-adherent compartment of co-cultures with stromal cell lines from adult murine BM and embryonic AGM region-, liver- and gut mesentery-derived stromal cells [23]. We speculated as to whether stromal elaboration of chemoattractant activities contributes to the establishment of a distribution of (primitive) progenitors over adherent and non-adherent fractions in co-cultures. One potential chemoattractant for progenitors is the stromal cell-derived factor-1 (SDF-1, CXCL12), a ligand for the G-protein coupled CXCR4 receptor, produced by BM stromal cells and osteoblasts. This chemokine has been shown to strongly attract CD34+ and the more primitive CD34+CD38- hematopoietic cells. It activates β1-integrins, which induce gelatinase B (MMP-9) expression on hematopoietic progenitors, probably allowing these cells to migrate through endothelial cell layers [24-30].

We also wished to know whether changing environmental factors modulate the chemoattractive activity of stromal cells. Thus, we evaluated whether the early acting cytokines Flt3L and Tpo, which enhance maintenance of (primitive) progenitors in extended long-term cultures, would modulate the chemoattractive activity of stromal cells. To this purpose, we have prepared stromal cell-conditioned medium (SCM) in the absence or presence of Flt3-L and/or Tpo, and tested these for their ability to stimulate both chemotaxis and chemokinesis [31] of a human leukemic cell line (HL-60) and human CD34+ UCB cells. We here demonstrate that different stromal cells produce in their supernatants different levels of chemotactic activities and that Flt3-L and/or Tpo can modulate these activities by stromal cells. Our results suggest that these chemoattractive
Umbilical cord blood hematopoietic stem and progenitor cells

activities are involved in the localization of (primitive) progenitors in the adherent compartment of stromal co-cultures.

Material and methods

*Human umbilical cord blood cells.* Human UCB samples were collected from umbilical cord vein after full-term delivery by the nursing staff of the Department of Obstetrics and Gynecology at the Sint Franciscus Gasthuis (Rotterdam, The Netherlands). UCB was collected in sterile flasks containing 10 ml citrate-glucose as anticoagulant, stored at room temperature and processed within 24 hours of collection. Low-density cells were isolated using Ficoll Hypaque density centrifugation (1.077g/cm², Lymphoprep, Nycomed Pharma, Oslo, Norway). After centrifugation at 600 g for 15 minutes, the mononuclear cells (MNCs) at the interface were collected, washed twice with Hanks Balanced Salt Solution (HBSS, Gibco, Breda, The Netherlands) and resuspended in Iscove’s modified Dulbecco’s medium (IMDM, Gibco, Breda, The Netherlands). The MNCs were stored in 10% FCS, 20% DMSO in IMDM in liquid nitrogen until use.

*Isolation of CD34+ cells.* In all experiments human UCB were positively selected for the expression of CD34 using either Variomacs or Automacs Immunomagnetic Separation System (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers instructions. After thawing and before the CD34+ hematopoietic cells were isolated we pooled 2 to 21 different UCB samples. The purity of the CD34 selected cells from UCB, as determined by FACS after staining with a CD34 antibody, was >90%. The progenitor and stem cell numbers in the input and output suspensions were determined by CFC and CAFC assays. The absolute number of progenitors present in uncultured material was 14,194 ± 6,504 and 2,942 ± 1,047 per 10⁵ CD34+ cells for CFU-GM and CAFC respectively.

*Hematopoietic growth factors.* The following cytokines were used: recombinant human stem cell factor (SCF), recombinant human FT3-L and recombinant human Tpo, all kindly donated by C. Saris from Amgen (Thousand Oaks, CA, USA); recombinant human interleukin-6 (IL6), recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF), recombinant human granulocyte colony stimulating factor (G-CSF) and recombinant murine stem cell factor (SCF), all gifts from Genetic Institute (Cambridge, MA, USA); recombinant human erythropoietin (Epo, Boehringer, Mannheim, Germany) and recombinant human IL3 (Gist Brocades, Delft, The Netherlands).
Chapter 6

Stromal cells. The FBMD-1 cells were cultured as previously described [32]. MS-5 was kindly provided by Dr. L. Coulombel, Paris, France and cultured as FBMD-1 cells. The embryonic stromal clones were generated from subdissected AGM regions, livers and gastrointestinal tracts from E10 and E11 embryos of transgenic for LacZ inserted into the first intron of the Ly-6E.1 locus or transgenic for temperature-sensitive mutant SV40 large T antigen. Details about the establishment of these stromal clones have been described elsewhere [33]. The embryonic stromal clones were cultured in 25 or 75 cm² flasks (Costar, Cambridge, MA, USA) with 5 or 15 ml IMDM containing 10% fetal calf serum (FCS, Summit, Fort Collins, CO) and 10% horse serum (HS, Gibco), supplemented with penicillin (100 U/ml, Gibco), streptomycin (0.1 mg/ml, Gibco), β-mercapto-ethanol (10⁻⁴ M, Merck, Darmstadt, Germany). The stroma cells were grown to confluency at 33°C and 10% CO₂ and irradiated with 40 Gy prior to stromal cultures, or not as was the case with SCM.

Stromal cocultures. Irradiated confluent layers of different stromal cells in 25 cm² flasks were overlaid with 1.5 x 10⁴ or 2 x 10⁴ CD34⁺ UCB cells. These cells were cultured in 5 ml IMDM-cocktail containing 20% FCS supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), β-mercapto-ethanol (10⁻⁴ M), cholesterol (15 μM, Sigma), linolic acid (15 μM, Merck), iron-saturated human transferrin (0.62 g/l, Intergen, Uithoorn, The Netherlands), nucleic acids (cytidine, adenosine, uridine, guanosine, 2'-deoxycytidine, 2'-deoxyadenosine, thymidine, 2'-deoxyguanosine (all at 10⁻³ g/ml, Sigma)). Cultures were fed with the cytokine combination Flt3-L (50 ng/ml) and/or Tpo (10 ng/ml). Cultures of each group were set up in two-fold and maintained at 33°C and 10% CO₂ for two weeks with half medium change and removal of half of the non-adherent cells at week 1. At week 2 the CFC outputs of the adherent and non-adherent fractions from two co-cultures with FBMD-1, EL08-1D2, UG26-1B6, UG26-3D4, GI29-2B4, AM30-2A4, AM30-3A3, AM30-3F4, and AM30-1C6 were determined. In addition, we also determined the CAFC outputs from the co-cultures with FBMD-1, EL08-1D2, UG26-1B6, UG26-3D4, GI29-2B4. In a second series of experiments 1.5 x 10⁵ or 2 x 10⁵ CD34⁺ UCB cells were cultured on FBMD-1, MS-5, GI29-2B4 and EL08-1D2 in the absence or presence of Flt3-L (50 ng/ml) and evaluated for their adherent and non-adherent CFC fractions at week 2 of culture. In two additional experiments 1 x 10⁴ or 2 x 10⁴ CD34⁺ UCB cells were deposited on UG-26-1B6 and evaluated in the presence of Flt3-L (50 ng/ml), Tpo (10 ng/ml), SCF (100 ng/ml), IL6 (100 ng/ml) alone and in various combinations of these cytokines for the balance between CFCs in the adherent and non-adherent compartments of the cultures. These experiments were also performed with the cytokine combination G-SCF (20 ng/ml) and IL3 (10 ng/ml) and

158
without addition of any cytokines. The cultures were maintained for two weeks under the same conditions as above mentioned. To harvest the adherent layer, the non-adherent cells were first collected and the layer washed with phosphate-buffered saline (PBS, Life Technologies, Breda, The Netherlands) and subsequently incubated with trypsin-EDTA (Life Technologies, Breda, The Netherlands) at 37°C for 5-10 minutes. The digestion was stopped by adding 1 ml FCS.

**Stroma-conditioned medium.** Confluent layers of different stromal cells in 75 cm² flasks were washed twice with PBS and incubated for 7 days with 15 ml IMDM and 0.25% BSA supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml) and β-mercapto-ethanol (10⁻⁴ M): IMDM/BSA. The conditioning was also performed in the presence of either Flt3-L (50 ng/ml) and Tpo (10 ng/ml) alone or a combination of both cytokines. The conditioned media were harvested, centrifuged at 315 g for 5 minutes to get rid of non-adherent cells and used subsequently in migration assays.

**In vitro migration assays.** Migration of cells were performed using two chamber transwells (6.5 mm diameter, 24-well clusters, Costar, Cambridge, MA) with 5 μm pore size. The pore filter were pre-coated overnight with 20 μg/ml bovine fibronectin (Sigma F 1141). The HL-60 leukemic cell line (CCL-240, American Type Culture Collection, Manassas, USA) was cultured in IMDM with 20% FCS. For migration assays, either 10⁵ to 3x10⁵ HL-60 or 10⁵ CD34⁺ UCB cells were washed twice with PBS and resuspended in either 100 μl IMDM/BSA or SCM and placed in the upper chamber of the transwell. In the lower chamber, 600 μl of IMDM/BSA with or without the ligands SDF-1 (R&D Systems Europe, Oxon, UK), Flt3-L (50 ng/ml) and Tpo (10 ng/ml) alone or in combination was placed. In additional experiments 600 μl of cytokine-absent or cytokine-induced SCM was placed in the lower chamber. Finally, in one experiment the effect of CXCR4 antagonist (Becton & Dickinson, Alphen aan den Rijn, The Netherlands) on migration was evaluated. Herefore, HL-60 cells were incubated with or without 10 μg/ml for 30 minutes in culture medium at room temperature prior to migration assay. After 4 hours of incubation at 37°C 10% CO₂ the upper chamber was removed and the number of migrated cells was assessed using a CASY®1/TTC cellcounter (Schärfe System GmbH, Reutlingen, Germany) for HL-60 cells and Symex microcellcounter (Charles Goffin, IJsselstein, the Netherlands) for CD34⁺ UCB cells. Percentage of migration was determined by calculating percentage of input cells migrated into the lower chamber.

**Clonogenic assay.** Granulocyte-macrophage colony forming unit (CFU-GM) and burst forming unit-erythroid (BFU-E) progenitor cells were assayed using a semisolid culture medium (1.2% methylcellulose), containing IMDM supplemented with 30%
FCS, β-mercapto-ethanol (5 x 10⁻⁵ M), penicillin (100 U/ml), streptomycin (0.1 mg/ml), hu-Epo (1 U/ml), hu-IL3 (20 ng/ml), hu-GM-CSF (5 ng/ml), hu-G-CSF (50 ng/ml) and mu-SCF (100 ng/ml). Duplicate cultures were plated in 35 mm tissue culture dishes (Falcon) and incubated at 37°C and 10% CO₂ in a humidified atmosphere for 14 days. Colonies containing 50 cells or more were scored at day 14 using an inverted light microscope.

Cobblestone area forming cell assay. Confluent stromal layers of FBMD-1 cells in flat-bottom 96-well plates (Falcon) were overlaid with UCB cells in a limiting dilution set up as described [34]. Briefly, twelve successive two-fold dilutions were used for each sample with 15 wells per dilution. The percentage of wells with at least one phase-dark hematopoietic clone (cobblestone area) of at least five cells beneath the stromal layer was determined at week 6 (CAFC_wk6). At initiation of the cultures 250 CD34⁺ UCB cells per well were plated in the first dilution, while after cultures 857 CD34⁺ input equivalent cells per well were used in the first dilution. The CAFC frequencies were calculated using Poisson statistics.

Statistical analysis. The values are reported as mean ± 1 SD. The correlation coefficients are measured using Microsoft Excel 97 program.

Results

High proportion of (primitive) progenitors is found in the non-adherent compartment of stromal cocultures. To determine the progenitor subsets in the two compartments of different stromal co-cultures, CD34⁺ selected UCB cells were seeded on confluent layers of irradiated (40 Gy) stromal cells and maintained for two weeks. Interestingly, a high proportion of CAFC_wk6 and CFU-GM were observed in the non-adherent compartment of stromal co-cultures (figure 1a). These proportions remained high and even increased when cultured for extended periods of twelve weeks [23]. Furthermore, the percentage of non-adherent CFU-GM generally reflected that of the non-adherent CAFC_wk6 in these cultures (figure 1b), suggesting that common factors determined their distribution and that measurement of CFU-GM would give sufficient information for both progenitor subsets.

