Characterization of the human CD34+ hematopoietic progenitor cell compartment during the second trimester of pregnancy

Frans T.H. Lim
Humphrey H.H. Kanhai
J.H. Frederik Falkenburg

Background and Objectives. Characterization of the different sites of fetal hematopoiesis during the second trimester of pregnancy can provide important information for the timing of in utero stem cell transplantation (SCT), as an experimental treatment for congenital hematologic disorders.

Design and Methods. We analyzed the distribution of the different hematopoietic precursor cells in fetal blood, liver, bone marrow (BM), spleen and thymus from 66 fetuses between the ages of 13 to 23 weeks of gestation by flow cytometry and culture of hematopoietic progenitor cells (HPC) in semi-solid media.

Results. During the second trimester the percentages of CD34+ cells did not change and were 4.0% (1.0-12.0%) (median [min.-max.]) in blood, 16.5% (3.0-32.0%) in BM, 6.0% (2.0-16.0%) in liver, 5.0% (2.0-14.0%) in spleen, and 1.1% (0.9-3.0%) in the thymus. Each tissue contained all subsets of CD34+ cells at various levels. Within the CD34+ population, in BM the main sub-population was CD34+CD19+ (38% (11-67%)), in thymus CD34+CD7+ (83% [45-98%]), and in blood and liver CD34+CD33+ (57% (30-80%) and 48% (20-82%), respectively). In all tissues approximately 1 % of nucleated cells were non-committed CD34+CD38- cells. The frequencies of both committed CD34+ cells and non-committed CD34+CD38- cells were constant from 13 to 23 weeks in fetal blood, BM, liver and spleen. The frequencies of cultured HPC were high in fetal liver, low in fetal BM, and increasing in fetal blood.

Interpretation and Conclusions. During the second trimester of gestation, all CD34+ subsets were present in each hematopoietic compartment at different levels. An exchange of stem cells between organs is likely, but no major shift of the hematopoietic stem cell compartment from the liver to other hematopoietic organs was found during the mid-trimester. No arguments for a specific time window for performing in utero SCT were found, but if engraftment of donor stem cells in the human fetus is influenced by competition of endogenous stem cells or fetal immune competence, in utero SCT should be performed as early as possible during fetal development.

Key words: stem cell transplantation, fetus, human, ontogeny, hematopoiesis CD34+ CD38-.

Haematologica 2005; 90:173-179

From the Departments of Hematology (FTHL, JHFF) and Obstetrics (FTHL, HHHK), Leiden University Medical Center, The Netherlands.

Correspondence:
Frans T.H. Lim, Department of Obstetrics and Gynecology, Leyenburg Teaching Hospital, PO Box 40551, 2504 LN, The Hague, The Netherlands.
E-mail: f.lim@leyenburg-ziekenhuis.nl

Antenatal molecular analysis of fetal cells allows diagnosis of the majority of congenital hematologic disorders before 12 weeks of gestation.[1,2] Early correction of these disorders may allow normal development of the fetus without invasive therapeutic interventions after birth. *In utero* hematopoietic stem cell transplantation has been proposed as an alternative to postnatal stem cell transplantation for the treatment of these diseases.[3] However, only a few transplants performed for immunodeficiency disorders have provided significant degrees of persistent engraftment.[1,4,5] In postnatal stem cell transplantation, cytoreduction of the bone marrow and immunosuppression of the recipient permit engraftment of allogeneic donor stem cells.[6] Early *in utero* stem cell transplantation performed in an immune-immature fetus may allow donor hematopoietic cells to be introduced without having to condition the recipient. However, competition between the pre-existing hematopoietic compartment of the host and the hematopoietic graft may play an important role in establishing sufficient donor chimerism in the fetus. Characterization of the endogenous pool of hematopoietic stem cells during fetal growth is necessary for further development of *in utero* stem cell transplantation as an alternative to postnatal stem cell transplantation.

