9 Fetal Hematopoietic Stem Cells: In Vitro Expansion and Transduction Using Lentiviral Vectors

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9.1 Introduction

Umbilical cord blood (CB), remaining in the placenta at delivery, is rich in hematopoietic stem and progenitor cells. Compared with adult peripheral blood, the content of $CD34^+$ and $CD34^+CD38^-$ cells in CB is approximately tenfold higher and thus comparable to adult bone marrow (Broxmeyer et al. 1989, 1990; Hao et al. 1995). Moreover, CB progenitors have high plating efficiency in clonogenic assays, they respond rapidly to cytokine stimulation in vitro and generate progeny comparable to that derived from bone marrow precursors (Emerson et al. 1985; Cardoso et al. 1993; Lansdorp et al. 1993). Due to these characteristics, CB has been recognized as an attractive alternative source of hematopoietic stem cells for transplantation (Cairo and Wagner 1997). Several hundreds of patients with hematological malignancies and genetic diseases affecting the hematopoietic system have been treated with

CB from related as well as from unrelated donors (Gluckman et al. 1989, 1997; Rubinstein et al. 1998). Clinical results have shown that CB transplants engraft and sustain hematopoietic function. Survival rates are comparable to those after bone marrow transplantation. The main advantage of CB over adult hematopoietic tissue is immunologic immaturity of accessory cells in the graft, including a lower expression level of T cell-derived growth factors (Ehlers and Smith 1991; Harris et al. 1992; Sautois et al. 1997). In clinical practice, this results in reduced incidence and severity of graft-versus-host disease, and may allow a higher degree of human leukocyte antigen (HLA) disparity between donor and recipient (Rubinstein et al. 1998). While CB transplantation has proven feasible in pediatric patients, the number of stem cells in a CB graft may be insufficient to reconstitute an adult recipient. Therefore, efforts to expand pluripotent hematopoietic CB cells in vitro are under way. The most efficient amplification of hematopoietic cells from human CB has recently been described in cultures supplemented with thrombopoietin, and flt-3 ligand (Moore and Hoskins 1994; Piacibello et al. 1997).

CB-derived stem cells are an attractive target for somatic gene therapy of inborn defects of the lymphoid and the hematopoietic system. Engraftment of genetically modified $CD34^+$ cells has been achieved with CB of neonates with adenosine deaminase deficiency (Kohn et al. 1995; Bordignon et al. 1995). Advances in understanding the molecular background of hereditary diseases and progress in prenatal diagnostics have opened a therapeutic concept of in utero treatment of genetic diseases identified during pregnancy (Flake and Zanjani 1997). Feasibility of in utero transplantation has been documented by long-term multilineage chimerism in sheep, mice and monkeys transplanted with human tissue early during gestation (Flake et al. 1986; Fleischman and Mintz 1979; Harrison et al. 1989; Zanjani et al. 1992). Recently, first clinical reports described the successful treatment of immunodeficiency syndromes in human fetal recipients transplanted with parental bone marrow (Flake et al. 1996; Wengler et al. 1996) or allogeneic fetal liver cells (Touraine et al. 1989, 1992). A therapeutic option of in utero therapy could also be envisaged with autologous fetal stem cells if enough circulating progenitors could be acquired by cordocentesis during pregnancy, genetically modified ex vivo, and transplanted back to the affected fetus. This strategy would avoid limitations and risks associated

with immunological barriers of allogeneic grafts. So far, progress in human gene therapy has been hampered by inefficiency of gene transfer to immature hematopoietic progenitors with long-term repopulating capacity after transplantation. Recently, new generations of the replication-deficient lentiviral vectors have been shown useful for ex vivo gene delivery to non-dividing cells (Naldini et al. 1996; Uchida et al. 1998), thus opening a perspective for targeting quiescent hematopoietic progenitors.

