RAPID COMMUNICATION

Sustained, Retransplantable, Multilineage Engraftment of Highly Purified Adult Human Bone Marrow Stem Cells In Vivo

By Curt I. Civin, Grace Almeida-Forada, Mi-Jeong Lee, Johanna Olweus, Leon W.M.M. Terstappen, and Esmail D. Zanjani

Data from many laboratory and clinical investigations indicate that CD34+ cells comprise approximately 1% of human bone marrow (BM) mononuclear cells, including the progenitor cells of all the lymphohematopoietic lineages and lymphohematopoietic stem cells (stem cells). Because stem cells are an important but rare cell type in the CD34+ cell population, investigators have subdivided the CD34+ cell population to further enrich stem cells. The CD34+/CD38- cell subset comprises less than 10% of human CD34+ adult BM cells (equivalent to <0.1% of marrow mononuclear cells), lacks lineage (lin) antigens, contains cells with in vitro replating capacity, and is predicted to be highly enriched for stem cells. The present investigation tested whether the CD34+/CD38- subset of adult human marrow generates human hematopoiesis after transfer to preimmune fetal sheep. CD34+/CD38- cells purified from marrow using immunomagnetic microspheres or fluorescence-activated cell sorting generated easily detectable, long-term, multilineage human hematopoiesis in the human-fetal sheep in vivo model. In contrast, transfer of CD34+/CD38+ cells to preimmune fetal sheep generated only short-term human hematopoiesis, possibly suggesting that the CD34+/CD38- cell population contains relatively early multipotent hematopoietic progenitor cells, but not stem cells. This work extends the prior in vitro evidence that the earliest cells in fetal and adult human marrow lack CD38 expression. In summary, the CD34+/CD38- cell population has a high capacity for long-term multilineage hematopoietic engraftment, suggesting the presence of stem cells in this minor adult human marrow cell subset.

© 1996 by The American Society of Hematology.

EXPRESSION of the CD34 membrane phosphoglycoprotein provides a useful operational definition of the human lymphohematopoietic stem/progenitor cell population, and CD34+ stem/progenitor cells are commonly identified and purified for research and clinical applications.1,6 However, the CD34+ cell population is heterogeneous, including committed progenitor cells of all the lymphohematopoietic lineages, early multipotent progenitor cells, and lymphohematopoietic stem cells (stem cells).7 Stem cells are a rare cell subset, estimated at less than 1% of CD34+ bone marrow (BM) cells, yet they generate the entire cellular repertoire of the lymphohematopoietic system, and they are the normal counterparts of the abnormal cells in myeloid leukemias, myelodysplasias, and aplastic anemias.8 To further define human stem cells, the CD34+ cell population has been subdivided based on differential cellular expression of multiple membrane proteins, including leukocyte differentiation antigens of mature lymphohematopoietic cell lineages ("lin antigens"), HLA-DR, Thy-1, CD33, MDR-1 (pgp170), c-kit and CD45 isoforms, and CD38.9-23 Recent studies have shown that CD34+/CD38- cells comprise less than 10% of human CD34+ adult BM cells (equivalent to <0.1% of marrow mononuclear cells), lack lin antigens, and contain cells with in vitro replating capacity.14-22,24-26 Thus, it is predicted that the CD34+/CD38- cell population is highly enriched for stem cells.

However, the complete phenotype of human stem cells remains unclear, because no available in vitro assay directly measures human stem cell function. The aim of the present investigation was to test more directly whether the minor CD34+/CD38- subset of adult human marrow contains stem cells. Since it has been shown repeatedly that human marrow cells generate human hematopoiesis after transfer to preimmune fetal sheep,26-30 the human-sheep model system can be used as an in vivo assay for the presence of human stem cells in a purified CD34+ cell subset.

