Characterization of a Bipotent Erythro-Megakaryocytic Progenitor in Human Bone Marrow

By Najet Debili, Laure Coulombel, Laure Croisille, André Katz, Josette Guichard, Janine Breton-Gorius, and William Vainchenker

The aim of the present study was to determine if the human erythroid (E) and megakaryocytic (MK) lineages were closely linked to the existence of a bipotent burst-forming unit (BFU) E/MK progenitor. In methylcellulose cultures, BFU-E/MK colonies were observed at day 12 and closely resembled mature BFU-E with the exception that the erythroid component was surrounded by MK. These colonies were quite different from the colony forming unit (CFU)-GE/MKM-derived colonies, which were composed of a large number of erythroblasts and which developed later in culture. The existence of thesemultilineage colonies composed of 100 to 1,000 erythroblasts intermingled with a few MK and without granulocytic cells was confirmed by the plasma clot technique and immunoenzyme alkaline phosphatase labeling of the MK. To investigate if this bipotent progenitor belonged to the compartment of primitive progenitors, CD34+ marrow cells were subfractionated according to expression of the CD38 antigen. The bipotent BFU-E/MK progenitor as well as a large fraction of MK progenitors were found in the CD34+ CD38+ or in the CD34+ CD38- cell fractions. Growth of this bipotent BFU-E/MK progenitor required the combination of stem cell factor (SCF), interleukin-3 (IL-3), and Epo in serum free conditions. Addition of only monopotent progenitors is still Clonal Absolute Requirement, but slightly increased the plating efficiency of CFU-MK and of BFU-E/MK progenitors when combined with SCF, IL-3, and Epo. In contrast, when these cultures were performed in the presence of 30% fetal calf serum, no BFU-E/MK colonies were observed irrespective of the combination of growth factors used, including the presence of MGDF; however, inclusion of the MS-5 cell line restored the growth of this bipotent progenitor. In contrast, in cultures performed in the presence of human normal or aplastic plasma, MS-5 had only a slight effect on the cloning efficiency but improved MK cytoplasmic maturation and MK size, suggesting that the main effect of MS-5 is to diminish the inhibitory effect of the fetal calf serum on the MK differentiation. The clonal origin of bipotent BFU-E/MK colonies was demonstrated in liquid culture of single CD34+ CD38low cells by immunophenotyping individual clones. At day 12, 30% of the clones contained erythroblasts (glycophorin A +) and some MK (CD41+) without granulocytes (G) or macrophages (M) (CD14+ and CD15+). At day 20, clones containing erythroblasts and MK were rare (5%). In contrast multilineage clones could be frequently detected at this time without passage from BFU-E/MK clones at day 12 to GEMM at day 20. These results suggest that a bipotent BFU-E/MK progenitor may be a nonrandom step in the hierarchical development of stem cells.

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THE HEMATOPOIETIC system is composed of heterogeneous populations of cells that have been schematically divided into three compartments, 1 ie, a stem cell, a progenitor cell, and a terminally differentiating cell compartment. Regulation of cell proliferation and differentiation in the progenitor compartment is mainly controlled by identified cytokines, but molecular mechanisms involved in the commitment of a pluripotent stem cell toward one cell lineage are presently unknown. Several lines of evidence, most of them based on the analysis of the composition of colony forming unit (CFU)-S or of the progeny of paired daughter suggest that decisions at the level of early stem cells are stochastic. However, whether the restriction of stem cells potential occurs progressively through the production of intermediate oligopotent progenitors with all the various combinations of potentialities or only after one division with the generation of only monopotent progenitors is still unclear. 1,6 Clonal transfer experiments have demonstrated that a bipotent neutrophil/macrophage progenitor exists that retains biresponsiveness to both granulocyte macrophage colony stimulating factor (GM-CSF) and M-CSF. 7 The existence of such a tight linkage between two cell lineages could be an exception along the hematopoietic differentiation pathway, but it cannot be excluded that other types of bipotent progenitors also exist. For several years, numerous similarities between the erythroid (E) and megakaryocytic (MK) cell lines have been emphasized. In humans, almost all leukemic cell lines described as erythroleukemic or megakaryoblastic express E- and MK-specific genes and this dual expression is found in the same cell. 8,9 Similar observations have also been made in some acute leukemias. 10 Indeed, it has been shown that the regulation of E- and MK-specific genes share many features 11-14 and transgenic mice expressing thymidine kinase gene under the control of the GPIIA promoter have defective erythropoiesis and megakaryocyteopoiesis. 15 It is thus possible that restriction toward the E or the MK pathways occurs relatively late in the hematopoietic hierarchy and that a bipotent BFU-E/MK can be identified. 15,17 Mixed E-MK colonies have been described by several investigators in both humans and mice 16,18 but no detailed analysis of their properties have been provided. The present experiments used clonogenic assays and single cell cultures to document the existence of human bone marrow cells able to generate only erythroblasts and megakaryocytes, to determine their phenotype, relative frequency, and optimal growth requirements. This burst forming unit (BFU)-E/MK segregated in the CD34+ CD38low cell fraction, required 12 days to differentiate and produced exclusively erythroblasts and megakaryocytes.

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BONE MARROW CELLS

MATERIALS AND METHODS

Bone Marrow Cells

Bone marrow was obtained from patients undergoing hip surgery after informed consent was obtained in accordance with the institutional guidelines of the Committee on Human Investigation. Cells were collected by vigorous shaking of bone fragments in Iscove’s modified Dulbecco’s medium (IMDM; Gibco, Paisley, Scotland), supplemented with 100 μg/mL of deoxyribonuclease (DNase type I; Sigma, St Louis, MO), centrifuged once, counted, and separated on Ficoll-Hypaque (Seromed, Berlin, Germany). Light-density (<1.077 g/mL) marrow cells (LDMC) were recovered and after washing were used either for isolation of CD34+ cells by the immunomagnetic bead technique or flow cytometry.

