The aim of the present study was to determine whether SCF and EPO act differently on defined subsets of progenitor cells, and if potential differences correlate with the receptor density on each subset. To investigate this possibility directly, we optimized conditions for the identification and purification of homogeneous progenitor cell subpopulations from human bone marrow. Populations containing 40% and 44% colony forming cells (CFCs) with 99% and 95% purity for the granulomonocytic and erythroid lineage, respectively, were sorted on the basis of differential expression of CD34, CD64, and CD71. In addition, a population containing 67% CFUs, of which 29-43% were CFU-MIX, was sorted from CD34+CD38lowCD50+CD71+ cells. Purified progenitor cell subsets were compared directly for responsiveness to SCF and EPO using a short-term proliferation assay. Expression of the receptors for SCF and EPO were then examined on each subset using a flow cytometer modified for high-sensitivity fluorescence measurements. The results show that EPO induces extensive proliferation of erythroid progenitor cells, but has no effect on the proliferation or survival of primitive or granulomonocytic progenitors, even when used in combination with other cytokines. The majority of erythroid progenitor cells furthermore stained positively with anti-EPO receptor (EPO-R) monoclonal antibodies, whereas other progenitor cells were negative. SCF alone induced extensive proliferation of erythroid progenitor cells, and had a stronger synergistic effect on primitive than on granulo-monocytic progenitors. In spite of these differences in SCF activity, there were no significant differences in SCF-R expression between the progenitor subsets. These results suggest that the selective action of EPO on erythropoiesis is determined by lineage-restricted receptor expression, whereas there are additional cell-type specific factors that influence progenitor cell responsiveness to SCF.

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we optimized conditions for the identification and purification of homogeneous progenitor cell subpopulations. This was accomplished by examining the content of colony-forming cells in a large number of CD34+ cell subsets identified by quantitative multiparameter analysis. The response of purified populations of primitive, erythroid and granulomonocytic progenitors to SCF and EPO was examined using a short-term proliferation assay. Finally, we measured the expression of receptors for EPO and SCF on each subset. A flow cytometer modified for high-sensitivity fluorescence measurements was used to overcome the problems associated with the detection of low receptor numbers. Using this approach, we show that EPO selectively increases survival and proliferation of erythroid committed progenitors and that this corresponds to the specific expression of EPO-R on this subset. SCF induced extensive proliferation of erythroid progenitor cells, and had a stronger synergistic effect on primitive cells than on granulomonocytic progenitors. In spite of these differences in SCF activity, there were no significant differences in SCF-R expression between the progenitor subsets. These results suggest that there are additional cell-type specific factors that influence progenitor cell responses to SCF.

MATERIALS AND METHODS

Monoclonal Antibodies (MoAbs)

MoAbs to the EPO-R (mh2er 16.5.1 and mh2er 7.9.2, both mouse IgG1) were kindly provided by Dr Simon Jones (Genetics Institute, Cambridge, MA). Anti-SCF-R MoAB 1C1.1.F was generously supplied by Dr Hans-Jörg Bühring (Univ. Clinic of Tübingen, Tübingen, Germany), whereas anti-SCF-R MoAB YB5.B5 (mouse IgG1) and 17F11 (mouse IgM) were obtained from the 5th International Workshop on Leukocyte Differentiation Antigens. All the SCF-R MoAbs used were clustered as CD117 in the Workshop. MoAbs used to discriminate between noncommitted and various lineage-committed progenitor cells included CD34 (HPCA-2), CD38 (Leu-17), CD19 (SJ25C1), CD71 (transferrin receptor) (all from Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA), CD50 (140-11, kindly provided by Dr Ramon Vilella, Servi d’Immunologia, Hospital Clinic, Barcelona, Spain), CD64 (M22, generously supplied by MedaRex Inc, Annandale, NJ) and CD45RA (8d2, kindly provided by Dr Peter Lansdorp, Terry Fox Laboratory, Vancouver, BC, Canada). The MoAbs were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), Cyanine 5 (Cy5) or biotin. Fluorochrome and biotin conjugates that are not listed in the BDIS catalog were made through the custom conjugation program at BDIS. Polyclonal PE-conjugated F(ab’)2 goat-antimouse-IgG heavy + light chain and goat-antimouse IgM κ chain was purchased from Jackson ImmunoResearch (West Grove, PA), whereas streptavidin-perCP and streptavidin-PE were obtained from BDIS. Normal mouse serum was from Caltag Laboratories (South San Francisco, CA).

Cytokines and Media

The serum-free medium consisted of Iscove’s modified Dulbecco’s medium with 25 mmol/L Hepes buffer (Sigma Chemical Co, St Louis, MO) supplemented with 200 μg/mL iron saturated transferrin (ICN Biomedicals, Inc, Costa Mesa, CA), 10 μg/mL human insulin (Collaborative Biomedical Products, Bedford, MA), 2% bovine serum albumin (Sigma), 5 × 10⁻⁴ mol/L 2-mercaptoethanol and penicillin and streptomycin at 100 U/mL and 100 μg/mL, respectively (JRH Biosciences, Lenexa, KS). Immediately before use 50 μg/mL human low density lipoprotein (LDL) (Sigma) was added to this medium. For studies with serum-containing medium, 20% heat-inactivated fetal calf serum (FCS) (Sigma) was added instead of LDL. Recombinant human interleukin-3 (rIL-3), rIL-6 and rh granulocyte-macrophage colony-stimulating factor (GM-CSF) were from Collaborative Biomedical Products. Rh SCF was from Peprotech (Rocky Hill, NJ), rh EPO from CILAG AG (Schaffhausen, Switzerland) and rh granulocyte-CSF (G-CSF) from Amgen (Thousand Oaks, CA). Cytokines were added at final concentrations of 10 ng/mL rhGM-CSF, 10 ng/mL rIL-3, 500 U/mL rhIL-6, 40 ng/mL rhSCF, 10 ng/mL G-CSF, and 2.5 U/mL rhEpo.

