Improved Engraftment of Human Hematopoietic Cells in Severe Combined Immunodeficient (SCID) Mice Carrying Human Cytokine Transgenes

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Summary

We have generated immunodeficient *scid*$^-$/*scid*$^-$ (SCID)–transgenic mice expressing the genes for human interleukin 3, granulocyte/macrophage-colony stimulating factor, and stem cell factor. We have compared engraftment and differentiation of human hematopoietic cells in transgenic SCID mice with two strains of nontransgenic SCID mice. Human bone marrow cells carrying the CD34 antigen or human umbilical cord blood were injected into sublethally irradiated recipients. Human DNA was detected by polymerase chain reaction in peripheral blood and bone marrow of 14 of 28 transgenic SCID mice after transplantation, but in only 2 of 15 nontransgenic SCID littermates at a 10-fold lower level. Bone marrow cultures 8 wk after transplantation of cord blood gave rise to human burst-forming unit erythroid, colony-forming unit granulocyte/macrophage, or granulocyte/erythroid/macrophage/megakaryocyte colonies. Engraftment was observed for up to 6 mo in transgenic SCID mice, twice as long as nontransgenic littermates or previous studies in which transplanted SCID mice were given daily injections of growth factors. We conclude that the level and duration of engraftment of human cells in SCID mice can be improved by expression of human cytokine transgenes and that transgenic SCID mice are an efficient model system for the study of human hematopoiesis.

Most of our knowledge about human hematopoiesis has been derived from studies of human cells in vitro (for reviews see references 1–4). These studies generated information about the phenotype of primitive and committed progenitors, their lineage relationships, and their differentiation programs, and led to the identification of a broad spectrum of growth factors and biomolecules that modulate and regulate human hematopoiesis (5). In the murine system, multiple in vivo assays have been established that define the properties of the most primitive hematopoietic cells (6). Because the study of human hematopoiesis would be enhanced by an in vivo model, multiple strategies have been pursued to develop an animal recipient for human hematopoietic cells. In sheep and monkey models, intrauterine injection of human fetal liver cells has reproducibly resulted in long-term engraftment, proliferation, and differentiation of human hematopoietic cells (7, 8). Genetically immunodeficient mouse strains provide a small animal model for the propagation of human hematopoietic cells. Mice homozygous for the SCID mutation (*scid*$^-$/*scid*$^-$; abbreviated SCID) are widely used for xenogeneic transplantation of hematopoietic cells (9). SCID mice are severely deficient in functional B and T lymphocytes because of impaired recombination of immunoglobulin and T cell receptor genes, which causes an early arrest in the development of B and T lineage–committed cells; other hematopoietic cells appear normal (9, 10). All cell types are highly sensitive to ionizing radiation, since the *scid* mutation also affects the DNA repair mechanism (11, 12). Mutations in the DNA-dependent kinase gene have been proposed as the underlying defect (11).

To provide support for the optimal growth and differentiation of human hematopoietic cells transplanted into SCID mice, several strategies have been pursued. In the SCID-hu model, human fetal liver cells are cotransplanted with human fetal thymus into SCID mice (13–15). It was proposed that the human fetal thymus graft provided a human microenvironment and cytokines. Human cells differentiated into multiple lineages, including T lymphocytes, and could be monitored for up to several months in the majority of hosts. In another model, transplantation of human bone marrow, fetal liver, or umbilical cord blood cells was combined with the injection of the recombinant cytokines human stem cell factor (hSCF;[1] steel factor, c-kit ligand, mast cell growth factor), PIXY 321, a fusion protein of human

GM-CSF (hGM-CSF), and human IL-3 (hIL-3; multi-CSF) (16, 17). These cytokines were selected because SCF, GM-CSF, and IL-3 are known to be species specific (18, 19) and because hSCF, hGM-CSF, and hIL-3 together can promote growth and differentiation of bone marrow progenitor cells in vitro (20). Repetitive injection of high doses of these cytokines supported engraftment of human bone marrow and umbilical cord blood cells in a large portion of transplanted animals routinely for 8 wk, and occasionally up to 14 wk (17, 21); cord blood also engrafted SCID mice without exogenous cytokines (21).