Effect of cytokines on the proportion of progenitor subsets in adherent and non-adherent compartments of stromal cocultures. In order to elucidate whether cytokines could modulate the distribution of primitive progenitors over adherent and non-adherent compartments of stromal co-cultures, CD34⁺ UCB cells were cultured in the presence
Umbilical cord blood hematopoietic stem and progenitor cells

Figure 1. The proportion of non-adherent CFU-GM and CAFC_{week6} in cocultures of CD34+ UCB cells and various stromal cells. Data represent the mean of two experiments with duplicate cultures. The cultures were maintained by half medium change at week 1 with removal of half of the non-adherent cells in the presence of Flt3-L+Tpo. In each experiment the cultured cells were assayed after pooling the corresponding cultures prior to CFC and CAFC assays. Bars represent the non-adherent CFU-GM fraction of several stromal cell lines at week 2 of culture (a). Also the non-adherent CAFC_{week6} fraction with respective CFU-GM fractions are plotted for five representative stromal co-cultures at week 2 (b).
of either single cytokines or combinations thereof for two weeks on UG26-1B6 stromal cells. The CFU-GM of the non-adherent compartment as percent of total is shown in table 1. Cytokine addition led to an increased proportion of non-adherent CFU-GM in the groups that contained exogenous Tpo, SCF, Flt3-L + Tpo + SCF, Flt3-L + Tpo + IL6, SCF + IL6 or IL3 + G-CSF, while the other cytokine groups did not significantly differ from the control cultures. This suggested that exogenous cytokine(s) can affect the distribution of CFU-GM over adherent and non-adherent compartments of stromal co-cultures.

Chemotactic activity of medium conditioned by different stromal cells. To determine whether the presence of primitive progenitors in the non-adherent compartment was due to chemotactic factors released by stromal cells, we measured the in vitro migratory capacity of HL-60 cells towards media conditioned by different stromal cells. We first compared the spontaneous and ligand induced migration of HL-60 cells in a transwell system with fibronectin-coated filters. HL-60 cells, like CD34+ UCB cells, express CXCR4 receptor and migrate towards SDF-1 [31, 35]. Hardly any spontaneous migration was observed over these filters (figure 2), while increasing percentages of HL-60 cells migrated towards increasingly higher SDF-1 concentrations. Some cytokines including Tpo have been reported to act as chemoattractant [36], while other cytokines including Flt3-L are associated with mobilization of CD34+ cells [37, 38]. Using the same cytokine concentrations as had been used under stromal co-culture conditions, the presence of the cytokines Flt3-L, Tpo or both in the lower chamber showed no additional effect on chemotactic activity (figure 2b). However, SDF-1 in combination with various cocktails including Flt3-L or Tpo showed consistent effects on chemotactic activity.

We next investigated the migration-inducing activity of SCM in the presence or absence of cytokines. In the first set of experiments we evaluated the migration of HL-60 cells in a positive chemoattractant gradient (directed movement, i.e. chemotaxis). The MS-5 stromal co-culture was used as reference because it produces high levels of SDF-1 [39]. The supernatant from the MS-5 cells attracted 10% of the HL-60 cells, while the FBMD-1 CM attracted 6.5%. The supernatants from the embryonic stromal cells showed similar chemoattractive activity (15-42%), except for three AM30 clones (-1C6, -2A4, and -3F4), which showed much higher chemoattractive activity for HL-60 cells (figure 3a).

We were interested to see whether the presence of Flt3-L and Tpo would affect the chemotactic activity in the medium of our stromal co-cultures. As shown in figure 3a, Flt3-L increased the chemotactic activity of the BM-derived stromal cells MS-5 and FBMD-1, whereas Tpo had no effect. In contrast, Flt3-L decreased the chemotactic
Table 1. Effects of exogenously added cytokines on the proportion of non-adherent CFU-GM in co-culture supported by UG26-1B6 stromal cells.

<table>
<thead>
<tr>
<th>Stromal co-culture conditions</th>
<th>Non-adherent proportion of CFU-GM as of total CFU-GM content</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cytokines</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>Flt3-L</td>
<td>77 ± 27</td>
</tr>
<tr>
<td>Tpo§</td>
<td>88 ± 8</td>
</tr>
<tr>
<td>SCF</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>IL6</td>
<td>79 ± 21</td>
</tr>
<tr>
<td>Flt3-L + Tpo</td>
<td>73 ± 23</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>Flt3-L + Tpo + IL6</td>
<td>79 ± 1</td>
</tr>
<tr>
<td>Flt3-L + Tpo + SCF + IL6</td>
<td>68 ± 2</td>
</tr>
<tr>
<td>SCF + IL6</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>IL3 + G-CSF</td>
<td>85 ± 1</td>
</tr>
</tbody>
</table>

CD34+ UCB cells were cultured with UG26-1B6 stromal cells and maintained in the presence of cytokines for two weeks. A half medium change at week 1 was performed by removal of half of the non-adherent cells. Data represent the mean (± 1 SD) of two independent experiments with duplicate cultures. Abbreviations: UG, urogenital ridge. §, mean of four experiments.

Figure 2. The effect of SDF-1, Flt3-L and/or TPO on migration of HL-60 cells. Data represent the mean (± 1 SD) percentage of one representative experiment performed in duplicate. Migration of HL-60 cells was determined in response to various concentration of SDF-1 (a). The effect of Flt3-L (50ng/ml) and/or Tpo (10 ng/ml) with or without SDF-1 (250 ng/ml) were also determined (b). IMDM/BSA without any supplements was included as control. Abbreviations: F, Flt3-L; T, Tpo; S, SDF-1.
Figure 3. The chemotactic activity in SCM of adult, fetal liver and embryonic stromal cells measured using HL-60 cells. Data represent the mean (±1 SD) percentage of migrating HL-60 cells in one experiment performed in duplicate or quadruplicate. Migration of HL-60 cells in response to cytokine-absent or cytokine-induced SCM was determined after 7 days medium conditioning on different stromal cells in the absence or presence of Flt3-L and/or Tpo (a). Effect of exogenous added cytokines on migration in response to SCM prepared in the absence of cytokines (b) and the chemokinetic activity of this latter SCM group (c) were also determined. The mean percentage of migration towards control was 0.7 ± 0.3 and towards SDF-1 21.2 ± 19.5 in case of chemotaxis and 2.3 ± 1.6 in case of chemokinesis, respectively. Abbreviations: FL, fetal liver; AM, aorta-mesenchyme; UG, urogenital ridge; GI, gastrointestinal tract; EL, embryonic liver. ** mean of 2 experiments performed in four- or six-replicates.
activity of the AM30-1C6, -2A4, EL08-1D2, and GI29-1B4 embryonic stromal cells (figure 3a), as well as all of the embryonic stromal clones tested (not shown). Since both Flt3 and Tpo as single cytokine additions decreased chemotactic activity of the embryonic stromal clones, we were surprised to find that the presence of both cytokines during medium-conditioning induced a dramatic stimulatory effect on migration ability of all four AM30 clones (figure 3a). In control experiments, we evaluated whether Flt3-L or Tpo would directly affect the HL-60 migratory activity. As is shown in figure 3b, exogenous addition of either Flt3-L, Tpo or both to the lower chambers during the chemotaxis assay did not affect HL-60 migration. Thus, the presence of exogenous cytokines in the stromal co-cultures strongly affects the secretion of chemotactic activity by stromal cells.

We also tested whether the various SCMs induced chemokinetic activity (random movement, i.e. chemokinesis). We observed variable chemokinesis of HL-60 cells between CM from different stromal cells (figure 3c). Except for AM30 clones, no significant effect in random migration of HL-60 cells was observed as compared to the reference stromal activity of MS-5.

### Table 2. Effects of blocking CXCR4 receptor on migration of HL-60 cells towards stroma-conditioned medium.

<table>
<thead>
<tr>
<th>Conditions in the lower well</th>
<th>Migration (% of input)</th>
<th>% of migration inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without CXCR4 antagonist</td>
<td>With CXCR4 antagonist</td>
</tr>
<tr>
<td>Control</td>
<td>1 ± 0.3</td>
<td>0.9 ± 0.7</td>
</tr>
<tr>
<td>SDF-1</td>
<td>64 ± 3</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>MS-5</td>
<td>20 ± 1</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>FBMD-1</td>
<td>10 ± 0.7</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td>AM30-3F4</td>
<td>50 ± 2</td>
<td>9 ± 0.4</td>
</tr>
<tr>
<td>AM30-2A4</td>
<td>41 ± 6</td>
<td>5 ± 0.08</td>
</tr>
<tr>
<td>AM30-1C6</td>
<td>15 ± 2</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td>AM30-3A3</td>
<td>11 ± 3</td>
<td>5 ± 0.6</td>
</tr>
<tr>
<td>UG26-1B6</td>
<td>5 ± 1</td>
<td>3 ± 0.8</td>
</tr>
<tr>
<td>UG26-3D4</td>
<td>4 ± 0.2</td>
<td>3 ± 0.1</td>
</tr>
<tr>
<td>EL08-1D2</td>
<td>5 ± 0.7</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td>GI29-2B4</td>
<td>6 ± 0.9</td>
<td>3 ± 0.02</td>
</tr>
</tbody>
</table>

Migration of HL-60 cells in response to medium conditioned by stromal cells in the absence of cytokines was determined in the absence or presence of a CXCR4 antagonist (10 µg/ml) in the upper well. Percentage of migration represents the mean of one experiment performed in duplicate. Abbreviations: AM, aorta-mesenchyme; UG, urogenital ridge; GI, gastrointestinal tract; EL, embryonic liver.
Table 3. The chemotactic activity in Flt3-L and Tpo-induced SCM of adult and embryonic stromal cells measured using UCB CD34+ cells.

<table>
<thead>
<tr>
<th>Conditions in the lower well</th>
<th>Migration (% of input)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.4 ± 0.04</td>
</tr>
<tr>
<td>SDF-1</td>
<td>34 ± 14</td>
</tr>
<tr>
<td>MS-5</td>
<td>14 ± 0</td>
</tr>
<tr>
<td>FBMD-1</td>
<td>8 ± 0</td>
</tr>
<tr>
<td>AM30-3F4</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>AM30-2A4</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>AM30-1C6</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>AM30-3A3</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>UG26-1B6</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>UG26-3D4</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>EL08-1D2</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>GI29-2B4</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

Migration of CD34+ UCB cells in response to medium conditioned by stromal cells in the absence of cytokines was determined. Percentage of migration represents the mean of one experiment performed in duplicate. Abbreviations: AM, aorta-mesenchyme; UG, urogenital ridge; GI, gastrointestinal tract; EL, embryonic liver.

The chemotactic activity released by stromal cells can be partly blocked by CXCR4 antagonist. To examine whether the chemotactic activity of SCM could be due to the presence of SDF-1, we added a CXCR-4 antagonist to the upper chamber of the chemotaxis chambers. Addition of this antagonist inhibited the SDF-1 induced migration of HL-60 cells by >70% (table 2). Indeed, the SCM-induced migration was suppressed to a great extent for all SCM elaborated from the various stromal lines (42% to 87%), indicating that the chemotactic activity of the different SCMs was largely due to the presence of SDF-1 in these SCMs.

