Immature Human Cord Blood Progenitors Engraft and Proliferate to High Levels in Severe Combined Immunodeficient Mice

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Unseparated or Ficoll-Hypaque (Pharmacia, Piscataway, NJ)–fractionated human cord blood cells were transplanted into sublethally irradiated severe combined immunodeficient (SCID) mice. High levels of multilineage engraftment, including myeloid and lymphoid lineages, were obtained with 80% of the donor cells as assessed by DNA analysis, fluorescence-activated cell sorting (FACS), and morphology. In contrast to previous and concurrent studies with adult human bone marrow (BM), treatment with human cytokines was not required to establish high-level human cell engraftment, suggesting that neonatal cells either respond differently to the murine microenvironment or they provide their own cytokines in a paracrine fashion. Committed and multipotential myelo-erythroid progenitors were detected using in vitro colony assays and FACS analysis of the murine BM showed the presence of immature CD34+ cells. In addition, human hematopoiesis was maintained for at least 14 weeks providing further evidence that immature hematopoietic precursors had engrafted the murine BM. This in vivo model for human cord blood–derived hematopoiesis will be useful to gain new insights into the biology of neonatal hematopoietic cells and to evaluate their role in gene therapy. There is growing evidence that there are ontogeny-related changes in immature human hematopoietic cells, and therefore, the animal models we have developed for adult and neonatal human hematopoiesis provide useful tools to evaluate these changes in vivo.

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Transplantation of cord blood cells into SCID mice. The SCID mice were bred and maintained under defined flora conditions at the Ontario Cancer Institute (Toronto, Ontario, Canada). The animal experiments were approved by the Animal Care Committee of the Hospital for Sick Children and the Ontario Cancer Institute. In accordance with our transplantation protocol for adult BM, 6- to 8-week-old SCID mice were sublethally irradiated with 400 cGy from a 137Cs source immediately before tail vein injection of either 1 mL of unseparated cord blood or 15 to 50 x 10^6 cells separated on Ficoll-Hypaque as described above. For injection, Ficoll-separated cells were resuspended in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS; Sigma Chemical Co, St Louis, MO). Growth factor-treated mice received human mast cell growth factor (huMGF, kit ligand) and a fusion protein of human interleukin-3 (huL3)human granulocyte-macrophage colony-stimulating factor (huGM-CSF) (PIXY321) (provided by D. Williams, Immunex Corp, Seattle, WA) every other day as intraperitoneal (IP) injections at a concentration of 10 μg and 6.8 μg per mouse, respectively. If not indicated otherwise, the mice were killed 4 weeks after transplantation.

DNA analysis. High molecular weight DNA was obtained from BM and other murine tissues by phenol extraction using standard protocols. DNA (5 μg) was digested with EcoR1, run on a 0.6% agarose gel, blotted onto a nylon membrane and hybridized with a human chromosome 17-specific α-satellite probe (pI7H8). To quantify the presence of human cells in various tissues, the intensity of the characteristic 2.7-kb band in the samples was compared with human/mouse mixtures (0, 0.1, 1.0, 10, 50, and 100% human DNA) as previously described. Multiple exposures of the autoradiographs were taken to ensure sensitivity down to 0.01% human DNA.

Flow cytometry. Two- or three-color flow cytometry was performed on a FACScan (Becton Dickinson, Mountain View, CA) as described. For erythrocyte lysis, mouse and human BM samples were diluted with 14 volumes of an NH₄Cl lysis buffer (10−4 mol/L EDTA, 10−3 mol/L KHCO₃, 0.17 mol/L NH₄Cl in water [pH 7.3]) and after gentle mixing kept at room temperature for 3 to 5 minutes. Thereafter, BM cells and single cell suspensions of lungs were

MATERIALS AND METHODS

Cord blood samples. Cord blood samples were obtained from umbilical and placental tissues scheduled for discard as described according to procedures approved by our Institutional Review Boards. Cells were either left unseparated or low-density (less than 1.077 g/mL) cells were collected after separation on Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and shipped overnight from Indianapolis, IN to Toronto, Ontario, Canada.