In spite of these achievements, the strategies described have drawbacks. The availability of fetal tissue is often restricted, and transplant surgery risks the health of the infection-prone recipient animals. Finally, there is a wide variability in the engraftment of human cells into different strains of SCID mice. As an alternative to earlier models, we have generated a transgenic SCID mouse that carries the genes for hIL-3, hGM-CSF, and hSCF. We hypothesized that the transgenes would support the long-term engraftment of human hematopoietic cells in these mice by providing human-specific cytokines. Our data indicate that SCID mice transgenic for hIL-3, hGM-CSF, and hSCF can support long-term engraftment of human hematopoietic cells and can be considered a suitable in vivo model for human hematopoiesis.

Materials and Methods

Generation of Transgenic SCID Mice. BALB/cBy, BALB/cBy *scid*$^-$/*scid*$^-$, and nonobese diabetic (NOD)/LtSz *scid*$^-$/*scid*$^-$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and the breeding colony was maintained under specific pathogen–free conditions. Plasmid constructs containing the hIL-3, hGM-CSF, and hSCF cDNA under the control of the adenovirus major late promoter (AMLP) (see Fig. 1) were kindly provided by Steven Clark (Genetics Institute, Inc., Cambridge, MA). The constructs were isolated from vector sequences and coinjected into the male pronucleus of fertilized mouse eggs by standard techniques (22). Transgenic founder mice were identified by DNA Southern blot and PCR amplification of the human cytokine genes from tail DNA. To generate immunodeficient transgenic mice, transgenic founders were crossed with homozygous SCID mice, and the progeny carrying the transgenes were backcrossed with homozygous SCID mice up to five times.

Detection of the Transgenes. Transgenes were amplified using an oligonucleotide primer for the AMLP (A: 5′-CAGGGTGTGAAGACACATGTC-3′) and one for each cytokine construct (GM-CSF: 5′-TCATCTGGCCGGTCTCACTC-3′; IL-3: 5′-GTCTGCTGAGCCTGAGCATTC-3′; SCF: 5′-GTGACACTGACTCTGGAATC-3′). PCR was performed using conditions recommended by the manufacturer (Perkin-Elmer Cetus Instruments, Norwalk, CT), including [^{32}P]dCTP for 35 cycles with each cycle at 94°C for 1.5 min, 58°C for 2.5 min, and 72°C for 3 min (DNA Thermal Cycler model 480; Perkin-Elmer Cetus Instruments). As an internal control, a 401-bp sequence specific for the murine hemoglobin β gene was amplified as described (23). Southern blotting of genetic DNA digested with BamHI or HindIII was performed as described (24).

Expression of transgene messenger RNA (mRNA) was demonstrated by reverse transcriptase (RT)-PCR. Total cellular RNA was prepared from peripheral blood, bone marrow, liver, spleen, thymus, kidney, testes, and brain by extraction with guanidinium isothiocyanate and treatment with DNase I. RNA was reverse transcribed using random oligonucleotide primers to cDNA, which was then amplifed according to the conditions recommended (Perkin-Elmer Cetus Instruments). Posttranscriptional splicing of an immunoglobulin gene between the promoter and the cytokine sequences resulted in distinct PCR bands from DNA and RNA. Northern blot was performed as described (24) using [^{32}P]dNTP-labeled probes specific for the human cytokine constructs.

Preparation of Human Cells for Transplantation. Bone marrow: Hematopoietic progenitors and stem cells expressing the human CD34 antigen were immunoselected from healthy volunteers and patients undergoing autologous transplantation for multiple myeloma using an immunoabsorption column (CEPRATE LC 34; CellPro, Inc., Bothell, WA) as described by the manufacturer. CD34 antigen–positive cells were enriched to 80–95% purity as confirmed by flow cytometry. Cells were washed in PBS, resuspended in PBS containing 10% FCS (Life Technologies, Inc., Gaithersburg, MD) and injected. Cord blood: Umbilical cord blood was obtained from Indiana University Medical Center (Indianapolis, IN) and sent overnight after density cut separation on Ficoll–Hypaque (<1.077 g/cm^3; Pharmacia Inc., Piscataway, NJ) to the National Institutes of Health (Bethesda, MD) on wet ice. Upon arrival, cord blood cells were washed in PBS, resuspended in PBS containing 10% autologous human plasma, and injected.