Antibodies

FITC-anti-CD41 (anti-Gplb/IIa; Immunotech, Luminy, France), FITC-anti-CD61 (GpIIIa; Dako, Glostrup, Denmark), FITC-anti-glycophorin A (GPA) (Dako), R-PE anti-GPA (Immunotech), FITC-anti-CD15 (Lewis, granulocytic and monocytic differentiation, Dako), FITC anti-CD38 (106) (Immunotech), FITC-CD11b (Mac-1, granulocytic and monocytic differentiation, Immunotech), FITC-anti-HLA-DR (Immunotech), R-PE anti-CD41 (Dako), R-PE-HPCA-2 (CD34; Becton Dickinson, Mountain View, CA), and R-PE anti-CD14 (Gp55, granulocytic and monocytic differentiation, Becton Dickinson) were used either for cell sorting or analysis by flow cytometry. The unconjugated antibody Y2/51 (anti-CD61) was a generous gift from Dr D. Mason (Oxford, UK).

Isolation of CD34 by the Immunomagnetic Bead Technique

CD34+ cells were recovered from LDMC (2 × 10^6 cells) by the immunomagnetic bead technique using the Dynal kit (Dynabeads M-450 CD34) according to the manufacturer’s instructions (Dynal, Oslo, Norway). CD34+ cells were detached from the beads using the DETACHa BEAD solution (Dynal).

Cell Sorting

Subpopulations of CD34+ cells were sorted according to their expression of the CD38 antigen starting from LDMC or magnetic bead isolated CD34+ cells. Cells were incubated simultaneously with the R-PE-HPCA-2 (CD34) and FITC-IOB6 (CD38). After one washing, cells were suspended in IMDM at a concentration of 5 × 10^7 cells/mL and separated by cell sorting. In other experiments, triple staining with R-PE-HPCA-2, FITC-anti-CD38, and incubation with Hoechst 33342 dye (Sigma at a concentration of 5 μg/mL for 90 minutes at 37°C) was performed to study the cell cycle status of hematopoietic progenitors.

Cells were sorted on an ODAM, ATC 3000® cell sorter (ODAM/Brucker, Wissembourg, France) or a FacsVantage (Becton Dickinson) equipped with two argon ion lasers (INNOVA 70-4 and 90-5; Coherent Radiation, Palo Alto, CA) tuned to 488 and 360 nm, respectively, and operating at 500 mW. A morphologic gate including all the CD34+ cells was determined on two-parameter histograms (side scatter [SSC] versus electric measurement of the cell volume [ODAM] or the forward Scatter [FSC, FacsVantage]). Compensation for two-color labeled samples was set up with single stained samples. Negativity for the CD34 among the CD34+ cells was determined using control cells labeled with the PE-HPCA-2 and an irrelevant IgG1 monoclonal antibody (MoAb).

For limiting dilution experiments, CD34+ CD38low cells were directly sorted into 96-well tissue culture plates (Falcon, France) with an automatic cloning device unit.

Assessment of Hematopoietic Progenitor Cells

Quantitation of clonogenic progenitors in semi-solid assays. Erythroid (CFU-E and mature and immature BFU-E), granulocytic (CFU-GM), megakaryocytic (CFU-MK), and mixed (CFU-GEMM) progenitors were quantified using previously described methylcellulose assays. Each cellular fraction selected was plated at a concentration of 0.5 to 2 × 10^3 cells/mL of complete methylcellulose medium (0.8% methylcellulose in IMDM, 30% fetal calf serum [FCS], 1% deionized bovine serum albumin [BSA] and 10^-4 mol/L, β-mercaptoethanol). Cultures were also performed in the presence of sera from aplastic patients or in serum-free medium as previously reported. Briefly, the medium contained 1.5% deionized serum albumin (Cohn fraction V; Sigma Chemical Co), iron saturated human transferrin (300 μg/mL; Sigma), calcium chloride (28 ng/mL), a mixture of sonicated lipids, 7.5 × 10^-4 mol/L α-thioglycerol, 100 ng/mL insulin, and 0.8% chemically pure methylcellulose in IMDM. The lipid mixture was produced by sonication 7.8 mg cholesterol, 6.14 mg oleic acid, and 7.4 mg dipalmitoyl lecithin (all obtained from Sigma) in 10 mL of IMDM (without sodium bicarbonate) containing 1% albumin. Aliquots (20 μL/mL) of this mixture were

Fig 1. BFU-E/MK colonies in methylcellulose and plasma clot cultures. (A) A typical BFU-E/MK colony observed with an inverted microscope in cultures containing 30% FCS and MS-5 cells. This type of colony contained erythroblasts and megakaryocytes. The other cells observed on the figure correspond to MS-5 cells. (B) A similar colony in plasma clot. MK appeared in pink color after immunolabeling with an anti-CD61 MoAb. (C) A typical BFU-E/MK colony in cultures where FCS has been replaced by AP.
added to the culture medium. Colony-stimulating factors were provided either as agar-leukocyte conditioned medium (agar-LCM; 10% vol/vol) or as recombinant human (rh) growth factors: rhStem cell factor (SCF) (50 ng/mL), G-CSF (20 ng/mL), and megakaryocyte growth and development factor (MGDF) (10 ng/mL) also called Mpl-ligand (Mpl-L), or thrombopoietin (TPO) (all three kindly provided by AMGEN, Thousand Oaks, CA), rhIL-6 (Sigma), 0.01 mol/L e amino caproic acid, and horse thrombin (6 mU/mL, Stago). Cultures were incubated as described above and studied after 12 days. Colonies were quantified by an indirect immunophosphatase alkaline labeling technique using an anti-GplIla MoAb (CD61, Y2-51). Dishes were scanned completely under an inverted microscope at 40 or 100 × magnification.

