Presence and Characteristics of Circulating Megakaryocyte Progenitor Cells in Human Fetal Blood

By Giorgio Zauli, Luisa Valvassori, and Silvano Capitani

The in vitro growth of early (burst-forming unit-megakaryocyte [BFU-meg]) and late (colony-forming unit-megakaryocyte [CFU-meg]) megakaryocyte progenitors was investigated in midtrimester human fetal blood and compared with adult bone marrow. Most of the experiments were performed in a serum-free fibrin clot assay, using purified hematopoietic progenitor (CD34+) cells. High BFU-meg and CFU-meg levels were found in human fetal blood, with a clear prevalence of BFU-meg (BFU-meg:CFU-meg ratio, 2:5:1), at variance with adult bone marrow, in which mature CFU-meg predominates (BFU-meg:CFU-meg ratio, 0:6:1). Fetal and adult megakaryocyte progenitors had a similar phenotypic profile for the expression of CD34, HLA-DR, and glycoprotein-complex IIB-III A. However, fetal BFU-meg were larger in size (number of megakaryocytic elements per colony) than adult BFU-meg, but were usually composed by only one or two foci of development. On the other hand, fetal and adult CFU-meg were similar in both morphology and size. Fetal megakaryocyte progenitors appeared earlier in culture and had an increased proliferative activity as demonstrated by the higher number of megakaryocyte progenitors in S phase with respect to adult CFU-meg and BFU-meg. Finally, fetal megakaryocyte progenitors displayed a higher sensitivity to stimulatory cytokines, in particular recombinant interleukin-3, than adult megakaryocyte progenitors, whereas they were inhibited by purified transfoming growth factor-β1 in a similar fashion to adult megakaryocyte progenitors.

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During the course of fetal development, hematopoiesis begins in the yolk sac and transfers to the liver and spleen from 6 weeks of gestation. Bone marrow hematopoiesis is established between weeks 11 and 22 of gestation, increasing in the third trimester, when hepatic hematopoiesis progressively declines. Erythropoiesis predominates in the yolk sac and fetal liver, whereas myelopoiesis only occurs in significant amounts in fetal bone marrow. The migration of hematopoietic progenitor cells is possibly due to the presence of circulating stem and progenitor cells that populate the different fetal organs in various periods of gestation. At birth, cord blood is still rich in hematopoietic progenitor cells, whereas the number of hematopoietic progenitors in adult peripheral blood is very low.

Most studies of hematopoietic progenitor cells in human fetal tissues have concentrated on erythropoietic progenitors due to the prevalence of erythropoiesis in the first and second trimesters of gestation. Yet, to our knowledge, no reports are available on megakaryocyte progenitors in human fetal blood, probably because optimal assays for human megakaryocyte progenitors were developed only recently.

Because the number of circulating platelets in human fetal blood is similar to that of adult peripheral blood at the end of the second trimester, fetal megakaryocytopoiesis should already be developed at this time of gestation.

This study investigated the presence of both classes of megakaryocyte progenitors, colony-forming unit-megakaryocyte (CFU-meg) and burst-forming unit-megakaryocyte (BFU-meg), in human fetal blood and their in vitro growth characteristics compared with adult bone marrow megakaryocyte progenitors. The studies were performed in cultures deprived of fetal bovine serum using purified CD34+ cells.

Materials and Methods

Samples. Human midtrimester (from 18 to 22 weeks of gestation) fetal blood was obtained by aspiration from an umbilical vessel under fetoscopic control (performed to exclude β-thalassemia major; 9 cases) and before legal termination of pregnancy for microcephaly (7 cases) using intra-amniotic prostaglandin E2. Only one fetus, not included in this study, was affected by β-thalassemia major and was later aborted.

Adult bone marrow was taken from the posterior iliac crest of 15 hematologically normal individuals. All individuals gave their informed consent to the study according to the Helsinki declaration of 1975.

