Persistence of Human Multilineage, Self-Renewing Lymphohematopoietic Stem Cells in Chimeric Sheep

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We have previously reported the ability of uncharacterized human bone marrow (BM) cells to engraft into preimmune fetal sheep, thereby creating sheep-human chimera suitable for in vivo examination of the properties of human hematopoietic stem cells (HSC). Adult human bone marrow CD34+ HLA-DR- cells have been extensively characterized in vitro and have been demonstrated to contain a number of primitive hematopoietic progenitor cells (PHPC). However, the capacity of such highly purified populations of human marrow CD34+ HLA-DR- cells to undergo in vivo self-renewal and multipotential lymphohematopoietic differentiation has not been previously demonstrated. To achieve that, human CD34+ HLA-DR- cells were transplanted in utero into immunoincompetent fetal sheep to investigate the BM-populating potential of these cells. Long-term chimerism, sustained human hematopoiesis, and expression of human cells belonging to all human blood cell lineages were demonstrated in two animals for more than 7 months' posttransplantation. Chimeric BM contained erythroid, granulocytic/monocytic, and megakaryocytic hematopoietic progenitor cells, as well as the primitive high proliferative potential colony-forming cell (HPP-CFC). Under a variety of in vitro experimental conditions, chimeric BM cells gave rise to human T cells expressing T-lymphocyte-specific markers, human natural killer (NK) cells, and human IgG-producing B cells. In vivo expansion and possibly self-renewal of transplanted PHPC was confirmed by the detection in chimeric BM 130 days' posttransplantation of CD34+ HLA-DR- cells, the phenotype of human cells constituting the stem-cell graft. These studies demonstrate not only the BM-populating capacity, multipotential differentiation, and most likely self-renewal capabilities of human CD34+ HLA-DR- cells, but also that this BM population contains human HSC. Furthermore, it appears that this animal model of xenogeneic stem-cell transplantation is extremely useful for in vivo examination of human hematopoiesis and the behavioral and functional characteristics of human HSC.

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Submitted March 30, 1993; accepted August 10, 1993.

Supported by Grants No. HL4-6556, HL4-9042, and HL4-8378 from the Department of Veterans Affairs, the G. Harold and Leila Y. Mathers Charitable Foundation (E.D.Z.), National Cancer Institute Grant No. PO1 CA59348-01, a Phi Beta Psi Sorority research award (E.F.S.), and by National Institutes of Health Grantees No. RO1 HL46548-01 (RH), E.F.S. and K.C. are recipients of Junior Faculty Research Awards from the American Cancer Society.

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0006-4971/93/8211-0024$3.00/0
for substantial periods of time in sheep transplanted in utero early in gestation with human adult BM cells or fetal liver cells without the need of cotransplantation of other human tissues into the recipient animals. The success of our sheep model is dependent on the incompetent immunologic status of the recipient fetus and the presence, within the graft, of functional pluripotent HSC. Using this animal model, we have previously shown that a graft of human PHPC consisting of phenotypically uncharacterized BM cells sustained the engraftment and multilineage differentiation of human hematopoietic cells in host BM.

We now report that the in utero transplantation of grafts containing $4 \times 10^6$ to $10 \times 10^6$ purified adult human CD34$^+$ HLA-DR$^-$ BM cells into developing sheep fetuses resulted in the creation of chimeric sheep that express a full complement of human hematopoietic cell lineages. Over a period of 220 days’ posttransplantation, chimeric BM harbored human progenitor cells capable of differentiation into the myeloid, erythroid, megakaryocytic, and lymphoid lineages. In addition, transplanted CD34$^+$ HLA-DR$^-$ cells were capable of undergoing in vivo expansion as evidenced by the continued presence of CD34$^+$ HLA-DR$^-$ cells, as well as assayable primitive colony forming cells such as HPP-CFC within these animals, indicating that this population of BM cells contains significant numbers of adult human pluripotent stem cells.

**MATERIALS AND METHODS**

**Cell separation.** Human BM was collected from the iliac crest of adult healthy donors after obtaining informed consent according to guidelines established by the Human Investigation Committee of the Indiana University School of Medicine. Low-density cells were separated over Ficoll/Hypaque and suspended in phosphate-buffered saline (PBS) containing 0.01% EDTA wt/vol and 1.0 g/L D-glucose. Cells were then fractionated in a standard chamber of a counterflow centrifugal elutriation (CCE) system (Beckman Instruments, Palo Alto, CA) sterilized with 70% ethanol to yield fractions eluting at 12 and 14 mL/min (Fr 12 to 14) as previously described.