We have previously reported that addition of murine stromal cells from the MS-5 cell line to hematopoietic colony assays selectively stimulates the expression of very early clonogenic progenitor cells (imature BFU-E, CFU-GEMM) in synergy with growth factors. The MS-5 stromal cell line was kindly provided by K. Mori and maintained in αMEM supplemented with 10% FCS. Its growth-promoting effect was assessed by adding 10,000 MS-5 cells to both methylcellulose and plasma clot assays.

*Single cell cultures.* In limiting dilution experiments, individual CD34+ CD38− cells were sorted into 96-well plates using serum-free medium and a combination of seven cytokines (SCF, IL-3, Epo, IL-6, G-CSF, GM-CSF, and MGDF) or of AP and six cytokines (all except MGDF). Plates were examined at day 11 to 13, day 18 to 20, and later after incubation at 37°C in an air atmosphere supplemented with 5% CO₂.

Individual clones were cytocentrifuged or divided into two parts for flow cytometric analysis. The first half was doubly labeled with
BIPOTENT ERYTHRO-MEGAKARYOCYTIC PROGENITOR

Fig 3. Kinetics of development of BFU-E, CFU-MK, and BFU-E/MK colonies from different CD34' cell subfractions. Experiments were designed as in Fig 2 and cultures were studied at day 7, 12, and 18.

Fig 4. Analysis of the cell composition of individual clones derived from CD34' CD38<sup>+</sup> at days 11 to 13 of culture and at day 20 of cultures. The results presented are those of five independent experiments that have been pooled representing the analysis by flow cytometry of 93 and 91 clones, respectively. Culture conditions were serum-free plus a combination of SCF, IL-3, Epo, GM-CSF, G-CSF, IL-6, and MGDF in two experiments, and in three others AP plus a combination of SCF, IL-3, Epo, GM-CSF, G-CSF, and IL-6. ( ) E; ( ) E/MK; ( ) E/MK/GM; ( ) GM/MK; ( ) GM; ( ) E/GM.

Identification of a Bipotent Erythroid-Megakaryocytic (BFU-E/MK) Progenitor Cell

In preliminary experiments aimed at optimizing culture conditions for the growth of marrow-derived CD34<sup>+</sup>/CD38<sup>-</sup> primitive progenitors in methylcellulose, we observed that the addition of the murine MS-5 stromal cell line to the culture synergized with added human cytokines (see below) to promote the development of primitive progenitors. Of note, we observed that at early time points (day 12) during the culture a fraction of small (<100 cells) hemoglobinized colonies contained MK (Fig 1A). The erythroid compartment of these colonies was similar in size and time required for maturation to that of mature BFU-E-derived colonies. Some typical colonies were studied by morphology and only contained E and MK cells. These progenitors were easily distinguished from standard CFU-GEMM and primitive BFU-E both by the size of the colonies generated and by the time (>18 to 20 days) required for their differentiation. These progenitors were also identified in plasma-clot assays (Fig 1B) and the presence of MK in these mixed colonies was confirmed by immunolabeling with an anti-CD41 or an anti-CD61 MoAb. Usually 2 to 30 MK were counted together with 500 to 1,000 erythroblasts. No other cell type was detected by morphologic criteria. These colonies were not a simple overlap of two types of colonies since they were still observed at low cell concentration where even a single colony was grown. This result was confirmed by single cell assay (see below). These progenitors were designated BFU-E/MK.

In another series of experiments, we precisely defined the properties of this progenitor cell with respect to its CD34/CD38 phenotype and response to cytokines. We also tried to define its place in the hierarchy of hematopoietic progenitor cells by investigating if this bilineage association was purely random or reflects a preferential association between E and MK differentiation.

Phenotype of the Erythroid-Megakaryocytic Progenitor Cell

Expression of CD34 and CD38 antigens on the BFU-E/MK progenitor was done by plating different subpopulations of marrow cells in plasma clot and methylcellulose assays. When bone marrow cells were simultaneously labeled with anti-CD34 and anti-CD38 antibodies, two discrete populations were clearly identified among CD34<sup>+</sup> cells: cells brightly stained with CD38 antibodies (referred to as CD38<sup>+</sup> cells), which represent 70% of the CD34<sup>+</sup> cell population, and cells expressing lower levels of the CD38 antigen.
Fig 5. Flow cytometry analysis of clones containing erythroblasts and MK. Dot plot analysis of two typical BFU-E/MK clones (A and B). These clones contained a great majority of GPA' cells and a minority of CD41' cells. The CD41' cells have very high forward and side scatter properties that distinguish them from erythroblasts. More than 99% of the cells were labeled by both antibodies. CD15 and GPA controls IgG1 FITC and IgG1 PE are shown, one on a clone negative for the CD41 marker (C) and the other on an immature erythroid clone expressing low level of GPA (D).

(CD38(high)). This last subpopulation was very heterogeneous including CD38 negative (CD38−, 10% of the CD34+ cells) cells that fell within the limits defined by control IgG, and CD38+ cells (20% of the CD34+ cells), although the limit between these two populations was empirical. These three subpopulations, CD34+/CD38+, CD34+/CD38−, and CD34−/CD38−, were sorted and analyzed for their content in BFU-E/MK progenitors, simultaneously in plasma clot and methylcellulose assays.