Enrichment in hematopoietic progenitor (CD34+) cells. Heparinized (20 U/mL) fetal blood and bone marrow samples were diluted 1:3 with Iscove’s modified Dulbecco’s medium (IMDM; GIBCO, Grand Island, NY) plus 10% fetal calf serum (FCS), layered over Ficoll Histopaque (d = 1.077 g/mL; Pharmacia, Upssala, Sweden), and centrifuged at 1,500 rpm for 30 minutes. Light-density mononuclear cells were collected, washed twice in IMDM supplemented with 10% FCS, and counted. The number of total mononuclear cells per milliliter was nearly three times higher in bone marrow aspirates than in fetal blood samples. Usually, 18 to 40 × 106 and 30 to 170 × 106 mononuclear cells were obtained from fetal blood and bone marrow samples, respectively. Light-density mononuclear cells were used for kinetic experiments or further enriched in CD34+ cells. Mononuclear adherent cells were then removed by two successive 1-hour incubation steps in plastic Petri dishes (Costar, Cambridge, MA) at 37°C in a humidified atmosphere of 5% CO2 in air.

Mononuclear nonadherent cells were then pelleted at a concentration of 5 × 106 cells per tube and 50 µL of the following monoclonal antibodies (MoAbs) were added to each tube: anti-CD2, anti-CD3, anti-CD4, anti-CD8, anti-CD11, anti-CD14, anti-CD19, and anti-CD20 (Becton Dickinson, Mountain View, CA). These antibodies were added in the presence of 1% bovine serum albumin (BSA; fraction V Chon, Sigma, St Louis, MO). After two washings, 50 × 106 immunomagnetic beads coated with IgG antimouse (MPC 450 Dynabeads; Dynal, Oslo, Norway) were then added to each tube to enrich the cell population of CD34+ hematopoietic progenitor cells.
obtain an immunomagnetic bead to cell ratio of 10:1 in a final volume of 0.4 mL for 30 minutes in ice, under continuous agitation. Lineage-positive cells were removed by a magnet (MPC1, Dynabeads) and the remaining cells were pelleted at a concentration of 5 × 10⁶ cells/tube for subsequent CD34⁺ cell selection.

After these negative selections, CD34⁺ cells were positively selected using AIS Microelector flasks (AIS, Menio Park, CA) coated with an Myl0² anti-CD34 antibody, according to the manufacturer's instructions.

When the phenotypic profile of this enriched CD34⁺ cell population was analyzed by FACScan using an MoAb that recognizes a separate epitope of CD34 molecule, a positive reaction to anti-CD34 (Tecnicogenetic, Milan, Italy) ranging from 90% to 97% was observed in fetal blood and bone marrow samples. CD34⁺ cells represented 0.5% to 4% and 0.9% to 3.5% of the initial number of light-density mononuclear cells from adult bone marrow and fetal blood, respectively. The purification procedure allowed a 60- to 70-fold enrichment in both adult bone marrow and fetal blood progenitors, calculated as the percentage of megakaryocytic progenitor number obtained from 100,000 light-density mononuclear cells (data not shown).

Kinetic investigation of fetal and adult megakaryocyte progenitors. The fraction of BFU-meg and CFU-meg in DNA synthesis (S phase) in both fetal blood and adult bone marrow was assessed, as previously described, by the suicide technique after exposure to high specific activity tritiated thymidine (¹H-Tdr) before performing semisolid cultures. The fetal blood and adult bone marrow light-density mononuclear cells were resuspended in 0.5 mL of IMDM plus 10% FCS, at a concentration of 2 × 10⁶ cells/mL. Five tubes were prepared and incubated with medium (in duplicate), 0.5 mg/mL cold thymidine (Sigma), and ¹H-Tdr (100 μCi/mL, specific activity: 20 Ci/mmol/L; Amersham International, Buckinghamshire, UK, in duplicate). After 30 minutes of incubation at 37°C, the reaction was stopped by the addition of 5 mL ice-cold IMDM containing 0.5 mg/mL unlabeled thymidine. After three washes, cells were resuspended in IMDM and megakaryocyte progenitor cell assay was performed by seeding 1 × 10⁵ cells/dish.

The percentage of progenitor cells in the S phase of the cell cycle (Ns) was determined by applying the following formula: Ns = Nc - Nt/Nc, where Nc is the number of BFU-meg and CFU-meg in the controls, and Nt is the number of BFU-meg and CFU-meg in the samples treated with high-dose ¹H-Tdr.