MATERIALS AND METHODS

Human marrow cell separation using immunomagnetic microspheres. Human BM was collected from the posterior iliac crests of consenting healthy adults under an Institutional Review Board approved protocol. Because large numbers of marrow cells were needed, in some experiments marrow was obtained from multiple donors; each donor's cells were separated individually by density centrifugation on ficoll-Hypaque (Pharmacia, Piscataway, NJ) to remove potentially incompatible serum. CD34+ cells were then isolated using immunomagnetic microspheres as previously described.11,32 Briefly, CD34+ cells were purified by indirect immunofluorimetric and detached from immunomagnetic microspheres using chymopapain.9,33 Residual mature cells were then depleted using immunomagnetic microspheres coated with monoclonal antibodies (MoAbs) recognizing multiple lin antigens (CD15, CD3, CD5, CD19, CD20, CD36, glycoporphin). The CD34+/lin- cells were

From the Departments of Oncology and Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD; Immunicon, Huntingdon Valley, PA; Becton Dickinson Immunocytometry Systems, San Jose, CA; and Veterans Administration Medical Center, University of Nevada, Reno.

Submitted July 1, 1996; accepted September 10, 1996.

Supported in part by a Grant No. ACS DHP-64 from the American Cancer Society (C.I.C.); and National Institutes of Health Grants No. HL49042 (E.D.Z.), HLS2955 (E.D.Z.), and DK51427 (E.D.Z.).

Financial Disclosure: The Johns Hopkins University holds patents on CD34 monoclonal antibodies and related inventions. C.I.C. is entitled to a share of the sales royalty received by the University under licensing agreements between the University, Becton Dickinson Corporation, and Baxter Healthcare Corporation. C.I.C. has also served as a consultant for these companies. The terms of these arrangements have been reviewed and approved by the University in accordance with its conflict of interest policies.

Presented in part at the December 3-7, 1993 Annual Meeting of the American Society of Hematology, St. Louis, MO.

Address reprint requests to Curt I. Civin, MD, Oncology 3-109, Johns Hopkins Oncology Center, 600 N Wolfe St, Baltimore, MD 21287-5001.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

0006-4971/96/8811-0039$3.00/0
CD34+/38- MARROW ENGRAFTS IN FETAL SHEEP

Further purified using immunomagnetic microspheres coated with CD38 and the CD13 lin antigen to obtain CD34+/lin+/CD38- cells. The reciprocal cell fraction, CD34+/lin-/CD38+ and CD34+/lin-/CD38, was also obtained, but the cell counts of these cells could only be estimated: it was not possible to count these cells accurately because the isolation procedure agglutinates these cells with immunomagnetic microspheres.

Transplantation of human marrow cells into preimmune fetal sheep and analysis of engraftment. Human donor cells were injected into preimmune fetal sheep (<65 days old; term gestation: 145 days) recipients using the amniotic bubble technique, as previously described. The two immunomagnetic bead-purified cell populations, CD34+/lin+/CD38- and CD34+/lin-/CD38+, were shipped overnight from Johns Hopkins to the University of Nevada in RPMI 1640 culture medium (RPMI) containing 10% fetal bovine serum (FBS). In addition, CD34+ stem/progenitor-depleted cells were irradiated (5.3 Gy, 170Cs source) to ablate their proliferative capacity and shipped in RPMI containing 10% FBS, 50 nM interleukin-3 (IL-3; Montsanto, St Louis, MO), and 200 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA), for use as carrier cells. A total of six fetuses were transplanted with cells isolated using immunomagnetic beads (5 × 10^6 cells suspended in 3 × 10^6 irradiated carrier cells). One fetus was absorbed into the ewe during the gestation period. Three fetuses (B2A, B2B, and B9) were killed before birth at 63 days posttransplant, and the remaining two fetuses were allowed to complete gestation. One fetus died at delivery and was lost to study. Lamb B10 was born alive.

A total of 12 preimmune fetal sheep were transplanted with adult human BM CD34+ cells further purified by fluorescence-activated cell sorting (FACS) based on expression of CD38 (see text below and Figs 3 through 5). Each fetus in this latter group received a single intrauterine injection of either 3 × 10^6 CD34+/CD38- cells or 3 × 10^6 CD34+/CD38+ cells suspended in 10^7 maternal sheep red blood cells (SRBCs). All were born alive.

