Differential Kinetics of Primitive Hematopoietic Cells Assayed In Vitro and In Vivo During Serum-Free Suspension Culture of CD34$^+$ Blood Progenitor Cells

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ABSTRACT

So far, blood progenitor cells (BPC) expanded ex vivo in the absence of stromal cells have not been demonstrated to reconstitute hematopoiesis in myeloablated patients. To characterize the fate of early hematopoietic progenitor cells during ex vivo expansion in suspension culture, human CD34$^+$-enriched BPC were cultured in serum-free medium in the presence of FLT3 ligand (FL), stem cell factor (SCF) and interleukin 3 (IL-3). Both CD34 surface expression levels and the percentage of CD34$^+$ cells were continuously downregulated during the culture period. We observed an expansion of colony-forming units granulocyte-macrophage (CFU-GM) and BFU-E beginning on day 3 of culture, reaching an approximate 2-log increase by days 5 to 7. Limiting dilution analysis of primitive in vitro clonogenic progenitors was performed through a week 6 cobblestone-area-forming cell (CAFC) assay, which has previously been shown to detect long-term bone marrow culture-initiating cells (LTC-IC). A maintenance or a slight (threefold) increase of week 6 CAFC/LTC-IC was found after one week of culture. To analyze the presence of BPC mediating in vivo engraftment, expanded CD34$^+$ cells were transplanted into preirradiated NOD/SCID mice at various time points. Only CD34$^+$ cells cultured for up to four days successfully engrafted murine bone marrow with human cells expressing myeloid or lymphoid progenitor phenotypes. In contrast, five- and seven-day expanded human BPC did not detectably engraft NOD/SCID mice. When FL, SCF and IL-3-supplemented cultures were performed for seven days on fibronectin-coated plastic, or when IL-3 was replaced by thrombopoietin, colony forming cells and LTC-IC reached levels similar to those of control cultures, yet no human cell engraftment was recorded in the mice. Also, culture in U-bottom microplates resulting in locally increased CD34$^+$ cell density had no positive effect on engraftment. These results indicate that during ex vivo expansion of human CD34$^+$ cells, CFC and LTC-IC numbers do not correlate with the potential to repopulate NOD/SCID mice. Our results suggest that ex vivo expanded BPC should be cultured for limited time periods only, in order to preserve bone-marrow-repopulating hematopoietic stem cells. ***Stem Cells*** *1999;17:152-161*

INTRODUCTION

Peripheral blood progenitor cells (BPC) have been used for both autologous, and more recently, allogeneic hematopoietic stem cell transplantation [1, 2]. Hematopoietic cytokines have been used to expand hematopoietic cells in ex vivo culture [3, 4]. The main rationales for ex vivo expansion have been: A) to generate amplified numbers of progenitor cell populations such as colony-forming cells of the granulocyte-macrophage or megakaryocytic lineages or their progeny, in an attempt to shorten neutrophil or platelet regeneration during the recovery phase from bone marrow aplasia [3-5]; B) to provide a prolonged time period for

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retroviral infection in the course of developing improved protocols for gene transfer into hematopoietic progenitors [6-8], or C) to purge contaminating tumor cells from autografts [5, 9, 10].

Clinical protocols have previously shown that bone marrow transplantation with cultured hematopoietic cells is principally feasible; *Barnett et al*. [11] and *Chang et al*. [12] transplanted bone marrow cultured under Dexter-type conditions in patients with acute or chronic leukemia. Purging of leukemic cells was observed, and numbers of Philadelphia-chromosome positive colony forming cells (CFC) decreased in patients with chronic myelogenous leukemia; however, hematopoietic regeneration was generally delayed compared with transplantation using noncultured cells. Later, cytokine-supported stroma-free suspension culture protocols were developed [3, 13-15]. The efficiency of the progenitor expansion as well as the maintenance of the transplantation potential have been assessed using semisolid clonogenic assays, numbers of $CD34^+$ cells, or the incidence of cells capable of initiating long-term bone marrow cultures [3, 13-15]. In mice, the ability of ex vivo-expanded hematopoietic progenitor cells to repopulate the bone marrow has been studied in congenic recipients [16-18]. More recently, immunodeficient mice have been used as a model for the bone marrow repopulation by human ex vivo expanded hematopoietic cells [19-21].

For the clinical use of cultured progenitor cells in hematopoietic transplantation, serum-free culture medium has been proposed to avoid some disadvantages of serum, such as the risk of infection by viruses, variation between individual batches, and regulatory problems [15, 17, 22]. Data comparing the incidence of primitive cells assayed in vitro and their value to predict for the presence of cells capable of repopulating the bone marrow in vivo are scarce but are of primary importance when developing clinical protocols. We used transplantation into NOD/SCID mice in addition to CFC and long-term culture-initiating cells (LTC-IC) assays to determine the levels of progenitor cells during a serum-free expansion protocol. We used stem cell factor (SCF), FLT3-ligand (FL), and interleukin 3 (IL-3) as cytokines, since they have been shown to be efficient mediators of primitive cell maintenance and expansion in studies employing in vitro progenitor cell assays [15, 23]. Our data indicate that hematopoietic bone-marrow-repopulating cells follow different kinetics in cytokine-supported suspension culture as CFC and LTC-IC.

