Reversibility of CD34 expression on human hematopoietic stem cells that retain the capacity for secondary reconstitution

Mo A. Dao, Jesusa Arevalo, and Jan A. Nolta

The cell surface protein CD34 is frequently used as a marker for positive selection of human hematopoietic stem/progenitor cells in research and in transplantation. However, populations of reconstituting human and murine stem cells that lack cell surface CD34 protein have been identified. In the current studies, we demonstrate that CD34 expression is reversible on human hematopoietic stem/progenitor cells. We identified and functionally characterized a population of human CD45$^+$/CD34$^-$ cells that was recovered from the bone marrow of immunodeficient beige/nude/xid (*bnx*) mice 8 to 12 months after transplantation of highly purified human bone marrow–derived CD34$^+$/CD38$^-$ stem/progenitor cells. The human CD45$^+$ cells were devoid of CD34 protein and mRNA when isolated from the mice. However, significantly higher numbers of human colony-forming units and long-term culture-initiating cells per engrafted human CD45$^+$ cell were recovered from the marrow of *bnx* mice than from the marrow of human stem cell–engrafted nonobese diabetic/severe combined immunodeficient mice, where 24% of the human graft maintained CD34 expression. In addition to their capacity for extensive in vitro generative capacity, the human CD45$^+$/CD34$^-$ cells recovered from the *bnx* bone marrow were determined to have secondary reconstitution capacity and to produce CD34$^+$ progeny following retransplantation. These studies demonstrate that the human CD34$^+$ population can act as a reservoir for generation of CD34$^-$ cells. In the current studies we demonstrate that human CD34$^+$/CD38$^-$ cells can generate CD45$^+$/CD34$^-$ progeny in a long-term xenograft model and that those CD45$^+$/CD34$^-$ cells can regenerate CD34$^+$ progeny following secondary transplantation. Therefore, expression of CD34 can be reversible on reconstituting human hematopoietic stem cells. (Blood. 2003;101:112-118)

Introduction

Evidence that CD34$^-$ cells can have regenerative capacity in a transplant setting has been established. Osawa et al published the first definitive study on this topic, which defined a murine CD34$^-$ cell that had full engrafting capacity.[1] The first evidence that there was a human correlate to the engrafting CD34$^-$ murine stem cell was published by Bhatia et al in 1998.[2] In an elegant study using transplantation of highly selected human hematopoietic cell populations into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, this team of investigators demonstrated that there was a low level of engraftment and hematopoietic capacity in human CD34$^-$ cells,[2] and Fujisaki et al confirmed this finding.[3] In those initial studies, the CD34$^-$ cells with engrafting capacity were extremely rare, and the levels of engraftment in the NOD/SCID mice were very low. Nakamura et al later demonstrated that preculture of the human CD34$^-$ cells with cytokines in vitro prior to transplantation greatly increased their capacity for homing and engraftment.[4]

Using another long-term xenograft model, the preimmune fetal sheep system, it was demonstrated that human CD34$^-$/lineage-negative (lin$^-$) cells had multilineage reconstitution capacity.[5-7] Goodell et al characterized a primitive population of "side population" (SP) cells in mouse, monkey, and man, that excluded the Hoechst 33342 dye, lacked CD34 expression, and had reconstitution capacity.[8,9] Sato et al next demonstrated that murine CD34$^-$ stem cells could be induced to express CD34 and to increase engraftment capacity, following in vivo treatment of the mice with 5-fluorouracil (5-FU).[10] The authors hypothesized that the CD34$^-$ stem cells are extremely quiescent and that some type of activation is required to cause up-regulation of CD34 expression and to induce the capacity to engraft a recipient mouse. In the studies by their group, activation was promoted by in vivo treatment of donor mice with 5-FU.[10] These studies demonstrated that CD34$^-$ cells can generate CD34$^+$ cells. However, whether human CD34$^-$ cells with reconstituting capacity could be derived from CD34$^+$ cells has not been known until now, and has been a topic of great interest, due to the immense applicability to clinical stem cell transplantation regimens, as well as to furthering the field of basic stem cell biology.