Cell Separation

Human BM from aborted fetuses of gestational age 17 to 22 weeks was obtained from Advanced Bioscience Resources (Alameda, CA) and The Anatomic Gift Foundation, Folkston, Georgia, both non-profit corporations, which provide tissue in compliance with state and federal laws. The intramedullary cavities of upper and lower extremity bones were flushed with PBS-FCS, and the cell suspension centrifuged. The cell pellet was resuspended in PBS-FCS. Human adult BM from healthy donors was generously provided by Dr Ping Law (Stem Cell Laboratories, Division of Hematology and Oncology, University of California San Diego). Adult BM and cell suspensions from fetal BM was diluted 10-fold in solution containing 0.9% NaCl, 0.1% KHCO₃, and 0.0037% tetrasodium EDTA in double-distilled H₂O (pH 7.2, 20°C) to lyse mature red blood cells. After 5 minutes of lysing, cells were centrifuged, washed twice in PBS-FCS and filtered through a 70 μm nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ) for subsequent staining.

Cell Staining

To minimize nonspecific antibody binding, cells were preincubated with PBS containing 0.5% human gamma globulin (Sigma). For indirect immunofluorescence staining, nucleated BM cells were incubated with an unlabeled MoAb, washed twice in PBS-FCS and stained with fluorochrome-conjugated secondary antibodies. Before additional labeling with fluorochrome-conjugated MoAbs the cells were washed twice and incubated for 20 minutes with 30% normal mouse serum to block nonspecific binding sites for mouse IgG. In some experiments, biotin-conjugated MoAbs were used, and labeling was followed by a final incubation step with fluorochrome-conjugated streptavidin. When only directly fluorochrome-conjugated MoAbs were used, they were all added simultaneously. All incubation steps were performed on ice for 25 minutes. Immunostained cells were either kept on ice and analyzed immediately by flow cytometry or fixed in 0.5% paraformaldehyde and analyzed within 48 hours.

Flow Cytometric Analysis

Immunostained cells were analyzed using an experimental high-sensitivity flow cytometer designed by Dr Robert Hoffman at BDIS. This flow cytometer is equipped with three lasers; a 488-nm Argon ion laser (15 mW) for excitation of PerCP and FITC, a 633-nm Helium-Neon laser (10 mW) for excitation of APC or Cy5 and a
352-nm YAG laser (20 mW) for excitation of PE. The 352-nm excitation of PE yields six times the signal-to-noise ratio obtained with 488-nm excitation (not shown). For each sample 10,000 to 50,000 events were acquired in list mode with Cell Quest software (BDIS). Forward light scatter, orthogonal light scatter and four fluorescence signals were determined for each cell, and the listmode data files were analyzed on a Macintosh computer (Apple Computer, Cupertino, CA) with Paint-A-GatePRO software (BDIS). The instrument was calibrated using fluorescent particles to allow direct comparison between staining intensities measured in different experiments. Dead cells were identified by positive staining with propidium iodide (PI) (Molecular Probes Inc, Eugene, OR) at a final concentration of 1 \( \mu \text{g/mL} \).

**Immunomagnetic Purification of CD34+ Cells**

Before cell sorting, CD34+ cells were purified from nucleated BM using a separation kit from Miltenyi Biotech (Auburn, CA) according to the description from the manufacturer. The purification method routinely gave CD34+ cells that were more than 90% pure, and the yield was typically 70% to 80% (results not shown). Purified cells were then stained with CD34 FITC (HPCA-2, BDIS) and fluorescein- or biotin-conjugated MoAbs to other antigens to allow flow cytometric sorting of progenitor cell subsets.

**Cell Sorting and Culture**

Immunostained cells were sorted by a fluorescence-activated cell sorter (FACS) Vantage flow cytometer equipped with an Automated Cell Deposition Unit. FITC and PE fluorescence was excited by a 488-nm Argon ion laser and APC or Cy5 fluorescence by a 90 mW Spectrum laser tuned at 647 nm. To eliminate doublets and cell aggregates, a pulse processor module was used on the forward light scatter parameter. The gates defining cells staining positively with MoAbs were set to include less than 2% of cells stained with isotype control. For determination of colony-forming potential, cells were sorted singly into 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) containing serum supplemented medium and the concentrations of SCF, IL-3, IL-6, GM-CSF, G-CSF, and EPO previously described. For determination of cytokine effects on populations, each cell subset was sorted into polypropylene tubes containing medium without cytokines or serum. The cells were then equally distributed between individual wells in a 24-well plate (Becton Dickinson Labware) and serum and cytokines were added as indicated at the concentrations previously described.