Fig 1. Summary of the DNA analysis of BM from SCID mice transplanted with either whole cord blood or Ficoll-Hypaque-fractionated (ficolled) cells. Data are presented from 12 different donors leading to high human cell engraftment in the majority of the transplanted mice. All mice were killed 4 weeks posttransplantation except for 8 mice from 2 donors that were analyzed after 8 weeks. The level of engraftment in the BM was determined by Southern analysis as described in Materials and Methods. Each circle represents a single mouse. Eighteen mice were transplanted with whole cord blood (O) and 34 mice with ficolled cells (C). The horizontal line indicates the mean level of engraftment.

Fig 2. DNA analysis of the organs of SCID mice transplanted with human cord blood. This representative Southern blot (experiment #CB1) shows 1% to 10% human cell engraftment in the lungs (Lu) and only low levels of human cells (less than 1%) in the spleen (Sp) and thymus (Thy) 4 weeks posttransplantation.
Fig 3. DNA analysis of BM from cord blood-transplanted SCID mice with or without cytokine treatment. (A) Summary of the DNA analysis from 6 independent experiments in which mice transplanted with cord blood were kept with and without cytokine treatment for 4 weeks (five donors) or 8 weeks (one donor). Human engraftment was determined as described in Materials and Methods. Each circle represents a single mouse. Seventeen mice received cytokine treatment (0) and 18 mice did not receive any growth factor injections (O). The horizontal line indicates the mean level of engraftment. (B) Representative Southern blots from mice transplanted with cells from two different donors are shown. Mice from experiment #CB5 were transplanted with whole cord blood (14 x 10^6 nucleated cells/mL). Mouse #CB8.13 was transplanted with 35 x 10^6 Ficoll-Hypaque-fractionated cells whereas the other mice from this experiment were transplanted with whole cord blood (5 x 10^6 nucleated cells/mL) from the same donor. All mice except #CB8.18 through #CB8.23 received injections of huMGF and PIXY321 on alternate days and were analyzed 4 weeks after transplantation.

washed in phosphate-buffered saline (containing 0.02% NaN₃) and incubated for 30 minutes on ice with saturating amounts of human-specific monoclonal antibodies. Data acquisition and analysis was performed with LYSYS II-software (Becton Dickinson). Dead cells were gated out by their staining with propidium iodide (PI). In each experiment, cells from a nontransplanted mouse were stained with the same antibodies as a negative control. IgG1 isotype controls conjugated to phycoerythrin (PE), fluorescein isothiocyanate (FITC), and PerCP were also included.

Anti-CD45 peridinin chlorophyll protein (PerCP) (HLel, Becton Dickinson) and anti-CD45 FITC or PE (KC56, Coulter Immunology, Hialeah, FL) were used for identifying the human cells. T cells were detected by staining with anti-CD2 PE (T11, Coulter), anti-CD3 FITC (T3, Coulter), anti-CD4 FITC (T4, Coulter) and anti-CD8 PE (T8, Coulter); myeloid cells were stained with anti-CD13 PE (MY7, Coulter) and anti-CD16 FITC (Leu-11a, Becton Dickinson); B cells with anti-CD19 FITC (B4, Coulter); and immature cells with anti-CD34 FITC (HPCA-1, Becton Dickinson).

Hematopoietic progenitor assay. Semisolid cultures were performed as previously described. Briefly, 0.5 to 2 x 10^5 cells mixed with IMDM were plated in 0.9% methylcellulose, 15% FCS, 15% pretested human plasma, 5 x 10^-5 mol/L 2-mercaptoethanol, 50 ng/mL huMGF, 5 ng/mL PIXY321, 10 U/mL huIL-3, 9 U/mL huG-MSCF, and 2 U/mL human erythropoietin. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 10 to 14 days, the plates were scored for macrophage colony-forming unit (CFU-M), granulocyte-macrophage colony-forming unit (CFU-GM), granulocyte colony-forming unit (CFU-G), erythroid burst-forming unit (BFU-E) and multilineage colony-forming unit (CFU-GEMM) by morphologic criteria and cytologic staining of selected colonies. These conditions are specific for the growth of human colonies as has been shown by different methods including morphol-
Fig 4. FACS analysis of the BM and lungs of SCID mice transplanted with human cord blood. (A) This figure shows three-color FACS analysis of the BM from a representative highly engrafted mouse (#CB17.4) 4 weeks posttransplantation (left panel), isotype controls (middle panel), and an untransplanted control SCID mouse (right panel). Dead cells were excluded by their intense staining with PI, which is easily distinguishable from staining with the anti-CD46PerCP antibodies, as shown. For lineage-specific analysis, the human cells were gated by intensity of CD45 staining and size as indicated within the figure (gates R1 through R3). In the lymphoid (R2) and early progenitor (R3) window, cells with high side-scatter properties (less than 5% and 20% of the cells in windows R2 and R3, respectively) were excluded for further analysis. The percentages given in the different quadrants represent the percentage of cells in the total ungated population (10,000 to 30,000 events).
CORD BLOOD PROGENITORS ENGRAFT SCID MICE