Complement-mediated cytotoxicity. In some experiments, a complement-dependent cytotoxicity with B331 anti-HLA-DR antibody was performed after the positive selection of CD34⁺ cells. Fetal and adult CD34⁺ cells were first incubated at 4°C at a concentration of 1 × 10⁶/mL in 100 μL IMDM for 1 hour with an equal volume of anti-HLA-DR antibody at a plateau killing (dilution, 1:50) concentration in sterile tubes. Controls were represented by cells incubated in a similar fashion with medium alone, antibody alone, or complement alone.

After two washings, the pellet was resuspended with 150 μL medium containing 20% rabbit complement and incubated at 37°C for 1 hour. Cells were then washed twice in IMDM plus 10% FCS and assayed for BFU-meg and CFU-meg growth. The number of cells plated was determined by cell counts made before treatment with antibody and complement.

Megakaryocyte progenitor cells assay. Serum-free fibrin clot assay was performed as previously described. CD34⁺ cells (10⁶) were seeded in IMDM supplemented with 300 mg/mL iron saturated transferrin (Sigma), 3 mg BSA, 280 μg/mL CaCl₂, 1 × 10⁻⁶ mol/L- L-asparagine, 0.1 mL of 0.2% (wt/vol) purified fibrinogen (Kabi, Stockholm, Sweden) resuspended in phosphate-buffered saline (PBS) and 0.1 mL of 0.2 U/mL purified human thrombin (95%) (Sigma) in PBS. As a source of megakaryocyte colony-stimulating activity, different combinations of human recombinant growth factors, displaying either stimulatory or inhibitory activity on adult megakaryocytopoiesis, were used: interleukin-1α (IL-1α; Genzyme Co, Boston, MA), IL-6 (Genzyme), granulocyte-macrophage colony-stimulating factor (GM-CSF; Genzyme), IL-3 (Genzyme), erythropoietin (Epo; Cilag, Milan, Italy), purified transforming growth factor-β1 (TGF-β1; R&D Systems, Minneapolis, MN).

After 2, 4, 8, 12, 14, 20, 22, and 28 days of incubation, fibrinclots were fixed in situ with methanol-acetone (1:3) for 20 minutes, washed with PBS and double-distilled water, and then air dried. Fixed plates were stored at −20°C until immunofluorescence staining was performed. Megakaryocyte colonies were composed of cells intensively fluorescent to anti-CD41 antibody, according to the manufacturer’s instructions.

Statistical analysis. The results were expressed as mean ± standard deviation (SD) of the data obtained from three or more experiments performed in duplicate. Statistical analysis was performed using the two-tailed Student’s t-test.

RESULTS

For a reliable comparison between the growth of fetal blood and adult bone marrow megakaryocyte progenitors, hematoapoietic progenitor (CD34⁺) cells were extensively purified, mainly because of the different cellular composition of light-density mononuclear cells in fetal blood and adult bone marrow.

Both classes of megakaryocyte progenitors (BFU-meg and CFU-meg) were identified in fetal blood, using the same criteria adopted for adult BFU-meg and CFU-meg in limiting dilution experiments (Fig 1). In culture supplemented with optimal concentrations (2 ng/mL) of IL-3, CD34⁺ cells
MEG PROGENITORS IN FETAL BLOOD

Table 1. In Vitro Growth of Fetal Blood and Adult Bone Marrow
Megakaryocyte Progenitors Reported as the Number of BFU-meg and CFU-meg per Milliliter

<table>
<thead>
<tr>
<th></th>
<th>BFU-meg/mL</th>
<th>CFU-meg/mL</th>
<th>BFU-meg/CFU-meg Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal blood</td>
<td>942 ± 314</td>
<td>378 ± 157</td>
<td>2.5:1</td>
</tr>
<tr>
<td>Adult bone marrow</td>
<td>670 ± 240</td>
<td>1,050 ± 270</td>
<td>0.6:1</td>
</tr>
</tbody>
</table>