During ontogeny, the development of the human hematopoietic system follows a series of co-ordinated changes. In the human yolk sac hematopoiesis occurs as

early as week 4 of gestation.[7-9] In the 5-week old human embryo, a major population of hematopoietic stem cells is associated with the ventral endothelium of the dorsal aorta.[10-12] From the sixth week of gestation hematopoiesis is mainly found in the fetal liver,[13-15] and to some extent in the fetal spleen.[16] From 10 weeks of gestation hematopoietic cells can be detected in the bone marrow cavities.[17,18] In the third trimester, the bone marrow develops as the definitive site of hematopoiesis. It has been suggested that during the mid-trimester of gestation the hematopoietic stem cell pool migrates from the liver to the bone marrow.[19-21] Alternatively, if a defined set of hematopoietic stem cells develops in each fetal tissue,[22] the timing of *in utero* stem cell transplantation may determine the final destiny of the stem cells and direct bone marrow homing may then be necessary for sustained engraftment after transplantation.

The human hematopoietic stem cells reside in the fraction of cells expressing the CD34 antigen. The $CD34^+$ population contains primitive stem cells with self-renewal and repopulating potential,[23] as well as progenitor cells committed to the myeloid, lymphoid, and erythroid lineages.[24] Committed hematopoietic progenitor cells (HPC) can be functionally characterized *in vitro* by their ability to form colonies in semi-solid media upon stimulation with specific hematopoietic growth factors.[25]

In this study we investigated whether major shifts in the $CD34^+$ compartments occur in hematopoietic tissues during the mid-trimester by analyzing the distribution of the different populations of hematopoietic precursor cells in fetal blood, liver, bone marrow, spleen and thymus from 66 fetuses between the age of 13 to 23 weeks of gestation by flow cytometry and culture of HPC in semi-solid media. We show that during the second trimester of gestation, all $CD34^+$ subsets, including immature precursors and lineage committed progenitor cells were present in each fetal hematopoietic compartment, but that the lineage-committed $CD34^+$ subpopulation showed organ specific differentiation. The non-committed $CD34^+$ $CD38^-$ subpopulation was present approximately equally in all tested organs. The frequencies of $CD34^+$ subpopulations were constant during the second trimester, indicating no major migration of the hematopoietic stem cell compartment from the liver to other hematopoietic organs.

Design and Methods

This study was approved by the Medical Ethical Committee of the Leiden University Medical Center. After informed consent from the pregnant women, fetal tissue was obtained from human fetuses after legal abortions. Gestational age was defined by the date of the first day of the last menstrual period, and checked by ultrasound fetal biometry of the biparietal distance or the femur length.

Sample collection

Abortion was performed by dilatation of the cervix and mechanical evacuation of the uterus. Fetal blood was aspirated from the umbilical vein during the abortion procedure into a syringe with 0.2 mL Hank's balanced salt solution (HBSS) containing 100 IU preservative-free heparin/mL. After the completion of the abortion fetal liver, spleen and thymus were selected and dissected using a microscope. Under sterile conditions, tissues were minced and washed in RPMI 1640 supplemented with penicillin (20 U/mL) and streptomycin (20 µg/mL) (BioWhittaker, Verviers, Belgium) and 2% heat-inactivated fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA) within 2 hours after harvest, and single cell suspensions were made. To obtain fetal bone marrow, femur, fibula, tibia, humerus, ulna or radius were used when identified. Fetal bone marrow was obtained by repeated flushing of the marrow cavities of the long bones with RPMI.