Materials and Methods

Patients and Cells

Patients with a diagnosis of a solid tumor or a lymphoid neoplasia were included after informed consent according to the declaration of Helsinki and underwent BPC mobilization by administration of combination chemotherapy and daily s.c. injection of G-CSF. Patients had received previous chemotherapy consisting of between two and eight cycles of doxorubicin, cyclophosphamide, vincristine, bleomycin, etoposide, prednisolone (VACOP-B); cyclophosphamide, adriamycin, vincristine, prednisolone (CHOP) in lymphoma; high-dose dexamethasone in multiple myeloma; cyclophosphamide, methotrexate, 5-fluorouracil (CMF); or cyclophosphamide, epirubicin, cis-platinum (PEC) in mammary carcinoma; etoposide, ifosfamide, cis-platinum, epirubicin (VIP) in sarcoma; cis-platinum, etoposide, ifosfamide (PEI); or cis-platinum, etoposide, bleomycin (PEB) in carcinoma testis (Table 1). Mobilization was achieved by once daily s.c. application of 300 or 480 µg G-CSF on days 1-12 and a one-day course of etoposide, ifosfamide, cis-platinum with or without eipirubicin on day 0. BPC were separated from whole blood after anticoagulation with acid citrate dextrose by leukapheresis in a CS-3000 cell separator (Baxter; Munich, Germany). Aliquots taken

Table 1. Donor characteristics and total mononuclear cell expansion of $CD34^+$ cells serum-free medium supplemented with FLT3-ligand, stem cell factor and IL-3. Symbols equal as in Figures 1, 3, and 5.

Donor #	Symbol	Diagnosis	Previous chemotherapy (# of cycles)	Fold expansion cell number (d7)
1	◊	Non-Hodgkin's lymphoma	2	3.1
2	∇	Carcinoma testis	6	1.1
3	Δ	(Healthy donor)	0	0.7
4	●	Multiple myeloma	5	29.8
5	○	Sarcoma	2	9.5
6	♦	Mammary carcinoma	3	7.0
7	⊕	Mammary carcinoma	8	3.5
8	▲	Non-Hodgkin's lymphoma	2	22.6

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from the leukapheresis harvests were separated on Ficoll gradients (density 1.077 g/ml, Biochrom KG; Berlin, Germany). Anti-CD34-antibody (CellPro; Bothell, WA) was added to the cell suspensions and CD34+ separation was subsequently performed on Ceprate™ LC columns (CellPro) according to the manufacturer's instructions. In some cases, CD34+ cells were aliquots from clinical scale CD34+ cell separations (Ceprate™ SC columns). CD34+ cell purity was above 70% in all cases. Viability of CD34+ BPC preparations was >95% as determined by flow cytometric analysis of propidium iodide (Sigma; Deisenhofen, Germany) stained cells using a FACScan® flow cytometer (Becton Dickinson; Heidelberg, Germany). CD34+-enriched BPC were suspended at a concentration of 2 to 6 $\times 10^6$/ml in the CD34+ column elution buffer containing phosphate-buffered saline. Ex vivo expansion was started immediately after cell isolation.

Culture Expansion of CD34+ Cells

A serum-free culture medium developed for the expansion of human hematopoietic cells (CellGro®, CellGenix; Freiburg, Germany) modified from [22, 24] was used. Growth factor suppliers and concentrations used were: recombinant human (rh)-SCF 100 ng/ml, rh-FL 300 ng/ml, and rh-IL-3 100 ng/ml (all from Genzyme; Cambridge, MA). CD34+-enriched BPC were seeded into T25 cell culture flasks (Falcon; Heidelberg, Germany, culture volume 10 ml), or in some experiments, into U-bottom 96-well plates (Becton Dickinson) at an input CD34+ cell concentration of 3×10^4 cells/ml and incubated at 37°C, 5% CO_2 in a humidified atmosphere for up to seven days. Also, in that series, some flasks were precoated with human plasma fibronectin (Sigma) at a concentration of 10 μg/cm^2 [25], or performed with rh-thrombopoietin (TPO) (100 ng/ml, Genzyme) instead of IL-3. Before reseeding expanded cells into methylcellulose clonogenic assays and long-term bone marrow cultures (LTBMC) at different time points of the incubation period, cell viability was determined by trypan blue exclusion and found to be above 90% in all cultures. For flow cytometric analysis, aliquots of cell samples were labeled for 30 min at 4°C with either fluorescein (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies directed against CD45 (clone 2D1), CD34 (clone 8G12), CD14 (clone MϕP9), CD15 (clone MMA), CD19 (clone 4G7), CD38 (clone HB7), HLA-DR (clone L243), and isotypic IgG control antibodies (all from Becton Dickinson) or monoclonal antibodies directed against CD3 (clone UCHT-1, from Immunotech; Marseille, France). After two washes with phosphate buffered saline, 10,000 cells were subsequently analyzed on a FACScan® analyzer (Becton Dickinson). Data were evaluated using FACScan Lysys II research software. Dead cells were stained with propidium iodide and excluded from analysis.