As shown in Table 1 and Fig 2, BFU-E/MK identified in plasma clot and methylcellulose assays were present in the CD34+/CD38+ or CD34+/CD38− fraction and were not found in the CD38' fraction. This contrasted with the distribution of mature BFU-E, 90% of which were detected in the CD38' fraction, an observation that fits with previous observations showing that expression of CD38 discriminates between mature (CD38') and immature (CD38−) progenitor cells. If one considers as a single category colonies hemoglobinized at days 10 to 12, a significant proportion of these (19% to 60%) contained MK, whereas mature BFU-E–derived colonies scored in assays initiated with CD38+ cells were exclusively erythroid. There was some variability in the frequency of the BFU-E/MK–derived colonies among CD38+ and CD38− cells. In some, the BFU-E/MK progenitors were predominantly in the CD38', whereas in others they were found in the CD38+ cell fraction (Fig 2). Although immature BFU-E (imBFU-E) were enriched in the CD38+ and CD38− cell fraction, approximately 40% of them were contained in the CD38+ cell fraction. Therefore, taking into account only the expression of the CD38 antigen, BFU-E/MK appears more immature than the imBFU-E. In plasma clot and methylcellulose, MK progenitors were also enriched in the CD34+ CD38low cell population with significant heterogeneity in the size of the colonies (from 3 to more than 100 MK). In the CD34+ CD38− cell fraction, 30% to 50% of the MK colonies were bipotent. In contrast, in the CD34+ CD38− cell fraction MK progenitors gave rise to pure MK colonies.

Kinetics of development of MK-containing progenitors were next analyzed in plasma clot assays. As shown in Fig 3, the kinetics of BFU-E/MK and CFU-MK were comparable. MK colonies were the first colonies to be recognized, and at day 7 of culture, MK colonies were almost the only type of hematopoietic colonies present with some colonies containing up to 50 MK. CFU-MK were counted at day 12 and contained up to a few hundred MK. BFU-E/MK–derived colonies developed with very similar kinetics; they were first detected at day 7 of culture and their number was maximum at about day 12. Although the absolute number of BFU-E/MK–derived colonies was low, these mixed colonies represented up to 60% of the total number of pooled MK and E colonies. At day 18 of culture, CFU-MK and BFU-E/MK–derived colonies were still present but began to lyse and were not detected after day 20. In contrast, large E colonies that included granulocytes, monocytes, and more rarely MK were detected at this time. By day 18, in methylcellulose cultures, lysing BFU-E/MK colonies and growing CFU-GEMM–derived colonies were observed in the same dish, but they clearly corresponded to two distinct types of colonies.

**Single Cell Cultures**

The existence of a bipotent BFU-E/MK progenitor was clearly established in single cell cultures. Furthermore, this approach also allowed us to validate previous observations, which suggests that even late in differentiation the E and MK programs are preferentially associated. CD34+/CD38low cells from five different bone marrows were directly sorted into wells of 96-well microtiter plates. After sorting, we checked that a single cell was present in more than 95% of the wells. Five hundred wells were initiated per experiment in IMDM supplemented in three experiments with 10% AP and a combination of six cytokines (SCF, Epo, IL-3, GM-CSF, G-CSF, and IL-6) and in the other two in serum-free medium supplemented with the same cytokines plus MGDF. This combination of cytokines was chosen to ensure optimal differentiation in E, MK and granulo/macrophagic pathway. Cultures were studied after 10 to 12, 18 to 20, and in two experiments after 30 days at 37°C, 5% CO2. Clones were selected that contained at least 100 cells and only those were analyzed. Those represented 8% ± 2% and 20% ± 6% of the initial wells at days 12 and 20, respectively. In a preliminary experiment, individual clones were pipetted off, cytocentrifuged, and morphologically analyzed. However, this procedure resulted in partial loss of MK during cytocentrification, and in subsequent experiments clones were analyzed by flow cytometry. Each clone was divided into two equal cell fractions. Part of the cells was labeled simultaneously with antibodies against GPA and either CD61 or CD41 and the other half with antibodies directed against CD14 and either CD15 or CD11b. Under these conditions, at day 12, 61% of the 93 analyzed clones contained some erythroblasts (ranging from 15% to 100% of the total population) and thus arose.
from a progenitor capable of erythroid differentiation (Fig 4). Twenty-one percent of the clones (pooled experiments) contained only GPA+ cells, while 30% of the clones contained both GPA+ cells and cells highly labeled with the antibodies against the CD61 or CD41 antigen (Fig 5). These cells positive for MK markers had high forward and side scatter properties characteristic of cultured MK, which allow easy discrimination between erythroblasts and MK on the basis of light scatter properties. In all these clones, GPA+ cells represent more than 85% and MK 1% to 8% of the events analyzed (Fig 5). None of the cells present in these clones expressed granulocytic markers (CD14 and CD15). Clones containing granulocytes and MK without erythroblasts or granulocytes and erythroblasts without MK were also observed but at a low frequency (3% and 4%, respectively). Since we selected only wells containing over 100 cells, pure small MK clones could not be detected by flow cytometry. However, these were detected by microscopic observation and their MK nature was further documented by CD41 immunolabeling (data not shown).