Cell counts and FACS analysis

Nucleated cells were counted using a Sysmex® K1000 (Toa Medical Electronics Co. Ltd., Kobe, Japan). Red blood cell (RBC) lysis was performed by incubating the cell suspensions with NH_4Cl (0.155 mol/L) for 10 minutes at 0°C. The cells were washed twice and resuspended in RPMI containing 2% FCS. After RBC lysis, the phenotype of the nucleated cells was analyzed using a Becton Dickinson FACScan flow cytometer. The following combinations of directly fluorescein isocyanate (FITC)- or phycoerythin (PE)-labeled monoclonal antibodies were used: anti-CD45 FITC/anti-glycophorinA PE, anti-CD45 FITC/anti-CD14 PE, anti-CD45 FITC/anti-CD34 PE, anti-CD34 FITC/anti-CD38 PE, anti-CD34 FITC/anti-CD19 PE, anti-CD34 FITC/anti-CD7 PE and anti-mouse IgG1 FITC/anti-mouseIgG1 PE as isotype controls. All the monoclonal antibodies were obtained from Becton Dickinson (Mountain View, CA, USA). After 30 minutes of incubation at 4°C, cells were washed in PBS with 1% deionized bovine serum albumin (BSA). For each sample 10,000 events were analyzed, defining white blood cells (WBC) as $CD45^+$ cells, and lymphocytes as $CD14^-CD45^+$ cells with the characteristic forward-sideward scatter profile of lymphocytes. Within this gated lymphocyte cell population, the different $CD34^+$ subsets were defined in the various FITC/PE dot plots. Frequencies were expressed as percentages of nucleated cells, counted by the Sysmex® cell counter.

Hematopoietic progenitor cell cultures

Samples were cultured at three concentrations of 5×10^3, 1×10^4 and 3×10^4 nucleated cells/mL in Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker, Verviers, Belgium), containing 30% fresh-frozen AB-heparin plasma, 0.5% BSA, human transferrin (0.47 g/L) (Behringwerke AG, Marburg, Germany) saturated with $FeCl_3H_2O$, 5×10^{-5} M mercaptoethanol (Sigma Chemicals, Saint Louis, MO, USA), and 1.1% methylcellulose (Methocel 4,000 cps, Fluka, Freiburg, Germany) in the presence of recombinant granulocyte-colony stimulating factor (G-CSF, 10 ng/mL) and stem cell factor (SCF, 50 ng/mL) both kindly provided by Amgen (Thousand Oaks, CA, USA), human granulocyte macrophage-colony stimulating factor (GM-CSF, 10 ng/mL) and interleukin-3 (IL-3, 25 ng/mL) gifts from Novartis (Basel, Switzerland), and human recombinant erythropoietin (Epo, 2 U/mL) a kind gift from Organon Technica N.V. (Turnhout, Belgium). After 14 days of culture in 35-mm tissue culture dishes (37°C, fully humidified atmosphere, 5% CO_2), colonies were counted. Colonies were defined as aggregates of more than 50 cells. Erythroid burst-forming unit (BFU-E) colonies were defined as burst of colonies consisting of hemoglobinized cells, granulocyte-macrophage colony-forming units (CFU-GM) as colonies containing granulocytes or macrophage or both, and granulocyte-erythoid-macrophage-megakaryocyte colony-forming unit (CFU-GEMM) colonies were defined as aggregates containing at least both erythroid and myeloid cells.

Statistics

Results are expressed as a median with the minimum and maximum. For the statistical analysis, a non-parametric method, the Kruskal-Wallis test, was used to compare groups. The Jonckheere-Terpstra test in SPSS version 12.0 was used to detect differences in medians.

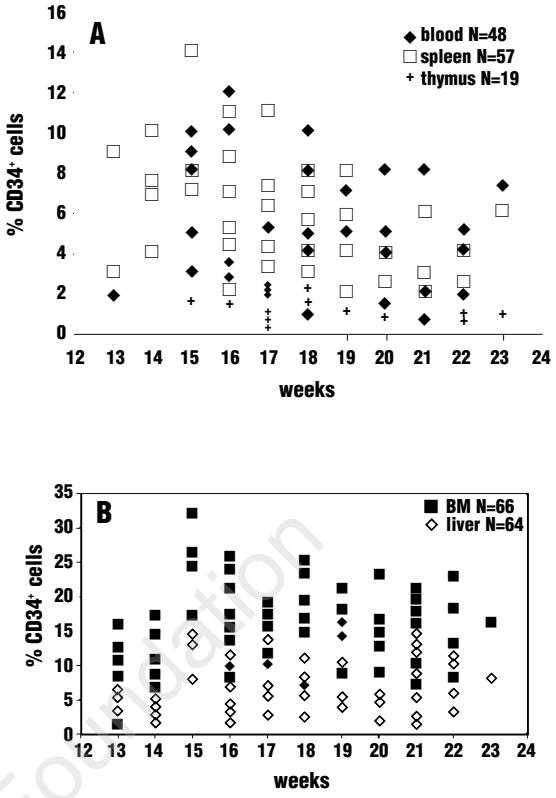

Figure 1. Frequencies of CD34+ cells in fetal blood, spleen and thymus (A) and in fetal BM and liver (B) during the second trimester. The percentages of CD34+ cells did not change in blood, BM, liver, spleen and thymus during this period.