**Determination of Colony Size and Type**

96-well plates containing singly sorted cells were examined for the presence of progeny after 14 days of culture by examination through an inverted light microscope. The colonies were initially classified in four categories according to size. Type I colony: 40 to 100 cells. For colony types II-IV, colony sizes were compared to cell numbers by counting cells from individual colonies by flow cytometry using a fixed number of fluorescent latex beads as standards. Type II colony: more than 100 cells, but covering less than 25% of the area of the well (average cell number = 1,871, range 102 to 10,595, \( n = 48 \)). Type III colony: covering 25% to 50% of the area of the well (average cell number = 41,924, range 5,162 to 84,483, \( n = 32 \)). Type IV colony: covering more than 50% of the area in the well (average cell number = 316,640, range 80,593 to 823,034, \( n = 26 \)). Wells that by microscopic examination contained more than approximately 300 cells were harvested and the cells stained with CD14 FITC, CD34, CD15 PE, CD45 APC, and CD71 PerCP. Cell sorting experiments have shown that this combination of markers allows the discrimination of granulocytic, monocytic and erythroid cells after culture (Olweus and Lund-Johansen, unpublished observations, November 1994). The immunophenotyping was correlated to morphology by staining of cells displaying a homogeneous phenotype with Wright-Giemsa followed by morphological examination. In wells containing fewer than approximately 300 cells, the cells usually had homogeneous morphological characteristics when examined in the wells (not shown). Clone II and colonies were pooled according to their morphologic characteristics before antibody-staining to determine the cell type. The immunophenotype confirmed that these colonies consisted of solely granulomonocytic or erythroid cells (not shown).

**Measurement of Cytokine Effects on Cell Proliferation**

For determination of cytokine effects on specific cell populations, each population was sorted and equally distributed between individual wells in a 24-well plate containing medium as previously described. After 5 days of culture the cells were harvested and counted by flow cytometry using a fixed number of fluorescent latex beads as standard. Live and dead cells were identified by combined measurement of cellular light scatter and PI fluorescence.

**RESULTS**

**Identification of CD34+ Subpopulations by Three-Color Immunofluorescence**

**Primitive progenitor cells.** Primitive progenitors are enriched in the population expressing the highest levels of CD34 (CD34+low) and low levels of CD34 (CD34+). In fetal BM, a subset of CD34+CD38+ cells give rise to stromal cells. The hematopoietic cells can be discriminated from stromal progenitors by their positive staining with CD50 MoAb. The population identified as CD34+ in the present study was set to include on average 12 ± 1.2% of the CD34+ cells (\( n = 5 \)) (Fig 1A). In the present study the phenotype CD34+CD38+CD50+ was used to sort the most primitive hematopoietic progenitors. These cells were those that simultaneously satisfied the criteria defined by regions 1, 2, and 3 in Fig 1. The CD34+CD38+CD50+ population constituted on average 0.9 ± 0.2% of the CD34+ cells (\( n = 5 \)).

**Intermediate stage progenitor cells.** Intermediate stage progenitors were sorted as CD34+CD34+low. These cells simultaneously satisfied the criteria defined by regions 1, 2, and 4 in Fig 1 and constituted 8.5 ± 5.2% of the CD34+ cells (\( n = 5 \)).

**Granulomonocytic progenitor cells.** CD34 is specifically expressed by cells committed to the granulomonocytic lineage. In this study CD34+CD64+ cells were sorted as CD34+CD64+ and CD34+CD64- cells, respectively, in an attempt to obtain more homogeneous progenitor cell populations. The CD34+CD64+ population was defined by regions 5, 6GM and 7GM (2.2% ± 1.0% of the CD34+ cells, \( n = 5 \)), whereas the CD34+CD64- cells were defined by regions 5, 6GM and 8GM in Fig 2 (9.3 ± 3.1% of the CD34+ cells, \( n = 5 \)).

**Erythroid progenitor cells.** Erythroid progenitor cells are enriched among CD34+CD71+ cells. In the present study, the gate for sorting erythroid progenitors was therefore set to include this population and to exclude CD64+ cells (region 5 in Fig 2). To examine whether differences in CD34 expression could be used to obtain more pure erythroid progenitors from human BM, CD34+CD64+ cells were divided into CD34+CD64+ and CD34+CD64- fractions (Fig 2D). The CD34+CD64+ fraction (0.3 ± 0.3% of the CD34+ cells, \( n = 5 \)) consisted of cells that simultaneously...
Fig 1. Identification of CD34⁺ progenitor subsets by phenotypic criteria. Nucleated fetal BM cells enriched for CD34⁺ cells by immunomagnetic separation (see Materials and Methods) were stained with CD34 FITC, CD38 APC (A through D), and CD50 PE (A through C) or CD64 PE (D). Black dots represent CD34⁻ SSC⁻ cells. Regions 1 through 3 represent the criteria used to sort the population containing the CD34⁺CD38⁻CD50⁺ cells. Regions 1, 2, and 4 represent the criteria used to sort CD34⁺CD38⁺CD64⁻ cells. FSC, forward light scatter; SSC, side scatter.

satisfied the criteria for regions 5, 6E, and 7E. The CD34⁺ fraction (3.1 ± 1.3% of the CD34⁺ cells, n = 5) consisted of cells within regions 5, 6E, and 8E in Fig 2.

The CD34⁺ cells not included in any of the populations above were sorted as CD34⁺CD64⁺. These cells constituted 74.3 ± 3.7% of the CD34⁺ cells (n = 5). 43.5 ± 13.8% of these cells were CD19⁺ (n = 3, not shown).