While hematopoietic properties of neonatal CB from full-term pregnancies have been well characterized, less is known about CB from early gestational ages. Previous studies revealed the presence of hematopoietic cells in the fetal circulation: it has been demonstrated that the content of $CD34^+$ progenitors in CB is higher during fetal life than at birth (Thilaganathan et al. 1994; Shields and Andrews 1998; Surbek et al. 1998), and that preterm CB is rich in colony-forming precursors (Clapp et al. 1989; Jones et al. 1994; Migliaccio et al. 1996). In our recent work, we analyzed the primitive hematopoietic progenitors, defined phenotypically as $CD34^+CD38^-$ cells and functionally as long-term culture-initiating cells (LTC-IC). Using different combinations of recombinant growth factors, we have also established culture conditions for short- and long-term expansion of circulating progenitors from preterm CB. The results demonstrated that CB from fetuses at early gestational age contains a significantly higher number of both committed as well as early progenitor cells than term CB, with profound proliferation capacity in vitro (Wyrsch et al. 1999). In this work, we used lentiviral vectors for transfer of the enhanced green fluorescence protein (GFP) gene to fetal progenitors. We show that the efficiency of transduction of hematopoietic cells from preterm and term CB is comparable. Furthermore, transduced cells can be extensively amplified in vitro. Owing to these properties, preterm fetal CB may be a potential source of progenitors for in utero treatment of disorders amenable to transplantation of genetically corrected stem cells.

9.2 Material and Methods

9.2.1 Study Population

The samples from term healthy newborns (n=20; weeks ≥35) included uneventful vaginal births and cesarean sections. Preterm CB included samples from weeks 13 and 14, from second trimester (n=18; weeks 16–28), and early third trimester (n=20; weeks 29–34) of pregnancy. Premature deliveries followed idiopathic preterm labor or prelabor rupture of membranes, as well as spontaneous and elective abortions in the second trimester. Exclusion criteria were clinically overt chorioamnionitis and preeclampsia. There has been no evidence of hematopoietic abnormalities of the fetuses. Informed consent was obtained from the mothers prior to delivery, and the Ethical Committee of the University Hospitals in Basel, Switzerland approved the investigations.

9.2.2 Cord Blood Cells

CB was harvested aseptically by umbilical vein puncture, and up to 10 ml was collected in heparin-containing tubes. The delay between collection of CB samples and their subsequent processing did not exceed 12 h. Flow cytometry was performed with 250 µl of heparinized full blood. For cell cultures, cord blood mononuclear cells (CBMNC) were isolated by centrifugation on Histopaque (density <1.077 g/ml; Sigma Diagnostics, St. Louis, Mont., USA), and used either freshly to initiate methylcellulose cultures (see below), or were cryopreserved in liquid nitrogen in a freezing mixture containing Iscove's modified Dulbecco's medium (IMDM), 20% fetal calf serum (FCS; both Gibco, Gaitherburg, Md., USA) and 10% DMSO and used for long-term cultures or for transduction with lentiviral vectors. Prior to the experiment, cells were thawed and allowed to adhere overnight in culture dishes containing IMDM-25% FCS; nonadherent cells were collected to initiate the cultures (see below).

9.2.3 Flow Cytometry (FACS) Analysis

Aliquots of 50 µl of heparinized CB were stained with anti CD34-phycoerythrin (PE; Becton Dickinson, San Jose, Calif., USA) and anti CD38-fluoroscein isothiocyanate (FITC; Immunotech, Marseille, France) or with the corresponding isotype control antibodies (Becton Dickinson) at concentrations recommended by the manufacturers. After staining, the samples were treated with lysing buffer (Ortho, Neckargemünd, Germany) to lyse red blood cells. Dual color analysis was performed on a FACScan (Becton Dickinson) acquiring 10–50,000 events. Lymphocyte gates were set according to forward and sideward light scatter; dead cells were stained with propidium iodide and excluded from the analysis. Analysis was performed with CellQuest software (Becton Dickinson).

9.2.4 Growth Factors

The following recombinant human growth factors were used: stem cell factor (SCF; AMGEN, Thousand Oaks, Calif., USA), megakaryocyte growth and development factor (PEG-rHuMGDF, a truncated Mpl ligand, AMGEN), flt-3 ligand (FL; Immunex, Seattle, Wash., USA), Interleukin (IL)-3, -6, granulocyte-macrophage colony-stimulating factor (GM-CSF; all from Novartis, Basel, Switzerland), granulocyte colony-stimulating factor (G-CSF; Rhone-Poulenc, Antony, France), and erythropoietin (Epo; Böhringer Mannheim, Mannheim, Germany). Growth factors were used at a concentration of 100 ng/ml, except for MGDF at 50 ng/ml and Epo at 36 U/ml.