Megakaryocyte progenitors were assayed in fibrin-clot cultures supplemented by 2 ng/mL of IL-3, at optimal cell density (10^5 CD34+ cells/plate). Data are expressed as the mean ± SD of seven experiments performed in duplicate. No significant differences were observed in the number of BFU-meg per milliliter between fetal blood and adult bone marrow. On the other hand, the number of CFU-meg per milliliter was significantly (P < .01) lower in fetal blood than in adult bone marrow.

purified from fetal blood between weeks 18 and 22 gestation gave rise to significantly (P < .01) higher levels of BFU-meg than CFU-meg, with a BFU-meg:CFU-meg ratio of 2.5:1 (Table 1). Moreover, although fetal BFU-meg displayed a greater plating efficiency than adult BFU-meg (45 ± 15 ± 11 ± 4/10^3 CD34+ cells, respectively; P < .01), the number of BFU-meg per milliliter was comparable in fetal blood and adult bone marrow due to the higher number of light-density mononuclear cells per milliliter recovered in adult bone marrow. On the other hand, fetal and adult CFU-meg showed a similar plating efficiency (18 ± 7.5 v 17.5 ± 4.5). Therefore, the number of CFU-meg per milliliter was significantly lower (P < .01) in fetal blood than in adult bone marrow, which showed a BFU-meg:CFU-meg ratio of 0.6:1.

While no significant differences were noted in the morphology and size (number of megakaryocytic cells/colony) of fetal and adult CFU-meg, fetal BFU-meg showed an enlarged (P < .01) colony size (sometimes greater than 500 megakaryocytic cells/colony) with respect to adult BFU-meg. Fetal BFU-meg were usually composed of 1 or 2 foci of development, whereas adult BFU-meg always had 2 or more distinct foci of development (Table 2).

To establish whether the large megakaryocytic aggregates observed in fetal blood could be considered homologues of adult BFU-meg, complement-dependent cytotoxicity experiments with an anti–HLA-DR MoAb were performed (Table 3). Similar to adult bone marrow and peripheral blood BFU-meg,10 fetal BFU-meg were only partially affected by anti–HLA-DR treatment, but fetal and adult CFU-meg were drastically reduced by anti–HLA-DR treatment.

Because an earlier appearance in cultures has been described for fetal erythroid progenitors11,14,15 with respect to adult precursors, we explored the time-course in culture of fetal megakaryocyte progenitors. Both fetal BFU-meg and BFU-meg appeared significantly earlier in culture, with a peak at day 8 for fetal BFU-meg versus day 12 to 14 for adult BFU-meg (Fig 2A) and a peak at day 12 to 14 for fetal BFU-meg versus day 20 to 22 for adult BFU-meg (Fig 2B).

We next analyzed the sensitivity of fetal BFU-meg (Fig 3A) and BFU-meg (Fig 3B) to increasing concentrations of IL-3. Both classes of fetal blood megakaryocyte progenitors showed a higher (P < .05) sensitivity to IL-3, with respect to adult precursors. GM-CSF also supported the growth of fetal blood CFU-meg and BFU-meg, although to a lesser extent than IL-3 (Table 4). On the other hand, IL-1α, IL-6, and Epo, used alone, did not support the in vitro growth of fetal megakaryocyte progenitors. Combinations of optimal doses of IL-3 plus GM-CSF and IL-3 plus IL-1α significantly (P < .05) increased the number of fetal BFU-meg and BFU-meg with respect to IL-3 alone. Combinations of IL-6 plus IL-3 enhanced the number of fetal megakaryocyte progenitors with respect to IL-3 alone, but not significantly, whereas combinations of IL-1α plus IL-3 plus GM-CSF failed to further increase the colony yield with respect to IL-3 plus GM-CSF. Epo did not show any synergistic effect with IL-3.

Purified human TGF-β1, one of the main negative regulators of megakaryocyte and platelet homeostasis, showed a clear dose-dependent inhibition (0.001 to 10 ng/mL) on fetal megakaryocyte progenitors (Fig 4), similar to that previously observed on adult bone marrow and peripheral blood megakaryocyte progenitors.10,21,28

In a further series of experiments, we evaluated the percentage of megakaryocytic progenitor cells in S phase (Table 5), demonstrating that the number of both CFU-meg and BFU-meg in S phase was significantly (P < .05) higher in fetal blood than in adult bone marrow.