Methylcellulose Clonogenic Assays and Long-Term Bone Marrow Cultures

Nucleated cell counts were obtained using a hemocytometer and trypan blue dead cell exclusion. Colony-forming cells were assayed in 0.9% methylcellulose (WAK Chemie; Bad Homburg, Germany) in Iscove's modified Dulbecco's medium (IMDM), 20% fetal calf serum (FCS), 10% bovine serum albumin in the presence of 100 ng/ml rh-IL-3, rh-GM-CSF (both from Genzyme) and 2 U/ml erythropoietin (Janssen-Cilag; Neuss, Germany). Numbers of inoculated cells per ml were 2,000 to 5,000 (for CD34+) or 2,000 to 10,000 (for expanded cells). Cultures were incubated at 37°C, 5 % CO_2 in a humidified atmosphere for 14 days in 35-mm petri dishes (Nunc; Wiesbaden, Germany). Colonies >50 cells were counted under an inverted microscope and classified according to the presence of GM cells and red hemoglobinized BFU-E. For generation of preformed stroma, LTBMC were established as described, using bone marrow aspirations from healthy donors [24, 26]. Briefly, red cells were depleted from bone marrow by density gradient sedimentation at 1*g* in 0.1% methylcellulose/IMDM for 1 h, and the remaining cells were seeded at 2×10^6/ml into 96-well plates (Becton Dickinson) in IMDM, 10% preselected horse serum (Sigma), 10% FCS (GIBCO; Paisley, UK), and 5×10^{-7} M hydrocortisone. Cultures were incubated at 33°C in an atmosphere of 5% CO_2 in air. When a confluent stroma layer had formed after three to four weeks, cultures were irradiated using a ^{137}Cs-gamma source with 15 Gy at a dose rate of 3-4 Gy/min. After removal of most of the culture medium, irradiated cultures were reseeded with CD34+ or expanded cells as starting cell populations in fresh medium at limiting dilution using 10-20 replicates per dilution step. Dilutions were chosen at 1,000, 300, 100, 30, and 10 cells per well for CD34+ fractions, and at 30,000, 10,000, 3,000, 1,000, 300, and 100 cells for expanded cells. After six weeks' incubation at 33°C with weekly half medium exchanges, cultures were evaluated under a light microscope by scoring for the presence or absence of at least one cobblestone area consisting of more than 15 closely attached, homogeneously formed bright cells. Previous experiments have demonstrated that under these culture conditions, the LTC-IC/week 6 cobblestone area forming cell (CAFC) frequencies measured are equivalent to the incidences obtained by replating microcultures into a methylcellulose progenitor assay at week 6 [14, 27] and have therefore been termed "LTC-IC" here. The incidence of LTC-IC was calculated by plotting the percentage of positive wells against the values of cell numbers, fitting a linear regression through the data points and choosing the 37% intercept, according to Poisson statistics.

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Mice

A NOD/LtSz-scid/scid mouse colony (originally obtained from *Dr. Leonard Schultz*, Jackson Laboratories; Bar Harbor, ME) was expanded and maintained under pathogen-free conditions in the animal facility of the Max Delbrück Center of Molecular Medicine (Berlin, Germany). They were fed a standard diet purchased from Sniff (Soest, Germany) and acidified drinking water ad libitum. Mice were irradiated at six to eight weeks of age with a dose of 250 to 300 cGy of gamma irradiation and transplanted with human cells within 3 to 5 h. For transplantation, 0.2-ml samples of BPC at graft doses of 3-5 × 10^5 cells or the expanded progeny thereof were injected into the lateral tail vein. Mice with BPC grafts were additionally transplanted with a stably transfected rat fibroblast cell line (Rat-hIL-3) expressing the human IL-3 gene [28]. Briefly, transfected cells were propagated in Dulbecco's modified Eagle's medium (GIBCO) with 10% FCS. Trypsinized cells were washed, resuspended in cold medium without additives, and mixed with Matrigel (Basement Membrane Matrix 40234; Becton Dickinson; Bedford, MA) as previously described [29]. Five million cells were injected s.c. into the lateral abdomen.

At five to eight weeks post-transplantation, mice were killed by cervical dislocation, and blood as well as bone marrow cells were collected for analysis. Single-cell suspensions were prepared, cell counts were performed, and viability was determined by trypan blue exclusion. Following blocking of Fc receptors by pretreatment of the cells with human serum and an anti-mouse IgG receptor antibody (2.4G2, Pharmingen; San Diego, CA), cell staining was carried out as described earlier [29]. Human- or mouse-specific monoclonal antibodies conjugated with either FITC or PE were used in combinations to identify cells of human and murine origin: mouse anti-human CD45 (clone HI30) and anti-human HLA class I (clone G46-2.6, Pharmingen), anti-CD10 (clone ALB1), anti-CD13 (clone SJ1D1), and anti-CD33 (clone D3HL60.251, Immunotech), anti-CD14 (clone MϕP9, Becton Dickinson; Heidelberg, Germany); rat anti-mouse CD45 (LCA; Pharmingen). As a control, in each experiment, cells from a NOD/SCID mouse not transplanted with human cells were used and stained with the same antibodies. Background fluorescence was assessed by including isotype-matched antibodies conjugated with FITC or PE. Cell analysis was performed with a FACSCalibur system (Becton Dickinson; Heidelberg) using CellQuest software. Each measurement contained 10,000 events. Dead cells were excluded by outgating of propidium-iodide-stained cells.