Human CD34+ UCB cell migration towards SCMs. To validate some of the above findings obtained with the HL-60 culture line we also performed experiments with primary human CD34+ UCB cells. Since the stromal co-cultures testing for CFU-GM were performed in the presence of the cytokines Flt3-L and Tpo, we used Flt3-L + Tpo induced SCM to assess the chemotactic activity on CD34+ UCB cells. These cells showed hardly any spontaneous migration, whereas chemoattractant activity towards SDF-1 resulted in migration of 34% ± 14 of input cells (table 3). SCM from various stromal cell lines demonstrated a significant chemoattractive activity for CD34+ UCB cells, particularly the AM30 clones that showed the highest percentage of activity (31%...
Umbilical cord blood hematopoietic stem and progenitor cells

to 70%). The chemotactic activity of the various SCMs for the HL-60 cells and CD34+ cells showed a high correlation coefficient (figure 4a), strongly suggesting that the use of HL-60 cells as target cells in migration assays is predictive for CD34+ UCB cell migration.

Figure 4. Correlation between the non-adherent CFU-GM content of stromal cocultures and the chemotactic activity on HL-60 and UCB CD34+ cells. CFU-GM data represent the mean (± 1 SD) with duplicate cultures and are measured as percentage of total at week 2 of culture (see figure 1b). The chemotactic data represent the mean (± 1 SD) percentage of migrating cells towards SCM in one experiment performed in quadruplicate for HL-60 cells (data are the same as in table 2, first column) and duplicate for CD34+ UCB cells (data are the same as in table 3). The stromal co-cultures and the conditioning of stroma were performed in the presence of the cytokines Flt3-L and Tpo. The chemotactic activity of SCMs measured as migration of HL-60 or CD34+ UCB cells are plotted (a). Also the non-adherent CFU-GM or CAFCweak (b) proportions with corresponding chemotactic activity measured as migration of CD34+ UCB cells are depicted. The correlation coefficients are given as the R squares.
When we compared the migration results obtained with HL-60 cells (table 2) or CD34+ UCB cells (table 3) with the non-adherent CFU-GM fractions (figure 1a), we observe some relationship between these parameters (figure 4b), suggesting that chemotactic activity plays a modest role in the distribution of progenitors over adherent and non-adherent compartment of stromal co-cultures. To test this, we added Flt3-L to co-cultures of C34+ UCB cells and four representative stromal lines and assessed CFU-GM in the non-adherent compartment. As shown in figure 5, the presence of this cytokine diminished the non-adherent CFU-GM percentage in cultures with GI29-2B4 and EL08-1D2 stromal cells and increased its percentage with MS-5 and FBMD-1 (figure 5). Although these changes are similar to, but not as robust as, what we observed for chemoattractive activity (figure 3a), these data suggest that Flt3-L affects the chemotactic activity elaborated by stromal cells, which in turn affects the non-adherent progenitor fractions.

![Figure 5](image)

**Figure 5.** The non-adherent proportion of CFU-GM in the presence of Flt3-L. Data represent the mean of two experiments with duplicate cultures. The cultures were maintained by half medium change at week 1 with removal of half of the non-adherent cells in the presence of Flt3-L + Tpo. In each experiment the cultured cells were assayed after pooling the corresponding cultures prior to CFC and CAFC assays. Abbreviations: GI, gastrointestinal tract; EL, embryonic liver.
Discussion

The interaction between hematopoietic stem cells and the microenvironment requires a close association of these entities. In order for the stem cells to home to the proper microenvironment, chemotactic factors are produced. The most potent of these factors is SDF-1. We here describe that embryonic stromal cells, particularly those derived from the dorsal AM, strongly elaborate chemotactic activities for HL-60 and UCB CD34+ cells. The presence of Flt3-L and/or Tpo during the stromal conditioning phase of medium modulated this chemotactic activity in a stromal cell specific way. Flt3-L or Tpo increased the chemotactic activity of MS-5 and FBMD-1 stromal cells, while these cytokines had an inhibitory effect on production of chemotactic activity by most other stromal cells. Surprisingly, the combination of Flt3-L and Tpo had the opposite effect, as it dramatically increased the chemotactic activity of AM30 clones. These effects were not due to residual Flt3-L and/or Tpo during the transwell migration assay, as addition of these cytokines to the medium in the migration assay did not affect HL-60 cell migration in the absence or presence of various SCM.

In vitro culture systems with stromal cells from BM, yolk-sac, fetal liver or AGM region offer unique opportunities to investigate the interactions between progenitor subsets and stromal cells or factors. The mechanisms underlying these interactions have still not been clearly established. Most studies indicated that the majority of primitive progenitors, which appear to be multifactor-responsive for their survival, adhere directly to the stromal cells, presumably through mediation of various adhesion proteins [40, 41]. Murine and human stromal cells are capable of producing a large number of hematopoietic cytokines [42-47], some of which, like SCF and Tpo, have been shown to have chemoattractive capabilities in addition to their activity on progenitor growth. Other cytokines such as IL8 and MCPs function mainly as regulators of proliferation for progenitors [48-50], even though on mature blood cells they mainly act as chemoattractant and activating factors. In addition, various chemoattractant factors have been described to regulate (primitive) progenitor mobilization and homing. SDF-1, which is released by BM stromal cells and osteoblasts [26, 28, 39], has been shown to be the major chemoattractant for progenitors. In our laboratory, somatostatin (ligand of somatostatin receptor-2) has also been identified as chemoattractant for normal and malignant hematopoietic progenitors [51, 52]. The role of other chemokines, like MIP-3β and WECH which are also expressed by marrow and embryonic stromal cells [53, 54] in HSC and progenitor chemotaxis is less clear. Changing the growth requirements of stem cells is also likely to influence their homing behaviour. Indeed, it is known
that chemoattraction of progenitor and stem cells can be modulated by cytokines. For instance, CD34+ cells have been reported to show enhanced SDF-1 directed chemotaxis by either pre-treatment or co-incubation with IL3 or SCF [26, 31, 55]. Our data show that stromal cells from different origins produce different levels of chemoattractant activities. In particular the AM clones elaborate high levels of CXCR4-dependent chemoattractant activity. More interestingly, we found that Flt3-L inhibited the elaboration of chemoattractive activity by the majority of stromal cells. This effect was entirely due to an effect on the stromal cells, because the fresh addition of this cytokine in the lower chamber of the transwell cultures, in combination with SDF-1 (figure 2) or cytokine-absent SCM showed no significant additive effect on migration of HL-60 cells (figure 3b). The effect of Flt3-L may explain the results of recent clinical analysis of plasma Flt3-L levels in mobilization of CD34+ cells, where high plasma levels of this cytokine were associated with poor mobilization outcome [37, 38].

The yolk-sac and intraembryonic area surrounding the dorsal aorta serve as the main hematopoietic tissues during early and mid-gestation. The intraembryonic AGM region autonomously generates the first adult HSCs at embryonic day 10.5 (E10.5), the number of which dramatically increases thereafter [56, 57]. At E11 the HSCs are generated only in the AM subregion, while the UGs contain HSC activity at E12 or after culture of E11 explants [58]. All these data suggest that AM cells may behave like a center for emergence of adult HSCs, and subsequently support their maintenance and expansion in their environment and surrounding tissues prior to their localization in the liver microenvironment. Our current results suggest that the localization of these adult stem cells at the ventral wall of the dorsal aorta may in part be due to the elaboration of chemotactic stimuli by the AM microenvironment, since the most highly chemotactic active stromal cells were derived from this particular region. However, it is still unclear as to whether these chemotactic factors act to attract or maintain stem cells following their emergence, or to attract cells from neighbouring regions.

There is evidence that cytokines can modify the chemotactic activity of SDF-1 on hematopoietic progenitors and cell lines. For example, preincubation of M07e and UT-7 mpl cell lines and CD34+ UCB progenitors with Tpo led to a significant reduction in SDF-1 induced migration [59]. It has also been shown that the migration towards SDF-1 was reduced for CD61+ cells and megakaryocytic progenitors when cultured in cytokine cocktails including IL6, IL11, Flt3-L and/or IL3 in combination with Tpo + SCF as compared to Tpo + SCF alone [60]. In another study, progenitors from CD34+ UCB cells cultured in the presence of Tpo, Flt3-L and SCF had a chemotactic response equivalent to uncultured cells, while addition of IL3 reduced this response by 20-50% [61].
contrast to these migration-diminishing effects, other cytokines may in fact enhance the migratory capacity of cells. For example, a cooperative interaction between SCF and SDF-1 enhances chemotaxis of CD34+ hematopoietic progenitors [31]. At present it is unclear how early-acting cytokines modulate SDF-1 responsiveness. One explanation might be that there are common downstream substrates where signaling pathways converge. For instance, it has been shown that the combined use of SDF-1 and SCF leads to a delayed activation of mitogen-activated protein kinase (MAPK) activation, suggesting that signaling pathways cooperate to enhance chemotaxis of progenitors [55]. In line with this, investigation of intracellular signaling pathways induced by SDF-1 and Tpo revealed some overlapping patterns of protein phosphorylation/activation (MAPK, protein kinase B) and some that were distinct or even opposite for Tpo and for SDF-1 [62]. In addition, simultaneous activation of three signals through c-mpl, c-kit and CXCR4 has been reported to induce in vitro proliferation and differentiation of megakaryocytic progenitors by signaling through protein kinases phosphatidylinositol 3-kinase, protein kinase C and MAPK [63]. All these data show that cytokines can interact functionally in modulating cell proliferation and migration. Thus, it is likely that treatment of stromal cells with Flt3-L and Tpo, which are produced by both stromal cells and hematopoietic progenitors [45, 64] may affect adhesion molecule expression, local cytokine and/or chemokine production. In fact, our observations would support such a view. However, though we have evidence that both Tpo and Flt3-L are produced by the AM cells [33], it is unclear whether their respective receptors, c-mpl and Flk2/Flt3, are also expressed. We are currently investigating this issue.

In conclusion, we have shown that marrow- and embryonic subregion-derived stromal cells elaborate factors, probably SDF-1, which chemoattract HL-60 and UCB CD34+ cells. More importantly, we show that treatment of stromal cells with early-acting cytokines alters their ability to elaborate chemotactic factors. This interesting observation demonstrates that stromal cells not only make early-acting cytokines, but also respond to those factors, revealing another layer of interactions between stem cells and the microenvironment.

Acknowledgements

We would like to acknowledge the nursing staff and midwives of St. Fransiscus Gasthuis for collection of UCB samples and M. Schoester for providing HL-60 cells.
References


Chapter 7

General discussion
Umbilical cord blood hematopoietic stem and progenitor cells

General discussion

Rational for HSC expansion

Adult HSC transplantation (SCT) has achieved significant therapeutic success over the last decades, providing a viable treatment option for many previously incurable diseases. However, several inherent limitations of the procedure have restricted its widespread use. These include lack of sufficient donors for all recipients, period of BM aplasia leading to severe and prolonged neutropenia and thrombocytopenia, and the potential for tumor contamination in autologous SCTs. Experimental studies suggest that umbilical cord transplantation might help eliminate the issue of donor availability and some post-transplant complications. Despite its theoretical advantages over SCT from adult donors, the lower number of HSCs in single UCB samples and the preliminary data showing the importance of cell dose for the outcome of UCB transplantations have been of major concern in adult patients. Indeed, as a consequence of limited HSC numbers, UCB transplants have a tendency for delayed time to platelet recovery as compared to adult ones. While the time required for myeloid recovery was similar in children receiving transplantation with UCB or unrelated BM, platelet reconstitution was significantly delayed, being 51 days for sibling and 75 for unrelated UCB transplants in comparison to 23 days for unrelated BM transplants [1-7]. These limitations of UCB raised a considerable interest in the development of ex vivo HSC and progenitor expansion strategies. In addition, the need for large-scale marrow harvesting could be reduced by ex vivo increase of sufficient number of HSCs and progenitors from a single marrow aspirate. Also small MPB grafts from heavily treated cancer patients might benefit from ex vivo expansion of these cells before clinical application. In another setting, expansion studies can contribute to the development of culture conditions that permit efficient gene transfer into primitive HSCs.

Which hematopoietic cell population should preferentially be expanded?