The current studies demonstrate that a human CD34$^-$ stem cell population can be generated from CD34$^+$ cells and that the CD34$^-$ cells can then regain expression of CD34 on secondary transplantation. We identified a population of long-term engrafting human hematopoietic stem/progenitor cells that express high levels of CD34 when transplanted into immunodeficient beige/nude/xid (*bnx*) mice, then absolutely lack CD34 expression at both the cell surface and mRNA levels when harvested 8 to 12 months later. The human CD45$^+$/CD34$^-$ cells recovered from the marrow of the mice retain extensive in vitro 30-day colony-forming and long-term culture-initiating activity, even though they lack CD34

From the Division of Research Immunology and Bone Marrow Transplantation, Children's Hospital of Los Angeles, CA.

Submitted January 17, 2002; accepted June 4, 2002. Prepublished online as *Blood* First Edition Paper, July 25, 2002; DOI 10.1182/blood-2002-01-0025.

Supported by the National Institutes of Health National Heart, Lung and Blood Institute (SCOR no. 1-P50-HL54850) and the National Institute of Diabetes and Digestive and Kidney Diseases (RO1DK 53041 and RO1DK 61848).

Reprints: Jan A. Nolta, Washington University School of Medicine, Division of Oncology/Stem Cell Biology, 660 S Euclid, Box 8007, St Louis, MO 63110; e-mail: jnolta@im.wustl.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

expression at the time of isolation. In secondary immunodeficient mouse reconstitution assays, human $CD34^+$ cells were produced from the transplanted $CD34^-$ cells. The data that we present in the current studies therefore demonstrate that highly purified human $CD34^+$ cells can generate $CD34^-$ cells in vivo, which retain the capacity to regenerate $CD34^+$ cells on secondary transplantation. Therefore, expression of CD34 is reversible on human multilineage engrafting stem/progenitor cells.

Materials and methods

Isolation of hematopoietic stem/progenitor cells

Human marrow cells were isolated from the screens used to filter marrow during harvest of healthy donors for transplantation. Use of these samples, otherwise discarded as waste, was approved by the Children's Hospital of Los Angeles (CHLA) Committee on Clinical Investigation according to the Declaration of Helsinki. Mononuclear cells were isolated using Ficoll Hypaque (Pharmacia, Piscataway, NJ) density centrifugation. The mononuclear fraction was pre-enriched for $CD34^+$ cells using the mini-Magnetic Activated Cell Sorter system (Miltenyi Biotec, Auburn, CA), which provides an 85% to 90% pure $CD34^+$ population. The cells were then incubated with fluorescein isothiocyanate (FITC)–labeled anti-CD34 (HPCA2-FITC; Becton Dickinson, San Jose, CA) and phycoerythrin (PE)–labeled anti-CD38 (Leu 17-PE; Becton Dickinson), and $CD34^+/CD38^-$ cells were isolated by FACSVantage (Becton Dickinson) to a purity of more than 99%. $CD34^+CD38^-$ cells were acquired strictly as those with high CD34 antigen expression and CD38 fluorescence less than half of the maximum PE fluorescence of the isotype control, as previously defined.[11,12]