Results

Frequencies of CD34+ cells

Hematopoietic tissues were harvested from 66 fetuses. Figure 1 shows the frequencies of CD34+ cells in fetal blood, spleen, thymus, BM and liver, in the second trimester of pregnancy and the numbers of samples obtained from the different organs at various gestational ages. During 48 procedures it was technically possible to take a blood sample. A thymus could be isolated from 19 fetuses.

To detect possible changes during this period, the second trimester was divided into three periods, 13-16 weeks, 17-19 weeks and 20-23 weeks, as shown in Figure 2. For the entire second trimester the percentages of CD34+ cells found in the fetal tissues were 4.0% (1.0-12.0%) (median [minimum-maximum]) in blood, 16.5% (2.0-32.0%) in BM, 6.0% (2.0-16.0%) in liver, 5.0% (2.0-14.0%) in spleen, and 1.1% (0.9-3.0%) in thymus. The percentages of CD34+ cells did not change in blood, BM, liver and spleen during the second trimester.

Phenotypic characterization of the CD34 compartment

To define the CD34+ cell subpopulations in each hematopoietic compartment further, the distribution of CD34+CD38- (non-committed), CD34+CD7+ (T-cell committed), CD34+CD19+ (B-cell committed), and CD34+CD33+ (myeloid committed) cells within the CD34 compartment was analyzed. As shown in Figure 3 each compartment contained all subsets of CD34+ cells, although in different amounts. In BM the predominant subset was CD34+CD19+ (38% [11-67%]) illustrating the B-cell commitment of this compartment. Predominant T-cell commitment, as determined by CD34+CD7+ cells, was found in the thymus

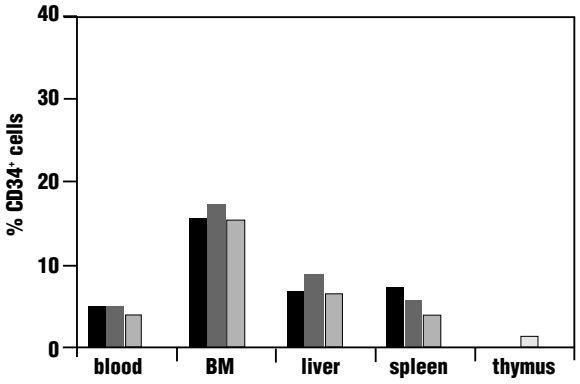

Figure 2. Frequency of CD34$^+$ cells in the fetal hematopoietic compartments. The frequencies of CD34$^+$ cells are expressed as median percentages of nucleated cells. Clusters of bars represent CD34 percentages in fetal blood, BM, liver and spleen. The second trimester was divided into three periods: ■ 13 – 16 weeks, ■ 17 – 19 weeks, □ 20 – 23 weeks of gestation, respectively. Frequencies of CD34$^+$ cells were relatively constant during the second trimester. Since only limited experiments could be performed for the thymus, all these data are presented in one bar, □ 13 – 23 weeks of gestation.