**Immunofluorescence staining and cell sorting.** Fr 12 to 14 BM cells obtained by CCE were stained over ice with fluorescein isothiocyanate (FITC)-conjugated CD34 and phycoerythrin (PE)-conjugated HLA-DR (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) for 20 minutes. The cells were then washed and sorted on a Coulter Epics 753 flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a Cicero high-speed computer (Cytomation, Fort Collins, CO) as previously described. Positivity for each fluorochrome was defined as fluorescence greater than 99% of that of isotype controls. CD34$^+$ HLA-DR$^-$ cells were sorted separately at a rate of 3,500 cells/s. Cell viability after sorting was greater than 98%. To examine whether Fr 12 to 14 mL/min CD34$^+$ HLA-DR$^-$ cells contained cells expressing lymphoid commitment antigens, phenotypic analysis of CD34$^+$ HLA-DR$^-$ cells was performed. To that effect, biotinylated HLA-DR, which was subsequently developed with streptavidin-allophycocyanin (Molecular Probes, Eugene, OR), PE-conjugated CD34, and FITC-conjugated lineage markers, were used in three-color flow-cytometric analysis as previously described. These analyses indicated that the fraction of CD34$^+$ HLA-DR$^-$ cells found within the CCE fractions of 12 to 14 mL/min contained less than 1% CD7$^+$ and CD3$^+$ cells and no detectable numbers of cells expressing CD10, CD19, CD16, or CD56. However, approximately 18% of these cells expressed CD15 and only 23% expressed CD38 (data not shown). To isolate chimeric CD45$^+$ cells from sheep no. 273 and 306, BM cells from these animals were separated over Ficoll/Hypaque and were then stained on ice with purified CD45 for 20 minutes. After washing, the CD45 antibody was developed with a Texas red (TR)-conjugated goat anti-mouse IgG, second-step reagent. Stained cells were then sorted as described above, collecting TR-positive (CD45$^+$) cells and negative cells (CD45$^-$. Phenotypic analysis of chimeric BM or peripheral blood low-density cells, sorted CD45$^-$ and CD45$^+$ cells, or cultured cells was performed as previously described. Briefly, cells were stained on ice with combinations of FITC- and PE-conjugated monoclonal antibodies identifying CD45 (where applicable), CD14, CD3, CD4, CD8, CD7, CD25, CD19, HLA-DR, and a combination of CD16 plus CD56 (CD16/CD56) (BDIS) for 20 minutes. Nonspecific isotype-matched antibodies were used for the determination of background fluorescence. Cells were then washed with PBS containing 1% human serum albumin, resuspended in 0.5 mL of the same buffer, and analyzed on a FACScan (BDIS).

**In utero transplantation.** Human HSC grafts were transplanted into ten 45- to 50-day-old sheep fetuses as previously described after obtaining approval for the use of animals from the Animal Care and Use Committee of the University of Nevada. Briefly, the uterus of pregnant ewes was accessed through a midline laparotomy incision. A transverse incision was then made through the myometrium and chorion, which allowed for the manipulation of the fetus into an amniotic bubble. Human HSC were then injected intraperitoneally through a 22-gauge needle. The myometrium was then closed in a double layer and the pregnancy allowed to proceed to term. Five of 10 transplanted animals were born alive and were available for analysis. At different time points after birth, 5- to 10-mL BM samples from the iliac crests were collected from these animals for analysis.