Depending on clinical applications, expansion of different hematopoietic cell subsets can be required. It is thus important to define culture conditions that are relevant to the particular cell type being expanded. The relevant properties of cells in each of the different hematopoietic compartments can be summarized as (a) primitive HSCs for long-term engraftment and gene replacement therapy; (b) progenitors for intermediate and short-term engraftment; and (c) mature cells such as dendritic cells, and different T- and NK-cell subsets for immunotherapy. It is essential to define the aim of hematopoietic cell expansion and to adapt the experimental conditions to obtain the required cell subset.
Defining such cell-based culture systems and elucidating the molecular events, which permit the ex vivo expansion of HSCs from various sources, is additionally important to pursue. The major aim of this study is to address the conditions in which LTR HSCs derived from UCB can be expanded ex vivo.

The ex vivo expansion studies of HSCs and progenitors have used either total CD34+ cells or CD34+ cell subsets. Liquid cultures established either under stroma-free or exogenous cytokine lacking stroma-supported conditions clearly demonstrated that primitive subsets of CD34+ cells possess greater expansion potential than their more mature counterparts. CD34 antigen has been used as a convenient positive selection marker to enrich HSCs and progenitors, which in turn were used in SCT and research. However, new findings have added to the growing evidence that some HSCs in the BM do not express CD34. The capacity of human CD34- HSCs to give rise to large numbers of CD34+ cells that can mediate reconstitution [8-12], and raises the possibility that we may inadvertently discard significant number of stem cells by CD34+ selection of human samples. Several studies indicate that CD34 expression reflects the activation/kinetic state of HSCs and that it is reversible (reviewed in [13-15]). Injection of 150 mg/kg fluorouracil to mice showed that both CD34- and CD34+ populations of the BM may contain stem cells [8] and that CD34- stem cells can become CD34+ after ex vivo incubation [8], indicating that activated stem cells express CD34. Also the reversal of the phenotype from CD34+ to CD34- has been demonstrated in a in vivo model [16]. Thus the most appropriate cell population for clinical transplantation is an extremely important but controversial issue. Whether the CD34- cells should be included in clinical transplantation, or whether the CD34 expressing cells are enough, remains to be seen.

Expansion of HSCs and progenitors with in vitro repopulating ability
Previous attempts emphasized the role of early acting cytokines Flt3-L, Tpo, SCF and IL6 in regulation of HSC and progenitor growth and survival in vitro [17-23]. In line with this, considerable progress in ex vivo expansion of hematopoietic progenitors has been obtained when these early acting cytokines were used in combinations [24-26]. These four cytokines have also been reported to support primitive HSC and progenitor proliferation with maintenance of LTR ability [17-19, 21, 23]. Only one group has reported dramatic expansion of LTC-ICs and SRCs during stroma-free ex vivo propagation of more than three months, however, other laboratories to date have not reproduced these observations. Using the long-term production of CD34+CD38- and LTC-IC subsets as a stringent endpoint for HSC expansion, Piacibello and colleagues have uniquely achieved a dramatic expansion of primitive CD34+ UCB cells over periods
Umbilical cord blood hematopoietic stem and progenitor cells

as long as thirty weeks under cytokine-supplemented stroma-free conditions [25, 26]. These authors additionally demonstrated that the SRCs were markedly expanded using Flt3-L, Tpo, SCF and IL6 under the same conditions after ten weeks of culture [27].

The supportive capacity of the hematopoietic microenvironment on the maintenance of HSCs and their ability to generate progenitors in in vitro cultures has also been explored [28-30]. Initially, stromal support was evaluated in the absence of exogenous cytokines, which failed to significantly expand the in vitro and in vivo LTR cells in co-cultures [31-34]. The inclusion of Tpo in such stromal co-cultures has dramatically increased the longevity of hematopoietic activity and maintenance of repopulating ability of HSCs [35, 36]. Additionally, stroma-supported cultures of UCB and MPB HSCs have been reported to maintain and even moderately expand their numbers when at least Flt3-L and Tpo were included [37-39]. Ex vivo expanded UCB cells have also been shown to maintain their ability to differentiate along myeloid and lymphoid progenitors after two to five weeks under stroma-contact or non-contact conditions in the presence of at least Flt3-L and Tpo [39, 40]. Lastly, we have shown that stromal support was also required for optimal maintenance and transplant quality of CD34* selected MPB cells in short-term cultures [41, 42]. All these studies demonstrate only maintenance or a slight increase of HSCs after culture and suggest that at least both stromal cells and the exogenous cytokines Tpo and/or Flt3-L together might be required for expansion of HSCs and progenitors ex vivo.

Because a short culture period is favorable in a clinical setting, we firstly tried to expand UCB HSCs and progenitors during two-week stroma-free and stroma-supported cultures. A more than 10-fold expansion of CAFC_{week6} and of the potential of CD34* UCB cells to multilineage engraft the BM of sublethally irradiated NOD/SCID mice was obtained in the presence of at least Flt3-L + Tpo. Under stroma-free conditions, the results following two-week cultures showed Flt3-L + Tpo + SCF + IL6 to represent an optimum cytokine combination for expansion of early and late progenitors. Addition of SCF ± IL6 to Flt3-L + Tpo enhanced expansion of CFU-GM and CAFC subsets, indicating a synergistic effect. This is in contrast to the data of Piacibello and colleagues, which show no difference in the extent of HSC and progenitor expansion with addition of SCF ± IL6 at the early stage of the culture period [25, 26]. The sources of cytokines, the purification procedures of cell populations or the presence of uncharacterized factors in the sera may reflect these differences. In these two-week cultures, the stromal support could be replaced by adding additional SCF ± IL6 to the Flt3-L + Tpo stimulated short-term stroma-free cultures. In contrast to stroma-free cultures, addition of SCF ± IL6 to Flt3-L + Tpo in stroma-supported cultures did not substantially modify the extent
of CAFC and SRA. This observation suggests that the SCF and IL6 elaborated by the FBMD-1 stromal cells were sufficient to compensate for the exogenously added SCF and IL6.

Using combinations of early acting cytokines Flt3-L, Tpo, SCF and IL6 we observed synergistic effects in support of early HSCs and progenitors for extended periods of ten weeks. As can be seen in detail in chapter 3 we were able to dramatically improve the expansion of CAFC subsets in cultures exceeding a time period of two weeks and showed that stromal support with exogenous addition of at least Flt3-L + Tpo was required to do this. Stromal support expanded the CAFC_{week6} subset maximally from 11-fold to 89-fold, as compared to stroma-free conditions in the presence of the cytokine combination Flt3-L + Tpo + IL6. Also the CD34^{+}CD38^{-}CXCR4^{+} cells representing SRCs required both stromal support and cytokines to expand over a time period of five weeks. These data contrast with the previous data published by Piacibello and colleagues indicating no stromal requirement in expansion of LTC-ICs for more than six months and of SRCs for ten weeks. These authors used initially FCS in stroma-free LTCs for evaluation of optimal conditions, which resulted in massive and prolonged \textit{ex vivo} expansion of (primitive) progenitors from CD34^{+} UCB cells. Subsequently, they switched to pooled human serum as a substitute for FCS and assessed whether the cytokine combination Flt3-L + Tpo + SCF + IL6, which gave the best expansion results of LTC-ICs and CFCs, could maintain and amplify SRCs. This latter condition gave an increase of >70-fold the input SRCs after a culture period of nine to ten weeks. Therefore, the differences observed between these and our extended long-term data might be in part attributable to the culture conditions used with special reference to serum batches or the source of serum used.

We then studied the effect of a new fusion protein of IL6 and soluble IL6R, H-IL6, on the expansion of UCB CD34^{+} cells. The addition of H-IL6 to Flt3-L + Tpo substantially increased CD34^{+} generation and enhanced the proliferation of CFC and CAFC_{week6} during six-week stroma-free cultures of CD34^{+} or AC133^{+} UCB cells. This fusion protein also acted synergistically with Flt3-L + SCF and Flt3-L + Tpo + SCF for the expansion of CD34^{+} cells and CAFC_{week6} both under stroma-free and stroma-supported conditions. These data support and extend the studies of two groups [43, 44], who tested a complex of the soluble IL6R and IL6 (IL6/sIL6R) in combination with SCF or Flt3-L in three-week stroma-free cultures and semisolid clonal assays and demonstrated an increased proliferation of multipotential and committed progenitors from UCB- and BM-derived CD34^{+} cells. In addition, Zandstra et al [45] reported a higher LTC-IC expansion in one-week serum-free cultures of CD34^{+}CD38^{−} UCB cells stimulated by Flt3-L and IL6/
sIL6R complex when compared to the combinations of Flt3-L + Tpo or Flt3-L, SCF, IL6, IL3 + G-CSF. Under the same conditions, stimulation of CD34+ UCB cell subsets with IL6/sIL6R instead of IL6 resulted in a higher expansion of erythroid progenitors, mixed colonies (CFU-GEMM) and LTC-ICs [43, 46, 47]. In contrast, though the H-IL6 fusion protein enhanced the expansion of AC133+ hematopoietic progenitors with a variety of cytokine combinations, IL6 synergized more potently than the fusion protein with Flt3-L and Tpo for the expansion of CD34+, CD34+AC133+ cells as well as CAFCs in our culture system. This may in part be due to the use of AC133+ cells, which have been found to possess a lower expansion potential in our culture system than CD34+ cells. AC133 is expressed on a subfraction of CD34+ cells, containing committed progenitors and LTC-ICs as well as LTR cells. However, CD34+ cells lacking AC133 antigen have no LTR ability and are enriched for erythroid progenitors [48, 49], which have been shown to be IL6Ra+ [46]. Therefore, it might be that AC133 selection results in an enrichment of IL6Ra+ cells which in turn gives an enhanced response to IL6. Stromal support greatly enhanced the progenitor expansion induced by Flt3-L + Tpo or Flt3-L + Tpo + H-IL6. However, the highest proliferation was again obtained in the presence of IL6, whereas addition of SCF in these stroma-supported cultures resulted in increased differentiation.

**Expansion of the ability of the cells to multilineage repopulate the BM of NOD/SCID mice**

Several data support the contention, that in contrast to CFU-GM, SRC are difficult to expand, and are rapidly lost in stroma-free cultures for five to nine days [50-52]. It is at present unclear whether the loss of SRC following culture is due to loss of their proliferative ability, or to decreased ability to home to the murine BM. In the present study, the expansion of the ability of CD34+ UCB cells to multilineage engraft the BM of sublethally irradiated NOD/SCID mice following two-week stroma-free cultures was observed in the presence of various cytokine permutations including Flt3-L + Tpo and SCF + IL6. Remarkably, addition of SCF ± IL6 to the cytokine combination Flt3-L + Tpo showed no significant effect on the estimated expansion of the engraftment ability. In addition, Piacibello and coworkers showed that more than 70-fold SRC increase could be obtained after nine to ten weeks culture of CD34+ UCB cells under stroma-free conditions in the presence of the cytokines Flt3-L, Tpo, SCF and IL6 [27]. However, it is not clear from these studies whether SRC were expanded after two weeks of culture. Another study showed that two-week expanded CD34+ UCB cells retain their ability to support long-term hematopoiesis in serum-free cultures containing Flt3-L + Tpo + SCF + G-CSF as shown by their in vivo engraftment of the NOD/SCID mice and ability
to undergo multilineage differentiation along all myeloid and B-, NK, and T-lymphoid pathways \textit{in vitro} [40]. Increase of SRA after culturing CD34+ UCB cells may be very likely due to an increase of the total SRC number, however, it is also possible that quiescent SRC, or pre-SRC, are activated or recruited, respectively. Alternatively, it could be that cultured cells show an increased ability to home either to, or expand in the BM of NOD/SCID mice, or that there is a numerical or functional gain of facilitating cells that is possibly required for engraftment of the NOD/SCID BM by SRC. According to previously published data [53], the use of at least SCF + IL6 in our culture system might have caused increased homing potential of the CD34+ UCB cells by upregulation of CXCR4 receptor on expanded cells, which in turn contributed to the increased SRA. Interestingly, our results (chapters 2 and 3) also indicated that the stromal microenvironment has a significant capacity to improve the SRA expansion in two-week cultures with at least Flt3-L + Tpo. These data contrast with several previous studies indicating that proliferation of HSCs in the presence of stroma is associated with loss of most \textit{in vivo} repopulating ability [31, 33]. It should, however, be realized that Flt3-L and Tpo were not exogenously added to the stroma-supported cultures in the latter studies. In agreement with our findings, stromal cells and a combination of Flt3-L + Tpo and/or SCF resulted in 13- to 80-fold expansion of late CAFCs and at least several fold-increase of SRA in two to four-week cultures [54, 55]. Also the type of stromal cells used in these cultures can contribute to variable results as it has been demonstrated that the type of stromal cells used in LTC-IC assay affected the frequency assessment of primitive progenitors [56]. Recently, Lewis \textit{et al.} demonstrated that AFT024 stroma non-contact cultures with addition of Flt3-L + Tpo + SCF + IL7 or Flt3-L + Tpo can also maintain long-term engrafting cells, defined by their ability to engraft secondary or tertiary hosts [57]. However, while at week 2 the expansion of SRA was similar under our stroma-free and stroma-supported conditions, after five weeks of culture cells were hardly or not at all able to give detectable levels of human engraftment in the mice. Possibly, this low SRA might be due to a) loss of SRCs; b) differentiation of SRCs; c) dilution of SRCs as limited numbers of input equivalent cells were infused at five- and ten-week cultures when compared with two-week time point, or d) decreased ability to home to and to expand in the BM of NOD/SCID mice when cultured for extended periods.