9.2.5 Cell Culture Assays

Colony forming Unit Assay. CBMC from term and preterm samples (5×10^4 and 2.5×10^4, respectively) were plated into 1% methylcellulose cultures (Wodnar-Filipowicz et al. 1992) supplemented with the growth factors indicated in the figure legends. Secondary methylcellulose cultures, performed with cells harvested from liquid cultures (see below), were supplemented with Epo, G-CSF, GM-CSF, IL-3, and SCF. He-

matopoietic colonies derived from granulocyte (G)-colony forming units (CFU), macrophage (M-CFU), erythroid (BFU-E), granulocyte-macrophage (GM-CFU), granulocyte-macrophage-erythroid-megakaryocyte (GEMM-CFU), and megakaryocyte (Mk-CFU) precursors were counted after 14 days of culture. The identity of Mk-CFU was confirmed by cell morphology after differential staining (Dade Diff-Quik, Baxter Diagnostics, Düdingen, Switzerland) of individually picked colonies.

Long-Term Culture-Initiating Cell Assay. Determination of long-term culture-initiating cell (LTC-IC) content was performed according to Sutherland et al. (1990). The murine fibroblast cell line M210B4 (American Type Culture Collection) was used as a feeder layer. Before initiation of the co-culture with human cells, M210B4 cells were trypsinized, irradiated with 80 Gy, seeded into 96-well plates and allowed to readhere over night. Term or preterm CBMNC (6×10^3 and 3×10^3, respectively) were seeded in quadruplicate wells over the feeders in 200 µl of IMDM containing 12.5% horse serum, 12.5% FCS, 0.016 mm folic acid, 0.16 mm i-inositol (all from Gibco) and 10^{-6} m hydrocortisone (Sigma Diagnostics). If limiting dilution analysis was carried out, a minimum of 12 replicate wells per cell concentration ($50–3\times10^3$ CBMNC/well) were initiated. The cultures were maintained at 33°C for 5 weeks with weekly half-medium changes. At the end of the culture period, nonadherent cells were combined with the corresponding trypsinized adherent cells, washed and assayed in secondary methylcellulose cultures for CFUs as described above.

Long-Term Liquid Cultures. Cultures were carried out according to Piacibello et al. (1997). Briefly, 1×10^5 term or preterm CBMNC were seeded into 1 ml of IMDM containing 10% FCS, 380 mg/ml iron saturated human transferrin, 1% bovine serum albumin (BSA; Behring, Marburg, Germany). Cultures were supplemented with MGDF and FL or SCF. Weekly half-medium changes were performed. At 1, 3, 6, and 10 weeks, harvested cells were counted, and aliquots were plated into secondary methylcellulose cultures to determine the content of CFUs. In addition, at 1, 3 and 6 weeks, cells were seeded over M210B4 feeder layers, and the content of LTC-IC was determined.

9.2.6 Transduction with Lentiviral Vectors

Lentiviral vectors were produced by co-transfection of 293 T cells with three plasmids, as described in Costello et al. (1999), and concentrated by ultracentrifugation to 5×10^7 IU/ml. Prior to transduction, 1×10^5 $CD34^+$ CB cells were precultured for 3 days with IL-3, -6, SCF, and FL. For transduction, cells were resuspended in 50 µl IMDM with 5 µg/ml protamine sulfate (Sigma, St. Louis, USA) in V-bottom tubes previously coated with 20 µg/cm^2 fibronectin (Retronectin, TaKaRa, Shiga, Japan) or BSA. Lentivirus-containing supernatant (30 µl) was added, and cells were centrifuged at 3000 rpm for 3 h (spinoculation). Cells were then resuspended in 1 ml of IMDM containing 20% FCS and growth factors as above, and incubated without washing for 24 h at 37°C. The next day, cells were washed twice with IMDM supplemented with 2% FCS. In some experiments, transduction was repeated. Samples were spinoculated with fresh lentiviral supernatant, incubated and washed as described above. Transduced CB cells were counted and used for CFU-, LTC-IC- and liquid cultures. GFP-expression was analyzed by flow cytometry starting from day 3 after transduction.