At day 18 to 20 of culture, 20% of the total number of wells contained more than 100 cells and 56% of the 91 analyzed clones contained cells that expressed either CD14 or CD15 or both. A minority of the clones (8%) only contained GPA+ cells. BFU-E/MK clones were also uncommon (5%) with very few MK (<2%). In contrast, clones containing GPA+, CD14+, CD15+, and CD41+ cells were much more frequent (22%) suggesting that they were derived from CFU-GEMM. These clones contained on average 5-fold more cells than those studied at day 12. Later in culture (around day 30), all the clones analyzed contained CD14+ and CD15+ cells but no GPA+ or CD41+ cells.

Two BFU-E/MK clones identified at day 12 were subsequently studied at day 18, and no granulocytic cells could be detected.

**Definition of Optimal Conditions for the Detection of BFU-E/MK Progenitors**

The BFU-E/MK requirement for cytokines was first assessed in plasma clot cultures, which are optimized for the expression of MK differentiation. Cultures were ended at days 10 to 12 and stained with an anti-CD61 MoAb as

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### Table 2. Assessment of Different Progenitor Cell Types in Serum-Free Semi-Solid Clot Assay Supplemented With Different Combinations of Cytokines

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>BFU-E</th>
<th>CFU-MK</th>
<th>BFU-E/MK</th>
<th>CFU-GM</th>
<th>G/MK</th>
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<tr>
<td>MGDF</td>
<td>32 ± 2</td>
<td>0</td>
<td>0</td>
<td>5</td>
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<tr>
<td>Epo</td>
<td>1.33 ± 1.5</td>
<td>0</td>
<td>1.57 ± 0.57</td>
<td>0.56 ± 0.57</td>
<td>8 ± 3</td>
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<tr>
<td>MGDF, Epo</td>
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<td>38.3 ± 5</td>
<td>29.3 ± 16</td>
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<td>1.5 ± 5</td>
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<tr>
<td>IL-3, Epo</td>
<td>12.3 ± 0.57</td>
<td>3.6 ± 0.57</td>
<td>6.6 ± 0.57</td>
<td>8 ± 3</td>
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<tr>
<td>MGDF, IL-3, Epo</td>
<td>13.3 ± 3.7</td>
<td>29.3 ± 16</td>
<td>2.3 ± 1.1</td>
<td>6.3 ± 1.5</td>
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<tr>
<td>SCF, Epo</td>
<td>7.3 ± 0.57</td>
<td>1 ± 1</td>
<td>0.66 ± 0.57</td>
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<tr>
<td>MGDF, Epo, SCF</td>
<td>12 ± 3.4</td>
<td>76.6 ± 6.6</td>
<td>1.6 ± 1.5</td>
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<td>IL-3, SCF, Epo</td>
<td>16.3 ± 0.57</td>
<td>15.3 ± 0.57</td>
<td>6 ± 1</td>
<td>12.3 ± 4.9</td>
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<td>MGDF, IL-3, SCF, Epo</td>
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<td>13.3 ± 1</td>
<td>12 ± 2</td>
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<td>IL-3, Epo, SCF, IL-6</td>
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<td>11.3 ± 2.5</td>
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<td>13.3 ± 3.7</td>
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<tr>
<td>MGDF, IL-3, Epo, SCF, IL-6</td>
<td>13.3 ± 4.5</td>
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<td>IL-3, Epo, SCF, IL-6, G-CSF</td>
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<td>5 ± 1.7</td>
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<tr>
<td>MGDF, IL-3, Epo, SCF, IL-6, G-CSF</td>
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<td>22.3 ± 5</td>
<td>14 ± 4.5</td>
<td>20 ± 3</td>
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</table>

CD34+/CD38™ cells (ie, CD38+ + CD38 cells) (1,000 per plate) were plated in serum-free semi-solid medium with the indicated combinations of cytokines. Colonies were scored at days 10 to 12 after immunolabeling of the plates with anti-CD61 antibody as described (see Materials and Methods). Numbers represent the mean ± SD of counts performed in three different plates. Results are from one experiment.

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### Table 3. Effect of the Addition of MS-5 Cells on the Growth of Different Types of Progenitor Cells in Methycellulose Assays

| Additive | 
|----------|---|
|          | n | MS-5 | mBFU-E | Total Pure Erythroid E/MK | Total Pure Erythroid CFU-GEMM |
| A-LCM + Epo | 3 | 34 ± 17 | 34 ± 17 | 0 | 3 ± 2 | 3 ± 2 |
| SCF + IL-3 + Epo | 5 | 27 ± 7 | 21 ± 4 | 6 ± 1 | 20 ± 7 | 13 ± 5 | 7 ± 2 |

One thousand CD34+/CD38™ (ie, CD38+ + CD38 cells) were plated in methycellulose colony assays in the presence of either A-LCM + Epo or SCF + IL-3 + Epo as growth factors and 30% FCS. MS-5 cells were added at the concentration of 10,000 cells per dish. E/MK were enumerated in the mBFU-E and scored at day 12 and CFU-GEMM in the imBFU-E and scored at days 20 to 25. Duplicate plates were initiated in each condition. Numbers refer to the mean ± SEM of counts obtained in the indicated number of experiments (n).
surrounded by two MS-5 cells. Note that MK develops blebs in the contact with MS-5 cells. Magnification x 2,900.

There is a harmonious distribution of demarcation membranes and α-granules. Magnification x 19,600.

A rosette with a large MK and an MS-5 cell. MS-5 cells possess numerous large granules and cisternae of endoplasmic reticulum. The cell membrane extends its pseudopods between the large blebs of MK, which are always devoid of organelles. Note the tight contact between the two cells. Magnification x 7,700.