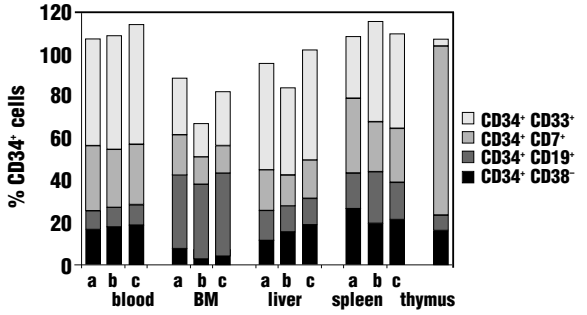

Figure 3. Distribution of CD34$^+$ subsets in the fetal hematopoietic compartments. Each bar represents the CD34 compartment in the different fetal hematopoietic organs. Within these compartments the frequencies of CD34$^+$CD38$^-$ (non-committed), CD34$^+$CD19$^+$ (B-cell committed), CD34$^+$CD7$^+$(T-cell committed) and CD34$^+$CD33$^+$ (myeloid committed) are expressed as median percentages of CD34$^+$ (hematopoietic progenitor) cells. The second trimester was divided into three periods, a. 13-16 weeks, b. 17-19 weeks and c. 20-23 weeks, represented by each bar. Clusters of bars represent fetal blood, BM, liver and spleen. Since only limited experiments could be performed for the thymus, all these data are presented in one bar. All subsets are found in fetal blood, BM, liver, spleen and thymus, but each in different amounts. The frequencies of all CD34$^+$ subsets were constant during the second trimester in the different hematopoietic compartments.

(83% (45-98%) of the CD34$^+$ cells). In blood and liver relatively high percentages of myeloid committed CD34$^+$CD33$^+$ cells were found (57% (30-80%) and 48% (20-82%), respectively). In the spleen the distribution of myeloid, B-cell and T-cell committed CD34$^+$ cells was relatively equal. Similar percentages of non-committed CD34$^+$CD38$^-$ cells were found in all tissues except BM, which contained only 2.7% (0.5-26.4%) of CD34$^+$CD38$^-$ cells. When the frequencies of CD34$^+$CD38$^-$ cells were expressed as percentage of nucleated cells, they were similar in blood (0.8% [0.1-3.5%]), BM (0.4% [0.1-2.9%]), liver (0.8% [0.1-4.9%]) and spleen (1.1% [0.2-5.8%]). To analyze whether there was a developmental change in the CD34$^+$ subsets during the second trimester in the various hematopoietic compartments, we analyzed the distribution of CD34$^+$ subsets during the three periods within this trimester. No significant changes in the frequencies of either committed CD34$^+$ cells or non-committed CD34$^+$CD38$^-$ cells were observed from 13 to 23 weeks in fetal blood, BM, liver and spleen.

Functional characterization of hematopoietic progenitor cells

To determine whether a shift in functionally characterized HPC took place within the myeloid and erythroid precursor cell compartments during the second trimester of gestation, CD34$^+$ cells were analyzed for their capacity to form colonies in semi-solid culture media. For the entire second trimester, the median (min-max) plating frequency of cultured HPC, expressed as a percentage of CD34$^+$ cells, was 15.5% (3.5-128.0%) in fetal blood, 5.4% (1.2-13.2%)

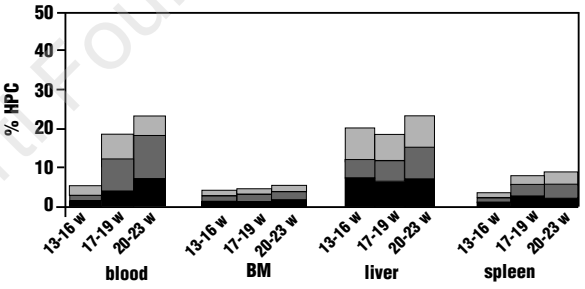

Figure 4. Frequencies of HPC in fetal blood, BM, liver and spleen, as determined by culture in semi-solid medium, and expressed as median percentages of CD34$^+$ cells. The second trimester was divided into three periods, represented by the bars. Each bar shows the distribution of HPC into ■ BFU-E, ■ CFU-GM, and ■ CFU-GEMM. This distribution was similar in the different fetal organs during the second trimester. The frequencies were relatively constant in liver and BM (difference of medians were not significant). An increase of HPC was found in fetal blood and spleen, (Kruskal-Wallis test 15.9, $p<0.001$ and 15.7, $p<0.001$, respectively).