**Hematopoietic progenitor-cell assays.** Cells to be assayed for their clonogenic potential were suspended at different cell concentrations in 35-mm plastic tissue culture dishes (Costar Data Packaging, Cambridge, MA) containing 1 mL of 30% fetal calf serum (FCS), 1.5×$10^{-5}$ mol/L 2-mercaptoethanol, 1.1% methylcellulose, 2.5 ng interleukin (IL)-3, 2.5 ng granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 ng c-kit ligand, and 2 U erythroid-stimulating factor (EPO) in Iscove's modified Dulbecco's medium. Culture dishes were incubated at 37°C in a 100% humidified atmosphere of 5% CO$_2$ in air. Erythroidic bursts (BFU-E), granulocyte-macrophage colony-forming units (CFU-GM), and mixed lineage (CFU-GEMM) colonies were scored after 14 to 17 days, while HPP-CFC-derived colonies were scored after 28 days of culture according to previously established criteria. Cells were assayed for CFU-megakaryocyte (CFU-MK) colonies in a serum-depleted method previously detailed by Bruno et al. Cells were suspended in a 1-mL serum-substituted fibrin clot with 1 ng of IL-3 in 35-mm culture dishes and incubated at 37°C in a 100% humidified atmosphere containing 5% CO$_2$ in air. Two weeks to 18 days later, the cultures were fixed in situ and stained using rabbit anti-human platelet glycoprotein antigen, and FITC-conjugated goat F(ab')$_2$-specific anti-rabbit IgG (Tago, Burlingame, CA). The rabbit anti-human platelet glycoprotein antigen was shown to be nonreactive with sheep BM cells (data not shown). Megakaryocyte colonies were enumerated on a fluorescence microscope and a positive colony was defined as a cluster of three or more fluorescent cells. Colony counts were standardized to reflect number of colonies per 5×10$^6$ plated cells.

**Propagation of T lymphocytes in vitro.** Chimeric BM cells obtained 190 days' posttransplantation were stained with CD45 and sorted as described above to yield CD45$^+$ and CD45$^-$ cells. Both phenotypes alongside BM cells from a control sheep were then cultured at 300 cells per well in round-bottomed 96-well plates in
RPNI 1640 medium supplemented with 10% FCS and 1% L-glutamine containing 1% phytohemagglutinin (PHA; Wellcome Diagnostics, Dartford, England) and 5% T-cell growth factor (TCGF; 5,000 rad) cells of the Epstein-Barr virus (EBV)-transformed B-cell line JY. The plates were incubated at 37°C in a 100% humidified atmosphere of 5% CO₂ in air and were fed with 10 μL/well of TCGF on day 7. After 14 days in culture, wells within a group that contained harvested colonies were harvested and the cells were washed and stained for flow-cytometric analysis as described above.

Immunoglobulin synthesis in vitro. Production of human immunoglobulins by CD45+ and CD45- chimeric BM cells was induced in vitro with pokeweed mitogen (PWM). Chimeric BM cells from sheep no. 273 and 306 collected 190 days posttransplantation were stained with CD45 and sorted to yield CD45+ and CD45- cells as described above. Between 15 × 10⁴ and 5 × 10⁴ sorted cells along with control human and sheep low-density BM cells were delivered into six to 12 wells of a round-bottomed 96-well plate containing 200 μL of RPMI 1640 medium supplemented with 20% FCS and containing PWM (Gibco, Detroit, MI) at a final concentration of 50 μg/mL. Cells were incubated at 37°C for 7 days, after which the supernatants were collected and assayed for the presence of human immunoglobulin in an enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottomed 96-well plates were coated with 4 μg/mL of either goat anti-human IgG or IgM and incubated overnight at 4°C. The wells were washed three times with PBS containing 0.05% Tween-20 and blocked for 1 hour at 25°C with 2% goat serum. The plates were incubated at 37°C for 2 hours with a 1:6,000 dilution of goat anti-human IgG or IgM conjugated with horse radish peroxidase (Southern Biotechnology, Birmingham, AL). After washing, tetramethyl benzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and the reaction allowed to develop in the dark at room temperature. The reaction was terminated when appropriate by the addition of 0.2 mol/L of H₂SO₄, and the color intensity was measured on a Titertek Multiskan (Labsystems, Finland) microtiter plate reader.

Detection of human β-globin sequences. The presence of human β-globin DNA sequences in chimeric BM cells and in hematopoietic colonies was detected by the polymerase chain reaction (PCR). To prepare DNA, cells were suspended in PBS and pelleted by centrifugation. The cells were then resuspended in 40 μL of water and heated for 10 minutes at 95°C followed by proteinase K digestion (1 hour at 55°C) and inactivation (15 minutes at 95°C). For PCR amplification, 10 μL of each DNA suspension was added to the prescribed reaction mixture (GeneAmp, Perkin Elmer Cetus, Norwalk, CT) in the presence of 1.5 mmol/L of MgCl₂ and 100 pmol/L of each primer (CAATCCAGCTACCCATTCTGC and GGCTTGGACATCCTGGATTC). Thirty cycles of denaturation (94°C, 3 minutes), annealing (56°C, 3 minutes), and polymerization (72°C, 3 minutes) were performed. Samples were prepared for Southern blotting as previously described using a 32P-labeled β-globin probe. Signal detected corresponds to the expected 345-bp β-globin PCR product.