RESULTS

**High-level human hematopoiesis in SCID mice after cord blood transplantation.** To determine whether immature cells capable of engrafting SCID mice were present in cord blood and to establish an experimental in vivo model for human cord blood-derived hematopoiesis, unseparated human cord blood (1 mL with $4.6 \times 10^6$ to $19 \times 10^6$ nucleated cells) or Ficoll-Hypaque–fractionated cells (15 to $50 \times 10^6$) were injected into the tail vein of sublethally irradiated SCID mice. The transplanted mice were kept with or without cytokine treatment. After 4 weeks, the engraftment of human cells in the BM was analyzed by molecular methods as well as morphology. Whole cord blood and/or Ficoll-Hypaque–fractionated cells from 16 of 20 donors (80%) successfully engrafted the BM of the majority of the transplanted mice. A summary of the Southern analysis of mice transplanted with cells from 12 of the donors that successfully engrafted SCID mice is shown in Fig 1. The BM from 39 of 53 mice (74%) contained greater than 10% human cells indicating a highly reproducible transplant system (Fig 1) (for a representative Southern see Fig 3B). In addition, high levels of human cells were found in the bone marrow from mice transplanted with cells from the other 4 donors using cytology and/or immunohistology with CD45 staining.

There have been reports suggesting that significant losses of progenitor cells occur after Ficoll-Hypaque separating cord blood. Therefore, we initially injected 1 mL of heparinized whole cord blood into irradiated SCID mice. In subsequent experiments, no significant differences were found between transplantation of unseparated whole cord blood or Ficoll-Hypaque–fractionated cells; 13 of 18 (72%) mice transplanted with whole cord blood had greater than 10% human cells detectable in the BM compared with 26 of 35 (74%) mice transplanted with Ficoll-Hypaque–fractionated cells (Fig 1).
If immature hematopoietic cells had engrafted the SCID mice, human hematopoiesis should be maintained for longer periods of time. Accordingly, one mouse (CB8.11) transplanted with cord blood was kept for 14 weeks posttransplant; the BM from this mouse contained 25% human cells as estimated by Southern analysis (data not shown).

The distribution of the human cells in engrafted SCID mice was assessed by Southern analysis of DNA extracted from the BM, lungs, liver, spleen, thymus, and kidney from mice transplanted with 5 different donors. The highest levels of human cells were consistently found in the mouse BM as shown in Fig 1. However, 1% to 10% human cells were also detected in the lungs (Fig 2 and data not shown). In contrast, only low levels (0.1% to 1%) of human cells were present in thymus, spleen, liver, or kidney (Fig 2 and data not shown).

Cytokine treatment is not required for human engraftment. After our experience with the transplantation of adult BM into SCID mice, the majority of cord blood transplanted mice received IP injections of PIXY321, a fusion protein of huIL-3 and huGM-CSF, and huMGF every other day. Interestingly, subsequent control experiments indicated that there was no difference in the level of engraftment in cord blood-transplanted mice treated with or without growth factors. This was in contrast to our previous and concurrent experiments (data not shown) with adult BM, which always showed a clear growth factor effect. The average level of engraftment in the marrow of cord blood-transplanted mice with or without cytokine treatment was 15% (Fig 3A) as estimated by Southern analysis. The analysis included mice transplanted with six different donors. The independence from exogenous human growth factor supplement was observed whether whole blood or Ficoll-Hypaque-separated cells were transplanted into SCID mice (data not shown). As few as 5 x 10^6 nucleated cells/mL whole blood were sufficient to lead to high levels (in some cases greater than 50%) of human hematopoiesis 4 weeks posttransplantation without administration of human growth factors (Fig 3B).