in BM, 21.6% (3.1-95.0%) in liver, and 6.7% (0.5-36.0%) in spleen. As shown in Figure 4, early in the second trimester (13-16 weeks of gestation) the frequency of HPC was highest in fetal liver (median 21.3% [3.1-48.0%]) and lowest in BM (median 4.1% [1.6-10.5%]). During the second trimester, the frequencies remained relatively low in fetal BM, and relatively high in fetal liver (differences were not significant). In contrast, in fetal blood the percentages of cultured HPC increased from a median of 6.9% (4.6-14.7%) at 13–16 weeks to a median of 27.1% (3.5-128.0%) at 20–23 weeks (Kruskal-Wallis test 15.9, df 2, $p<0.001$; Jonckheere-Terpstra test 4.126, $p<0.001$),

and in the fetal spleen, the percentages increased from a median of 2.9% (1.1-19.0%) to a median of 10.2% (4.0-28.0%) (Kruskal-Wallis test 15.7, df 2, $p<0.001$; Jonckheere-Terpstra test 3.857, $p<0.001$). The distribution of erythroid, myeloid and mixed progenitor cells in each hematopoietic organ was similar (approximately 30% BFU-E, 40% CFU-GM and 30% CFU-GEMM), and did not change during development.

Discussion

In utero hematopoietic stem cell transplantation is a novel approach to the treatment of fetuses affected by congenital hematologic or immunological disorders.[1,4,5] Antenatal diagnosis can be accomplished at 10 to 12 weeks of gestation[2,26] and, therefore, *in utero* intraperitoneal or intravenous infusion of cells for therapeutic purposes can be performed as early as the second trimester of gestation. Until now *in utero* stem cell transplants have produced successful persistent engraftment in only a minority of cases. Several factors appear to play a key role in successful engraftment of allogeneic stem cells. First, the developing immune system may be capable of rejecting the allogeneic graft. Several studies have indicated that the immune system matures during the second trimester of gestation, and is capable of developing an alloreactive response.[27-29] Therefore, *in utero* stem cell transplantation must be performed early in gestation. Secondly, the hematopoietic compartments must be available to receive and host the hematopoietic stem cells infused. We recently described the development of the bone marrow compartment in the second trimester of gestation,[18] illustrating that early during the second trimester all hematopoietic compartments are developing, and appear to be capable of hosting hematopoietic precursor cells. Thirdly, since no ablation of the endogenous hematopoietic system is performed, the transplanted stem cells have to compete with the endogenous stem cell compartment. Therefore, characterization of the composition of the different hematopoietic sites of the fetal host, and their changes during the second trimester can provide important information for the timing of *in utero* stem cell transplantation.

Only limited information is available on the characterization of CD34 subpopulations in the various fetal hematopoietic organs, or the possible changes of hematopoietic progenitor cells in the different fetal organs during the second trimester of gestation.[30-32] It has been suggested that hematopoietic stem cells translocate from the liver to other hematopoietic tissues during the second trimester of gestation, and that this process is associated with a loss of multipotentiality and with tissue commitment.[33] Although a tissue specificity of lineage commitment could be demonstrated, the development and sequential changes of $CD34^+$ subsets during the second trimester were not documented. Other reports indicated that the $CD34^+$ content of fetal liver did not appear to diminish from 9-22 weeks of gestation.[15] The frequency of $CD34^+$ cells in blood was also found to be stable during the second trimester, and only gradually diminished in the third trimester.[19-21,34,35] Recently, we showed that also in the bone marrow compartment the percentages of $CD34^+$ cells did not alter, and only increased with the expanding size of the compartment.[18]