RESULTS

Establishment of chimerism in sheep transplanted in utero with human CD34+ HLA-DR+ bone marrow cells. Chimerism in three animals transplanted in utero at 45- to 50-day gestational age with isolated human CD34+ HLA-DR+ BM cells was documented by the detection of cells expressing the human common leukocyte antigen, CD45 (Fig 1). At 120 days posttransplantation (~20 days after birth), BM samples from the three sheep contained 8.5% (sheep no. 273), less than 1% (sheep no. 298), and 11% (sheep no. 306) human CD45+ cells (Fig 1). Karyotypic analysis of peripheral blood samples from these animals demonstrated the presence of cells with human karyotypes in two animals only, sheep no. 273 and 306 (data not shown). Furthermore, peripheral blood of sheep no. 273 and 306, but not that of sheep no. 298, contained CD45+, CD14+, CD4+, CD8+, CD19+, and CD16+/CD56+ human cells (Fig 2). Chimerism in animals no. 273 and 306 was reconfirmed again 140 days postengraftment through the detection of CD45+ cells. However, at this time, no human cells could be detected in the BM of sheep no. 298. It is not apparent why long-term chimerism in sheep no. 298 was not established. Sheep no. 273 and 306 continued to be chimeric at day 220 posttransplantation.

Detection of myeloid, erythroid, and megakaryocytic lineages in chimeric sheep. The ability of chimeric BM cells to form human hematopoietic colonies was assessed 190 days following in utero transplantation. Using flow-cytometric cell sorting, CD45+ (human in origin) and CD45- (sheep in origin) cells were isolated from chimeric BM samples and assayed along with human and control sheep BM cells in hematopoietic progenitor cell assays. These assays allowed for the determination of the range of human progenitor cells of various cell lineages present in the transplanted sheep
Flow-cytometric analysis of chimeric peripheral blood from sheep no. 273 obtained 120 days' posttransplantation. Only cells contained within the gate shown in the side-scatter (SSC) versus forward-scatter (FSC) histogram were considered for analysis. The positions of the vertical and horizontal cursors establishing positivity for any of the selected markers were determined by the use of isotype-matched, fluorochrome-conjugated nonspecific antibodies. The percentage of positive cells for any given marker is indicated in the appropriate quadrant. Similar staining patterns were obtained with chimeric BM cells from sheep no. 306 (data not shown).

(Table 1). Only control human BM cells and CD45+ cells from sheep no. 273 contained human erythroid, megakaryocytic, and multilineage hematopoietic progenitor cells. In addition, CD45+ cells from sheep no. 306 contained both human erythroid and megakaryocytic progenitor cells. Such progenitors were not detected in control sheep BM cells and the CD45- fraction of chimeric marrow cells from either animal. Of interest, is that chimeric BM cells from sheep no. 273 gave rise to HPP-CFC, colonies arising from primitive progenitor cells capable of both self-renewal and multilineage (monocytic and granulocytic) differentiation.11,28 In these experiments, HPP-CFC were present in the CD45+ fraction of sheep no. 273 BM and in normal human BM cells only. Culture conditions used in these studies did not support the growth of HPP-CFC when assayed from any of the control sheep BM samples.

As can be seen in Table 1, the number of hematopoietic colonies detected in the CD45+ fraction of the marrows of sheep no. 273 and 306 was equal to, or greater than, those detected in normal human low-density BM cells. This observation is likely due to the percentage of CD34+ cells detected in chimeric CD45+ cells (Fig 3; see below) being higher than the percentage of CD34+ cells normally found in human BM.2,25,29,30 As a result, it appears that more CD34+ cells (and as a consequence, more hematopoietic progenitor cells) were present in a given number of isolated CD45+ chimeric BM cells than in normal human low-density BM cells.