Phenotypic characterization of the engrafted cells. To characterize the human cells in the mouse BM, three-color fluorescence-activated cell sorter (FACS) analysis was performed on selected mice with a panel of antibodies directed against the human myeloid and lymphoid cell surface epitopes CD2, CD13, CD16, CD19, and CD34. The human origin of the analyzed cells was established by their staining for the panleukocyte antigen CD45 (Fig 4A). Isotype controls and staining of a nontransplanted mouse confirmed the specificity of the staining conditions (Fig 4A). Further analysis of the gated CD45^+ cells showed the presence of human cells expressing the panmyeloid surface antigen CD13 (Fig 4A); however, no CD13^+CD16^- or CD13^-CD16^+ mature granulocytes could be detected. Human CD45^+CD19^- B cells were also seen in the BM (Fig 4A). In addition, human CD34^+CD45^-CD13^- early hematopoietic progenitors were found (Fig 4A). FACS analysis of highly infiltrated lungs (Fig 4B) confirmed the presence of CD2^+ and CD3^+ human T cells. Staining for CD4 and CD8 showed that the majority of the cells were mature CD4^+CD8^- T cells with a smaller proportion of mature CD4^+CD8^+ T cells (Fig 4B). No human cells of other lineages were found in the lungs by FACS analysis.

Touch preparations from the BM of transplanted mice were used to quickly assess human cell engraftment. In concordance with the FACs data, engrafted mice showed infiltration with early myeloid cells, mainly myeloblasts and promyelocytes (Fig 5A). A significant percentage of the human cells differentiated into the cosinophilic lineage up to the level of mature bilobulated eosinophils. Occasionally, mature basophils and lymphoid cells were detected. In highly engrafted mice, the human cells nearly completely replaced the murine cells. Residual mouse myelopoiesis could be distinguished by the characteristic ring-shaped nuclei of the granulocytes (Fig 5B) and the fine granulation of murine eosinophils and basophils. Histologic evaluation of the sternum of engrafted mice confirmed the BM infiltration with early myeloid cells that stained positive for the human specific panleukocyte antigen CD45 (Fig 5C) and the myeloid antigen CD15 (Fig 5D). CD68^+ cells of the macrophage lineage could also be detected in the BM by immunohistology. Histologic evaluation of the lungs showed perivascular and peribronchial infiltration of CD45^- human cells (Fig 5, E and F) in the lungs of several mice in accordance with the results from the Southern and FACS analysis. In mouse #CB12.5, interstitial and alveolar infiltration with CD68^- human macrophages was also observed (data not shown).

The plasma of mice transplanted with cells from four donors was tested for the presence of human IgG and IgM to determine if mature B cells had engrafted the mice. Low levels of human IgG ranging from 5.7 to 233 μg/mL could be measured in 8 of 19 mice, and 2 mice also had 1 to 2 μg/mL human IgM detectable. Only highly engrafted mice (greater than 10% human cells) contained detectable levels of human Igs.

Progenitor cell activity of the transplanted human cells. To determine whether human hematopoietic progenitor cells were present, 0.5 to 2 x 10^5 BM cells from engrafted mice were plated in methylcellulose cultures under conditions supporting only the growth of human cells. The BM contained an average of 200 human colonies per 1 x 10^5 plated BM cells including CFU-M, CFU-GM, CFU-G, BFU-E, and multilineage CFU-GEMM (Fig 6). No significant difference in the overall number or colony type was seen comparing mice treated with or without growth factors (data not shown). Human colonies, including CFU-GEMM, were detected in the marrow of mouse #CB8.11 14 weeks posttransplantation.