DNA Analysis

The presence of human-specific DNA within the bone marrow of transplanted mice was confirmed by polymerase chain reaction (PCR) using primers complementary to sequences of an 850-bp DNA fragment of the α-satellite DNA of the human chromosome 17 [30]. Genomic DNA was extracted through a QIAmp tissue kit (Qiagen; Hilden, Germany). Briefly, the DNA of lysed cells was adsorbed to a silica matrix, washed and eluted with QIAAmp elution buffer by centrifugation. Amplification of the centromere-specific human fragments of chromosome 17 was performed using primers corresponding to the primer pair 17a1/17a2 as described by *Warburton et al.* [31]. The primers were elongated to 25 nucleotides each for use at a higher annealing temperature. The 5′ primer (5′ GGG ATA ATT TCA GCT GAC TAA ACA G 3′) covers the positions 15 to 39, and the 3′primer (5′ TTC CGT TTA GTT AGG TGC AGT TAT C 3′) covers the positions 867 to 891 of the sequence HSSATA17 (Gene Bank #M13882). For PCR, the AmpliTaq-Gold polymerase and related reagents from Perkin Elmer (Applied Biosystems GmbH; Weiterstadt, Germany) were used. The PCR reaction contained 200 μM each of the respective nucleotides, 250 nM of each primer, 2 mM MgCl2, and 250 ng of genomic DNA were reacted. Following an initial DNA denaturation and Taq activation at 94°C for 10 min, 35 1-min cycles of denaturation at 94°C and annealing/extension at 60°C were performed followed by a final elongation step at 72°C for 10 min. Amplified DNA fragments were electrophoresed through 1.75% agarose gels and subsequently visualized through ultraviolet light after staining with ethidium bromide. Genomic DNA samples from both a human breast carcinoma line (MaCa 3366) as a positive control and NOD/SCID mouse liver tissue as a negative control were processed in parallel.

Results

Analysis of Cellular Phenotypes During Ex Vivo Expansion

We have analyzed the potential of ex vivo expanded peripheral blood CD34$^+$ progenitors to initiate clonogenic semisolid assays, LTBMC, and human hematopoiesis in xenografted NOD/SCID mice. The CD34$^+$ cell expansion cultures resulted in variable increases in total cell numbers over the culture period of one week (Table 1). The proportion of CD34-expressing cells decreased during this culture period (Fig. 1). The majority of the CD34$^+$ cells also initially expressed CD38 and HLA-DR antigens. During the seven-day expansion period, numbers of CD34$^+$ CD38$^+$ cells fell to almost zero levels, whereas a CD38$^-$ population still expressing CD34 remained (Fig. 1). HLA-DR expression decreased from highly positive to lower levels (Fig. 1). Flow cytometric determination of expanded cells also revealed development of CD14$^+$ and CD15$^+$ monocyte/macrophage and granulocytic cells (approximately 10%) during ex vivo expansion; the

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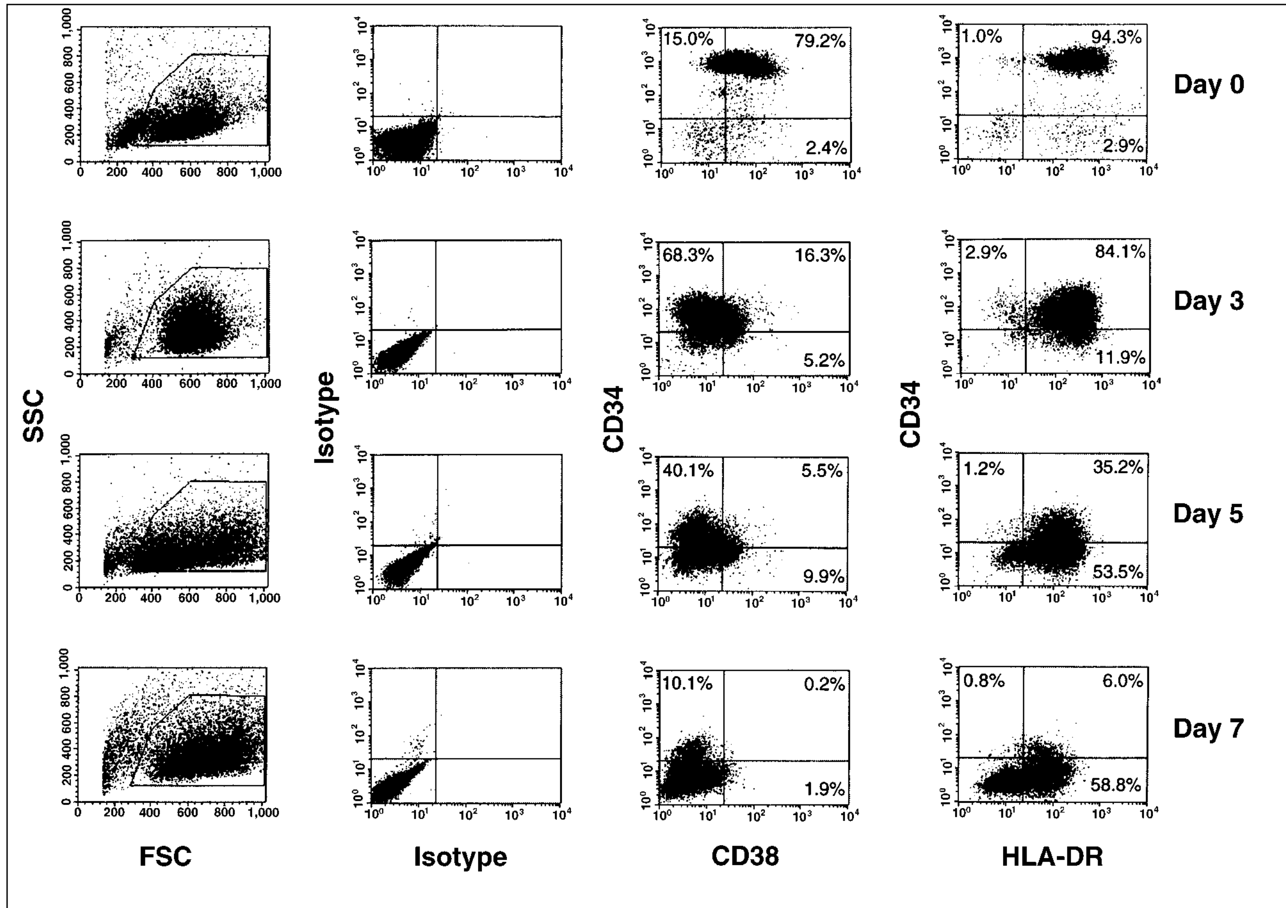