**Content of Colony-Forming Cells in CD34⁺ Subsets**

The content of colony forming cells in each of the 7 populations shown in Figs 1, 2, and 3 was determined by single cell sorting and culture. As shown in Fig 3, all CD34⁺ cell populations contained high frequencies of colony-forming cells. The highest frequency, in total 67 ± 10% (n = 4), was observed for the CD34⁺CD38⁻CD50⁺ population, followed by the CD34⁺CD38⁺CD64⁻ subset (Fig 3). A decrease in cellular expression of CD34 simultaneously with an increase in the granulo-monocytic lineage marker CD64 was associated with a reduction in clonogenic potential as well as a decrease in colony size (Fig 3). In contrast, the CD34⁺CD71⁺ cells maintained high levels of clonogenicity and formed large colonies (Fig 3). The majority of the large colonies obtained from the CD34⁺CD71hi and CD34⁺CD71hi Cells were visibly red, indicative of hemoglobin content. The population of CD34⁺CD64⁺CD71hi cells contained less than 0.5% clonogenic cells (n = 3, not shown).

The cellular content of the colonies from each population was determined by flow cytometric staining of individual colonies as described in Materials and Methods. Of the colonies obtained from CD34⁺CD64⁻ and CD34⁺CD64⁺ cells 98% to 100% were granulo-macrocytic (Table 1). Approximately a third of the granulo-macrocytic colonies in the
CD34+ population were recovered from the CD64+ subset, whereas the majority were derived from the CD34hi CD38hi CD64- fraction, containing a mixture of colony-forming cells (Table 2). Ninety-four percent or more of the colonies from CD34hi CD7hi cells were erythroid (Table 1), and 74% to 80% of the erythroid colonies were recovered from this population (Table 2). Of the colonies obtained from CD34hi CD38hi CD50hi cells 29% to 43% contained both erythroid and granulo-monocytic cells (Table 1). A small number of mixed colonies were also observed in cultures originating from the CD34hi CD38hi CD64+ population, but not from the other subsets sorted. The larger fraction of CD34hi CD38hi CD64+ as compared to CD34hi CD38hi CD50hi cells within the CD34+ population resulted, however, in higher recovery of mixed colonies from the former population (Table 2). The relative content of myeloid and erythroid cells in mixed colonies varied with a factor of up to 200 (not shown). This result is in keeping with those of Suda et al43,45 and Mayani et al9 and suggests that there is considerable asymmetry in the division of cells during differentiation.

Short-Term Expansion of CD34 Subsets

The CD34hi CD38hi CD50hi population expanded 140-fold ± 65 (n = 4) during a 5-day period in liquid medium containing 20% FCS and SCF, EPO, IL-3, IL-6, GM-CSF, and G-CSF. Increasing levels of CD64 together with lower levels of CD34 was accompanied by a decrease in the expansion potential (Fig 4). In contrast, higher levels of the CD71 antigen correlated with an increase in expansion, consistent with the high number of large colonies observed from cultured CD34hi CD71hi cells in the single cell assay (Fig 4 and 3). This could be explained by the higher content of erythroid progenitors in the CD34hi CD71hi as compared with the CD34hi CD71hi subset (Table 1). Staining of the progeny from
RECEPTORS FOR SCF AND EPO ON CD34+ SUBSETS

![Flow cytometry chart]

Colonies per 100 cells

**Fig 3.** Frequency of colony-forming cells in sorted subpopulations of progenitor cells cultured for 14 days. The indicated subpopulations of CD34+ cells were sorted as single cells into 96-well plates containing liquid medium supplemented with serum, SCF, EPO, IL-3, IL-6, GM-CSF and G-CSF, as described in Materials and Methods. The plates were scored for the presence of progeny after 14 days of culture by examination through an inverted light microscope. Type I colony: 40 to 100 cells. For colony types II-IV, colony sizes were compared to cell numbers by counting cells from individual colonies by flow cytometry using known numbers of latex beads as a reference, as described in Materials and Methods. Type II colony: more than 100 cells, but covering less than 25% of the area of the well (average cell number = 1,871, range 102 to 10,595, n = 48). Type III colony: covering 25% to 50% of the area of the well (average cell number = 41,924, range 5,162 to 84,483, n = 32). Type IV colony: covering more than 50% of the area in the well (mean cell number = 316,640, range 80,593 to 823,034, n = 26). The bars indicate the standard deviation (SD) for the total number of colonies obtained in the experiments.

Each subset with CD34 MoAb showed that only the CD34hi CD38lo CD50- cells gave rise to a substantial amount of CD34+ progeny. The cultures from these cells contained on average 27.6 ± 6% (n = 4) CD34+ cells, corresponding to a 34 ± 9-fold expansion of CD34+ cells (n = 4). In cultures from the CD34hiCD38loCD64- population, the expansion of CD34+ cells was 3.4 ± 2.5, whereas no expansion of CD34+ cells was observed in cultures containing the other CD34+ subsets (n = 4, not shown).

**Effects of SCF and EPO on the Survival and Proliferation of CD34+ Subsets**

The effects of EPO and SCF on the survival and proliferation of isolated progenitor subsets were examined. To simplify the interpretation of the data, only the populations containing the most homogeneous populations of CFCs were included in the experiments. These were the CD34hiCD71lo subset (erythroid progenitors), the CD34hiCD64+CD71lo population (granulo-monocytic progenitors) and the CD34hiCD38loCD50+ cells (primitive progenitors) (Fig 3 and Table 1). Sorted cells from each of the three CD34+ subsets were cultured for 5 days in liquid medium containing 20% FCS, IL-3, IL-6, GM-CSF, G-CSF, EPO and SCF, or in otherwise identical medium where either SCF or EPO was omitted. The effects of omitting EPO or SCF were examined by counting the viable cells 5 days after initiation of the culture, as described in Materials and Methods. Omitting EPO from the medium reduced the number of cells obtained from CD34hiCD71lo cells by a factor of 4, but did not have a significant effect on the other two subsets (Fig 5).