9.2.7 Statistical Analysis

Growth of term and preterm CB was compared by the Student's unpaired *t*-test, and a linear correlation test was performed using StatView software (Abacus).

9.3 Results

9.3.1 Content of $CD34^+$ and $CD34^+CD38^-$ Progenitors

The frequency of $CD34^+$ cells and their $CD34^+CD38^-$ subset was determined in CB from fetuses at different gestational ages, ranging from 13th week to term (35th week) pregnancies. We were unable to detect hematopoietic progenitors in samples derived from late first trimester of pregnancy (weeks 13 and 14). In contrast, high content of progenitors was measured in samples from the second trimester, starting from

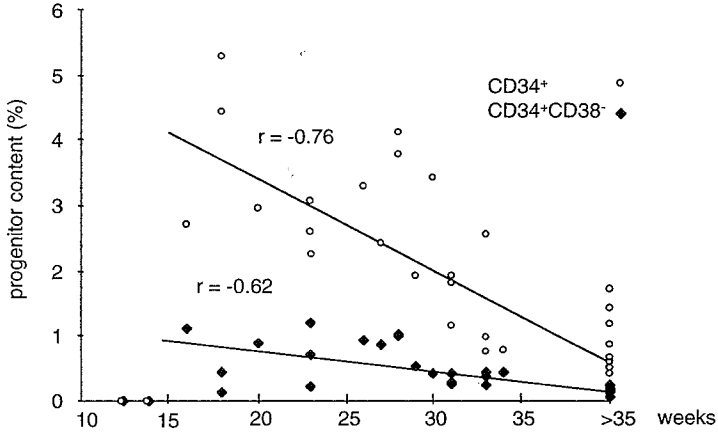

Fig. 1. Frequency of $CD34^+$ and $CD34^+CD38^-$ progenitor cells along the progression of pregnancy

week 16 (Fig. 1). The percentage of $CD34^+$ and $CD34^+CD38^-$ cells decreased steadily, in an inverse correlation to gestational age (r=–0.76 and –0.62, respectively). On average, the content of $CD34^+$ cells in preterm CB was 2.51±0.28%, which was significantly higher in both second and early third trimester than in CB from newborns delivered at term (0.88±0.17%; $P<0.001$). Preterm CB was also richer in the most primitive $CD34^+CD38^-$ progenitors (0.56±0.08 vs 0.13±0.02%; $P<0.002$) (Wyrsch et al. 1999).

9.3.2 Content of CFU and LTC-IC Progenitors

To define the frequency of committed CFU progenitors, we cultured CBMNC of various gestational ages in methylcellulose supplemented with a combination of growth factors containing Epo, IL-3, G-CSF, GM-CSF and SCF (Wodnar-Filipowicz et al. 1992) (Table 1). Colony numbers were approximately twofold higher in preterm than term CB (240±27 vs 142±12/10^5 CBMNC; $P<0.05$), with a prevalence of erythroid and mixed colonies (BFU-E and GEMM-CFU). The amount of CFUs correlated with the frequency of $CD34^+$ cells in CB (r=0.73;

Table 1. Frequency of hematopoietic colony-forming cells in term and preterm CB

CB	CFU[a]	LTC-IC[b]
Term	142±12	2.6±1.2
Preterm	240±27	6.7±2.9

Mean±SEM is indicated. Statistical significance of the differences between CFU and LTC-IC content in term and preterm CB, $P<0.05$.

[a] Frequency of CFUs per 1×10^5 CBMNC from term and preterm CB. CBMNC were cultured in methylcellulose in the presence of Epo, IL-3, G-CSF, GM-CSF and SCF.

[b] Frequency of LTC-ICs per 1×10^5 CBMNC from term and preterm CB. Results are based on limiting dilution analysis.

$P<0.0004$). For detection of more primitive LTC-IC precursors, stroma-supported long-term cultures were established. LTC-IC-derived colonies were detected in every preterm CB sample (Table 1). Their frequency was $6.7\pm2.9/10^5$ CBMNC, which was higher than in term CB (2.6 ± 1.2; $P<0.05$).