DISCUSSION

The experiments described here show that human cord blood cells can engraft sublethally irradiated SCID mice with high efficiency resulting in high levels of human multilineage hematopoiesis in the BM of transplanted mice. Mice could be engrafted with unseparated and/or Ficoll-Hypaque-fractionated cells showing that 1 mL of cord blood with a cell count as low as 5 x 10^6 nucleated cells contained enough immature hematopoietic cells to reconstitute an SCID mouse. In some mice, more than 50% of the cells in the mouse BM were of human origin nearly completely replacing endogenous mouse myelopoiesis. Enhanced extramedullary murine hematopoiesis in the spleen appeared to compensate for the reduced BM myelopoiesis. Morphologic and
Fig 5. Cytologic and histologic analysis of the BM and lungs of SCID mice 4 weeks after transplantation with human cord blood. (A) Wright stain of the BM touch preparation of a highly engrafted mouse (#CB17.1) showing infiltration with early human myeloid cells. (B) Wright stain of a control SCID mouse BM showing the characteristic ring-shaped mouse granulocytes. (C) CD45 stain of the sternum of a highly engrafted mouse (#CB16.3) confirming the human origin of the cells. (D) CD15 stain showing myeloid differentiation of the human cells in the marrow (#CB12.2). (E and F) CD45 stain of the lungs (#CB2.2) showing peribronchial (E) and perivascular (F) infiltration with human cells. Organs from nontransplanted control mice stained negative for CD45 and CD15.

FACS analysis of the BM of the engrafted mice showed high-level repopulation with mainly immature human cells of multiple myeloid lineages. Committed and multipotential progenitors for all of the myeloid and erythroid lineages were present at high numbers in the BM. In addition, CD19+ B cells could be detected in several mice. Comparison of the engrafted cells in different organs showed that human myelopoiesis is predominantly confined to the murine BM indicating that the engrafting cells home to and proliferate in the murine BM microenvironment.

 multilineage hematopoiesis was maintained in SCID mice for at least 14 weeks suggesting that immature hematopoietic
cells had engrafted the murine BM microenvironment and were maintaining the human progenitor pool. Furthermore, FACS analysis of engrafted mice showed immature CD34+ human hematopoietic cells in the BM of transplanted mice. However, at present we cannot distinguish between the engraftment of pluripotent stem cells or committed progenitors that give rise to the different lineages. Future experiments including retroviral marking and secondary transplants will clarify the nature of the cell that engrafts the SCID mice.

Mature human T cells were frequently detected in the parabronchial and paravascular lymphoid microenvironment of the lungs from engrafted mice. This appears to reflect engraftment of mature T cells from the original donor sample and may be correlated with the high number of T cells present in cord blood. In one mouse, interstitial and alveolar infiltration with human CD68+ macrophages was observed similar to the distribution of normal CD68+ alveolar macrophages in human lungs. The presence of human T cells in the appropriate location in the murine lungs suggests that some aspects of the interaction between the human T cells and the murine microenvironment may be conserved.

Surprisingly, our results show that high levels of human hematopoiesis can be achieved in cord blood-transplanted SCID mice without exogenous treatment with human growth factors. Our previous and concurrent experiments using adult BM transplanted into SCID mice have shown that immature human hematopoietic cells can home to and survive in the murine microenvironment for as long as 3 months without growth factor treatment. However, they do not proliferate and differentiate to high levels in vivo unless the mice are treated with human cytokines. Therefore, human cord blood cells may be able to provide their own cytokines in vivo in a paracrine fashion, thus rendering themselves independent from the exogenous supply of human growth factors. Potential sources for growth factor secretion include activated human T cells that produce IL-3 and GM-CSF24,25 and mature CD8+ T cells that are known to play an essential role in allogeneic engraftment independent from their potential function in overcoming residual host resistance,26 probably by providing necessary growth factors. Alternatively, the cord blood progenitors may have a higher proliferative potential than progenitors from adult BM25,26 and/or may be more responsive to the murine microenvironment. Future studies will distinguish between the different possibilities and evaluate the role of human cord blood T cells in the engraftment process.

In conclusion, this model will be useful in gaining new insights into the biology of early human cord blood progenitors and in studying developmental changes in the human hematopoietic system by comparing hematopoietic cells from cord blood and adult BM. Such information is of particular importance as cord blood is increasingly considered as an alternative stem cell source for both autologous and allogeneic transplantation.27 As this model does not require manipulation of the cord blood or exogenous human growth factor treatment, it is easily applicable. In addition, cord blood cells are highly infectable with retroviral vectors26; this approach will be useful in studying gene transfer and long-term in vivo expression into human hematopoietic cells.

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