Human donor cell engraftment in BM was assessed after spontaneous live birth or premature surgical delivery. Mononuclear cells were prepared from marrow by density gradient separation and tested for human cells by flow cytometry as previously described. Values for CD antigen expression indicate the percent of the lamb's marrow leukocytes expressing the indicated human-specific antigen. The samples were also evaluated for the presence of human hematopoietic progenitors by karyotyping of individual progenitor-derived colonies, as previously reported. To stimulate optimal growth of both human and sheep hematopoietic colony-forming cells from the chimeras, BM mononuclear cells (BMMCs) were cultured in methylcellulose stimulated by a combination of human erythropoietin, IL-3, and GM-CSF, plus sheep phytohemagglutinin-leukocyte conditioned medium (PHA-LCM). The colonies were counted and processed for karyotyping on days 9 and 19 of incubation. Under these conditions, maximal numbers of sheep colonies develop by day 9. By day 19, most of the sheep colonies degenerate, whereas human colonies are maintained. The percentage of progenitors with human karyotype was determined by the following formula: 100 × (actual number of plucked day 19 colonies with human karyotype)/(total number of colonies on day 9 + total number of colonies on day 19).

Flow cytometric immunophenotyping of purified marrow human cells. The data presented in Table 4 and Figs 1 and 2 were obtained using the MoAb conjugates CD34-PerCP, CD38-APC made by Barney Abrahams and Quadrat Nasarati in the Research Department of Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA). Cell analysis was performed on a modified FACSscan configured by Bob Hofman in the research department of BDIS. This flow cytometer was equipped with a 12-mW Argon ion laser (488 nm), a 30-mW YAG laser (532 nm) and a 10-mW HeNe laser (633 nm). Phycoerythrin (PE) was excited with the YAG laser which results in a sixfold increase in sensitivity compared with excitation at 488 nm. Immunostaining for marrow cell types was done as previously described. The data presented in Tables 1 through 3 and Figs 3 through 5 were obtained using commercial MoAbs and a commercial FACS flow cytometer (BDIS). For the experiment described in Figs 3 through 5, aspirated human BM low-density mononuclear cells were enriched for CD34+ cells (to 75% to 85% purity, based on prior experience) using an immunoaffinity column (CellPro, Bothel, WA) according to manufacturer's instructions. The cells were suspended in phosphate-buffered saline containing 1% bovine serum albumin (Sigma, St Louis, MO) and labeled with CD34-FITC and CD38-PE, then sorted on a FACS Vantage flow cytometer (BDIS). Six preimmune fetal sheep were transplanted by intraperitoneal injection of 3 × 10^6 adult human BM CD34+/CD38- cells (purified by FACS; FACS Vantage; BDIS) into 10^7 maternal SRBCs. An additional six preimmune fetal sheep received 3 × 10^6 CD34+/CD38+ cells suspended in 10^7 maternal SRBCs. All were born alive.

RESULTS

CD34+/lin-/CD38- adult human marrow cells provide sustained multilineage human hematopoiesis in the human-fetal sheep model. Marrow was aspirated from two healthy adult human donors (3.9 × 10^9 total nucleated cells obtained). Each donor's cells were separated individually on ficoll-Hypaque to remove potentially incompatible serum (1.1 × 10^9 marrow mononuclear cells obtained). Next, CD34+ cells were purified by indirect immunoaffinity, yielding 2.0 × 10^7 cells. Residual mature cells were then depleted using immunomagnetic microspheres coated with MoAbs recognizing multiple lin antigens, resulting in 1.2 × 10^7 CD34+/lin- cells, which were further purified using immunomagnetic microspheres coated with CD38 and the CD13 lin antigen. 1.3 × 10^6 CD34+/lin-/CD38- cells were obtained: −0.1% of starting cells, 90% viable by trypan blue dye exclusion. The reciprocal cell fraction, CD34+/lin+/CD38+ cells, was estimated at 10^7 cells. The two purified cell populations, CD34+/lin-/CD38- and CD34+/lin+/CD38+, were shipped overnight from Johns Hopkins to the University of Nevada, along with irradiated CD34+ carrier cells as described in Materials and Methods.

Lamb B10, when a preimmune fetus (day 58 gestation), had received CD34+/lin-/CD38- cells intraperitoneally. BM was aspirated from this animal on postnatal days 9, 40, 81, 146, and 289, and tested by flow cytometry and karyotyping of individual hematopoietic colonies. Over a 1-year period after transplant, 0.4% to 5% of the lamb's marrow cells expressed the human-specific CD45 common leukocyte antigen and 5% to 15% of the hematopoietic colonies had human karyotypes (Table 1).