9.3.3 Comparison of Hematopoietic Progenitor Cells from Various Sources

Table 2 summarizes published results on the content and growth properties of progenitors from various hematopoietic tissues. The frequency of $CD34^+$ cells in preterm CB, although highly variable, is higher than in traditional sources of transplantable stem cells such as "mobilized" peripheral blood or bone marrow. Significantly higher is also the content of $CD34^+CD38^-$ cells, as well as of progenitors scored in the functional hematopoietic in vitro assays, CFU and LTC-IC. Preterm CB has not yet been examined in terms of the content of SCID repopulating cells (SCR), which represent primitive human hematopoietic cells capable of in vivo repopulating the bone marrow of sublethally irradiated mice with nonobese diabetic/severe combined immunodeficiency (NOD/SCID) syndrome (Larochelle et al. 1996).

Table 2. Comparison of the frequency and growth properties of hematopoietic progenitor cells from different tissues

Source	CD34+ (% MNC)	CD34+CD38− (% MNC)	CFU /10^6 MNC	LTC-IC /10^6 MNC	SRC /10^6 MNC	References
PB	0.1	<0.1	40	0.5	<	Civin et al. 1987
PB "mobilized"	0.5–1.0	0.1	820	60	0.2	Wang et al. 1997, Hogge et al. 1996
BM	1.0–4.0	0.1	1400	30	0.3	Civin et al. 1987, Wang et al. 1997
CB term	0.5–2.0	0.1	1300	70	1	Hao Q-L et al. 1995, Wang et al. 1997
CB preterm	1.0–15.0	0.6	2300	150	?	Wyrsch et al. 1999

PB, peripheral blood; *BM*, bone marrow; *MNC*, mononuclear cells; *CB*, cord blood.

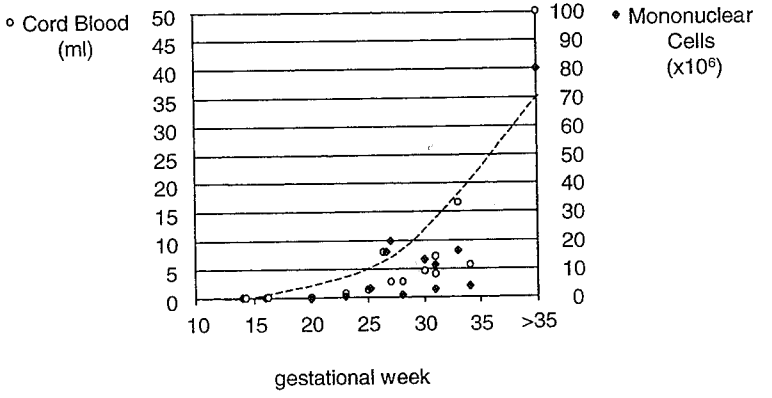

Fig. 2. Yield of cord blood and of mononuclear cells along the progression of pregnancy. Increasing weight of the fetus is marked with the *dotted line*

9.3.4 Retrieval of Hematopoietic Progenitor Cells from Preterm CB

We assessed the yield of hematopoietic progenitors from fetal compared with term CB, taking into account the total volume, the recovery of mononuclear cells, and the number of progenitors measured in samples from various gestational ages. The volume of CB collected from the umbilical cord and the total content of nucleated cells paralleled the increasing weight of the fetus (Fig. 2). On average, the CB volume and the cell content in second and third trimester samples were approximately 25- and 8-fold lower than in CB from term pregnancies (Ta-

Table 3. Total retrieval of hematopoietic progenitors from preterm and term CB

	Volume (ml)	Mononuclear cells ($\times 10^6$)	$CD34^+$ cells ($\times 10^5$)	CFUs
2nd trimester CB (until week 28)	2.3	3.3	1.0	8000
Early 3rd trimester CB (week 29–35)	8.0	10.3	2.1	21100
Term CB (>week 35)	50.0	80.0	7.4	106,000

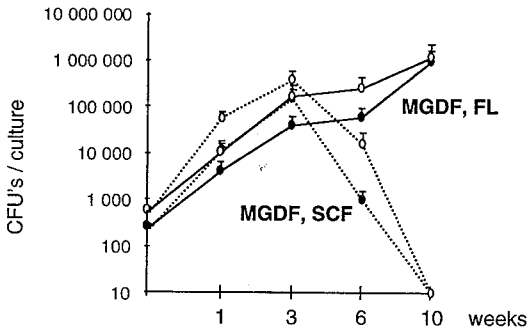

Fig. 3. Long-term expansion of CFU progenitors in cultures containing MGDF and FL or MGDF and SCF, as indicated. (o) preterm CB; (●) term CB

ble 3). Therefore, despite the high frequency of hematopoietic progenitors in fetal CB, the total yield of $CD34^+$ cells and CFU-derived colonies was significantly below the yield of progenitors from term CB (Table 3).