In contrast, the percentage of CD34+ cells in the progeny was not affected when EPO was omitted (not shown).

In the experiments previously described, the effects of SCF and EPO may be masked by other cytokines. To examine the ability of SCF and EPO to support cell survival and proliferation in the absence of other cytokines, sorted cells were cultured in serum-free medium containing EPO or SCF or both. The number of viable and dead cells after 5 days of culture was determined by flow cytometric analysis of cellular light scatter and propidium iodide staining (see Fig 7). The results in Fig 6 show that there was a difference between the progenitor subsets with regard to survival in

**Table 1. Progenitor Content of Phenotypically Defined CD34+ Subsets**

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>% MIX Exp 1</th>
<th>% MIX Exp 2</th>
<th>% Ery Exp 1</th>
<th>% Ery Exp 2</th>
<th>% GM Exp 1</th>
<th>% GM Exp 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34hi CD38lo CD50-</td>
<td>43</td>
<td>29</td>
<td>9</td>
<td>5</td>
<td>48</td>
<td>67</td>
</tr>
<tr>
<td>CD34hi CD38lo CD64-</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>7</td>
<td>89</td>
<td>87</td>
</tr>
<tr>
<td>CD34hi CD64-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>100</td>
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<tr>
<td>CD34hi CD71lo</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>CD34hi CD71hi</td>
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<td>0</td>
<td>71</td>
<td>68</td>
<td>29</td>
<td>32</td>
</tr>
</tbody>
</table>

The indicated cell subsets were sorted singly into 96-well plates containing medium and growth factors as described in Materials and Methods. After 14 days of culture, colonies were analyzed by flow cytometry as described in the legend to Fig 3 and Materials and Methods. The numbers indicate the percentage of colonies containing populations with phenotypic characteristics of granulo-monocytic cells (GM), erythroid cells (Ery), or both (MIX). The data are obtained from two individual experiments.
serum-free medium. Whereas the number of viable cells in the cultures of granulo-monocytic progenitors remained constant after 5 days in the absence of cytokines, there was a significant loss of cells from the cultures containing erythroid (P = .01) and primitive progenitors (P = .002, n = 4) (Fig 6). Measurement of cellular light scatter and PI uptake showed that the cultures of primitive and erythroid progenitors contained a higher frequency of dead cells than those of granulo-monocytic progenitors (not shown). Addition of EPO alone induced extensive expansion of erythroid progenitor cells, whereas no effects of EPO were noted in the other two subsets (Fig 6). In the presence of SCF, there was a twofold expansion of the cells originating from granulo-monocytic and primitive progenitor cells, respectively (P < .05). In the cultures of erythroid progenitor cells SCF induced more than a 10-fold expansion, and immunophenotyping confirmed that the expanded cells were erythroid (not shown). EPO acted synergistically with SCF on the erythroid subset, as proliferation in the presence of both factors was higher than the sum of the responses in the presence of each cytokine (Fig 6) (P = .01). No significant effect was observed when EPO was added in addition to SCF to the other two progenitor cell populations (Fig 6).

The colonies derived from the CD34CD38CD50 population were more heterogeneous than those obtained from the populations of lineage-committed progenitors (Fig 3 and Table 1). The SCF-induced increase in cell number in the cultures from CD34CD38CD50 cells could therefore be due to selective expansion of a subset of lineage-committed progenitors, such as early erythroid cells. Furthermore, the cells surviving in the absence of growth factors could represent a subset of more primitive cells. To investigate this possibility CD34CD38CD50 cells were stained with CD34, CD45RA, and CD71 after 5 days of culture in serum-free medium with or without SCF. Earlier studies have shown that primitive progenitor cells can be detected as

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>% Recovery Mix</th>
<th>% Recovery Ery</th>
<th>% Recovery GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+ CD38+ CD60+</td>
<td>36</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>CD34+ CD38+ CD64-</td>
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<td>0</td>
</tr>
<tr>
<td>CD34+ CD71+</td>
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<td>1</td>
</tr>
<tr>
<td>CD34+ CD71+</td>
<td>74</td>
<td>80</td>
<td>1</td>
</tr>
</tbody>
</table>

The indicated subsets were sorted singly, cultured, and analyzed as described in the legend to Table 1. The percentages of recovery indicate the number of colonies with phenotypic characteristics of granulo-monocytic cells (GM), erythroid cells (Ery), or both (MIX) derived from each subset relative to the total number of these colonies from all the subsets. The data are obtained from two individual experiments.

The colonies derived from the CD34CD38CD50+ population were more heterogeneous than those obtained from the populations of lineage-committed progenitors (Fig 3 and Table 1). The SCF-induced increase in cell number in the cultures from CD34CD38CD50+ cells could therefore be due to selective expansion of a subset of lineage-committed progenitors, such as early erythroid cells. Furthermore, the cells surviving in the absence of growth factors could represent a subset of more primitive cells. To investigate this possibility CD34CD38CD50+ cells were stained with CD34, CD45RA, and CD71 after 5 days of culture in serum-free medium with or without SCF. Earlier studies have shown that primitive progenitor cells can be detected as

![Fig 4](image-url)  
**Fig 4.** Expansion of CD34+ subsets after 5 days of liquid culture. The indicated subsets were sorted as described in Figs 1 and 2 and cultured in liquid medium containing 20% FCS and SCF, EPO, IL-3, IL-6, GM-CSF, and G-CSF. The cells were counted by flow cytometry using a constant number of latex beads as internal standard. The bars represent average number of viable cells divided by the number of input cells. Error bars indicate SD of four experiments.