To investigate further the nature and origin of isolated CD45+ cells and progenitor cells present within chimeric animals, PCR was used to detect the presence of the human β-globin gene in CD45+ cells and in cells contained in individual hematopoietic colonies.29 As expected, cells from an individual BFU-E cloned from human BM cells, but not from control sheep BM cells, contained the human β-globin gene (Fig 4). Furthermore, chimeric CD45+ cells, and individual BFU-E cloned from these cells contained the human β-globin gene, demonstrating the human origin of the hematopoietic colonies generated from chimeric CD45+ cells (Table 1).

Establishment of lymphopoiesis in transplanted animals. Having demonstrated the presence of myeloid, erythroid, megakaryocytic, and multilineage progenitor cells, as well as HPP-CFC, in the BM of sheep no. 273 and 306, we next investigated whether these marrows also contained functional human lymphoid elements. CD45+ and CD45- fractions of chimeric BM cells obtained 190 days' posttransplantation were used to examine the mitogen re-

### Table 1. Colony Formation by Chimeric BM Cells

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Day 12 CFU-MK</th>
<th>Day 17 BFU-E</th>
<th>Day 17 CFU-GM</th>
<th>Day 17 CFU-GEMM</th>
<th>Day 17 HPP-CFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human BM</td>
<td>2.3 ± 0.4</td>
<td>16.0 ± 2.8</td>
<td>101 ± 4.2</td>
<td>3.0 ± 1.4</td>
<td>1.0 ± 1.4</td>
</tr>
<tr>
<td>Control sheep</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>31.5 ± 3.5</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Sheep no. 273</td>
<td></td>
<td></td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>6.0 ± 8.4</td>
</tr>
<tr>
<td>CD45+</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>13.0 ± 1.4</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>CD45-</td>
<td>4.5 ± 2.1</td>
<td>50.0 ± 16.8</td>
<td>372 ± 16.8</td>
<td>6.0 ± 8.4</td>
<td>6.2 ± 8.4</td>
</tr>
<tr>
<td>Sheep no. 306</td>
<td></td>
<td></td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>CD45+</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>8.5 ± 0.7</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>CD45-</td>
<td>8.0 ± 0.0</td>
<td>16.0 ± 0.0</td>
<td>96.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

* Cells were assayed 190 days following in utero transplantation of fetal sheep with adult human CD34+ HLA-DR BM cells. Percent chimerism at this time point: sheep no. 273, 4.5%; sheep no. 306, 7.5%.

† Cells were plated in a fibrin clot assay for the determination of CFU-MK and in a methylcellulose assay for the determination of BFU-E, CFU-GM, CFU-GEMM, and HPP-CFC as described in Materials and Methods.
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**Fig 3.** Assessment of the expression of CD34 and HLA-DR by chimeric BM cells from sheep no. 306 collected 130 days' posttransplantation. The distribution of CD34 (along the X axis) and HLA-DR (along the Y axis) among sorted sheep (CD45⁻) and human (CD45⁺) cells is shown in the left and right histograms, respectively. The percentage of CD34⁺ HLA-DR⁺ and CD34⁺ HLA-DR⁻ cells detected in CD45⁺ cells is given in the upper right and lower right quadrants of the right histogram, respectively.

Response capability of chimeric human T lymphocytes and to assess the capacity of chimeric human B cells to produce human immunoglobulins.

In response to PHA and IL-2 stimulation, human T cells expressing CD3, CD4, and CD8, as well as natural killer (NK) cells (cells positive for CD16 and CD56) were generated in vitro from CD45⁺ cells derived from sheep no. 273 and 306 (Fig 5A and B). As expected, the majority of these activated T lymphocytes expressed HLA-DR and, in the case of sheep no. 273, approximately 20% of the cells expressed CD25. Cells generated in these assays from control sheep BM cells and from sheep no. 273 and 306 CD45⁻ cells failed to react with any of the cell lineage-specific monoclonal antibodies used in these experiments.

The in vitro immunoglobulin production by CD45⁺ and CD45⁻ chimeric BM cells, as well as control sheep and human BM cells, is shown in Fig 6. Whereas human BM cells produced both IgG and IgM isotypes, control sheep BM cells, as well as CD45⁻ cells from sheep no. 273 and 306, failed to produce any detectable human immunoglobulin. In contrast, CD45⁺ cells from both animals produced detectable amounts of human IgG. However, it is possible that IgM was also produced by the chimeric CD45⁺ cells in quantities below the sensitivity threshold of the ELISA used in these experiments. It is important to point out that despite the detection of what appears to be functional NK cells and T lymphocytes in chimeric animals, no signs of graft-versus-host disease were detected in either animal.