In a first set of experiments performed before the cloning of MGDF (Mpl-L, TPO), we compared the effect of normal human and aplastic human plasma on the development of CD34+/CD38low (including both CD38- and CD38+). In the presence of normal plasma, the growth of BFU-E/MK and CFU-MK was optimum in the presence of the combination of SCF, IL-3, and Epo with 4 ± 1.4 BFU-E/MK per 1,000 cells, and 6 ± 2.8 CFU-MK per 1,000 cells. Similar results were found with AP, but cloning efficiency was slightly higher (5 ± 1.4 BFU-E/MK per 1,000 cells, and 9.5 ± 1.9 CFU-MK per 1,000 cells). In a second set of experiments, we tested the effect of MGDF in serum free conditions (Table 2). Best results were obtained when MGDF was added to SCF, IL-3, Epo with 13 to 17 BFU-E/MK per 1,000 CD34+/CD38low (including CD38- + CD38+) cells, and 25 to 27 CFU-MK, whereas six BFU-E/MK progenitors were observed without MGDF. Withdrawal of either IL-3 or SCF even though high concentrations of MGDF were kept constant decreased the number of BFU-E/MK progenitors, but not of CFU-MK, which fits well with the known requirement of either IL-3 or SCF for the proliferation of early E cells. Addition of IL-6 either to a combination of three (SCF, IL-3, Epo) or four (the same three + MGDF) did not further increase plating efficiency. Precise quantitation of the E compartment was hampered in plasma clots by the early termination required for MK quantification.

Methylcellulose assays were also performed with CD34+/CD38low cells in the presence of 30% FCS, a concentration that is optimum for E development but selectively inhibits MK development. Addition of A-LCM or the combination of SCF, IL-3, and Epo and even MGDF did not neutralize this inhibition. In contrast, the addition of MS-5 cells to the assay (independently of the cytokine combination) dramatically counteracted FCS effect both on BFU-MK and CFU-E/MK (Table 3 and Fig 1C). Thus, on average eight BFU-MK were scored per 1,000 CD34+/CD38low cells (Table 1). Most interestingly, even though the total number of small E colonies scored as mBFU-E in the CD38low fraction did not change when MS-5 cells were added (Table 3), 20% to 25% of these now clearly included MK, which suggests that a proportion of E progenitors were in fact not restricted to the erythroid lineage. The observation that 90% of the mBFU-E are in the CD38 low fraction and generate only E cells independently of the presence of MS-5 cells reinforced the idea that the BFU-E/MK progenitor represents a unique step in the hierarchy of progenitors. However, expression of the MK potential requires specific conditions, and in the absence of MS-5 cells, this progenitor would be scored as a mature BFU-E only capable of differentiating along the E pathway.

Of note is the fact that MS-5 cells also increased the size of MKs suggesting that they may also act on MK terminal differentiation (see EM studies below).

In contrast to their lack of proliferative effect on small E colonies, the number and size of which did not change (Table 1 and ref 26), MS-5 cells stimulated the proliferation of immature BFU-E and CFU-GEMM as 20 to 30 of these progenitors were detected per 1,000 CD34+ CD38- cells, but almost none in the absence of MS-5 cells (Table 3).

Similar results were observed in plasma clot, after immuno-labelling. BFU-E/MK and pure MK colonies were absent when cultures contained 30% FCS. MK differentiation was observed when MS-5 cells were added.

Ultrastructural studies confirmed the action of MS-5 cells in promoting terminal MK maturation. For these studies, purified CD34+/CD38low cells were cultured for 7 to 10 days in liquid medium in the presence of MS-5 cells plus AP with or without SCF and IL-3 and studied by EM. These cultures were compared with control cultures performed in the absence of MS-5. In 10 distinct experiments, MK differentiation occurred in close contact with MS-5 (Fig 6). MK were generally surrounded by MS-5 in a rosette-like shape. In the areas of contact with MS-5, MK developed numerous blebs that were absent outside this zone. Contacts between the two membranes were very tight but without junction. In some rosettes, MS-5 cells extended their pseudopods between each bleb. The quality of maturation was equivalent to that observed in vivo with the regular distribution of demarcation membranes and α-granules. No such degree of maturity was observed in control cultures (without MS-5). Adhesion between MK and MS-5 cells occurred very early during maturation since small promegakaryoblasts with large nucleoli were associated with MS-5 cells. No such intimate contacts were seen with cells from other lineages, granulocyte or erythroblastic.

Cell Cycle Status of the BFU-E/MK Progenitor

Results from the experiments described above suggest that BFU-E/MK progenitors are more immature than the CD38- E-restricted progenitors based on their lack of expression of CD38 and ability to differentiate into two lineages. A third indicator of primitiveness is quiescence. In this series of experiments, we determined the cell cycle status of BFU-E/MK by three-color labeling using R-PE anti-CD34 MoAb, FITC anti-CD38 MoAb, and Hoechst dye as shown in Fig 7A and B. In an attempt to increase the sensitivity of the procedure and detect subpopulations within the G0/G1 population, which may correspond to more primitive populations, incubation with the Hoechst
Hoechst 3342 staining of CD34+ cells. CD34+ cells were purified by the immunomagnetic bead technique (Dynal) and subsequently labeled by R-PE-anti-CD34, an FITC-anti-CD38, and the Hoechst dye. (A) A dot plot analysis between the CD34 and CD38 labeling is shown. Only the CD34high cells were analyzed for Hoechst staining and sorted into two cell fractions, CD38+ and CD38−. (B) A dot blot analysis between the CD38 staining and the Hoechst labeling in the CD34high gate is shown. Three populations as shown in the histogram (C) can be observed according to the Hoechst staining: Hoechstlow, G0/G1, and S/G2/M. Of note, cycling cells are essentially in the CD38− cell fractions, whereas Hoechsthigh cells are in the CD38high cell fraction.