DISCUSSION

Circulating hematopoietic progenitors in midtrimester fetuses probably serve to colonize the developing bone marrow,
which becomes populated at 15 to 16 weeks of gestation. In this respect, it is noteworthy that not only erythroid but also granulocyte-macrophage progenitor cell levels are very high in fetal blood, even though mature granulocytes are not formed in large numbers in fetuses until shortly before birth. On the other hand, no reports are available on the characteristics of megakaryocyte progenitors in midtrimester fetuses. Indirect evidence that an active megakaryocytogenesis has already developed at this point of gestation comes from the work of Gruel et al., who reported that platelet counts in fetuses in weeks 18 to 26 of gestation were similar to those of adults, whereas circulating cells resembling mature platelets already existed in 11-week-old fetuses. Moreover, GPIIb/IIa and GPIb were normally expressed on fetal platelets as early as 18 weeks of gestation and Hegyi et al. reported a progressive shift to higher ploidy and an increase in megakaryocyte size during fetal development.

Taking into account that fetal BFU-E differ in several aspects from adult BFU-E, we compared here the in vitro growth characteristics of megakaryocyte progenitors in fetal blood and adult bone marrow. Most of these studies were performed on purified hematopoietic progenitor (CD34+) cells in a serum-free assay to avoid the presence of significant amounts of T lymphocytes, natural killer cells, and mono-
cytes, which can influence the in growth of hematopoietic progenitors\(^1\),\(^2\) and endogenous TGF-\(\beta 1\) as well as other serum-borne inhibitors. The number of CD34\(^+\) cells was extremely high in fetal blood (0.9% to 3.5% of total light-density mononuclear cells) compared with adult peripheral blood (approximately 0.01% of total light-density mononuclear cells),\(^5\),\(^6\) reaching values similar to those observed in adult bone marrow (0.5% to 4% of total light-density mononuclear cells).

Both classes of megakaryocyte progenitors, BFU-meg and CFU-meg, previously described in adult bone marrow\(^20\),\(^21\) and peripheral blood\(^10\) were also found in high levels in mid-trimester human fetal blood. Although the number of light-density mononuclear cells per milliliter was approximately three times higher in adult bone marrow than in fetal blood, the number of BFU-meg per milliliter was comparable in fetal blood and adult bone marrow due to the higher plating efficiency of fetal blood BFU-meg versus adult bone marrow BFU-meg. On the other hand, the number of CFU-meg per milliliter was significantly lower in fetal blood than in adult bone marrow.

Similar to adult BFU-meg, fetal BFU-meg also showed absent or low-level expression of HLA-DR, whereas fetal CFU-meg already expressed high levels of HLA-DR because they were extremely sensitive to the cytotoxic effect of anti-HLA-DR antibody. Besides an identical phenotypic profile, fetal megakaryocyte progenitors displayed several differences with respect to adult megakaryocyte progenitors. (1) Although usually larger than adult BFU-meg, fetal BFU-meg were mainly unifocal or bifocal at variance with adult BFU-meg, which were always composed of 2 to 7 distinct foci of development. On the other hand, fetal CFU-meg morphology and size were similar to adult CFU-meg. (2) Both fetal CFU-meg and BFU-meg appeared in culture earlier than adult CFU-meg and BFU-meg. (3) Fetal megakaryocyte progenitors showed a significantly increased proliferative activity, as demonstrated by the higher number of fetal megakaryocyte progenitors in S phase. (4) Finally, fetal megakaryocyte progenitors displayed a higher sensitivity to stimulatory cytokines, particularly IL-3, whereas they were markedly inhibited by TGF-\(\beta 1\), in a similar way to adult megakaryocyte progenitors.