\textbf{Role of embryonic hematopoietic microenvironment on HSC and progenitor expansion}

The microenvironment of the AGM region, where definitive adult HSCs first appear during mammalian embryonic development, has been proposed to play an important role in HSC recruitment, maintenance and/or expansion [58]. A few stromal cell lines from
the AGM region have been reported to maintain HSCs with competitive repopulating activity [37, 59] in vitro. However, this support did not seem to be better than that of earlier established stromal cell lines from fetal liver or adult BM ([60, 61], chapters 2 and 4). As the co-culture of HSCs with stromal cells has been shown to promote the hematopoietic activity and expansion of human HSCs and progenitors in vitro and in vivo, our second main topic in this thesis was aimed at analyzing whether our previous adult stromal support data could be improved by stromal cells derived from different embryonic regions. It is very likely that the neonatal HSCs and progenitors collected from UCB, which are a step closer to the fetal hematopoietic system, might also benefit from co-cultures with stromal cells derived from an earlier stage in the development than adult microenvironment. In the study presented in chapter 5 we have identified four embryonic stromal clones that support a dramatic expansion of CFU-GM and CAFC\textsubscript{week6} during a twelve-week co-culture with human CD34\textsuperscript{+} UCB cells. This expansion was higher than that obtainable with the well established stromal line FBMD-1 known for its capability to maintain or expand HSC activity. As was the case in our previous co-cultures with FBMD-1 (chapters 2-4), the majority of these embryo-derived stromal cells also required additional addition of cytokines, i.e. Tpo +/- Flt3-L, to achieve maintenance or expansion of human HSCs and progenitors. The addition of Tpo to different stromal co-cultures often dramatically prolonged the hematopoietic activity, indicating the importance of this cytokine in long-term survival of human HSCs. These data support and extend the requirement of Tpo to maintain murine HSCs in stromal co-cultures [35, 36]. We observed also a large variability in support of human HSCs and progenitors not only between clones derived from different embryonic regions, but also between clones derived from the same region. Such variability is most likely a reflection of the complex interplay between stroma and hematopoietic cells, regulated by adhesion molecules, extra-cellular matrix molecules, membrane-bound cytokines and cytokines receptors. Chemotactic factors such as SDF-1\alpha may additionally help to guide and maintain HSCs in their niches [62-64].

**Stroma-specific distribution of HSCs and progenitors over adherent and non-adherent compartments**

We found that the majority of primitive human progenitors (CAFC\textsubscript{week6}) localize in the non-adherent compartment of co-cultures with stromal cells derived from adult murine BM or embryonic AGM region-, liver- and gut mesentery (chapters 5 and 6). This is in contrast to co-cultures with primary BM-derived stroma, in which the majority of primitive progenitors can be recovered from the adherent stromal compartment [65, 66].

185
Our finding is in line with several previous studies suggesting that FBMD-1, M2-10B4 and AFT024 stroma non-contact support of LTC-ICs/CAFCs as assessed in transwell experiments differed between co-cultures supported by different stromal cells [42, 67]. In another study a direct interaction between progenitors and stromal cells appeared not to be required for the maintenance of long-term in vitro hematopoiesis [68]. All these data indicate that the distribution of (primitive) progenitors over adherent and non-adherent compartments in stromal co-cultures might very likely depend on the type of stromal cells used. In our case we investigated whether this distribution could be in part due to the secretion level of chemotactic activity by stromal cells. Indeed, different stromal cells contained variable levels of chemotactic activities in their supernatants that could be modulated by Flt3-L and/or Tpo. Various chemoattractant factors have already been described to regulate (primitive) progenitor mobilization and homing. Stromal cell-derived factor-1 (SDF-1, CXCL12), a ligand of the G-protein coupled CXCR4 receptor, produced by BM stromal cells and osteoblasts [64, 69, 70], has been shown as the major chemoattractant for progenitors. In our laboratory somatostatin (ligand of somatostatin receptor-2) has also been implicated in chemotaxis of normal and malignant hematopoietic progenitors [71, 72]. A new chemoattractant, WECH, expressed amongst others in dorsal aorta, yolk-sac, fetal liver and umbilical vein, has recently been identified [73]. Additionally, it is known that chemoattraction of cells can be modulated by cytokines. For instance, CD34+ cells have been reported to show enhanced SDF-1 chemotaxis by either pre-treatment or co-incubation with IL3 or SCF [69, 74, 75]. Thus, in view of the existence of many chemokines and seven-transmembrane G-protein coupled receptors, it is reasonable that the interaction of human cytokines or human hematopoietic cells with stroma could result in release of chemoattractants or other yet unidentified factors by stromal or hematopoietic cells which in synergy with cytokines might affect (primitive) progenitor motility and guide them in the proper stromal environment, i.e. either in the adherent or non-adherent culture compartments. Our results obtained with the CXCR4 antagonist suggest that the chemotactic activity in SCMs is to, a great extent, due to the interaction of this receptor and its ligand SDF-1.

**Which factors contribute to maintenance and/or expansion under stromal conditions?**

At present it is largely unknown which mechanisms are involved in the supportive effect of stroma on ex vivo maintenance and expansion of HSCs and progenitors. Cell adhesion molecules and extracellular matrix molecules most likely contribute to a specific contextual interaction between stem cells and the stromal environment, and chemotactic factors as SDF-1 and specific lipids help to guide and maintain HSCs and progenitors.
in their niches [62-64, 76, 77]. HSCs and progenitors are usually in a quiescent or slow cycling state under stroma-supported conditions and TGF-β has been indicated as a candidate for controlling this quiescence. Indeed, it has been observed that primitive CD34+CD38- cells show a high sensitivity to cell-cycle inhibition by TGF-β, whereas more mature CD34+CD38+ cells are hardly affected or are even stimulated by TGF-β [78-80]. MIP-1α, interferon (INF) and tumor necrosis factor (TNF) have also been shown to inhibit hematopoiesis in in vitro assays, and in some cases when administered to mice they inhibited the cycling activity of progenitors in vivo [78, 81-84]. In addition, our group emphasized the use TGF-β antagonist in expansion of MPB cells. Exogenous addition of IL3, SCF, IL6 and anti-TGF-β gave expansion of CAFCwook 6 in one-week FBMD-1 stroma-supported cultures. One possible explanation might be that the addition of neutralizing antibodies directed against TGF~ could have abrogated the proliferation inhibition of stroma-contact, because TGF-β is an important inhibitor of primitive stem cell proliferation and is produced by FBMD-1 stromal cells [85].

Whether HSCs require direct contact with stromal cells for their survival and/or proliferation or are regulated by stroma-derived factors exclusively remains unclear. While Verfaillie and coworkers reported an inhibitory effect of stroma-contact on CFC and LTC-IC proliferation as compared to stroma-noncontact conditions [68, 86-88], Bennaceur-Griscelli et al. demonstrated that MS-5 stromal cells counteracted differentiation events triggered by cytokines and promoted self-renewal divisions [89]. Different stromal cells may vary in the extend with which molecules are elaborated into the medium or in their expression of adhesion molecules or chemotactic molecules affecting the ability of HSCs and progenitors to co-localize and adhere to stroma. Unpublished observations in our lab show that stromal cells differed in their protein expression of various cell adhesion molecules (CAMs), membrane-bound cytokines and cytokines receptors. No correlation of these parameters with stromal support for HSCs and progenitors was observed. It should be noted that these latter experiments were performed in the absence of human HSCs and progenitors. The presence of hematopoietic cells might very likely result in higher or lower expression of proteins affecting hematopoietic-stromal cell interaction, and in turn affecting hematopoietic cell maintenance or expansion. It is also reasonable that this interaction might result in induction or release of factors from either stromal cells or themselves differently affecting the support provided by different stromal cells. Among many cytokines and chemokines that could be possibly involved, murine IL6, IL10, EGF, GM-CSF, LIF, IFN-β and γ and M-CSF do not cross-react with human cells, while human IL-4, IL-9, IL-12, EGF, GM-CSF and IFN-β and -γ are not able to activate the corresponding
receptors on mouse cells. In this context the study of Punzel et al. is of relevance [67]. These authors showed that addition of human SCF, G-CSF, GM-CSF, LIF, MIP-1α, and IL-6 in concentrations found in human bone marrow stroma-conditioned medium significantly increased LTC-IC maintenance. Other factors as O-sulphated GAGs have been shown to aid the long-term maintenance of LTC-IC under stroma non-contact conditions [90], while exogenous addition of LIF have been suggested to be responsible for maintaining and expanding transplantable CD34+Thy1+ cells in stroma-supported cultures through an action on the stroma [91]. We also observed that while the use of cytokines alone expanded early progenitors for twelve weeks and the use of stroma alone decreased their numbers within the same time period, the combination of stroma and cytokines greatly increased the maintenance and production of HSCs and progenitors for extended periods. The effect of cytokines can thus partly be explained by a direct effect on HSCs and progenitors. However, they are also likely to act indirectly by an effect on stroma, indicating that the addition of cytokines may have reversed the proliferation block in stroma-contact cultures. Human Flt3-L, Tpo and IL6 are known to exhibit cross-species activity on mouse cells. Unpublished observations in our lab indicate that the murine adult BM-derived FBMD-1 stromal cells and embryonic stroma cells do not express murine c-kit (receptor for SCF) and Flt3 (receptor for Flt3-L). However, they express the murine IL6Rα and c-mpl (Tpo receptor), and human Tpo down modulates this IL6Rα expression on these cells. These data substantiate our contention that at least the cytokines Tpo and IL6 used in the present study might have reacted on both hematopoietic cells and stroma. Of further interest was the observation that in SCF + IL6 stimulated extended LTCs, stroma decreased the expansion magnitude of CD34+ and CD34+CD38− cells, CFU-GM and CAFC subsets as compared to the stroma-free conditions. A possible explanation for this observation is that the exogenous soluble ligand SCF may compete with the stroma membrane-bound SCF for c-kit receptors on HSCs and progenitors, assuming that the interaction with the membrane-bound SCF, rather than soluble SCF, results in the modification of the most effective signaling pathways in maintaining these cells.