Human hematopoietosis in fetal sheep transplanted with CD34+/lin-/CD38- or CD34+/lin+/CD38+ adult human marrow cells purified using immunomagnetic microspheres. A second similar marrow cell purification from two adult human donors resulted in 1.2 × 10^6 CD34+/lin-/CD38- cells (−0.1% of starting cells, 90% viable) and 6 × 10^6 CD34+/lin+/CD38+ cells. Two twin preimmune...
Table 1. CD34+/lin-/CD38- Adult Human Marrow Cells Provide Sustained Multilineage Human Hematopoiesis in the Human-Fetal Sheep Model

<table>
<thead>
<tr>
<th></th>
<th>Day 9</th>
<th>Day 40</th>
<th>Day 81</th>
<th>Day 146</th>
<th>Day 289</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-Mix</td>
<td>0</td>
<td>2/47</td>
<td>1/13</td>
<td>1/19</td>
<td>0/20</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>7/64</td>
<td>9/56</td>
<td>6/48</td>
<td>3/32</td>
<td>4/58</td>
</tr>
<tr>
<td>BFU-E</td>
<td>3/70</td>
<td>2/43</td>
<td>2/31</td>
<td>0/22</td>
<td>1/19</td>
</tr>
<tr>
<td>CD45*</td>
<td>3.1%</td>
<td>4.2%</td>
<td>5.1%</td>
<td>1.1%</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

This lamb (no. B10), when a preimmune fetus (58 days gestation) received 5 x 10^6 CD34+/lin-/CD38- cells plus 3 x 10^6 irradiated CD34+ carrier cells intraperitoneally. BM was aspirated from this animal on postnatal days 5, 40, 81, 146, and 289, and tested by flow cytometry and karyotyping of individual hematopoietic colonies. Values for colony-forming cells indicate the percent of the plated colonies with human karyotypes, as described in Materials and Methods. Values for CD45 expression indicate the percent of the lamb’s marrow leukocytes expressing human-specific CD45 common leukocyte antigen.

Table 2. Human Hematopoiesis in Fetal Sheep Transplanted With CD34+/lin-/CD38- or CD34+/lin-/CD38, CD13+ Adult Human Marrow Cells

<table>
<thead>
<tr>
<th></th>
<th>CD34+/lin-/CD38-</th>
<th>CD34+/lin-/CD38, CD13+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 wk</td>
<td>50 wk</td>
</tr>
<tr>
<td>CFU-Mix</td>
<td>14/53</td>
<td>6/46</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>15/50</td>
<td>10/47</td>
</tr>
<tr>
<td>BFU-E</td>
<td>6/74</td>
<td>4/37</td>
</tr>
<tr>
<td>CD45+</td>
<td>4.9%</td>
<td>2.3%</td>
</tr>
<tr>
<td>CD34+</td>
<td>0.8%</td>
<td>0.7%</td>
</tr>
<tr>
<td>CD22+</td>
<td>0.7%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Immunomagnetic marrow cell purification from two adult human donors resulted in 1.2 x 10^6 CD34+/lin-/CD38- cells (~0.1% of starting cells, 90% viable, 80% CD34+) and 6 x 10^5 CD34+/lin-/CD38, CD13+ cells. Two twin preimmune fetal sheep (B2A and B2B) each received 5 x 10^6 CD34+/lin-/CD38- cells intraperitoneally on day 56 gestation, as in Table 1. A single preimmune fetus (B9, 54 days gestation) was transplanted with all of the estimated 5 x 10^6 CD34+/lin-/CD38, CD13+ cells. These three fetuses were killed 9 weeks after transplant, and marrow was obtained for flow cytometry and colony-forming assays, as in Table 1.