It is noteworthy that in fetal blood, ancestral megakaryocyte progenitors predominate with a BFU-meg:CFU-meg ratio of 2.5:1, whereas in adult bone marrow the BFU-meg:CFU-meg ratio is approximately 0.6:1. In this respect, we previously observed that BFU-meg are also predominant in adult peripheral blood.\(^10\) However, all the above characteristics of circulating and bone marrow megakaryocyte progenitors are identical in adults. Because we did not study megakaryocyte progenitors in other fetal tissues (fetal liver or fetal bone marrow), it is unclear whether the higher BFU-meg:CFU-meg ratio in fetal blood represents a true difference of the megakaryocyte progenitor pool composition in midtrimester fetuses. Alternatively, the higher ratio might reflect a fetal blood

### Table 4. Effect of Different Combinations of Recombinant Growth Factors on the In Vitro Growth of Fetal Blood Megakaryocyte Progenitors

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>CFU-meg/10(^3) CD34(^+) Cells</th>
<th>BFU-meg/10(^3) CD34(^+) Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.4 ± 0.6</td>
<td>0</td>
</tr>
<tr>
<td>IL-3 (2 ng/mL)</td>
<td>19 ± 7</td>
<td>43 ± 12</td>
</tr>
<tr>
<td>GM-CSF (2 ng/mL)</td>
<td>10 ± 3.5</td>
<td>23 ± 6</td>
</tr>
<tr>
<td>IL-1(\alpha) (0.1-10 ng/mL)</td>
<td>1.3 ± 0.8</td>
<td>0</td>
</tr>
<tr>
<td>IL-6 (0.2-20 ng/mL)</td>
<td>2 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td>Epo (0.5-6 U/mL)</td>
<td>1 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>IL-3 (2 ng/mL) plus GM-CSF (2 ng/mL)</td>
<td>26 ± 6.5</td>
<td>58 ± 14</td>
</tr>
<tr>
<td>IL-3 (2 ng/mL) plus IL-1(\alpha) (2 ng/mL)</td>
<td>27 ± 7</td>
<td>61 ± 16</td>
</tr>
<tr>
<td>IL-3 (2 ng/mL) plus IL-6 (2 ng/mL)</td>
<td>21 ± 7</td>
<td>47 ± 18</td>
</tr>
<tr>
<td>IL-3 (2 ng/mL) plus Epo (2 U/mL)</td>
<td>19 ± 6.5</td>
<td>42 ± 14</td>
</tr>
<tr>
<td>IL-3 (2 ng/mL) plus GM-CSF (2 ng/mL) plus IL-1(\alpha) (2 ng/mL)</td>
<td>28 ± 12</td>
<td>55 ± 17</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of 10 separate experiments performed in duplicate.

### Figure 4. Effect of increasing concentrations of purified TGF-\(\beta 1\) on the in vitro growth of fetal blood (○) CFU-meg and (□) BFU-meg in cultures, stimulated by 2 ng of IL-3. Data are expressed as the mean ± SD of five separate experiments performed in duplicate.

Table 5. Evaluation of the Percentage of Fetal Blood and Adult Bone Marrow Megakaryocyte Progenitors in S Phase

<table>
<thead>
<tr>
<th></th>
<th>Fetal Blood</th>
<th>Adult Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-meg</td>
<td>45 ± 12</td>
<td>27 ± 11</td>
</tr>
<tr>
<td>BFU-meg</td>
<td>32 ± 14</td>
<td>18 ± 10</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of five different experiments performed in duplicate. The absolute number of megakaryocyte progenitors was 23 ± 9 CFU-meg and 52 ± 13 BFU-meg per 10\(^3\) light-density mononuclear cells. The percentage of both classes of fetal blood megakaryocyte progenitors in S phase was significantly higher (\(P < .05\)) in fetal blood with respect to adult bone marrow.
enrichment of ancestral megakaryocyte progenitors cells (BFU-meg), which are considered very close to pluripotent hematopoietic stem cells, which have the function of populating the developing fetal bone marrow.

In conclusion, we have demonstrated that high levels of both classes of megakaryocyte progenitors are present in midtrimester fetal blood, whereas the number of circulating platelets is similar to that observed in adults. It is conceivable that the higher cycling activity, the higher sensitivity to replatelets is similar to that observed in adults. It is conceivable both classes of megakaryocyte progenitors are present in circulating hemopoietic progenitor cells in human fetal blood. Blood 62:118, 1983


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