Incongruency of surrogate HSC and progenitor assays after prolonged cultures
Characterization of expanded cells for HSC and progenitor function is currently based on both in vivo (SRC and fetal sheep assays) and in vitro (CAFC, LTC-IC, and CFC) surrogate models. We found that important differences existed between the HSC and progenitor assays that have been used to quantitate their expansion after culture (chapters 3 and 5). Especially, the enumeration of CD34+ subsets and CFC generation
greatly overestimated the expansion of CAFC or SRA at the time points analyzed, confirming that the different surrogate methods to quantitate HSCs and progenitors are unlikely to detect identical populations after culture. These results were in agreement with previously published data indicating a dissociation between primitive CD34+CD38- phenotype, LTC-IC and SRC during cultures of UCB or BM cells [34, 38, 92]. We have subsequently wondered whether the dramatic CD34+CD38- expansion during culture arose from the input CD34+CD38- cells or by downregulation of CD38 antigen expression within a population of cultured CD34+CD38+ cells that no longer contained any LTR ability, both in vitro and in vivo. Remarkably, we found that although before culture the CD34+CD38- cells were all Lin-, after culture the majority of the CD34+CD38- cells co-expressed lineage-restricted markers resulting in an overestimation of the expansion of the CD34+CD38- population. Additionally, Von Lear et al. demonstrated that the purified CD34+CD38- cell population lost their CD38 antigen while remaining CD34+ during a four days stroma-free culture [93]. Very recently, Danet et al. demonstrated that a loss of CD38, HLA-DR and c-kit surface expression led to a dramatic increase in phenotypically primitive MPB progenitors indicating that the relationship between stem cell phenotype and function is also altered in cultured CD34+ MPB cells [94]. These observations clearly indicate that this phenotype is not a reliable indicator for HSC assessment after culture. Thus one must be careful when interpreting data on HSC expansion, as it is possible to generate increased numbers of cells that may have phenotypic properties of primitive HSCs but do not functionally meet the criteria for HSCs.

Clinical drawbacks in ex vivo expansion of HSCs and progenitors

It requires a relative long time to increase the number of primitive cells by ex vivo manipulation [95-97]. Prolonged cultures may inherently cause exhaustion of proliferative and lineage potential of most primitive cells [50, 98]. In addition, ex vivo manipulation may reduce the efficiency of specific migration and homing of in vivo repopulating cells to the BM, as it is known that this homing process can be affected by cytokine exposure [50, 51, 53, 99, 100]. Thus, an effective culture system in which primitive cells can be rapidly expanded is required for clinical settings. Rapid expansion is also economical, less time consuming and labour intensive while limiting the risk of infections and transformation than a long process.

As we and others show that cytokines and stomal cells are required for ex vivo expansion, the contamination of stromal cells into cultured hematopoietic cells is a potential clinical limitation of this culture system. It is also difficult to harvest cultured hematopoietic cells completely, since a number of cultured cells migrate under stromal
layers. Several groups looked for ways to circumvent these problems by the use of non-contact and low or high pore density culture systems [87, 101, 102]. In these experiments, human hematopoietic cells were physically separated from the stromal layer in culture inserts. Whether such a system will be applicable in a clinical setting remains a question and a future challenge. In addition, the use of murine stromal layers for ex vivo expansion of human primitive cells is another problem for clinical application. Although the murine stromal cells can produce a large quantities of cytokines with cross-species activity, the availability of human stromal cell lines would allow a more stable and better defined tool to study the mechanisms of the support and manipulation of human hematopoiesis in vitro. Recent developments in this area are promising. Two human stromal cell lines have been reported to allow proliferation and differentiation of primitive hematopoietic cells from human UCB [103].

Current clinical status in ex vivo expansion of HSCs and progenitors
The goal of improving the rate of engraftment, as well as speeding the time to recovery of marrow function, has spurred investigation of methods to numerically increase the HSCs and progenitors, not only from UCB but also with other sources. The potential clinical utility of ex vivo expanded HSCs and progenitors no longer remains a debate but has become feasible. On the basis of experimental findings and the development of automated closed-system for cell growth [104, 105], human clinical trials have began. Several studies have been performed using teflon bags containing defined media [106-109] or a perfusion culture system called the Aastrom system [110]. The human trials indicate the clinical benefit in post-transplant rate of the neutrophil recovery after the use of expanded cells, while the effect of expansion on long-term engraftment ability remains unanswered [106-116]. In the majority of the cases, ex vivo expanded cells were infused in combination with conventional cells. After infusion of these cells no prohibitive unfavorable immunological problems have been observed, demonstrating the feasibility of the expansion procedures and the clinical safety of the approach. In addition, no additional effect on platelet recovery was observed after infusion of expanded cells independent of their source. Possible explanations therefore may be that the culture conditions do not support progenitors that differentiate into platelets and/or that expanded cells need substantial cytokines as Tpo for platelet development. More interestingly, successful engraftment with solely expanded BM HSCs was also achieved using a stroma-based continuous perfusion method with addition of the cytokines GM-CSF/IL3 fusion protein, Flt3-L and Epo or Tpo [112, 115], verifying the importance of the combination stromal cells and cytokines in order to ex vivo expand clinically
transplantable HSCs. Previously, our group demonstrated the requirement of stromal support and cytokines for optimal maintenance of the graft quality during ex vivo expansion of CD34+ MPB cells [42]. These observations extend the results described in this thesis in which we emphasize the role of stroma and cytokines for ex vivo expansion of UCB-derived HSCs and progenitors. Although we know that our expansion system requires an extended culture period, a combination of stroma and cytokines may allow sufficient increase for HSCs and progenitors with maintenance of the single stem cell quality that solely can be used as a graft without infusion of conventional cells.

The regulation of homing, migration, engraftment and hematopoiesis is a complex process that presumably depends on microenvironmental factors, which are related to functional presence of stromal cells. It is very likely that the myeloablative regimens prior to SCT can affect the in vivo hematopoietic microenvironment of the recipient. The limitations on donor cell engraftment can thus be related to an inadequately receptive microenvironment. The cotransplantation of stromal cells might increase the level of engraftment by providing the necessary supportive microenvironment for hematopoiesis during the time when a relative abundance of donor HSCs is circulating. Indeed, cotransplantation of adult human stroma has been reported to result in increased human cell engraftment in a xenogeneic sheep transplantation model [117]. Also improved recovery of hematopoiesis after irradiation was demonstrated after stromal cell transplantation [118]. In addition to these functional effects of donor stroma infusion, homing of stromal cells has been demonstrated in other studies [119-121], however, many studies including BM transplantation in humans have suggested that the effectivity of stromal cell transplantation is very limited, if demonstrable at all [122-125].

Conclusions and future directions

The results of this thesis research concerning the expansion of HSCs in vitro, show a maximal 825-fold expansion of UCB-derived CAFC_{week6} and 14-fold expansion in the ability of cells to engraft the BM of NOD/SCID mice dependent on the culture conditions and/or time of culture, with particular reference to the cytokine combination Flt3-L and Tpo and/or the stromal layer used. In contrast to our demonstration for presence of stromal cells, Piacibello and coworkers demonstrate a more than 70-fold SRC increase after nine to ten weeks culture of CD34+ UCB cells under stroma-free conditions in the presence of the cytokines Flt3-L, Tpo, SCF and IL6 [27]. Despite these data, there is some evidence to suggest that cytokine exposure in vitro may reduce the efficiency of specific migration and homing of in vivo repopulating cells to the BM [50, 51, 53, 99, 100]. Thus, at this stage it is not safe to extrapolate these preclinical findings to clinical
UCB transplantation in general. Expansion of UCB cells in the clinical setting should only be undertaken in highly specialized centers.

We also demonstrate that the combination of cytokines and stromal cells are required for \textit{ex vivo} expansion of primitive progenitors exceeding a time period of two weeks. These data suggest that this combination may also be required for maximal engraftment and self-renewal of transplanted cells \textit{in vivo}. Thus, it is important to assess the use of stromal cells to enhance the engraftment rate and quality of the graft. A combined cytokine and stromal cell strategy will potentially maximize engraftment and vastly improve therapeutic outcomes. Another option, which at present remains technically challenging, will be the use of genetically engineered cytokine producing stromal layers to create a continuous level of cytokine production throughout the LTC in order to optimize the \textit{ex vivo} expansion. Also, the membrane-bound form of cytokines, such as SCF, may play an important additional role in the cell-cell interactions observed between stromal and hematopoietic cells in these cultures.

In summary, the results described in this thesis provide information regarding regulation of hematopoiesis within its microenvironment. However, it is still unclear which stromal factor(s) is (are) involved in HSC and progenitor expansion. As we continue to functionally characterize several stromal cell-lines derived from AGM-region, further investigations may include the identification of (novel) molecules/genes by subtractive analysis using genomics and/or proteomics.
Umbilical cord blood hematopoietic stem and progenitor cells

References

Chapter 7


Umbilical cord blood hematopoietic stem and progenitor cells


67. Punzel M, Gupta P, Reodell M, Mortari P, Verfaillie CM. Factor(s) secreted by AFT024 fetal liver cells following stimulation with human cytokines are important for human LTC-IC growth. Leukemia 1999; 13 (7): 1079.


Chapter 7


87. Verfaillie CM. Soluble factor(s) produced by human bone marrow stroma increase cytokine-induced proliferation and maturation of primitive hematopoietic progenitors while preventing their terminal differentiation. Blood 1993; 82 (7): 2045.


Umbilical cord blood hematopoietic stem and progenitor cells


Summary
Summary

Umbilical cord blood (UCB) is a most recent source of hematopoietic stem cells (HSCs) and progenitors, which has been used to treat especially young patients with malignant and nonmalignant hematological diseases and other severely ill patients whose immune systems have been decimated by high dose chemotherapy. However the reconstitution of hematopoiesis after transplantation with UCB cells is slower as compared to the use of BM or mobilized peripheral blood (MPB) grafts, which is most probably due to the low number of transplanted HSCs per kg bodyweight. This limited number of HSCs from one UCB unit may especially pose a problem when transplanting adult patients. In view of these existing limitations, there is a strong need for _ex vivo_ culture systems allowing numerical expansion of UCB HSCs and progenitors prior to their transplantation in order to facilitate rapid and durable engraftment.

The aim of this thesis was to define conditions that allow rapid and extensive numerical expansion of UCB HSCs and progenitors _ex vivo_ without a concomitant loss of the ability for long-term repopulation. The surrogate _in vitro_ and _in vivo_ long-term repopulating cell assays were used to allow quantitative analysis of uncultured and cultured UCB HSCs and progenitors.

In chapter 2 we tested whether HSC expansion would alter the _in vitro_ and _in vivo_ long-term repopulating potential of CD34+ UCB under stroma-free and stroma-supported culture during two weeks. Our data showed a more than 10-fold numerical expansion of cobblestone area forming cells (CAFC)_{week 5} and of the potential of CD34+ UCB cells to multilineage engraft the bone marrow (BM) of sublethally irradiated NOD/SCID mice in the presence of at least Flt3-L + Tpo. Among the cytokine combinations tested, stroma significantly improved the maintenance of CAFC_{week 6} with the cytokine combination Flt3-L + Tpo.

The experiments described in chapter 3 were designed to investigate whether HSC expansion could be improved when the UCB cells were cultured for more than two weeks, and whether the presence of BM-derived stromal cells, and combinations of specific cytokines, could affect the maintenance or expansion of the _in vitro_ and _in vivo_ long-term repopulating potential of CD34+ UCB cells. In these studies stromal support enhanced the expansion of CAFC_{week 6} (maximally 89-fold the input at week 10) in the presence of Flt3-L + Tpo + IL6. Cultures stimulated with at least Flt3-L + Tpo gave an estimated 10 to 14-fold expansion of the ability of CD34+ UCB cells to multilineage engraft the BM of sublethally irradiated NOD/SCID mice at two weeks of stroma-free and stroma-supported cultures, while at week 5 and later the estimated SRA decreased
to low or undetectable levels in all groups. Shortly, our results show that stroma and Flt3-L + Tpo greatly increase the long-term generation of HSCs and progenitors as measured by in vitro assays. However, the different surrogate methods to quantify the HSCs and progenitors (CD34⁺CD38⁻, colony forming cells (CFC), CAFC <sub>week6</sub> and NOD/SCID repopulating ability (SRA)) showed increasing incongruency with increasing culture time. Especially, the enumeration of CD34⁺ subsets and CFC generation greatly overestimated the expansion of CAFC or SRA at the time points analyzed, confirming that the different surrogate methods to quantitate HSCs and progenitors were unlikely to detect identical populations after culture.