Human hematopoiesis in fetal sheep serially transplanted with human CD45+ cells obtained from hematopoietic chimeras which had been transplanted previously with either CD34+/lin-/CD38- or CD34+/lin-/CD38, CD13+ adult human marrow cells. To test the human hematopoietic reserve of the chimeras described immediately above, human CD45+ hematopoietic cells were immunopurified from these animals and these cells were serially transferred to new pairs of twin fetal lambs (Table 3). Human CD45+ hematopoietic cells from the pooled fetal liver and marrow cells of these animals (B2A and 2B combined; B9 separately) were obtained by immune adherence and transferred serially to new pairs of twin fetal lambs (fetal liver was used, in addition to fetal marrow, to provide more human cells). Fetal lambs nos. 86 and 89 each received 6 x 10^7 CD45+ cells from the no. B2A/B animals which previously had been transplanted with CD34+/lin-/CD38- cells, and fetal lambs nos. 86 and 87 each received 2 x 10^7 CD45+ cells from the no. B9 animal previously transplanted with CD34+/lin-/CD38, CD13+ cells. At 14 and 50 weeks after these serial transplants, marrow was aspirated from the 2- and 36-week-old lambs and assayed as in Tables 1 and 2.

Table 3. Human Hematopoiesis in Fetal Sheep Serially Transplanted With Human CD45+ Cells From Hematopoietic Chimeras Previously Transplanted With Either CD34+/lin-/CD38- or CD34+/lin-/CD38, CD13+ Adult Human Marrow Cells

<table>
<thead>
<tr>
<th></th>
<th>CD34+/lin-/CD38-</th>
<th>CD34+/lin-/CD38, CD13+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 wk</td>
<td>50 wk</td>
</tr>
<tr>
<td>CFU-Mix</td>
<td>3/28</td>
<td>1/21</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>4/20</td>
<td>2/38</td>
</tr>
<tr>
<td>BFU-E</td>
<td>1/12</td>
<td>2/39</td>
</tr>
<tr>
<td>CD45+</td>
<td>1.4%</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

CD45+ (human) hematopoietic cells from the pooled fetal liver and marrow cells of these animals (#62A and B2B combined; B9 separately) were obtained by immune adherence and transferred serially to new pairs of fetal lambs. Fetal lambs nos. 88 and 89 each received CD45+ cells from the no. B2A/B animals primarily transplanted with CD34+/lin-/CD38- cells, and fetal lambs nos. 86 and 87 each received CD45+ cells from the no. B9 animal previously transplanted with CD34+/lin-/CD38, CD13+ cells. At 14 and 50 weeks after these serial transplants, marrow was aspirated from the 2- and 36-week-old lambs and assayed as in Tables 1 and 2.
Fig 1. Flow cytometric analysis of adult BM enriched for hematopoietic progenitor cells. Forward light scatter, orthogonal light scatter, CD34 PerCP, and CD38 APC expression of isolated CD34+ adult BM samples. The signals representing CD34+/CD38- cells are displayed in red, CD34+/CD38- cells in blue, and CD34- cells in gray. The arrows indicate a population which remained consistently present throughout the subsequent selection procedures. The figure was created with a custom-made Macintosh data representation software program made by Ben Verwer in the research department of BDIS.

Fig 2. Flow cytometric immunophenotyping of CD34+ and CD34+/CD38- cell subsets. Expression of CD38 APC and HLADR FITC (A, C, E, and G) and CD38 APC and CD13 PE (B, D, F, and H) of the isolated CD34+ adult BM samples. Only CD34+ cells are shown in the figure (colored black in Fig 1). The CD34+/CD38- cells are shown in red, CD38+/HLADR- cells green, and all other CD34+ cells gray. The dotted line represents the staining level of the PE-labeled IgG, control. In (B) and (D), the letter L indicates the location of the CD34+ lymphoid progenitors. The figure was created with a custom-made Macintosh data representation software program made by Ben Verwer in the research department of BDIS.
serial transplants, marrow was aspirated from the lambs at 2 and 36 weeks age and assayed as in Tables 1 and 2. The lambs that had received human cells derived from CD34+/lin/CD38- cells had easily detectable human hematopoiesis, although there was a decrease in the percent human hematopoiesis by 50 weeks after transplant. In contrast, human cells were not detected from the animals which received CD34+/lin/(CD38,CD13)- cells (Table 3).