9.3.5 Long-Term Expansion of Clonogenic Progenitors

Recently, a dramatic effect of TPO in combination with FL on the expansion of CB precursors ex vivo has been reported by Piacibello et al. (1997). We were able to reproduce this effect with preterm CB: in the presence of FL and MGDF, representing a functional equivalent of TPO (Hunt et al. 1995), inexhaustible production of CFUs was observed in stroma-free liquid cultures monitored for 10 weeks (Fig. 3). An approximately 1000-fold expansion of CFUs was achieved in preterm as well as term CB. At week 10, multipotential progenitors (GEMM-CFU) were still prevailing in both preterm and term CB (Wyrsch et al. 1999). The effect of MGDF and FL on the expansion of LTC-IC was monitored until week 6; an approximately 30-fold amplification was observed in preterm CB cultures (Fig. 4). When MGDF was used together with SCF, clonogenic progenitors started to decline at week 3 and were exhausted after 10 weeks (Fig. 3). These results indicate that preterm CB progenitors can be not only maintained but also extensively amplified in cultures in the presence of appropriate combinations of growth factors.

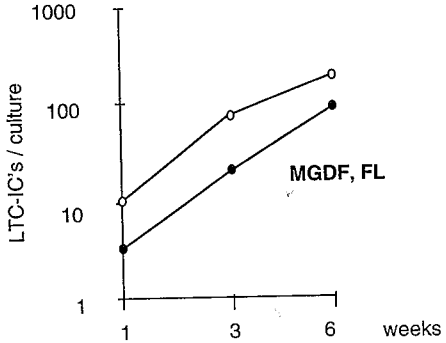

Fig. 4. Long-term expansion of LTC-IC progenitors in cultures containing MGDF and FL. (o) preterm CB; (●) term CB

9.3.6 Transduction of CB Cells with Lentiviral Vectors

We used preterm CB cells as targets for transfer of the enhanced GFP gene by lentiviral vectors. Preparation of the vectors has been described elsewhere (Costello et al. 1999). Various gene transfer protocols were tested for transfection of purified $CD34^+$ progenitors. Efficiency and longevity of GFP-expression were determined by flow cytometry and fluorescence microscopy of colonies in CFU assays. Best results were obtained by exposing cells to viral supernatant supplemented with protamine sulfate in 3-h spinoculation at 3000 rpm on 2 subsequent days (Fig. 5). In liquid cultures, 20–30% of $CD34^+$ cells was expressing GFP at 2–3 weeks after transfection, and this expression persisted for at least 5 weeks. The frequency of transduced progenitors from preterm and term CB was comparable. Likewise, the frequency of GFP-expressing CFU-derived colonies from preterm and term CB was similar (Table 4). About 25% of colonies obtained prior to expansion in MGDF/FL-containing cultures were GFP^+. This high frequency of transduced cells was maintained in cultures examined at 3 weeks. Simultaneously, a significant amplification of clonogenic progenitors was observed, in agreement with the results shown in Fig. 3.