In chapter 4, the effect of a designer fusion protein of IL6 and the soluble IL6R, H-IL6, on the ex vivo expansion of UCB progenitors was evaluated. This protein binds to Gp130, a transducer unit shared by a series of receptors including IL6, IL11, oncostatin-M and leukemia inhibitory factor. Gp130 is ubiquitously expressed on blood progenitors whereas the expression of IL-6R is limited to only 30-50% of CD34⁺ UCB cells. As a single agent, the fusion protein failed to induce the generation of UCB-derived CD34⁺ cells and CAFC, but synergized effectively and in a dose-dependent fashion with Flt3-L and Tpo for the expansion of hematopoietic progenitor cells under both stroma-free and stroma-supported conditions. We additionally showed that IL6 was more potent than the fusion protein in supporting the long-term generation of UCB CD34⁺AC133⁺ or CD34⁺ cells and CAFC in the presence of Flt3-L and Tpo under stroma-free and stroma-supported conditions.

In the embryo hematopoietic cells emerge and rapidly expand in the ventral wall of the dorsal aorta. To investigate the presumptive unique characteristics of the embryonic hematopoietic microenvironment and its surrounding tissues, we have generated stromal clones from subdissected day 10 and day 11 AGMs, embryonic livers (ELs) and gut mesentery, and tested many of them for their support of murine blood progenitors. Subsequently, nineteen newly established murine embryonic stromal clones were selected and investigated for their ability to sustain human UCB HSCs and progenitors in extended LTCs of CD34⁺ UCB cells in the absence or presence of the cytokines Flt3-L and Tpo. These embryonic stromal clones differed greatly in their support for human progenitors. Four embryonic stromal clones, two urogenital ridge (UG)-, one gastrointestinal tract (GI)- and one EL-derived, were identified that support a high numerical expansion of human UCB CFU-GM and CAFC <sub>week6</sub> in the presence of at least Tpo. This expansion was higher than that obtainable with the FBMD-1 stromal cell line known for its capability to maintain or expand HSC activity. Without addition of cytokines only one EL-derived cell clone maintained the CFU-GM generation for up to twelve
weeks, while in the presence of at least Tpo to the stromal co-cultures a continuous HSC and progenitor production was supported by twelve out of nineteen stromal clones. Overall, the limited number of stromal clones analyzed did not allow identification of the embryonic region that best supports the maintenance and expansion of human UCB HSCs and progenitors.

The interaction between hematopoietic stem cells and the microenvironment requires a close association of these entities. In order for the stem cells to home to the proper microenvironment, stromal cells produce chemotactic factors. The most potent of these factors is stromal derived factor-1. We found that embryonic stromal cells (chapter 6), particularly those derived from the dorsal AM, strongly elaborate chemotactic activities for HL-60 and UCB CD34+ cells. The presence of Flt3-L and/or Tpo during the stromal conditioning phase of medium modulated this chemotactic activity in a stromal cell specific way. Flt3-L or Tpo as single factors increased the chemotactic activity of MS-5 and FBMD-1 stromal cells, while these cytokines had an inhibitory effect on production of chemotactic activity by most other stromal cells. Surprisingly, the combination of Flt3-L and Tpo had the opposite effect, as it dramatically increased the chemotactic activity of AM30 clones. These effects were not due to residual Flt3-L and/or Tpo during the transwell migration assay, as addition of these cytokines to the medium in the migration assay did not affect HL-60 cell migration in the absence or presence of various stroma-conditioned medium.

The studies outlined in this thesis demonstrate that in vitro and in vivo long-term repopulating cells from UCB can be expanded ex vivo. However, this numerical expansion required at least 10 weeks of culture. Another point of attention is that the human progenitors are co-cultured with murine stromal cells that can not be fully depleted from the cultured graft to allow clinical use. At the moment the clinical utility of this approach remains to be fully defined as it is not feasible to perform these cultures on a routine basis for use in clinic. Expansion of UCB cells in the clinical setting should only be undertaken in highly specialized centers.
Samenvatting
Samenvatting

Navelstrengbloed (NB) is de meest recente bron van hematopoietische stamcellen (HSC) en voorlopercellen, die gebruikt worden voor de behandeling van met name jonge patiënten met maligne en non-maligne hematologische aandoeningen, en andere ernstig zieke patiënten met een defect immuunsysteem veroorzaakt door hoge-dosis chemotherapie. Het herstel van de bloedvorming na conditionering en daaropvolgende transplantatie met NB cellen is echter langzamer in vergelijking tot het gebruik van beenmerg (BM) en gemobiliseerd perifeer bloed (MPB) cellen. Dit is hoogst waarschijnlijk het gevolg van het lage aantal HSC dat wordt getransplanteerd per kg lichaamsgewicht. Door het lage aantal HSC uit een enkele NB verzameling kunnen zich met name problemen voordoen wanneer een volwassen individu ermee getransplanteerd wordt. Vanwege deze beperking is er een sterke behoefte aan ex vivo kweeksystemen die HSC en voorlopercellen uit NB numeriek kunnen vermeerderen alvorens ze te transplanteren teneinde een snel en duurzaam herstel te kunnen bewerkstelligen.

Het in dit proefschrift beschreven onderzoek was gericht op het definiëren van omstandigheden die een snelle en omvangrijke numerieke expansie van NB HSC en voorlopercellen ex vivo mogelijk maken zonder daarmee samengaand verlies van het lange-termijn repopulerend vermogen. Voor kwantitatieve analyse van ongekweekte en gekweekte NB HSC en voorlopercellen zijn er bekende kweekmethoden gebruikt voor in vitro en in vivo lange-termijn repopulerende cellen.

In de experimenten beschreven in hoofdstuk 2 hebben we bestudeerd of HSC expansie onder stroma-vrije en stroma-ondersteunde omstandigheden het in vitro and in vivo repopulerend vermogen van NB CD34+ cellen kan wijzigen gedurende twee weken kweek. In aanwezigheid van tenminste Flt3-L en Tpo werd er een meer dan 10-voudige numerieke expansie verkregen van CAFC<sub>week6</sub> en van het vermogen van CD34+ cellen om een multi-lineage herstel te geven van de bloedvorming in het BM van subletaal bestraalde NOD/SCID muizen. Naast de verschillende groeifactoren die gebruikt zijn bleek stroma in combinatie met Flt3-L en Tpo het behoud van CAFC<sub>week6</sub> verder te stimuleren.

De experimenten in hoofdstuk 3 waren ontworpen om te onderzoeken in hoeverre HSC expansie kan worden verbeterd wanneer de NB cellen langer dan twee weken waren gekweekt, en in hoeverre de aanwezigheid van stroma en bepaalde groeifactor combinaties het behoud of de expansie van het in vitro en in vivo lange-termijn repopulerend vermogen van CD34+ NB cellen konden bevloeden. In deze studies werd gevonden dat stromale ondersteuning de expansie van CAFC<sub>week6</sub> versterkt met op
week 10 een maximale 89-voudige toeename in aanwezigheid van Flt3-L + Tpo + IL6. Kweken die op zijn minst gestimuleerd waren met Flt3-L + Tpo resulteerden na twee weken in een geschatte 10 tot 14-voudige expansie van het vermogen van CD34+ cellen om het BM van subletaal bestraalde NOD/SCID muizen (SRA) te herstellen, terwijl na 5 weken en later de geschatte SRA fors daalde, zelfs tot ondetecteerbare niveaus in alle groepen. Kortom, onze gegevens laten zien dat stroma en Flt3-L + Tpo de lange-termijn productie van HSC en voorlopercellen (bepaald met in vitro kweekmethoden) enorm doet toenemen. Maar de verschillende kweekmethoden die gebruikt zijn om HSC en voorlopercellen te kwantificeren (CD34+CD38-, kolonie vormende cellen (CFC), CAFCweek6 en SRA) laten onderling een toenemende incongruentie zien met toenemende kweektijd. Met name de bepalingen van CD34+ subpopulaties en CFC productie hebben de expansie van CAFCweek6 en SRA sterk overgeschat. Deze gegevens laten zien dat na kweek de verschillende kweekmethoden waarmee HSC en voorlopercellen gekwantificeerd worden geen identieke populaties identificeren.

In het onderzoek beschreven in hoofdstuk 4 werd het effect onderzocht van een in ontwikkeling zijnd fusie eiwit van IL6 en de oplosbare IL6 receptor (IL6R), H-IL6, op de expansie van NB voorlopercellen. Het H-IL6 eiwit bindt aan Gp130, een ‘transducer unit’ dat als subunit fungeert in een aantal receptoren o.a. IL6, IL11, oncostatine-M en ‘leukemia inhibitory factor’. Gp130 wordt hoog geëxpressied op bloedvoorlopercellen, terwijl de expressie van IL6R beperkt is tot 30-50% van de CD34+ NB cellen. In de aanwezigheid van alleen het fusie eiwit wordt de productie van CD34+ cellen en CAFC uit NB niet gestimuleerd, maar het verhoogde synergistisch en in een dosis-afhankelijke wijze met Flt3-L en Tpo de expansie van hematopoietische voorlopercellen onder stroma-vrije en stroma-ondersteunde omstandigheden. In anvulling hierop hebben we laten zien dat IL6 meer doeltreffend werkte dan het H-IL6 fusie eiwit bij de ondersteuning van de lange-termijn productie van NB CD34+AC133+ of CD34+ cellen en CAFC in aanwezigheid van Flt3-L en Tpo onder stroma-vrije en stroma-ondersteunde omstandigheden.

Hematopoietische cellen in het embryo ontstaan en expanderen heel snel in de ventrale wand van de dorsale aorta. Om de vermoedelijk unieke karakteristieken van het embryonale hematopoietische micromilieu en zijn omringende weefseks te onderzoeken, hebben we stromale klonen ontwikkeld uit dag 10 en 11 aorta-gonado mesonephros (AGM), embryonale lever (EL) en darm mesenchym, en veel ervan zijn onderzocht op hun mogelijke ondersteuning van muize bloedvoorlopercellen. Volgend op deze experimenten zijn negentien van deze nieuw ontwikkelde muize embryonale stromale klonen geselecteerd en onderzocht op hun vermogen om humane NB HSC
en voorlopercellen te handhaven of de expanderen in verlengde lange-termijn kweken van CD34+ NB cellen in af- of aanwezigheid van de groeifactoren Flt3-L en Tpo. Deze embryonale stromale klonen verschillen grotendeels in hun support voor humane voorlopercellen. Vier embryonale stromale klonen, twee ervan afkomstig van urogenitale tractus (UG)-, een van gastro-intestinale tractus (GI)- en een van EL, zorgden voor een hoge numerieke expansie van humane NB CFU-GM and CAFCwook 6 in de aanwezigheid van tenminste Tpo. Deze expansie was hoger dan die welke verkregen werd met de FBMD-1 stromale cellijn die bekend staat om zijn capaciteit om HSC activiteit te handhaven of te expanderen. Terwijl zonder toevoeging van groeifactoren een stromale cellkloon uit de EL in staat was om de CFU-GM productie voor ongeveer twaalf weken te onderhouden, konden in aanwezigheid van tenminste Tpo twaalf uit een totaal van negentien stromale klonen dit bewerkstelligen. Vanwege het beperkt aantal onderzochte stromale klonen waren we statistisch niet in staat een embryonaal gebied met de beste ondersteunende capaciteiten voor het handhaven en expanderen van humane HSC en voorlopercellen uit NB aan te wijzen.