Transplantation Protocol. Transgenic and nontransgenic SCID mice at 4–8 wk of age were sublethally irradiated (350 rad), and 0.2–1 × 10^6 human CD34 antigen–positive bone marrow cells were intravenously injected. Alternatively, 0.5 × 10^8 human low-density umbilical cord blood cells were injected. Peripheral blood DNA was prepared for PCR at 2-wk intervals. At 8 wk after transplantation, genomic DNA was extracted from bone marrow, liver, spleen, and thymus of selected recipients, and bone marrow cells were cultured. In NOD-SCID mice, peripheral blood was tested for human cells by PCR and flow cytometry 5 and 6 wk after transplantation.

Detection of Human Cells in Chimeric Mice. PCR was performed using primers specific for human α-satellite sequences on human chromosome 17 as described (25). Internal controls were used as described above. The protocol was able to detect <0.01% human DNA in a murine DNA background, and the amplification of standardized mixtures of human and mouse DNA (100, 10, 1, and 0.1% human DNA) was linear. For flow cytometry, peripheral blood–nucleated cells were washed and incubated in PBS containing 10% rat serum and 10% FCS (Life Technologies, Inc.). Cells were stained with FITC-labeled Abs (1 μg/10^5 cells; Becton Dickinson & Co., Mountain View, CA) specific for human CD45 (panleukocytes) and PE-labeled antibodies specific for human CD14 (monocytes, neutrophils, eosinophils) or their corresponding isotype antibodies (IgG1-FITC and IgG2-PE). Cells were washed twice and analyzed by flow cytometry (Elite flow cytometer; Coulter Corp., Hialeah, FL).

Clonogenic Hematopoietic Progenitor Assays. 2 × 10^5 bone marrow cells were assayed for colonies derived from burst-forming unit erythroid (BFU-E), CFU-GM, or CFU granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEM) in methylcellu-

[1] *Abbreviations used in this paper:* AMLP, adenovirus major late promoter; BFU-E, burst-forming unit erythroid; CFU-GEM, CFU granulocyte/erythroid/macrophage/megakaryocyte; hGM-CSF, hIL-3, hSCF, human GM-CSF, IL-3, and stem cell factor, respectively; mRNA, messenger RNA; NOD, nonobese diabetic; RT, reverse transcriptase.

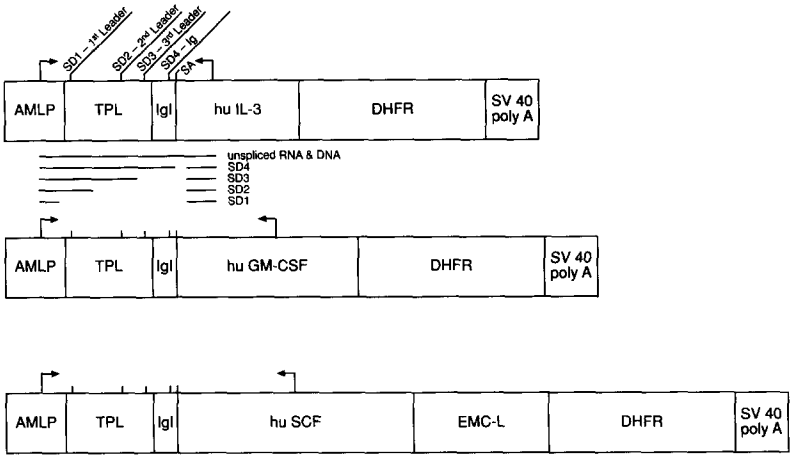

Figure 1. The constructs used to generate mice carrying the hIL-3, hGM-CSF, and hSCF transgenes. The AMLP with its tripartate leader (*TPL*) is indicated. The leader sequence contains three splice donor (*SD*) sites, and an Ig intron (*IgI*) is also shown. The approximate location of the primers used to amplify transgene cDNAs are indicated by arrows. The relative sizes of the possible mRNAs from the hIL-3 transgene are shown below the construct. The 3′ ends of the constructs include sequences from the 3′ end of the dihydrofolate reductase (*DHFR*) gene and an SV40 poly A signal (*SV40 poly A*).

lose medium selectively supporting growth of human cells as described (21, 26). Human colonies were counted after 10 and 20 d and were evaluated for human DNA by PCR.