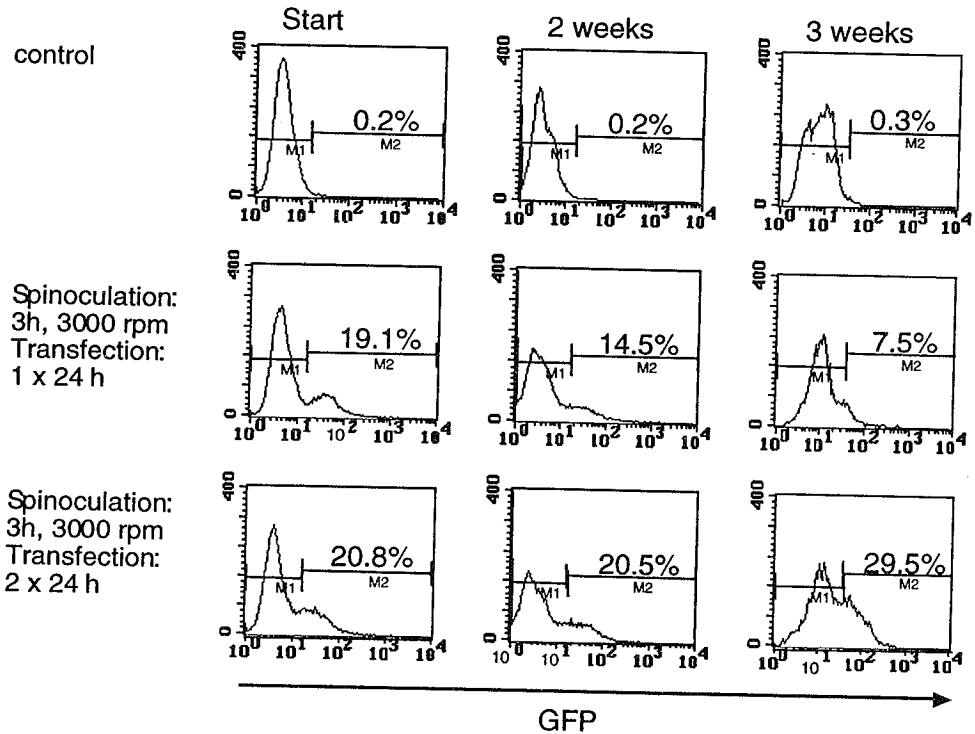

Fig. 5. Flow cytometry analysis of CB progenitor cells transduced with GPF gene using lentiviral vectors. Transduction conditions and the time of analysis are indicated

Table 4. GFP-expression in lentivirally transduced clonogenic CB progenitors in response to MGDF and FL

		Term CB (n=2)	Preterm CB (n=4)
Start	CFUs/10^3 $CD34^+$ cells	176±21	153±6
	GFP^+ CFUs	41±7	35±6
	%	23±1	23±5
3 weeks	CFUs/10^3 $CD34^+$ cells	10374±375	10100±2750
	GFP^+ CFUs	2475±175	4020±1150
	%	24±1	42±7

Transduced cells were cultivated in liquid cultures containing MGDF and FL, and assayed in secondary methylcellulose cultures at week 3. The content of GFP^+CFUs was determined by fluorescence microscopy.

9.4 Discussion

The first goal of this study was the phenotypic and functional characterization of committed and primitive hematopoietic CB progenitors from immature infants at weeks 13–34 of gestation, as compared with CB from newborns delivered at term. The frequency of circulating progenitor cells was the highest during the second trimester of pregnancy and declined linearly with progressing gestation. The content of $CD34^+$ cells decreased from an average of 3.11% to 0.88%, $CD34^+CD38^-$ from 0.69% to 0.13%, CFUs committed to granulocyte, macrophage and erythroid lineages from 230.2 to 133.2/10^5 CBMNC, and clonogenic LTC-ICs from 6.7 to 2.6/10^5 CBMNC. The gestation time-dependent changes in the content of circulating progenitors are in accordance with the sequential shifts of hematopoietic sites during ontogeny: At 6 weeks of gestation, hematopoiesis switches from yolk sac and aorta-gonad-mesonephros region to the fetal liver, which remains the major organ of blood cell production throughout pregnancy. During the second and third trimester, circulating stem cells start colonizing bone cavities and gradually establish adult hematopoiesis in the bone marrow (reviewed in Tavassoli 1991 and Péault 1996). According to our results, the highest concentration of progenitors is observed during transition from hepatic to bone marrow hematopoiesis, and the decline occurs with the termination of this process at birth.