Figure 1. Flow cytometric analysis of CD34+ BPC during ex vivo expansion in serum-free medium. *CD34+ BPC were cultured in the presence of SCF, FL, and IL-3 (100, 300, and 100 ng/ml, respectively) for the indicated time periods. Aliquots were analyzed after staining with FITC- or PE-conjugated antibodies, shown on the x-axis and y-axis, respectively, for the indicated cell surface markers, or IgG control antibodies (isotype). The gate of the analyzed cells is framed in the left panels. The analysis of a representative experiment is shown. (SSC = side scatter; FSC = forward scatter).*

development of myeloid cells was confirmed by light microscopical analysis of May-Grünwald-Giemsa stained samples (not shown). Megakaryocytic and erythrocytic cells, as determined by morphological analysis of May-Grünwald-Giemsa stained samples, were present at very low levels. In contrast, T and B lymphocytes, as assessed by expression of CD3 or CD19, respectively, did not develop. Therefore, CD34+-enriched blood progenitor cells from cancer patients downregulated expression of the CD34 antigen, and predominantly developed along a myeloid differentiation pathway in serum-free medium in the presence of SCF, FL, and IL-3 over a seven-day culture period.

CFU and LTC-IC Amplification During Ex Vivo Expansion

The numbers of CFC per ml of culture, which developed when seeded into methylcellulose-supported clonogenic assays containing IL-3, GM-CSF, and erythropoietin, increased steeply during ex vivo culture over a period of five to seven days (Fig. 2). This was observed for both granulocyte-macrophage and erythrocytic precursor cells. More primitive hematopoietic cell populations have been measured by their capacity to initiate LTBMC over a period of six weeks [32-33]. We have used a limiting-dilution assay seeding CD34+ cells onto allogeneic pre-established and preirradiated normal human bone marrow stroma. We observed that absolute numbers of LTC-IC, as assessed through formation of week 6 CAFC, were at least maintained or gradually increased over a period of up to seven days in culture about threefold (Fig. 2). These data show that culture of peripheral blood CD34+ cells in serum-free medium in the presence of SCF, FL, and IL-3 induces extensive amplification of lineage-committed progenitor cells and a more restricted expansion of LTC-IC.

Engraftment of Human Hematopoietic Cells in NOD/SCID Mice

To assess the development of bone marrow repopulation potential, ex vivo expanded cells were injected into NOD/SCID mice. Three to 5×10^5 noncultured CD34+ cells were used per mouse. For analysis of cultured cells in

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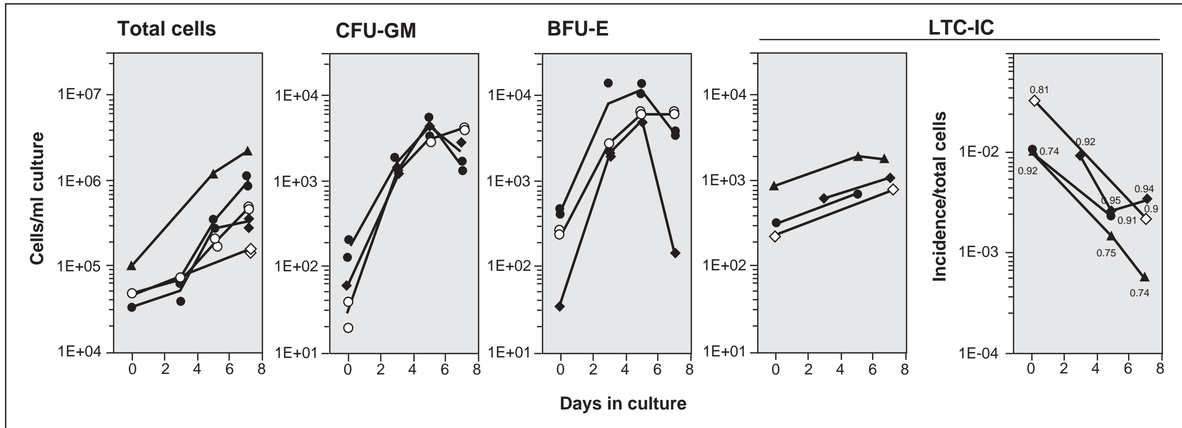