Fig 7. Hoechst 3342 staining of CD34+ cells. CD34+ cells were purified by the immunomagnetic bead technique (Dynal) and subsequently labeled by R-PE-anti-CD34, an FITC-anti-CD38, and the Hoechst dye. (A) A dot plot analysis between the CD34 and CD38 labeling is shown. Only the CD34high cells were analyzed for Hoechst staining and sorted into two cell fractions, CD38+ and CD38−. (B) A dot blot analysis between the CD38 staining and the Hoechst labeling in the CD34high gate is shown. Three populations as shown in the histogram (C) can be observed according to the Hoechst staining: Hoechstlow, G0/G1, and S/G2/M. Of note, cycling cells are essentially in the CD38− cell fractions, whereas Hoechsthigh cells are in the CD38high cell fraction.

Fig 8. Cell cycle status of BFU-E/MK progenitors. CD34+ CD38low cells were subfractionated according to the Hoechst staining in a Hoechstlow, G0/G1, and S/G2/M cell subfractions as shown in Fig 6. Cells were grown in fibrin clot in serum-free conditions using a combination of SCF, IL-3, Epo, and MGDF. Results are the number of colonies per 1,000 plated cells and are the average of one experiment made in triplicate. Two other experiments gave similar results.

stain was shortened, whereas usually 2 hours is required to reach the equilibrium. With this procedure, three CD34+ populations were identified, Hoechst+, which may identify more primitive progenitors,35 Hoechst− (2n), and Hoechst++ (S/G2/M) (Fig 7, B and C). There was a clear correlation between the expression of CD38 and the uptake of the Hoechst dye (Fig 7B). Among the CD38low subset, all cells were Hoechst− or Hoechst+ and only 1% to 4% of the cells were in the Hoechst++ (S/G2/M) fraction. In contrast, 10% to 15% of the CD38+ cells was in the S/G2/M phase. Since a similarly low proportion of CD38low cells was in the S/G2/M phase when the Hoechst staining period was prolonged for 2 hours, the number of cycling cells was not underestimated. In addition, this result is in agreement with a recent report using propidium iodide to stain DNA.36 Cell sorting experiments were held in which CD34+ CD38− Hoechstlow, CD34+ CD38− Hoechst− (G0/G1), and CD34+ CD38− Hoechst++ (S/G2/M) cells were assayed for their content in progenitors. The results showed that most BFU-E/MK progenitors were in the Hoechst− or Hoechst+ fractions and only 0.32% and 0.95% of BFU-E/MK and CFU-MK of the CD38− population was in the S/G2/M phase of the cell cycle, respectively. Cloning efficiency of BFU-E/MK was much higher in the CD34− CD38− G0/G1 cell population than in the two other cell fractions (1.2% versus 0.28% and 0.25%) (Fig 8). Further functional analysis of these different Hoechst fractions for their content of other clonogenic progenitors of varying maturity showed that 20% of the mBFU-E contained in the CD38− cell fraction were in the S/G2/M phase of the cell cycle. In contrast, only 1.2% of the mBFU-E and less than 0.5% of the CFU-GEMM contained in the CD38− cell fraction were in S phase.

DISCUSSION

Observations of mixed MK-E bursts in mice19 and also in humans20 have provided the initial basis suggesting the
existence of a bipotent progenitor restricted to both MK and E lineages. Further studies have suggested that divergence of both lineages was a late event in the hierarchy of stem cell differentiation even though no direct evidence supported this hypothesis. Since then, considerable evidence in support of such a common pathway has been obtained from studies that showed that several transcription factors such as GATA-1, Tal-1, and NF-E-2 are shared by both lineages. Detailed data on the phenotype and growth properties of this bipotent progenitor are lacking partly because most of the studies were performed before the isolation of the different hematopoietic growth factors and the development of cell purification techniques. Since then further similarities in the response to Epo and MGDF of committed progenitors from both lineages and shared expression of Epo-R also strengthened the hypothesis. In this study we provide strong evidence that a bipotent MK-E burst exists in human marrow, which can be distinguished from its more mature progeny, CFU-MK and mature BFU-E and also from its immature ancestor CFU-GEMM, by its phenotype, cell-cycle status, and cytokine response.

If one assumes that colony size and time taken to generate erythroblasts and mature MK both reflect the state of differentiation of the progenitor, BFU-E/MK appeared to be closer to mature BFU-E than to immature BFU-E or CFU-GEMM, based on the fact that mixed colonies were small containing at most 1,000 erythroblasts and a variable number of MK from 2 to 30. The number of BFU-E/MK was maximal after a period of 12 days in culture and colonies lysed a few days later. In contrast, 16 to 18 days must elapse before primitive BFU-E and CFU-GEMM can express their full potential and complete differentiation. If the kinetics of colony formation classifies BFU-E/MK as close to mBFU-E, they differ from these both by their phenotype and cell cycle status. In contrast to day 12 BFU-E, 90% of which express both CD34 and CD38 antigens, BFU-E/MK were in the CD34+ CD38- (either CD38+ or CD38−) fraction and the great majority (>90%) of BFU-E/MK were in G0/G1 phase of the cell cycle, two properties that usually characterize immature progenitors. Noteworthy, this was also true for a fraction of CFU-MK, therefore suggesting that CD38 may not be expressed by cells of the megakaryocytic lineage to the same extent as on other myeloid precursors.