Results

Transgenic Mice. Transgenic mice carrying the genes for the human cytokines IL-3, GM-CSF, and SCF were identified by PCR amplification of tail DNA using primers specific for both the AMLP and the cytokine sequences (Fig. 1). One founder strain was selected for further study. The presence of the transgenes was confirmed by Southern blot using construct-specific probes (Fig. 2 *A*). By comparison of the intensities of the hybridization signals to that of HeLa cell DNA, we concluded that the number of each individual transgene was approximately three to five copies per genome. The analysis of >100 animals from five back-cross generations revealed that all three transgenes were passed to ~50% of the animals of both sexes in the next generation, indicating that all three cytokine genes had integrated at the same site on an autosomal chromosome. RT-PCR revealed expression of all three transgene mRNAs in all tissues tested, including bone marrow, thymus, liver, spleen, kidney, brain, and testes (Fig. 3 and data not shown). Differential splicing of the region upstream of the cytokine cDNA generated products that were shorter than the genomic DNA fragment. No corresponding signal was observed in RNA samples from nontransgenic animals or from samples that were not treated with RT before amplification. Northern blot analysis of RNA from bone marrow confirmed expression of the human transgenes as shown in Fig. 2 *B* for hSCF. Transgenic mice appeared normal in phenotype, and no abnormalities were detected in peripheral blood differential counts or at autopsy in comparison with nontransgenic littermates. After crossing and back-crossing of transgenic BALB/cBy founders to homozygous SCID mice, absence of mature B and T lymphocytes was confirmed by flow cytometry in all animals used for the transplantation experiments.

Transplantation of Human Bone Marrow into Transgenic SCID Mice. Transgenic SCID mice were evaluated for their ability to support growth and differentiation of human hematopoietic cells. Populations of human bone marrow cells highly enriched for the presence of the human CD34 antigen were simultaneously transplanted into transgenic and nontransgenic littermates. Peripheral blood was collected every 2 wk and analyzed for the presence of human DNA by PCR using primers specific for human α-satellite DNA sequences on chromosome 17. During the first 6 wk after transplantation, human sequences could not be detected in either group. Subsequently, human DNA sequences were consistently amplified in 50% of transgenic animals between 6 and 24 wk (Table 1 and Fig. 4 *D*). Comparison of the signals from these mice with control samples diluted in mouse DNA allowed a semiquantitative estimate of the human DNA. Human DNA accounted for 0.5–1.5% of the total DNA extracted from peripheral blood. In contrast to transgenic recipients, only 1 of 12 nontransgenic SCID littermates was positive for human cells, which accounted for 0.1% of total cells and persisted for <10 wk (Table 1 and Fig. 4 *B*). Internal control signals could be amplified from all samples (data not shown).

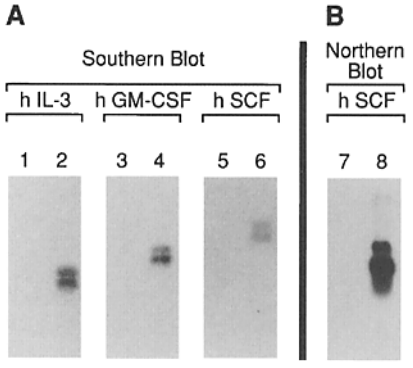

Figure 2. Characterization of mice carrying the hIL-3, hGM-CSF, and hSCF transgenes. (*A*) Southern blot analysis of DNA from transgenic mice (lanes *2*, *4*, and *6*) and nontransgenic littermates (lanes *1*, *3*, and *5*). The filters were hybridized with the probes indicated above the lanes. (*B*) Northern blot analysis of RNA extracted from the bone marrrow of transgenic mice (lane *8*) and nontransgenic littermates (lane *7*). The filter was hybridized with a probe for the hSCF mRNA (indicated above the lanes).