![Fig 5](image-url)  
**Fig 5.** Effects of SCF and EPO on proliferation of primitive and lineage-committed progenitor cells cultured for 5 days. The indicated cell populations were sorted and cultured in liquid medium containing 20% FCS and SCF, EPO, IL-3, IL-6, GM-CSF, and G-CSF ( ), cytokine mix ( ), cytokine mix -SCF ( ), cytokine mix -EPO ( ). The bars show cell expansion as the mean number of viable cells per input cell, and the error bars indicate the SD of four experiments. *P < .001.
CD34<sup>hi</sup>CD45RA<sup>−</sup>CD71<sup>b</sup> cells after culture in serum-free medium. Granulomonocytic and erythroid differentiation is associated with an increase in CD45RA or CD71, respectively.

Figure 7 shows light scatter and expression of CD34, CD45RA and CD71 on sorted CD34<sup>hi</sup>CD38<sup>−</sup>CD50<sup>−</sup> cells after 5 days in serum-free medium without cytokines (A through C) or with SCF (D through F). The plots show all the events that were measured from the wells containing the cultured cells, and the dots therefore give an indication about the absolute number of cells in each well (data for absolute numbers as determined by cell counting are shown in Fig 6). The cells inside the region in Fig 7A and 7D (black dots) were viable as determined by PI dye exclusion, whereas the cells with low forward scatter (grey dots) were permeable to PI (not shown). As shown in Fig 7A, only a small number of viable cells were detected after culture in the absence of cytokines, whereas the number of dead cells was comparable to or larger than that observed after culture in SCF-containing medium. To improve visualization of the data, the dots representing the viable cells in the medium-only cultures (i.e., inside region 7A) were enlarged in Fig 7B and C. On average 89 ± 4.5% of these cells were CD34<sup>hi</sup>CD45RA<sup>−</sup>CD71<sup>b</sup> (n = 3). This suggests a preferential survival of progenitor cells that committed to the granulomonocytic lineage either before or during the culture period. This interpretation is furthermore supported by the higher degree of survival observed for the isolated granulomonocytic progenitors (Fig 6). The absolute number and relative fraction of viable cells was considerably higher in the presence of SCF (Fig 7D). Of the viable cells 33 ± 5% were CD34<sup>hi</sup>CD45RA<sup>−</sup>CD71<sup>b</sup> (ie, primitive phenotype), 14 ± 1% were CD34<sup>hi</sup>CD45RA<sup>−</sup>CD71<sup>b</sup> (ie, erythroid phenotype) and the remaining cells were CD71<sup>b</sup> and showed variable levels of CD34 and CD45RA (Fig 7E and F). It therefore seems likely that SCF acts directly on the primitive progenitors, increasing their survival and inducing a low degree of proliferation. In addition, the high percentage of cells with high levels of CD45RA or CD71 suggests that a considerable fraction of the cells differentiated during the culture period.

**Expression of the EPO-R on CD34<sup>−</sup> Subsets**

Expression of the EPO-R on CD34<sup>−</sup> subsets was examined after staining of BM cells with anti-EPO-R MoAbs and CD38, CD50, CD64, CD71, and CD19. In Fig 8 the CD34<sup>hi</sup> cells are displayed as black dots for better visualization of the changes in EPO-R expression on early progenitor cells. (A) shows the EPO-R staining on unfractionated nucleated fetal BM (FBM) cells, whereas panels B through E represent CD34<sup>−</sup> cells gated from region R1, shown in (A). As shown in Fig 8A through E, the EPO-R was only detected on cells expressing high levels of CD71 (E) and low levels of CD64 (D). This is in agreement with the specific action of EPO on erythroid progenitor cells. In addition, there was a positive correlation between CD71 staining and EPO-R staining (Fig 8E). Two different anti-EPO-R MoAbs, 16.5.1 and 7.9.2, displayed similar staining profiles and were clearly positive when compared to all the 5 different isotype control MoAbs listed in Materials and Methods (98% of the cells stained with isotype controls were below the dashed horizontal lines in Fig 8A through E, not shown). The staining pattern was similar for adult BM cells (not shown). The mean fluorescence intensity of CD34<sup>−</sup>CD71<sup>b</sup> cells with anti-EPO-R was 20- to 68-fold higher than the staining of the same population with the isotype control (average 41.7, n = 4). The specificity of the anti-EPO-R antibodies was confirmed in that the staining of MoAb 16.5.1 was inhibited by more than 50% after cells had been incubated on ice for 60 minutes in PBS-FCS containing 500 U/mL rhEPO (not shown).