Evidence for the in vivo expansion of cells constituting the original human graft. With self-renewal being a defining functional characteristic of HSC, we investigated the self-regenerating potential of human BM cells present in chimeric animals. Chimeric CD45⁺ BM cells collected 130 days' posttransplantation were first isolated by flow-cytometric cell sorting using a TR probe, stained again, and analyzed flow cytometrically for the presence of CD34⁺ cells, as well as CD45⁻ cells from sheep no. 273 and 306.

**Fig 4.** Detection of human β-globin sequences by the PCR. DNA was prepared from a single human BFU-E colony (first lane), control sheep BM cells (second lane), sorted CD45⁺ BM cells from sheep no. 306 (third lane), and a single BFU-E colony cloned from chimeric CD45⁺ cells isolated from sheep no. 306 (fourth lane). BFU-E colonies were obtained using conditions favoring in vitro growth of human progenitor cells, as described in Materials and Methods.
HLA-DR− cells. Since human CD34+ HLA-DR− cells were capable of long-term engraftment in transplanted sheep, we assumed that cells expressing this phenotype contain PHPC and possibly human HSC and that the detection of chimeric BM cells with this phenotype could therefore be considered as evidence for expansion of human HSC and perhaps self-renewal. As can be seen in Fig 3, CD34+ HLA-DR− cells identical to those constituting the initial BM graft were de-
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Hence it was reasoned that through the use of this animal model it would be possible to investigate the lymphohematopoietic differentiation and in vivo expansion potentials of a group of human BM cells believed to contain, as evidenced by a number of in vitro assays, human HSC. These studies represent an important extension of a number of observations previously reported by our group. Engrafted sheep in our previous studies received, in addition to CD34+ HLA-DR- cells, uncharacterized BM cells (intended as carrier cells to facilitate the homing of HSC to the sheep BM), thus casting doubts about the identity of cells responsible for xenogeneic engraftment. Furthermore, our earlier studies did not examine expansion or self-renewal of the transplanted cells nor was the full range of human hematopoietic lineages generated from engrafted HSC studied. Through the use of grafts containing solely CD34+ HLA-DR- BM cells, it was possible not only to eliminate such concerns, but also to achieve a full and comprehensive picture of the multilineage hematopoietic differentiation potential of these cells.

The data presented here demonstrate for the first time the ability of a given phenotype of human BM cells to undergo multipotential lymphohematopoiesis and expansion in a single in vivo assay system. We and others, using several in vitro assays, have previously shown that CD34+ HLA-DR- cells represent a population of adult human BM cells highly enriched for PHPC displaying many of the functional characteristics associated with HSC. In this report, it was shown that CD34+ HLA-DR- BM cells, or possibly a number of subpopulations of cells present within this phenotype, gave rise in vivo to cells belonging to the erythroid, megakaryocytic, and granulocytic, as well as the B-, T-, and NK-lymphoid lineages. It is important to point out that fetal sheep transplanted with adult human CD34+ HLA-DR+ BM cells failed to engraft (E.F.S., unpublished observations), suggesting that only the HLA-DR- fraction of CD34+ cells contains PHPC and, arguably, true HSC. Obviously, these studies fail to address whether the observed pluripotential hematopoiesis resulted from clonal, multilineage differentiation of primitive stem cells or from a number of very early lineage-restricted PHPC. However, analysis of such an issue is possible in this animal model, since it lends itself to such investigations mainly through the use of genetically marked HSC, which can be then followed through their development in the animal.