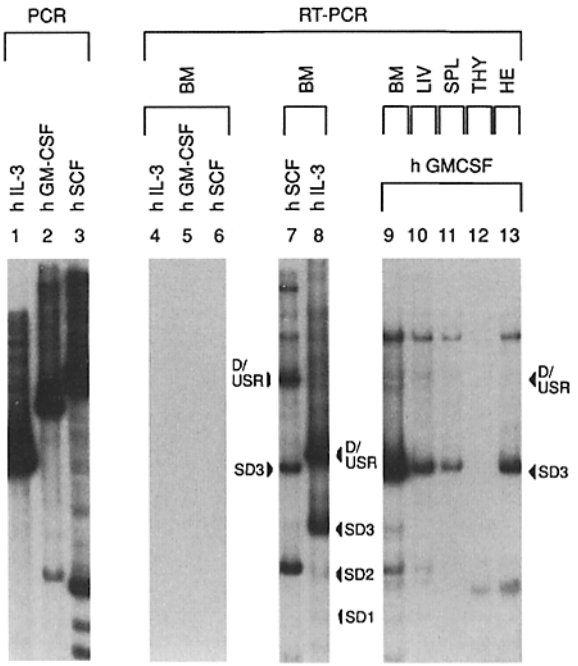

Figure 3. Splicing of transgenic mRNAs. DNA PCR was used to demonstrate the size of the unspliced mRNA indicated above the lane (lanes *1–3*). RT-PCR was used to evaluate mRNAs derived from the transgenes. No messages were detected in RNA from the bone marrow of nontransgenic littermates (lanes *4–6*) or in any from any other tissue, or in samples not treated with RT (data not shown). The mRNAs corresponding to expression of the hSCF and hIL-3 transgenes in the bone marrow (*BM*) are shown in lanes 7 and *8*, respectively. The splice donor corresponding to each message is indicated (see Fig. 1). Similar splicing generates several forms of the mRNA corresponding to the expression of the hGM-CSF transgene in BM, liver (*LIV*), spleen (*SPL*), thymus (*THY*), and heart (*HE*). The splice donor corresponding to each message is indicated (see Fig. 1).

Transplantation of Human Umbilical Cord Blood Cells into Transgenic SCID Mice. Because human umbilical cord blood cells have been shown to engraft readily into both human recipients (27) and SCID mice (21, 28), the engraftment of

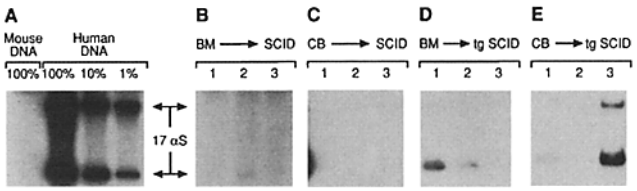

Figure 4. PCR detection of human α-satellite sequences in DNA extracted from the peripheral blood of SCID mice transplanted with human hematopoietic cells. (*A*) A dilution curve of human DNA diluted in increasing amounts of mouse DNA used to estimate the percentage of human cells. (*B*) Three representative samples from nontransgenic SCID mice transplanted with human bone marrow cells. Only a faint band (lane *2*) is detected. (*C*) Three representative samples from nontransgenic SCID mice transplanted with human cord blood cells. Only a faint band (lane *3*) is detected. (*D*) Three representative samples from transgenic SCID mice transplanted with human bone marrow cells. Signals are detected in lanes *1* and *2*. (*E*) Three representative samples from transgenic SCID mice transplanted with human cord blood cells. Signals are detected in lanes *1* and *3*.

human cord blood cells into transgenic SCID mice was compared with that of adult bone marrow. Populations of low-density cord blood cells were simultaneously injected into transgenic SCID mice and nontransgenic littermates. Analysis of peripheral blood DNA demonstrated the presence of human α-satellite sequences in 10 of 20 transgenic animals as early as 1 wk after transplant, whereas 6 wk were required for detection using adult $CD34^+$ bone marrow cells. Human DNA was detected for a minimum of 13 wk and accounted for an average of 2% of total DNA (Table 1 and Fig. 4 *E*), with occasional animals achieving levels ≥10% human cells. A trace amount of human DNA was detected in 1 of 3 nontransgenic recipients transplanted simultaneously (Table 1 and Fig. 4 *C*). 10 animals were killed 8 wk after transplantation. In 6 of 10 mice, human DNA was detected in the bone marrow, liver, and spleen, and accounted for 1–2% of the total DNA of extracted bone marrow and 0.1–2% in other tissues. Human hematopoietic progenitor cells in the bone marrow of transgenic mice in-