**Expression of the SCF-R on CD34<sup>−</sup> Subsets**

The expression of SCF-R on the CD34<sup>−</sup> subsets previously described was examined after staining of BM cells with anti-SCF–R MoAbs and CD38, CD50, CD64, CD71, and CD19. In Fig 9 the CD34<sup>hi</sup> cells are displayed as black dots for better visualization of SCF-R expression on early progenitors. (A) shows the SCF-R expression on unfractionated nucleated FBM cells, whereas panels B through E represent CD34<sup>−</sup> cells gated from region R1, shown in (A). As shown in Fig 9A through E, nearly all CD34<sup>−</sup> cells expressed high levels of SCF-R. There was no clear difference between CD34<sup>hi</sup> cells with low and high expression of CD38 (Fig 9B), suggesting that the SCF-R expression is not subject to extensive regulation during early hematopoiesis. CD34<sup>−</sup>CD19<sup>−</sup> cells, representing the earliest identifiable B-cell progenitors, showed 16 ± 4-fold (n = 4) lower SCF-R expression than the noncommitted cells, but still reproducibly more than 15 times the fluorescence levels of the isotype control (indicated by the line in [A]). The population...
containing a homogeneous population of granulo-monocytic progenitors (CD34"CD64" cells) expressed SCF-R at similar levels as the primitive cells (Fig 9D). The receptor expression was lower on CD64" cells in the CD34" population, suggesting downmodulation of SCF-R during granulo-monocytic differentiation (Fig 9D). Both CD34" and CD34" erythroid progenitor cells (CD71"CD54") displayed uniformly high levels of SCF-R (Fig 9E). The staining pattern shown for the MoAb 1C1.HF in Fig 9 was reproduced with two additional anti-SCF-R MoAbs, YB5.B8, and 17F11 and was similar for adult BM cells (not shown). The specificity of the staining was confirmed in that the labeling of 1C1.HF was reduced by more than 80% after incubation of the cells for 1 hour with 1 μg/mL rhSCF at 37°C (n = 3, not shown).

FIG 7. Phenotype of cultured CD34"CD38"CD50" cells after 5 days in culture in serum-free medium with (right) or without supplement of 40 ng/mL SCF. Cells were sorted as described in the legend to Fig 1 and cultured for 5 days in serum-free medium. The cells were then stained with the indicated fluorochrome-conjugated MoAbs and analyzed by flow cytometry. Grey dots in (A) and (D) indicate forward scatter low cells that were found to be positive for PI. (B and C) and (E and F) show viable cells only. The dots were enlarged in (B and C) for better visualization. The data illustrate data from one experiment representative of three performed.

DISCUSSION

Direct studies of changes in the expression and function of cytokine receptors during early hematopoietic development...
Fig 8. Staining of FBM cells with the anti-EPO-R MoAb mhter 16.5.1. Ammonium chloride lysed FBM cells were labeled with anti-EPO-R and goat-antimouse PE, followed by staining with CD34 FITC (A through E) and CD38 APC and CD50 PerCP (B), or CD19 APC (C), or CD64 APC and CD71 PerCP (D and E). (A) shows EPO-R staining on unfractoned nucleated FBM cells. (B through E) show staining of CD34+ cells only, gated from region R1 in (A). The cells expressing the highest levels of CD34 (CD34hi) are depicted in black on all plots for better visualization. Of the cells stained with five different isotype controls, 98% were below the dashed lines in (A through E). The results are representative of four experiments.

Thus, populations containing 44% and 40% CFCs, of which 95% and 99% gave rise to erythroid and granulo-monocytic colonies, respectively, could be defined and sorted by phenotypic criteria. The two subsets defined as more immature, contained high frequencies of multipotent progenitors, and the CD34hiCD38-CD50+ cells were clearly different from CD34hiCD38+CD64- cells in their ability to generate CD34+...
progeny in vitro. Therefore, the data presented here support the view that homogeneous populations of progenitor cells can be identified on the basis of gradual changes in the cell surface antigens CD34, CD38, CD64, and CD71.

It could be argued that the CD34hiCD38loCD50+ subset may represent a heterogeneous population of cells as they gave rise to a mixture of different colonies. However, this interpretation is based on the assumption that progenitor cells at a given stage of differentiation form highly similar colonies. Studies of paired progenitor cells have repeatedly shown that this is not true. The two daughter cells formed by the division of a primitive progenitor must be considered as representing the same differentiation stage immediately after cell division, and will most likely have similar cell surface phenotype. Yet, one daughter cell may give rise to a small single-lineage colony or no colony, whereas the other may lose its multipotential and proliferative capacity after a gradual differentiation process, generating a large, multi-lineage colony. These and similar results support the view that differentiation of primitive progenitor cells occurs in a stochastic and asymmetric fashion. In view of this model for progenitor cell differentiation, the populations defined by the markers used in the present study may therefore be more homogeneous at the time of sorting than apparent from the colonies generated. Some of the results on short-term effects of cytokines discussed below support this view.

The results on EPO-R expression and EPO-responsiveness of progenitor cell subsets suggest that surface expression of

Fig 9. Staining of FBM cells with anti-SCF-R antibody 1C1.HF. See legend to Fig 8. The results are representative of four experiments.
the EPO-R is induced shortly after erythroid lineage commitment. This view is different from that suggested by earlier reports. Wognum et al. and Sawada et al. suggested that EPO-R expression is a relatively late event in erythroid differentiation, starting at the late BFU-E or early CFU-E stage, whereas Testa et al. suggested that EPO-R is expressed on early progenitor cells. The reason for the discrepancies between the studies may lie in the problems associated with the detection of low receptor numbers. Based on an estimated expression of 1,050 EPO-Rs on CFU-E cells, the 20- to 68-fold increase in fluorescence intensity of erythroid BM cells stained with anti-EPO-R MoAbs compared to isotype control, suggests a detection limit between 20 to 50 receptors in our assay. It could be argued that primitive progenitor cells may express levels of EPO-R that are below this detection limit. However, the close association between detectable receptors and EPO effects on progenitor subsets supports the view that the negative staining of primitive and myeloid progenitor cells reflects a lack of physiologically relevant EPO-R expression. In view of the extensive expansion of erythroid progenitors in the presence of EPO and SCF, a small contamination of EPO-responsive cells among the primitive or myeloid progenitors would most likely be detected. Collectively our results therefore strongly suggest that primitive and myeloid cells are homogeneously negative for EPO-R, and that EPO-selectivity is regulated by restricted expression of the EPO-R on erythroid progenitors. The most likely explanation for the earlier reported stimulatory effects of EPO on CFU-MIX formation is therefore that the cytokine acted on cells that differentiated during the culture period. In addition, effects of EPO on myelopoiesis are likely to be indirect.