Figure 2. Analysis of total cell numbers, CFU-GM, BFU-E colonies, and LTC-IC (assessed through week 6 CAFC) during CD34⁺ cells in suspension culture. *CD34⁺ BPC were cultured in the presence of SCF, FL, and IL-3 (100, 300, and 100 ng/ml, respectively) for the indicated time periods, and aliquots were analyzed in the indicated assay systems. Different symbols indicate analysis of cells from different donors. Duplicate symbols for total cells, CFU-GM, and BFU-E values represent duplicate determinations. LTC-IC incidences were determined by limiting dilution analysis. LTC-IC/ml were calculated from the total cell number and the LTC-IC incidence values. Numbers attached to symbols of LTC-IC-incidences are* r^2 *values of the regression analysis. Each symbol represents an individual donor. Symbols represent the donors as shown in Table 1.*

NOD/SCID mice, the entire progeny of this number of CD34⁺ cells at the respective time points was transplanted, since we assumed that the repopulating cells would not expand to a much higher degree than the LTC-IC. Donor cells were detected five to eight weeks after transplantation in the bone marrow or blood of the animals using dual-color flow cytometry with human-specific monoclonal antibodies (Fig. 3). Analyses at various time points within seven days of CD34⁺ cell expansion demonstrated that the hematopoietic activity obtained in the mice could be maintained for only up to four days (Fig. 4). After this time point, hematopoietic engraftment in the bone marrow of the mice was very low; similarly, numbers of human cells detected in the circulation of the mice were reduced to low or undetectable levels. The establishment of human hematopoiesis in NOD/SCID mice has been confirmed by human-specific PCR using primers detecting an alpha-satellite sequence within human chromosome 17. In animals which had received seven-day expanded cells, human-specific PCR turned negative (data not shown). In addition, whenever human engraftment was detected, human cell populations expressing both lymphoid (CD10) and myeloid (CD13, CD14, CD33) cell surface markers were present irrespective of the culture time point investigated (Fig. 3).

Influence of Cell Density, Fibronectin Precoating, and TPO on Ex Vivo Expansion of Progenitor Cells

In a series of experiments, modifications of culture conditions were included. To investigate whether culture at the relatively low cell density of 5×10^4/ml used to inoculate the cultures might negatively influence the maintenance of

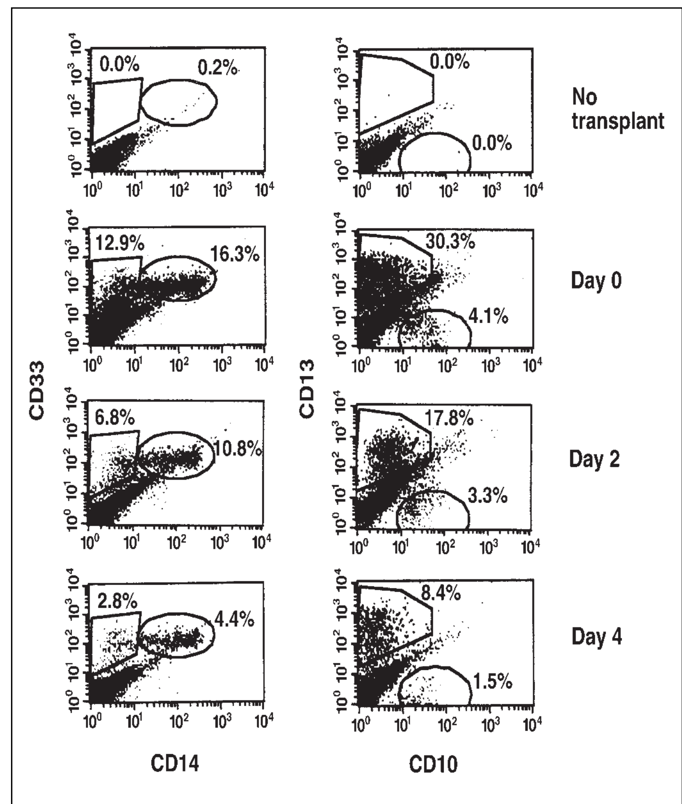

Figure 3. Flow cytometric analysis of human hematopoietic cells in the bone marrow of NOD/SCID mice. *A total of 3 to 5 × 10⁵ CD34⁺ BPC were cultured in the presence of SCF, FL, and IL-3 (100, 300, and 100 ng/ml, respectively) for the indicated time periods and injected i.v. into each animal. Engraftment was analyzed five to eight weeks after transplantation. Bone marrow cells from the mice were stained with the indicated FITC- or PE-conjugated antibodies, shown on the x-axis and y-axis, respectively, and analyzed by flow cytometry.*

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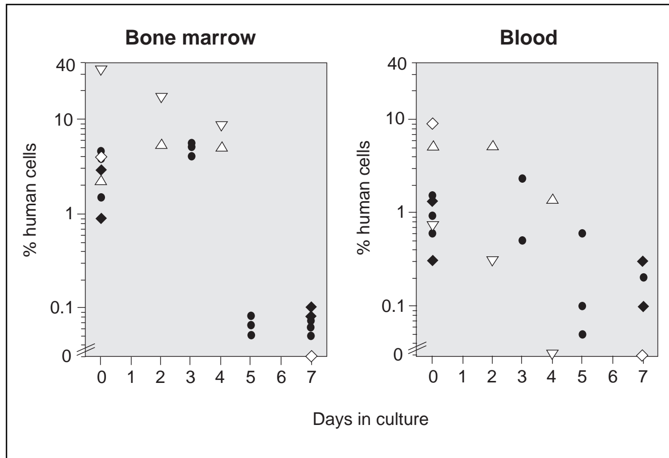