Immunophenotypic characterization of purified hematopoietic progenitor cells. To further characterize the types of cells obtained by each step in these purification steps, separate experiments were performed retrospectively and the resulting cells were subjected to detailed immunophenotypic comparisons. Four immunoaffinity procedures were used to select for hematopoietic stem/progenitor cells in these experiments: procedure 1, positive selection of CD34+ cells; procedure 2, positive selection of CD34+ cells followed by depletion with the lin MoAbs CD15,3,5,19,20,36 and glycoporphin; procedure 3, as in procedure 2 but with an additional depletion step with CD38; and procedure 4, as in procedure 2 but with an additional depletion step with both CD38 and CD13. After CD34+ selection and lin depletion of a single large marrow cell donation, the cells were split into two samples, one used for CD38 depletion and one used for depletion with the combination of CD38 and CD13. The purified cells of each of these four immunoaffinity selection procedures were stained with CD34-PerCP, HLADR-FITC, CD38-APC, and a IgGl-PE control antibody or CD13-PE antibodies. The lymphoid-committed CD34+ cells do not express CD13 and are indicated with L in panels B, D, F, and H indicate the levels of staining with the PE labeled IgGl control antibody. The percent CD34+/CD38- cells of each of the fractions clearly express CD15,3,5,19,20,36 and glycophorin clearly reduced the relative fraction of the lymphoid committed fraction (compare panels B v D). The depletion with the lin antibodies CD15,3,5,19,20,36 and glycoporphin clearly reduced the relative fraction of the lymphoid committed fraction (compare panels B v D), which is likely due mainly to the presence of CD19 in the lineage cocktail. As shown in Table 4, the addition of CD13 to the depletion protocol resulted in a slight decrease in the percent CD34+/CD38- cells. Comparison of panels F versus H showed a slight decrease in CD13 intensity, which suggests that both depletion procedures 3 and 4 are effective in eliminating the CD34+ cells expressing high levels of CD38 while retaining populations relatively pure for CD34+/CD38- cells.

**Table 4. Immunophenotypic Analysis of Adult BM Cells Purified By Four Different Immunomagnetic Bead Selection Procedures**

<table>
<thead>
<tr>
<th>% CD34+</th>
<th>% CD34+/CD38-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td>Unseparated marrow</td>
<td>1.4</td>
</tr>
<tr>
<td>CD34+ selected (procedure 1)</td>
<td>87.6</td>
</tr>
<tr>
<td>CD34+/lin selected (procedure 2)</td>
<td>90.4</td>
</tr>
<tr>
<td>CD34+/lin/CD38- selected (procedure 3)</td>
<td>74.9</td>
</tr>
<tr>
<td>CD34+/lin/(CD38,CD13)- selected (procedure 4)</td>
<td>75.6</td>
</tr>
</tbody>
</table>

In each of two repetitive experiments, the immunomagnetic cell purifications were done simultaneously on a single large marrow cell sample, as described in text. The values in this table derive from the same experiments as shown in Figs 1 and 2.
human cells present in the total BMMCs of the sheep transplanted with human CD34+/CD38- cells were: CD45%, 2.3%; CD34+, 0.34%; CD3~, 0.68%; CD22+, 0.52%.

DISCUSSION

These experiments indicate that immunomagnetic bead-purified CD34+/lin-CD38- or FACS-purified CD34+/CD38- cells from adult human marrow generate easily detectable, sustained, (re)transplantable, multilineage human hematopoiesis in the human-fetal sheep in vivo model. These sustained levels of human hematopoiesis are similar to results obtained after transplantation of the whole CD34+ cell population in this model system. It has been shown that the human component of chimERIC hematopoiesis can be expanded to much higher levels upon postnatal administration of human hematopoietic growth factors to the lambs. Based on their generation of short-term but not long-term human hematopoiesis after transfer, the immunomagnetic bead-purified CD34+/lin-CD38- cells and CD34+/CD38- cells which had been purified by FACS sorting, as described in text and Figs 2 and 3, were reanalyzed by flow cytometry. The top row of FACScan dot plots shows the light-scattering properties of the CD34+/CD38- cells (left column) and CD34+/CD38- cells (right column). The bottom row depicts the CD38 versus CD34 correlated immunofluorescence of the two purified cell populations. Both populations were greater than 99.9% pure by immunofluorescence.
marrow cell subsets appear to have lower specific activity of multilineage repopulating, putative stem cell function in this human-fetal sheep in vivo model.