Although SCF has a broader activity than EPO, the severe anemia and the lower numbers of primitive progenitor cells in mice with mutations in the Sl and W loci, suggest that the factor primarily targets primitive and erythroid progenitor cells. This was clearly shown in the present study by demonstrating a direct proliferative effect of SCF in erythroid cells and a stronger synergistic effect of SCF in primitive than granulo-monocytic progenitors. If these cell type-specific differences in activity were determined by receptor density, we would have expected erythroid and primitive progenitors to express the highest and granulo-monocytic progenitors the lowest levels of SCF-R. However, staining with three different MoAbs to SCF-R failed to demonstrate any differences in the expression on the cell types. This is in contrast to studies suggesting that functionally distinct progenitor cells may be discriminated on the basis of different SCF-R expression. However, most of the earlier studies have come to conflicting conclusions. Different investigators have found SCF-R on primitive progenitor cells in mice with mutations in the Sl and W loci, suggest that the factor primarily targets primitive and erythroid progenitor cells. Similarly, primitive progenitors have been suggested to be SCF-R on erythroid progenitors, respectively. Similarly, primitive progenitors have been suggested to be SCF-R on erythroid progenitors, whereas Testa et al. suggested that SCF-R expression on CD34'' cells. Interestingly, this expression pattern has close resemblance to that shown for murine hematopoietic progenitors, where the most primitive cells were found in the SCF-R'' fraction. However, whereas SCF-R MoAbs are used to purify primitive progenitor cells from murine BM, our results suggest that the markers used to discriminate between cell types here have significantly better resolution for human BM progenitor subsets than MoAbs to SCF-R.

The similarity in SCF-R expression between progenitor subsets that responded differently to SCF suggests that there may be other cell type-specific factors influencing SCF-mediated signals. One possibility is that the SCF-R function is determined by associated membrane components that are expressed in a lineage-restricted fashion. In certain erythroid cell lines, SCF-R associates directly with the EPO-R, and engagement of the SCF-R leads to rapid activation of EPO-R signaling. In view of the potent effects of EPO on proliferation and the restricted expression of the EPO-R, this type of receptor crosstalk could explain a higher responsiveness to SCF in erythroid cells. In addition to affecting signal transduction, SCF-R-associated molecules could also affect SCF-binding activity on different cell types. A recent study showed that erythroid progenitors in monkey bone marrow could be identified as a separate population of CD34'' cells with high SCF binding. We have failed to demonstrate this staining pattern for any of the eight MoAbs clustered as CD117 in the Vth Workshop of Leukocyte Differentiation antigens, although the MoAbs react with seven different epitopes. Studies on SCF-binding to human progenitor cells may determine whether there is a discrepancy between anti-SCF-R MoAb binding and SCF binding. In addition to differences in associated cell surface components, there could be differences in the intracellular signaling pathways activated through the SCF-R in erythroid and myeloid cells. Studies comparing SCF-R signal transduction in erythroid and myeloid cell lines, may provide information on this issue.

The difference in SCF responsiveness observed between primitive and granulo-monocytic progenitors is likely to be of another nature than that between granulo-monocytic and erythroid cells. When added alone, SCF induced a similar modest proliferation in primitive and granulo-monocytic progenitors. This suggests that both cell types are able to respond to the cytokine. However, when SCF was omitted from a mixture of multiple cytokines, the reduction in the proliferative response was significantly larger in the population of primitive cells. In the absence of SCF, primitive cells did not expand more than lineage-committed cells (Fig 5). These results may indicate that the seeming selectivity of SCF for primitive progenitors reflects a lower responsiveness of primitive cells to other cytokines rather than a higher responsiveness to SCF. Further studies on the expression and function of additional cytokine receptors on primitive and lineage-committed cells may clarify this. Alternatively, primitive progenitors are more dependent on an SCF-specific
survival signal than granulo-monocytic progenitors. Our data showed a clear difference in the ability of primitive and lineage-committed progenitors to survive in absence of any growth factors, whereas survival was similar in the presence of SCF. Although the extensive death of primitive progenitor cells in the absence of cytokines may seem surprising, the data are in agreement with an earlier report where primitive cells were cultured under similar conditions.9

In conclusion, the present study shows that highly homogeneous populations of lineage-committed and primitive hematopoietic progenitor cells can be identified and purified on the basis of phenotypic criteria. This allowed direct comparison of cytokine effects in short-term assays. Sorted cell populations responded differently to SCF and EPO and showed different abilities to survive in the absence of growth factors. The selective effect of EPO on erythroid progenitor cells could be explained by the specific expression of the EPO-R on these cells. Although primitive, erythroid and granulo-monocytic progenitor cells showed different responses and dependence on SCF, the cells had similar levels of the SCF-R. Therefore, it seems likely that there are additional cell type-specific factors that regulate responses to SCF.

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Expression and function of receptors for stem cell factor and erythropoietin during lineage commitment of human hematopoietic progenitor cells

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