Definite proof that the potential of BFU-E/MK was restricted to E and MK lineages only was obtained in single cell experiments. The frequency of BFU-E/MK was estimated from the proportion of wells that contained more than 100 cells and only GPA+ and CD41+ cells after 12 days in culture. An estimated 2% to 3% of total wells initiated with CD34+ CD38− containing BFU-E/MK based on these criteria, which represented 0.3% to 1% of normal marrow CD34+ cells. By comparison, the frequency of clones containing at least three lineages and thought to originate from standard CFU-GEMM was 4.4% in the CD34+ CD38− and 1% in the initial CD34+ cell fraction. Despite the fact that these numbers might be underestimated, as only clones containing >100 cells were analyzed by flow cytometry at day 12, they were in agreement with frequencies of BFU-E/MK calculated from colony assays. Finally, it remains to be determined whether some lineage markers (especially CD41 as suggested by a recent report) could be expressed on E/MK progenitors. Our preliminary results do not favor this hypothesis as the CD34+ CD41+ cell population was essentially enriched in MK progenitors.

In vitro appraisal of the real potential of progenitors is hampered by two difficulties. The first pertains to the fact that specific culture conditions and cytokine combinations that optimally support the differentiation of progenitor cells committed to one lineage may be inhibitory for progenitor cells committed to other lineages. Thus, myeloid and B lymphoid differentiations in long-term cultures require conditions that are mutually exclusive, but this is also true in colony assays where IL-3 has recently been shown to inhibit the emergence of B220+ cells in colonies. In this study, bipotent E/MK progenitors were not detected in the standard conditions of methylcellulose colony assays optimized for the growth of primitive BFU-E, ie, 30% FCS plus SCF, IL-3, and Epo. In the presence of FCS, the addition of MS-5 stromal cell line was absolutely required for the growth of E/MK progenitor and CFU-MK and no combination of cytokines could replace these stromal cells. In contrast, when normal plasma or AP were substituted for FCS, MS-5 cells improved terminal MK differentiation but had no significant effect on the number of MK and E/MK colonies. The inhibitory activity present in serum that inhibits MK growth is most likely accounted for by TGF-β. Whether this is accounted for by MGDF as is yet unclear; recent experiments showed that MS-5, which has reported for other stromal cells, synthesizes low amounts of MGDF (F. Wendling, manuscript in preparation). However, exogenous MGDF added to colony assays with 30% FCS in the absence of MS-5 supported the growth neither of BFU-E/MK nor CFU-MK. In serum-free plasma clot assays, MGDF alone optimally recruited CFU-MK but could not replace SCF or IL-3 in the E/MK progenitor assay. This, together with the observation that addition of MGDF to the combination of SCF, IL-3, and Epo increased the cloning efficiency of E/MK progenitors suggests that optimal development of E/MK progenitors requires combinations of cytokines that probably act sequentially on progenitors at different maturation stages. Further experiments will be required to characterize MS-5-derived molecules that affect MK maturation.

Two additional parameters may lead to underestimation of the real potential of progenitors in culture: asynchrony in the acquisition of specific differentiation markers identifying commitment to different lineages during the course of clone formation and discrepancy in the number of cells representative of each lineage. For example, emergence of lymphoid cells in colonies grown from lymphohematopoietic progenitor in the mouse is a very late event. Three arguments, however, rule out that E/MK clones were in fact multilineage
clones: (1) a similar phenotype was observed in clones that were sequentially studied at days 12 and 18; (2) time course studies in methylcellulose colony assays indicated that E/MK and CFU-GEMM represent two successive nonoverlapping waves of progenitor cells; (3) the sensitivity of immunolabeling of individual colonies, which allows the recognition of one to two MK cells within a colony.

Recent advances in the characterization of molecules associated with commitment to E and MK differentiation also strengthen the hypothesis of a tight linkage between both lineages. Expression of several transcription factors such as GATA-1, Tal-1, and NFE-2 is primarily found in both lineages. The recent experiments suggesting that GATA-I has an inductive role in the commitment of pluripotent stem cells as recently suggested in an avian cell line or if its lineage-specific effects may be related to its restricted pattern of expression is still an unsolved question. However, recent experiments suggesting that GATA-I is only required during the late stages of the E differentiation and the lack of a major defect in megakaryocytopoiesis in GATA-I knock-out mice, question the real requirement of GATA-I expression for early E and MK development. Whether GATA-I has an inductive role in the commitment of pluripotent stem cells as recently suggested in an avian cell line or if its lineage-specific effects may be related to its restricted pattern of expression is still an unsolved question. Our results suggest that in the near future, it may be possible to address these questions in specific cell subpopulations.

Such an assay, which allows simultaneous identification of a high number of bipotent progenitors as well as their monopotent progeny, will also be useful to characterize growth requirements of each compartment, with particular emphasis on the response to Epo and MGDF. The E and MK lineages are the only two hematopoietic cell lines that are regulated by humoral factors (Epo and Mpl-L/TPO/MGDF), the level of which is regulated by the mature cell mass. Several lines of evidence suggest that the Epo-R is expressed on MK and conversely it cannot be excluded that the Mpl-R is present on a subset of E progenitors. However, preliminary attempts to identify Epo-R and Mpl-R on CFU-E/MK progenitors by flow cytometry have failed (data not shown), but this does not exclude the possibility that E/MK progenitors express both the Epo-R and Mpl-R at very low, albeit biologically active, levels.

NOTE ADDED IN PROOF

After acceptance of this report, a report also demonstrating the existence of bipotent E and NK progenitors was published.

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Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow

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