Primary transplantation in immunodeficient mice

Fluorescence-activated cell sorted (FACS)–purified human $CD34^+/CD38^-$ cells were cotransplanted with interleukin 3 (IL-3)–producing human mesenchymal stem cell carriers into immunodeficient mice 1 hour after isolation or after 48 hours of culture on Retronectin (Takara Shuzo, Otsu, Japan) with IL-3, IL-6, and stem cell factor (SCF). Six- to 8-week-old homozygous bg.bg/nu.nu/xid.xid (*bnx*) mice bred at CHLA were used for the long-term primary reconstitution studies (8-12 months), and 9- to 12-week-old NOD/SCID mice, bred at CHLA, were used for short-term comparison studies. Sublethal conditioning of *bnx* mice was accomplished by administration of 400 rads from a Shepard Mark IV irradiator 48 hours prior to transplantation. NOD/SCID mice received only 300 rads, due to their sensitivity to DNA-damaging agents. Cotransplantation of 2000 FACS-purified human $CD34^+/CD38^-$ cells and 1 million human mesenchymal stem cells (MSCs) engineered to secrete human IL-3 (huIL-3) was done as previously described.[11,13-15] Transplantation of IL-3–secreting human MSCs alone never resulted in generation of human $CD45^+$ or $CD34^+$ cells in the blood or bone marrow of the recipient mice. Following 8- to 12-month engraftment of primary *bnx* mice, and 2- to 4-month engraftment of NOD/SCID control mice, marrow was harvested. One fourth of the marrow was used for FACS analysis, mRNA preparation to assess CD34 levels, colony-forming assays, and long-term culture-initiating cell (LTCIC) assays. The remainder of the cells (three fourths of the marrow from each *bnx* mouse) was cryopreserved for secondary transplantation.

Secondary transplantation in immunodeficient mice

Sublethal conditioning of NOD/SCID mice for secondary transplantation of the human hematopoietic cells from the primary *bnx* bone marrow was done 2 hours prior to injection of the marrow cell inoculum, by administration of 300 rads using a Shepard Mark IV irradiator with an attenuator to deliver the dose at the slowest possible rate. Faster delivery of the same dosage resulted in significant mortality to this strain. All mice were screened to rule out the presence of murine T and natural killer (NK) cells that can arise due to "leakiness," prior to use. For secondary transplantation, the 2-mL cryovials (Nunc, Naperville, IL) containing the human cells recovered from the primary *bnx* recipients were thawed, carefully diluted up to 35 mL in Iscove modified Dulbecco medium (IMDM) with 10% fetal calf serum (FCS), DNAse, and heparin as described,[16] and incubated overnight in 75-mL flasks (lying flat) with a combination of IL-6 and SCF (50 ng/mL each; R & D Systems, Minneapolis, MN) prior to transplantation. The frozen cells from one primary recipient, initially transplanted with human $CD34^+/CD38^-$ cells, then verified by RNA and FACS to contain no human $CD34^+$ cells at the time of harvest 8 to 12 months later, were used to transplant 2 NOD/SCID secondary recipients, in each set of experiments.

Determination of the presence of human hematopoietic cells in tissues recovered from the mice

Bone marrow was flushed from the 4 long bones of the hind legs and used immediately for FACS analysis and cell sorting, or cryopreserved for later analyses. To determine the total human hematopoietic cell content in the bone marrow, the single-cell suspensions were labeled with HLE-1 (antihuman CD45; Becton Dickinson), and then subjected to FACS analysis. In all secondary transplant analyses, samples were gated on human $CD45^+$ cells using antihuman CD45-allophycocyanin (APC; Pharmingen, San Diego, CA) against forward scatter, and the lineages within that gated population were then identified by 3-color analysis. Directly conjugated antibodies used to identify human-specific cell-surface antigens were My9-RD1 (anti-CD33; Coulter, San Francisco, CA), Leu-12 (anti-CD19; Becton Dickinson), Leu-3a (anti-CD4; Becton Dickinson), Leu-2a (anti-CD8; Becton Dickinson), HPCA-1 and HPCA-2 (anti-CD34; Becton Dickinson), and Leu-17 (anti-CD38; Becton Dickinson). Samples were acquired on a Becton Dickinson FACScan and analysis of 10 000 or 100 000 cells acquired from each tissue was done using CellQuest software (Becton Dickinson). The appropriate isotype controls were used in all analyses, and, because each lot of antibody can differ in its cross-reactivity to murine cells, parallel staining and FACS analyses were done on normal human and nontransplanted *bnx* mouse bone marrow controls, to confirm specificity for human cells.