Table 1. *Detection of Human Cells in Peripheral Blood and Bone Marrow after Transplantation of Human Bone Marrow and Umbilical Cord Blood Cells*

Recipient strain	Human cells transplanted	Detection of human cells in recipient mice (positive/transplanted)		
		Peripheral blood		Bone marrow
		0–6 wk	8–24 wk	>8 wk
			% *human*	
BALB/c SCID	$CD34^+$ BM	0/12 (ND)	1/12 (0.05)	0/12 (ND)
Transgenic BALB/c SCID	$CD34^+$ BM	0/8 (ND)	4/8 (1.0–1.5)	4/6 (1.0)
BALB/c SCID	Cord blood	1/3 (0.1)	1/3 (0.1)	0/3 (ND)
Transgenic BALB/c SCID	Cord blood	10/20 (1.0–10.0)	10/20 (1.0–10.0)	5/10 (1.0–5.0)

BM, bone marrow.

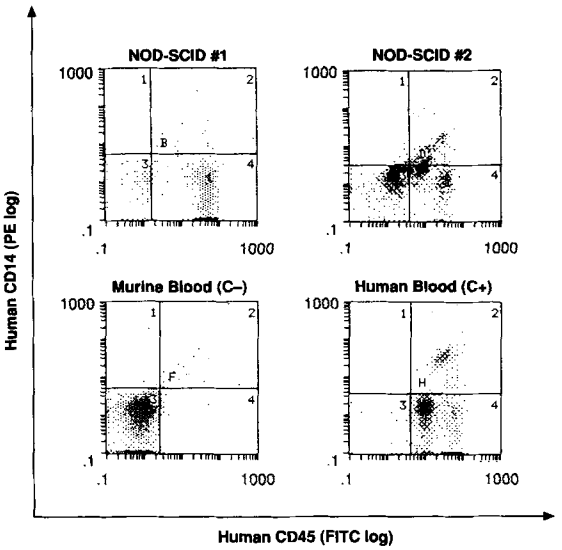

Figure 5. Expression of human CD14 and CD45 antigens on peripheral blood cells collected from NOD-SCID mice transplanted with human cord blood cells. The results from two transplanted mice are shown in the top half of the figure. The negative control (untransplanted mouse peripheral blood) and positive control (human peripheral blood) are shown in the bottom half of the figure.

jected with cord blood cells were grown in methylcellulose medium designed to support the growth of human BFU-E, CFU-GM, and CFU-GEM. All three colony types were observed in cultures 10–20 d after plating. The human origin of these colonies was verified by PCR amplification of human α-satellite sequences as in Fig. 4. The number of colonies was between 1 and 10% of the colony number that was obtained after plating an equivalent number of human bone marrow cells not passaged in mice.

Engraftment of Cord Blood Cells into NOD-SCID Mice. Engraftment of human bone marrow and spleen cells into NOD-SCID mice has been shown to be superior to other strains of SCID mice (29, 30). NOD-SCID mice contain very low rejection activity due to a deficiency in B cells, T cells, killer cells, and circulating complement (31–33). We tested the suitability of NOD-SCID mice for short-term engraftment using our cord blood protocol. Human cells were observed in peripheral blood in all of 11 NOD-SCID mice 6 wk after transplantation. PCR and flow cytometry of peripheral blood revealed an average of 30% human cells (range 5–95%) among total peripheral blood cells using monoclonal anti–human CD14 and anti–human CD45 antibodies, which specifically mark human monocytes and all leukocytes (Fig. 5).