Figure 4. Engraftment of human cells in the bone marrow or blood of NOD/SCID mice after culture in serum-free medium and SCF, FL, and IL-3 (100, 300, and 100 ng/ml, respectively). *Three to 5×10^5 CD34+ cells per mouse were precultured for the indicated time periods, and engraftment was analyzed five to eight weeks after transplantation by staining with human-specific antibodies for HLA-I (bone marrow cells) or CD45 (peripheral blood cells) and subsequent flow cytometric analysis. Each symbol represents an individual donor. Duplicate or triplicate use of a symbol represents analysis in different mice. The different symbols represent the donors as shown in Table 1.*

repopulating stem cells, which might require cellular contacts to survive, the expansion procedure was also performed in 96-well U-bottom plates. Since fibronectin precoating, which was found to improve retroviral gene transfer [6, 7], has also been suggested to confer maintenance of hematopoietic repopulation potential in NOD/SCID mice, we included cultures on fibronectin-precoated plastic. In addition, since high concentrations of IL-3 may negatively influence numbers of LTC-IC recovered after serum-free cytokine-supported culture of human highly enriched CD34+/CD38– progenitors from bone marrow, we intended to investigate the influence of IL-3 on the maintenance of CFC, LTC-IC, and marrow-repopulating cells after seven days of culture. As omission of IL-3 and culture in SCF and FL alone resulted in complete cessation of cell growth and decreased cell viability in preliminary experiments, we chose to replace IL-3 by TPO, which has been found to stimulate primitive hematopoietic progenitors [34]. Figure 5 shows the results obtained when analyzing CFC, LTC-IC, and human cells repopulating NOD/SCID mice after a culture period of seven days. Under all three conditions, similar yields of CFC and LTC-IC numbers were reached compared to controls that were cultured in SCF, FL, and IL-3 on an uncoated flat plastic surface. However, no significant human hematopoiesis was found in the bone marrow of the transplanted mice when injecting cells cultured under all three modifications, whereas mice transplanted with nonexpanded CD34+ cells showed human engraftment. These data indicate that no major improvement of the maintenance of in vivo

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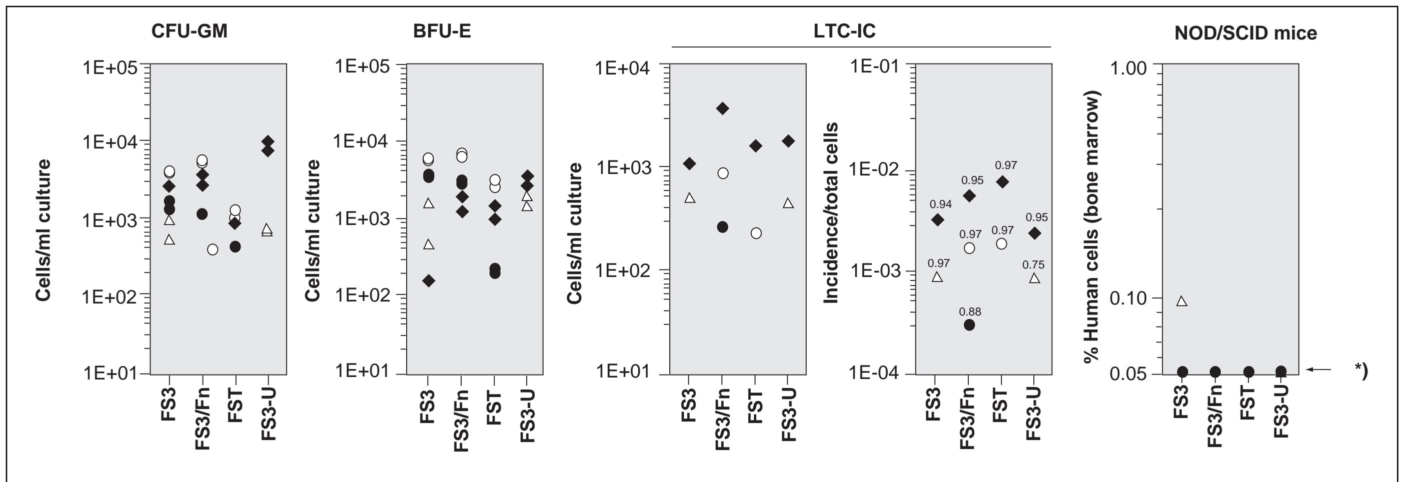

Figure 5. Development of CFC, LTC-IC, and hematopoietic repopulation potential in NOD/SCID mice after seven-day ex vivo culture of CD34+ BPC using variations in culture conditions. *FS3, control conditions as in experiments shown in Figures 2 to 4 using FL, SCF, and IL-3 (300, 100, and 100 ng/ml, respectively) in flat-bottom flasks; FS3/Fn, conditions as in FS3 but with fibronectin (10 µg/cm²) precoated culture surface; FST, conditions as in FS3, except that IL-3 was substituted by TPO (100 ng/ml); FS3-U, conditions as in FS3 except that culture was performed in U-bottomed 96-well plates. Positive control mice transplanted with noncultured CD34+ BPC (3 to 5×10^5 per mouse) were the same as shown in Figure 4 (day 0) and showed efficient human engraftment. Results represent values from individual cultures from different donors. Duplicate symbols for CFU-GM and BFU-E values represent duplicate determinations; LTC-IC incidences were determined by limiting dilution analysis. LTC-IC/ml were calculated from the total cell number and the LTC-IC incidence values. Numbers attached to symbols of LTC-IC incidences are r^2 values of the regression analysis. Engraftment in NOD/SCID mice was analyzed five to seven weeks after transplantation by staining of bone marrow cells with human-specific antibodies for HLA-I; values are means from two to three mice per experiment. *) values obtained from all experiments (n = 2-3) range from 0 to 0.1%. Each symbol represents an individual donor. Symbols represent the donors as shown in Table 1.*

hematopoietic activity is induced in serum-free cytokine-supported culture by altered local cell density, fibronectin precoating, or substitution of IL-3 by TPO.