The immunomagnetic microsphere technique used herein is similar to procedures currently in clinical use for stem/progenitor cell enrichment.\textsuperscript{3,6,7} Higher cell purity of the putative stem cell fractions can be achieved by FACS.\textsuperscript{18,22} Therefore, FACS was used to confirm the presence of multilineage long-term engraftment capacity of the CD34\textsuperscript{+}/CD38\textsuperscript{−} cell population. Interestingly, animals transplanted with human CD34\textsuperscript{+}/CD38\textsuperscript{−} cells had evidence of multilineage engraftment for several months, but human hematopoiesis eventually disappeared in these animals. These results are reminiscent of the results of Jordan and Lemischka,\textsuperscript{27} and may suggest that the CD34\textsuperscript{+}/CD38\textsuperscript{−} cell population contains relatively early multipotent hematopoietic progenitor cells, but not stem cells. An alternative interpretation is that the CD34\textsuperscript{+}/CD38\textsuperscript{−} cell population contains a much lower frequency of stem cells, compared with the CD34\textsuperscript{+}/CD38\textsuperscript{−} cell population (note that the animals transplanted with FACS-purified CD34\textsuperscript{+}/CD38\textsuperscript{−} cells received 100-fold higher numbers of cells than did the animals transplanted with CD34\textsuperscript{+}/CD38\textsuperscript{−} cells [Fig 5]).

Retroviral labeling of CD34\textsuperscript{+}/CD38\textsuperscript{−} cells would rigorously test for the presence of single stem cells generating the multiple lymphohematopoietic lineages (versus multiple long-lived lineage-restricted progenitor cells).\textsuperscript{39,38} Although the longevity of the human hematopoiesis obtained in our experiments to date argues against this alternative interpretation. Future experiments will attempt further purification of stem cells using HLADR or other markers differentially expressed within the CD34\textsuperscript{+}/CD38\textsuperscript{−} cell compartment.\textsuperscript{3,6,17,23,30} These small subsets can then be assessed for the capacity to generate human hematopoiesis in the human-fetal sheep model. Immunologic analysis of the purified CD34\textsuperscript{+}/CD38\textsuperscript{−} cells indicates that almost all CD34\textsuperscript{+}/CD38\textsuperscript{−} cells express HLADR (Fig 2).\textsuperscript{34,24,38}

Because most malignant solid tumors lack expression of CD34, purified CD34 stem/progenitor cells are currently used to provide hematopoietic rescue after myelosuppressive antineoplastic therapy.\textsuperscript{1,5,6,33} Although acute leukemias are commonly treated by BM transplant, use of purified CD34\textsuperscript{+} cell transplantation for acute leukemias is limited by the fact that about half of acute leukemia cases express the CD34 cell membrane antigen.\textsuperscript{40} Depletion of the CD34\textsuperscript{+} cells coexpressing CD38 (and/or leukocyte differentiation antigens) as done herein might extend the use of purified stem/progenitor cell transplantation to treatment of acute leukemias.\textsuperscript{40} A caveat is that the stem cells for acute myeloid leukemia may have the CD34\textsuperscript{+}/CD38\textsuperscript{−} phenotype, even in cases where the major population of leukemic cells from the patient are CD34\textsuperscript{−} and/or CD38\textsuperscript{−}.\textsuperscript{41} In addition, purified stem cells are ideal target cells for many gene therapy applications.\textsuperscript{14,22} This work extends the prior in vitro evidence\textsuperscript{16,22,23,25} that the earliest cells in fetal and adult human marrow lack CD38 expression. The CD34\textsuperscript{+}/CD38\textsuperscript{−} cell population has a high capacity for long-term multilineage hematopoietic engraftment, suggesting the presence of lymphohematopoietic stem cells in this minor adult human marrow cell subset.

ACKNOWLEDGMENT

The authors are grateful for the expert assistance of Shahina Amin, who performed the flow cytometric analysis of the immunomagnetic bead purified cells described in Table 4 and Figs 1 and 2.

REFERENCES

CD34+/38+ MARROW ENGRAFTS IN FETAL SHEEP


Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo

CI Civin, G Almeida-Porada, MJ Lee, J Olweus, LW Terstappen and ED Zanjani