Discussion

In this study we described a transgenic SCID mouse carrying hIL-3, hGM-CSF, and hSCF transgenes and compared it with other SCID models for the engraftment of human hematopoietic cells. We evaluated the effects of cytokine transgene expression in two different sources of human hematopoietic cells. In previous studies using exogenous cytokine support, human cells have survived for 8–10 wk and on rare occasions as long as 14 wk (17, 21). In SCID-hu mice, primitive fetal liver cells can differentiate into multiple lineages, including T cells (14, 17, 34), and can be successfully transferred to secondary recipients (35), a hallmark of hematopoietic stem cells (6). In our study, transgenic mice expressing the hIL-3, hGM-CSF, and hSCF genes supported long-term engraftment of human bone marrow and cord blood cells and supported human hematopoiesis routinely for a minimum of 13 wk, and in some animals for up to 24 wk, after transplantation. The presence of human cells over this period strongly suggests engraftment of very primitive hematopoietic progenitors (36). Although we have not demonstrated engraftment of pluripotent hematopoietic stem cells by detection of multiple hematopoietic lineages in secondary recipients, recovery of multipotent CFU-GEM colonies from bone marrow of transplanted mice provides evidence for primitive progenitor cell engraftment. We conclude that engraftment of human hematopoietic cells into SCID mice is facilitated and substantially extended by expression of the IL-3, GM-CSF, and SCF transgenes relative to nontransgenic SCID mice.

Both the source of hematopoietic cells and the strain of immunodeficient mouse have been shown to affect the engraftment of human cells into immunodeficient mice. Little engraftment of human hematopoietic cells into C57BL/6-SCID mice has been observed. Previous studies have used either C.B-17-SCID or BALB/cBy-SCID mice, in which repeatable but modest levels of engraftment of human bone marrow cells is seen when exogenous cytokines are supplied (16, 17). In this study we compared the engraftment of human cord blood and bone marrow into our transgenic BALB/cBy-SCID mice. We observed at least twofold higher levels of engraftment of human cord blood compared with bone marrow. Engraftment of cord blood cells may be enhanced by mature hematopoietic cells present in the injected low density fraction as opposed to the isolated $CD34^+$ bone marrow cells used for transplantation. Engraftment of cord blood cells into C.B-17-SCID mice has been reported to be cytokine independent (21), but our results indicate that while cord blood cells may not require cytokine supplementation to achieve short-term engraftment, more durable engraftment is seen in the transgenic BALB/cBy-SCID mice. We infer that while the initial engraftment may be cytokine independent, cytokine support can extend the period of engraftment by up to 10 wk. Other possible explanations for better engraftment of cord blood as opposed to bone marrow cells might be that cord blood is more enriched for primitive progenitors (37), that cord blood cells have an enhanced proliferative capacity based on their ability to generate expanded pools of progenitor cells in vitro (38, 39), and the efficient transduction of human cord blood progenitor cells with retroviral vectors (40, 41). Superior engraftment of human bone marrow cells has been reported in the NK cell–deficient NOD-SCID mouse as opposed to conventional C.B-17-SCID mice. In this study we show that cord blood cells engraft better in NOD-SCID mice

than in BALB/cBy-SCID mice. We feel that the high proliferative potential of cord blood cells may account for the cytokine-independent short-term engraftment we observed in NOD-SCID recipients. Currently, we are evaluating whether the expression of the human cytokine transgenes on a murine NOD-SCID genetic background will extend the high level of engraftment of human cord blood cells beyond 10 wk.

Contrary to reports using exogenous cytokine support (17, 21), we were able to detect human cells in peripheral blood of transplanted SCID mice. The only other study reporting human cells in peripheral blood was by Nolta et al. (42), who used immunodeficient *bnx* mice transplanted with human bone marrow and human stromal cells expressing hIL-3. The effects of local and continuous production of human cytokines from either transgenes or transplanted stroma appear to be different from those seen with exogenous cytokines, which provide varying levels of these factors.

In summary, complementation of the murine hematopoietic microenvironment by the introduction of human cytokine transgenes appears to be an efficient approach to the generation of an animal model for human hematopoiesis. This approach may eliminate the need for additional procedures, such as the injection of exogenous cytokines or cotransplantation of human fetal tissue, as are now used in other SCID mouse models. Transgenic SCID mice expressing human cytokine genes may therefore provide a useful in vivo assay for human hematopoietic cells and a system for the evaluation of retroviral gene transfer into hematopoietic stem cells.

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