With proper in vitro stimulation, chimeric human BM cells gave rise to hematopoietic colonies belonging to multiple lineages. In addition, PHPC such as HPP-CFC, which are believed to directly correlate with the in vivo hematopoietic repopulating potential of murine marrow cells and to contain cells capable of self-renewal, were detected in chimeric BM, which suggests that transplanted HSC had undergone normal multilineage differentiation giving rise to a wide array of both primitive and more differentiated progenitor cells. The fact that in the colony-forming assay performed with sorted CD45+ and CD45- cells some CFU-GM--derived colonies were observed in control sheep BM cells and in the CD45- fraction of sheep no. 273 and 306 BM cells is not surprising. Although sheep marrow cells do not respond maximally to stimulation with human recombinant cytokines, a partial response is frequently detected. Nevertheless, the number of CFU-GM--derived colonies observed in control sheep BM cells and in sheep no. 273 and 306 CD45+ cells was substantially less than that observed with human BM cells or with the CD45+ cells from these two animals. Although chimeric BM cells from sheep no. 306 contained approximately 20% CD34+ cells on day +130 (Fig 3), this population of cells did not contain high numbers of hematopoietic progenitor cells on day +190 (Table 1), possibly because of a declining degree of chimeraism as previously reported1,2,3 or due to an abnormal hematopoietic differentiation of such cells in the sheep BM microenvironment.

The likelihood that transplanted HSC had undergone self-renewal was suggested by flow-cytometric analysis of chimeric CD45+ BM cells. An unusually large percentage of CD34+ cells in chimeric BM with approximately one fourth of these cells expressing the phenotype CD34+ HLA-DR- was detected. Since the original human graft consisted of...
cells displaying this phenotype, the continued presence and expansion of chimeric cells expressing this phenotype could best be explained by self-renewal. Several plausible reasons may account for why as many as approximately 20% of human chimeric BM cells expressed CD34 compared with less than 3% normally detected in adult human BM.25,29,30 It is possible that a selective pressure to proliferate and expand was exerted on human CD34+ cells by the "nonpermissive" sheep BM microenvironment. The fact that a higher percentage of human CD34+ cells existed in the chimeric BM than in normal human BM may in turn explain why a higher number of human hematopoietic colonies was detected in the former when assayed in semisolid medium. Alternatively, this observation may suggest that human chimeric CD34+ HLA-DR- cells may undergo self-renewal yet their ability to differentiate further is somehow hampered, resulting in the accumulation of CD34+ cells in the BM.

In addition to their ability to differentiate along myeloid lineages, transplanted HSC also gave rise to lymphoid progenitors. Again, permissive in vitro stimulation of human hematopoietic lineages, transplanted HSC also gave rise to lymphoid progenitors. Again, permissive in vitro stimulation of human hematopoietic cells to achieve engraftment, our model has been consistently successful, even though the size of grafts used was considerably smaller than that reported by Lapidot et al42 and McCune et al.43 Peault et al44 have reported successful engraftment in SCID mice with a smaller number of graft cells. However, in these animals, engraftment of human hematopoietic cells was restricted to cotransplanted human thymic tissues and was not demonstrable in host BM or peripheral blood.45 Engraftment of transplanted human cells in immunodeficient mouse models has been, in general, short-lived and lineage-restricted.45 In contrast, chimerism in our sheep model has ranged from what we report here to as long as 2 years' posttransplantation in some animals23 and has resulted, as demonstrated here, in multilineage expression of myeloid and lymphoid cell types. Finally, the degree of chimerism achieved in these studies is equal to or higher than what can be achieved in immunoincompetent mice, which makes the analysis of engraftment and the resolation of human cells easier to approach.

The ability of adult human CD34+ HLA-DR- cells to engraft in a xenogeneic system raises the possibility of using these cells as stem-cell grafts for in utero transplantation in an attempt to correct a number of congenital metabolic and/or hematologic diseases prenatally. The absence of graft-versus-host disease in sheep examined in this and in previous studies by our groups31-33 suggests that grafts consisting of immunologically incompetent fetal liver cells or adult human CD34+ HLA-DR- BM cells may be incapable of inducing graft-versus-host disease. This suggests the possibility of using mismatched CD34+ HLA-DR- stem-cell grafts from related donors, therefore vastly expanding the pool of potential donors. The success of in utero allogeneic transplantation of sheep and monkeys has been previously demonstrated to be dependent on the number of cells constituting the graft,46 suggesting that, in the human setting, a higher degree of chimerism, as well as engraftment success, could be achieved with grafts that contain larger numbers of CD34+ HLA-DR- stem cells.

ACKNOWLEDGMENT

The authors thank Kathy Stambaugh of Genetic Therapy Inc for technical assistance with the PCR analysis.

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