The frequency of progenitors in fetal CB exceeded that reported in bone marrow and peripheral blood after "mobilization" (Civin et al. 1987; Udomsakdi et al. 1992; Hogge et al. 1996; Wang et al. 1997). However, the retrieval of CB from premature deliveries was low in terms of blood volume and mononuclear cell harvest. Consequently, despite the high frequency of hematopoietic progenitors in fetal CB, the total yield of $CD34^+$ cells and clonogenic progenitors was significantly below the yield observed with term CB. To overcome the limitations of the low cell numbers, we investigated the ability of preterm CB progenitors to undergo amplification ex vivo. We demonstrate that hematopoietic progenitors from preterm fetal CB can be expanded in vitro in cultures supplemented with early-acting hematopoietic growth factors. In short-term liquid cultures containing Epo, IL-1, -3, and -6, or G- and GM-CSF together with SCF or FL, expansion of CFUs was six- to eightfold at week 1 (Wyrsch et al. 1999). In long-term cultures containing MGDF and FL, an approximately 1000-fold expansion of multilineage progenitors was observed until week 10. The expansion of preterm progenitors was equally efficient as of term progenitors. Our results show that stem cells from CB of preterm deliveries preserved full proliferative capacity in vitro, although the fetuses might have suffered from unfavorable conditions before or during birth, either from a primary disease leading to premature termination of pregnancy, or from oxygen deprivation during induced abortion.

The second goal of this study was to examine the efficiency of retrovirally mediated gene transfer to fetal CB progenitors. Recently, new generations of retroviral vectors, including the replication-deficient lentiviral vectors, have been shown useful for ex vivo gene delivery to nondividing cells (Naldini et al. 1996; Uchida et al. 1998). CB progenitors are among the target cells in which sustained expression of transferred genes has been achieved (Conneally et al. 1998; van Hennik et al. 1998). This includes the highly efficient lentiviral transduction of CB-derived SRC progenitors capable of long-term repopulation of NOD/SCID mice (Miyoshi et al. 1999). We demonstrate that fetal CB progenitors can be efficiently transduced with lentiviral vectors carrying the GFP gene. GFP expression was found in approximately 25% of transduced $CD34^+$ cells, as well as CFU and LTC-IC-derived colonies. These high levels of GFP^+ cells were maintained during long-term in vitro expansion in MGDF/FL-containing medium. The frequency of transduced cells from preterm and term CB was comparable.

Studies on proliferation, expansion and transduction properties of CB progenitors from early gestational ages are relevant with regard to their potential use as autologous grafts in utero. Prenatal somatic gene therapy with autologous genetically corrected hematopoietic stem cells may have distinct advantages over postnatal therapy in genetic diseases such as thalassemias, immunodeficiency syndromes, or selected metabolic disorders. In these diseases, clinical symptoms of irreversible organ damage develop during pregnancy and could be prevented already before birth (Eddleman et al. 1996; Surbek et al. 1999). Human therapy with autologous CB stem cells harvested during pregnancy and genetically modified ex vivo has not yet been attempted. In more than 20 in utero transplantations of human fetuses which have been performed to date, bone marrow or fetal liver and thymus were used as sources of allogeneic stem cells. So far, success has been limited to recipients with immunodeficiency disorders (Touraine et al. 1989, 1992; Flake et al. 1996; Wengler et al. 1996). In other diseases such as hemoglobinopathies, engraftment has not been achieved either due to immunological intolerance or to occupation of bone marrow cavities with hematopoietic tissue (Slavin et al. 1992; Westgren et al. 1996). Since reduction of bone marrow cellularity by chemotherapy is not feasible in utero, improvement of repopulation with genetically modified cells may depend on methods to increase their number. Indeed, experiments in mice have demonstrated that engraftment of allogeneic cells without myelosuppressive conditioning can be achieved with a high dose of transplanted stem cells (Sykes et al. 1997).

The extensive in vitro proliferation of preterm CB progenitors in long-term cultures, as described in this work, may facilitate and broaden the clinical application of these cells. In the human fetus to be transplanted in utero with autologous CB progenitor cells, sufficient cell numbers may offer a feasible and promising outlook for treatment of a variety of hereditary disorders, not limited to immunodeficiencies and bone marrow failure syndromes. The biological properties of preterm CB, namely abundance of early and committed progenitors with high responsiveness to growth factors and ex vivo expansion and transduction potential equal to term CB, as well as high efficiency of purification, cryopreservation and recovery of viable $CD34^+$ cells described in our previous report (Surbek et al. 1998), are such that handling of fetal CB stem cells for transplantation should be as feasible as of term CB.

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