Discussion

We have compared the potential of human ex vivo expanded CD34+-enriched BPC to form or maintain lineage-committed progenitor cells assayed by CFC, progenitors able to confer sustained proliferation in LTBMC, or cells which can initiate a human lymphomyelopoiesis in NOD/SCID mice. Our data indicate that the incidence of the CFC and LTC-IC cell populations assayed in vitro does not develop in parallel with the in vivo hematopoietic repopulation potential in mice.

Ex vivo expansion of hematopoietic progenitors has been investigated in a number of previous studies, mostly using CD34-enriched cell populations [3-5, 13-15, 22, 35]. However, these studies have been restricted to in vitro endpoints (CFC, LTC-IC) to assess the transplantation potential of cultured cells, and systematic analyses of in vitro and in vivo repopulation potential over several time points have not been performed. In our serum-free expansion protocol, as well as in a study by *Zandstra et al.* [35], SCF and FL were present at relatively high concentrations (100 to 300 ng/ml). The resulting CFC amplification was in a range similar to that obtained by *Petzer et al.* [15], who also used these cytokines at high concentrations and obtained optimal LTC-IC expansion from bone marrow CD34+ CD38– cells. The relatively lower degree of LTC-IC expansion in our cultures compared with the study by *Petzer et al.* [15] coincides with the fact that we have not added a further enrichment procedure for primitive progenitors, such as the additional CD38– separation, which may preselect for a more primitive subpopulation of LTC-IC with higher expansion potential.

Gan et al. [19] have described a loss in the ability of human hematopoietic cells cultured on bone marrow stromal feeders to repopulate irradiated NOD/SCID mice. Our results after expansion of CD34+ BPC in the absence of stroma show that after a similar period of seven days in stroma-free suspension culture, the ability of CD34+ BPC to repopulate the bone marrow is also abrogated. Similar results were obtained by *Bhatia et al.* [36], who cultured human enriched CD34+CD38– cord blood cells in the presence of cytokines without stroma support. These authors noted that after a four-day suspension culture period, the repopulation potential of expanded cells in NOD/SCID mice was preserved or slightly augmented; however, after eight days it was generally lost. Interestingly, FL was also present in these cultures at high levels. *Dao et al.*, in their gene transduction work, noted that the presence of FL was required to maintain the repopulation potential of human CD34+ cells for a 72-h period (assessed in humanized bnx mice) [8] . *Larochelle et al.* found loss of human hematopoietic repopulating potential of CD34+CD38–-enriched cord blood cells within 48 h after cytokine incubation; in that study, all experiments except one were conducted in the absence of FL [7].

Murine progenitor cells have also been transplanted into lethally irradiated recipient mice after cytokine-supported stroma-free expansion cultures. These studies used either enriched or nonenriched cells, various cell densities, and different cytokine combinations. In some studies, maintenance of the repopulation potential was achieved [16, 37], but other authors showed that early loss of stem cells occurred during the expansion period [38]. Whereas the former researchers all used serum-supplemented media, *Rebel* and coworkers used serum-free expansion of murine highly enriched progenitors and showed that, although the numbers of cells with a primitive (lineage-negative wheat germ agglutinin-positive) phenotype expanded, the engraftment potential of the cultured product was not amplified but could at best be maintained [17]. Similar results were also reported by *Brown et al.* [39] after expansion of murine bone marrow cells in the presence of SCF and GM-CSF. Recently, however, an increase in murine reconstituting cells could be demonstrated in a culture system starting from Sca-1+ lin– bone marrow cells [40].

Our studies did not reveal a major advantage of using U-bottom vessels or fibronectin coating for the maintenance of transplantation potential. The substitution of IL-3 by TPO did not lead to major alterations in CFC or LTC-IC numbers within the expansion cultures on day 7, showing that substitution of IL-3 by TPO in SCF and FL-containing cultures is not sufficient to preserve NOD/SCID repopulating stem cells. Taken together, we have demonstrated that progenitor cells with the ability to engraft in vivo may be maintained ex vivo for a time period of three to four days with cytokines and serum-free suspension culture. The short-term culture protocol presented here should be of benefit for the clinical introduction of gene marking and tumor cell purging protocols, which both require maintenance of repopulating stem cells during in vitro culture. Other work has shown that for effective tumor cell purging with recombinant immunotoxins, short time periods may be sufficient [10]. Also, incubation of CD34+ Thy+ lin– progenitors in SCF, FL, and TPO has been shown to result in cell division in very primitive populations capable of repopulating immunodeficient mice during the first two to four days of culture [41], which leads to the expectation that retroviral gene transfer into primitive progenitors may be achieved under such conditions. Further studies will have to compare the repopulation in NOD/SCID mice by expanded BPC with human hematopoietic regeneration.

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