This study describes the development of hematopoietic precursor cell content of the various hematopoietic sites during the second trimester of gestation. Although recent studies suggest that cells not expressing CD34 and lineage commitment markers can also act as repopulating cells,[36,37] and CD133 is another marker used for hematopoietic stem cells,[38,39] in this study we focused only on the $CD34^+$ population. Immunophenotypic characterization of the hematopoietic fetal organs showed the presence of both non-committed $CD34^+$ $CD38^-$ precursor cells and lineage-committed progenitor cells in all compartments. CD38 is considered to be a differentiation marker, also present when cells express CD19, CD7 or CD33. In a few experiments we labeled cells with CD34FITC and CD19PE, CD7PE and CD33PE at the same time, and compared the percentage of $CD34^+/CD19^-CD7^-CD33^-$ cells with the percentage of $CD34^+CD38^-$ cells. These percentages were equal, assuming that $CD34^+CD38^-$ cells did not express one of the lineage commitment markers. Expressed as a percentage of total nucleated cells in each organ tested, the $CD34^+$ $CD38^-$ subpopulation was present in similar frequencies. These findings suggest that the $CD34^+CD38^-$ subpopulation corresponds with the circulating pool of stem cells that is equally distributed in the various hematopoietic fetal tissues.[40] However, each hematopoietic site shows a preference for different lineage-specific precursor cells, resulting in relatively organ-specific maturation. These findings suggest that hematopoietic stem cells in each tissue have the potential to differentiate into all lineage-committed precursor cells but the actual differentiation depends on the local microenvironment.[41-43] Alternatively, both hematopoietic stem cells and lineage-committed precursor cells easily travel throughout the developing fetus. The presence of hematopoietic cells with the most immature phenotype in all organs tested supports the present hypothesis that stem cells reside in many different tissues and that it is the local environment which determines the specific activation and induction of lineage-specific maturation in each organ.[44] Since the fetal bone

marrow already contributes significantly to the stem cell pool at the beginning of the second trimester,[45] it is not likely that the ontogenic change of hematopoiesis from liver to bone marrow is caused by a shift of the stem cell pool in these organs. In this study we show that the composition of the hematopoietic compartment is constant in both the fetal liver and BM during the second trimester of gestation. The absence of a major change in the hematopoietic precursor cell content of the circulating fetal blood does not support the theory of a gestation-dependent migration of hematopoietic cells from the liver to the BM.[46,47] The increase in the frequency of progenitor cells capable of forming erythroid, myeloid and mixed colonies in *in vitro* cultures, as determined in semi-solid media, may reflect the increasing demand of the growing fetus for mature erythroid and myeloid cells. The most marked increase of cultured HPC in peripheral blood may be the first sign of an increasing demand of committed progenitor cells in different tissues resulting in an altered distribution. The fact that lymphoid progenitors hardly respond to the hematopoietic growth factors added to the cultures may explain, in part, the discrepancy between the results of the phenotypic analysis of $CD34^+$ cells and the functional characteristics of HPC.

In conclusion, this study demonstrates no major shifts in the fetal hematopoietic compartments during the second trimester of gestation. The presence of similar non-committed $CD34^+$ $CD38^-$ immature cells, as expressed as percentages of nucleated cells, indicates a random distribution of these cells in the hematopoietic organs. Each hematopoietic tissue has a characteristic profile of committed $CD34^+$ subpopulations which is constant during the second trimester. The total hematopoietic stem cell compartment expands with fetal growth, but no clear indication of a major shift of hematopoietic progenitor cells from one fetal tissue to another was found. Since all future hematopoietic compartments already appear to host both immature and mature hematopoietic precursor cells, our data indicate that there is no specific time window for performing *in utero* stem cell transplantation during the second trimester. However, if maturation of the fetal immune system during the second trimester and competition with the stem cells of the host influence successful engraftment, *in utero* stem cell transplantation should be performed as early as possible during fetal development when the immune system of the fetus is most immature and the ratio between transplanted stem cells and endogenous stem cells is most favorable.

All authors participated in the conception and design of the study, analysis and interpretation of data as well as in drafting and revising the article for important intellectual content.

We wish to thank Dr. W. Beekhuizen from the Center of Human Reproduction in Leiden for providing the fetal tissue for this study, and Dr M.M. Bartelings from the department of Embryology and Anatomy of the University of Leiden for her help in identifying and selecting fetal hematopoietic organs. The authors declare that they have no potential conflicts of interest.

Manuscript received November 16, 2004. Accepted December 10, 2004.

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