De interactie tussen HSC en micromilieu vereist een nauwe samenhang. Om ervoor te zorgen dat de HSC zich via het homingsproces (het proces van migratie van HSC via de bloedbaan naar het beenmerg) in het juiste micromilieu vestigen, worden er chemotactische factoren geproduceerd. De meest potente van deze factoren is ‘stromal derived factor-1’. We hebben gevonden dat embryonale stromale cellen, met in het bijzonder de stromale cellen afkomstig van dorsale aorta (hoofdstuk 6), sterke chemotactische activiteiten produceren die HL-60 en NB CD34+ cellen aantrekken. De aanwezigheid van Flt3-L en/of Tpo tijdens de stromale conditioneringsfase reguleerde deze chemotactische activiteit in een stromale eel specifieke wijze. Het enkel gebruik van Flt3-L of Tpo deed de chemotactische activiteit van MS-5 en FBMD-1 stromale cellen toenemen, terwijl deze groeifactoren een inhibitoir effect hadden op de productie van chemotactische activiteit van de meeste andere stromale cellen. Onverwachts liet de combinatie van Flt3-L en Tpo een tegengesteld effect zien op de chemotactische activiteit van AM30 klonen door deze sterk te laten toenemen. Deze effecten waren niet het gevolg van overgebleven Flt3-L en/of Tpo tijdens de transwell migratie experiment, omdat de toevoeging van deze cytokinen aan het medium in het migratie assay de HL-60 cel migratie niet beïnvloedde in de af- of aanwezigheid van verschillende stroma geconditioneerd media.

De studies die in dit proefschrift staan beschreven, demonstreren dat in vitro en in vivo lange-termijn repopulerende cellen uit NB geëxpandeerd kunnen worden ex vivo. Deze numerieke expansie vraagt echter tenminste 10 weken kweek. Een ander aandachtspunt
is dat de humane voorlopercellen gekweekt zijn samen met muize stromale cellen, welke niet volledig verwijderd kunnen worden bij een eventuele klinische toepassing. Op dit moment dient het klinische gebruik van deze toepassing nog beter worden beschreven omdat het routine basis gebruik van deze kweekmethoden in de kliniek niet haalbaar is. Expansie van NB cellen voor het klinisch gebruik zal hierbij alleen kunnen plaatsvinden in hoog gespecialiseerde centra.
Abbreviations

AM  aorta/mesenchyme
AGM  aorta gonad mesonephros
APC  allophycocyanin
BFU-E  burst forming unit-erythroid
BM  bone marrow
BSA  bovine serum albumin
CAFC  cobblestone area forming cell
CD  cluster of differentiation
CFC  colony forming cell
CFU-C  colony forming unit in culture
CFU-GM  colony forming unit-granulocyte macrophage
CSF  colony stimulating factor
EL  embryonic liver
Epo  erythropoietin
FACS  fluorescent activated cell sorting
FBMD-1  flask bone marrow dexter-clone 1
FCS  fetal calf serum
FITC  fluorescent isothiocyanate
Flt3-L  fms-like tyrosine kinase-3 ligand
G-CSF  granulocyte-colony stimulating factor
GM-CSF  granulocyte-macrophage-colony stimulating factor
HS  horse serum
HSC  hematopoietic stem cell
HSPC  hematopoietic stem and progenitor cell
IL  interleukin
IMDM  Iscove’s modified Dulbecco’s medium
GI  gastrointestinal
Lin-  lineage marker negative
LTC  long-term culture
LTC-CFC  long-term culture colony forming cell
LTC-IC  long-term culture initiating cell
LTR  long term repopulating
MNC  mononuclear cells
MPB  mobilized peripheral blood
NC  nucleated cells
NOD/SCID  nonobese-diabetic/severe combined immunodeficient
PB  peripheral blood
PBS  phosphate buffered saline
PE  phycoerythrin
SCF  stem cell factor
SCM  stroma conditioned medium
SCT  stem cell transplantation
SD  standard deviation
SDF  stromal derived factor
SRA  NOD/SCID repopulating ability
SRC  NOD/SCID repopulating cell
Tpo  trombopoietin
UCB  umbilical cord blood
UG  urogenital-ridge
**Curriculum vitae**

Publications

Kuşadası N, Oostendorp RA, Koevoet WJ, Dzierzak EA, Ploemacher RE.
Flt3-L and Tpo modulate the SDF-1 mediated chemotactic activities of marrow and embryonic stromal cells.
_In preparation._

Kuşadası N, Oostendorp RA, Koevoet WJ, Dzierzak EA, Ploemacher RE.
Stromal cells from murine embryonic aorta-gonad-mesonephros region, liver and gut mesentery expand human umbilical cord blood-derived CAFC(week6) in extended long-term cultures.
_Leukemia._ 2002 Sep;16(9):1782-90.

Oostendorp RA, Medvinsky AJ, Kuşadası N, Nakayama N, Harvey K, Orelio C, Ottersbach K, Covey T, Ploemacher RE, Saris C, Dzierzak E.
Embryonal subregion-derived stromal cell lines from novel temperature-sensitive SV40 T antigen transgenic mice support hematopoiesis.

Oostendorp RA, Harvey KN, Kuşadası N, de Bruijn MF, Saris C, Ploemacher RE, Medvinsky AL, Dzierzak EA.
Stromal cell lines from mouse aorta-gonads-mesonephros subregions are potent supporters of hematopoietic stem cell activity.

Kuşadası N, Koevoet JL, van Soest PL, Ploemacher RE.
Stromal support augments extended long-term ex vivo expansion of hemopoietic progenitor cells.
_Leukemia._ 2001 Sep;15(9):1347-58.

Stroma-supported progenitor production as a prognostic tool for graft failure following autologous stem cell transplantation.
Kuşadası N, van Soest PL, Mayen AE, Koevoet JL, Ploemacher RE.
Successful short-term ex vivo expansion of NOD/SCID repopulating ability and CAFC week 6 from umbilical cord blood.

Gp130-signaling synergizes with FL and TPO for the long-term expansion of cord blood progenitors.

Ploemacher RE, Kuşadası N, Van Soest PL.
Ex vivo manipulation of human stem cells: Clinical applicability and research opportunities.

Breems DA, van Hennik PB, Kuşadası N, Boudewijn A, Cornelissen JJ, Sonneveld P, Ploemacher RE.
Individual stem cell quality in leukapheresis products is related to the number of mobilized stem cells.
Tot slot

Gekomen aan het begin van een einde, wil ik in dit gedeelte van het proefschrift nog een aantal mensen bedanken. Terugkijkend vind ik dit het leukste en tevens moeilijkste onderwerp om te verwoorden.

Prof. Dr. Löwenberg, mijn promotor, u wil ik bedanken voor de steun, het begrip en de mogelijkheid die u mij geboden heeft om te promoveren in een vakgebied die mijn interesse al vanuit de college banken veroverd heeft. Tevens wil ik u bedanken voor het kritisch doorlezen van dit proefschrift.

Dr. Ploemacher, mijn copromotor, veel heb ik van je geleerd, niet alleen op het gebied van hematopoietische stamcellen maar ook op allerlei andere gebieden. Rob, het was een leerzame tijd, bedankt.

Prof. Dziernak, jou wil ik bedanken voor de samenwerking, je steun en kennis die hebben bijgedragen tot dit proefschrift. Tevens wil ik je bedanken voor het kritisch doorlezen van dit proefschrift als lid van de kleine commissie. Dr. Wagemaker en Dr. Van der Schoot, overige leden van de kleine commissie, ook u wil ik bedanken voor de moeite en de tijd die u genomen heeft om dit proefschrift door te lezen.

In het bijzonder wil ik Dr. Oostendorp bedanken voor een erg leuke samenwerking en zijn bijdrage die hebben geleid tot gemeenschappelijke publicaties. Ik heb altijd met plezier naar je ideeën geluisterd en heel veel van je geleerd. Robert, jouw enorme kennis en enthousiasme bewonder ik. Ik wens jou en jouw familie heel veel geluk en veel succes in Duitsland.

Dr. Delwel, jou wil ik bedanken voor de samenwerking omtrent migratie experimenten. Jouw kennis en ideeën hebben me enorm geholpen in het laatste traject van dit promotieonderzoek.

Tevens wil ik Dr. Saris bedanken voor zijn steun en samenwerking.

Wendy, bedankt voor al jouw hulp in tijden van nood. Het was gezellig en plezierig om met jou samen te werken. Peter, bedankt voor jouw bijdrage en interessante gesprekken tijdens de eerste periode van mijn AIO-schap. Ik wens jou alle goeds. Elwin, jou wil ik in het bijzonder bedanken voor je steun en hulp op velerlei gebieden. Angelique, ook jou wil ik bedanken voor de samenwerking. Alexandra, Jose, Adrie, Irene, Robin en Erik, de overige leden van de groep Ploemacher, het was een leuke periode.

Cindy en Daphne, het was gezellig met jullie dames. Daphne nog bedankt voor jouw bijdrage. Het afstudeeronderzoek van jouw doctoraalfase heeft geleid tot het schrijven van een artikel.

Anoek en Susan, behalve dat het warm was in ons AIO-hokje, was het ook erg leuk, bedankt voor jullie vriendschappelijke collegialiteit, gezelligheid en steun. Ook Sandra en Dianne wil ik bedanken voor hun gezelligheid.

Karola en Monique bedankt voor jullie bijdrage.

De mensen van de afdeling verloskunde van het St. Fransiscus Gasthuis bedankt voor de moeite en het verzamelen van honderden samples navelstrengbloed. Niet te vergeten, de moeders die toestemming hebben gegeven voor het gebruik van het bloed voor wetenschappelijk onderzoek, hartelijk bedankt.


Ik wens jou alle succes met de voorbereidingen van jouw proefschrift. Orhan en Hüseyin ik wil jullie bedanken voor jullie steun, ik heb veel van jullie twee geleerd. Aysel Terkivatan, jou ben ik ook niet vergeten, ik zal mijn best doen om onze vakantie met de meiden nog een keer te regelen.

Nahim Avc1, mijn muziekleraar, jou wil ik bedanken voor je geduld met mij als leerling en voor het verruimen van mijn blik in muziek. Toen ik je uitlegde wat voor een idee ik over de kaft van dit proefschrift had wist jij precies het muziekstuk wat erbij hoorde.

Ik wil de cardiologen dr. Fels, dr. Stoel, dr. Ten Kate, de longarts dr. Van Uffelen, de leden van CCU, echo-cor, CAG en de afdeling cardiologie van het Albert Schweitzer ziekenhuis locatie Amstelwijk (kortom het Amstelwijk clubje) bedanken voor hun gezelligheid en hulp tijdens mijn eerste stappen van het artsenleven. Ik heb het werken nooit zo leuk gevonden als toen.

Türkan, een van mijn paranimfen, als ik moet opschrijven waarvoor ik jou moet bedanken heb ik meters papier nodig. Chirurgen houden van kort en bondig, daar jij een toekomstige chirurg bent zeg ik alleen maar bedankt.

Casanova, dit had jij niet gedacht maar ik ga het toch doen, bedankt dat jij een veilige haven bent geworden waar ik in alle rust kan verblijven. Ik wens jou alle goeds.

Mijn ouders, de eerste onderwijzers van mijn leven, wat ik van jullie heb geleerd kan ik nooit elders leren. Anne ve baba, hayattaki ilk ogretmenlerim, sizden ogrendiklerimi baska hic bir okul ogretemez, saygiyla ellerinizden opuyorum. Salim, Deniz, Emin en

217
Hilal, broers en zus, ik weet niet hoe ik jullie moet bedanken voor alles. Zonder jullie zorg, steun en liefde was dit proefschrift nooit waardevol geweest. Ik wil ook ons jongste familielid Fatma, mijn schoonzus, bedanken voor haar vriendschap en gezelligheid. Verder mijn grootouders, alle ooms en tantes, neefjes en nichtjes, kusjes.

Mocht ik iemand vergeten zijn... bedankt!

Selamlar ofwel gegroet allemaal!

[Signature]