LTCIC assay

The LTCICs were grown as described[12,17] in 96-well plates on preestablished monolayers of primary human stromal/mesenchymal stem cells. The stromal cells had previously been expanded to result in a relatively homogeneous monolayer of myofibroblastic cells that had less than 1% contaminating $CD45^+$ macrophages, when analyzed by FACS at passage 3, as we have described.[13,14] Within 1 week before addition of hematopoietic progenitors, the stromal cultures were trypsinized and irradiated with 20 Gy; then 1000 cells/well were plated in 96-well plates (Corning Costar, Cambridge, MA) in basal bone marrow medium (BBMM, which is IMDM with 30% FCS, 1% bovine serum albumin [BSA; Sigma, St Louis, MO], 100 μM 2-mercaptoethanol, 10^{-6} M hydrocortisone [Sigma], 50 U/mL penicillin G, 50 μg/mL streptomycin sulfate, and 2 mM L-glutamine). Human $CD45^+$ cells from the marrow of *bnx* or NOD/SCID mice were plated by FACS onto the stromal monolayers at 100 cells/well. Cultures were fed once a week by replacing 50% of the medium. Wells that showed growth at week 6 were collected and plated in human-specific colony-forming assay.

Human-specific CFU plating

To determine the number of clonogenic human hematopoietic progenitors derived from the LTCIC assay, or recovered from the murine bone marrow, cells were plated in human-specific colony-forming unit (CFU) assay as described.[13] Prior to plating, the *bnx*/human bone marrow cells were incubated in IMDM with 20% fetal bovine serum (Omega Scientific, Tarzana, CA) for 4 to 12 hours to remove (by adherence) murine stromal cells and monocyte/macrophages, which secrete murine cytokines and invalidate the specificity for growth of human hematopoietic colonies measured in the assay. The medium used for CFU plating was IMDM with 30% FCS (Omega Scientific), 1% BSA (Sigma), 1.3% methylcellulose (Sigma), 10^{-4} M 2-mercaptoethanol, 50 U/mL penicillin G, 50 μg/mL streptomycin sulfate, 2 mM L-glutamine, 10^{-6} M hydrocortisone, and

10 ng/mL recombinant human IL-3 (rhIL-3; Immunex, Seattle, WA). Recombinant human erythropoietin (Epo; Epoietin α; Amgen, Thousand Oaks, CA) was added to a concentration of 2 U/mL on day 4 of culture, after Epo-dependent murine erythrocyte CFUs (CFU-Es) had died off. Methylcellulose, FCS, and BSA were previously screened to provide maximal granulocyte-erythrocyte-macrophage-megakaryocyte CFU (CFU-GEMM) development from human $CD34^+$ cells. Then 5×10^4 and 1×10^5 plastic nonadherent cells from engrafted and control mice were plated in duplicate in 1 mL of the medium in gridded culture dishes (Nunc). Colonies were grown in a fully humidified incubator and enumerated on day 30 as previously described, because there is poor CFU development from primitive cells at day 14.[18] In this medium, growth of murine colonies, from nontransplanted control mice, was observed only when stromal cells had contaminated the CFU dish. Therefore, any plates containing adherent stromal or fibroblastic colonies were discarded.

RNA and cDNA preparation and testing

RNA was isolated from stimulated and nonstimulated cells using RNA STAT-60 (TEL-TEST, Friendswood, TX). Samples were quantitated using a spectrophotometer, and equal amounts of RNA from all samples were subjected to first-strand cDNA synthesis using the Superscript Preamplification System (Gibco BRL, Gaithersburg, MD). For some experiments where human $CD45^+$ cells were not reisolated from the *bnx* bone marrow, the amount of RNA contributed by the human, as opposed to murine $CD45^+$ cells in the sample was first calculated from FACS analysis. The same amount of RNA was then used from KG-1A cells, human umbilical cord blood (UCB), and bone marrow–positive controls for first-strand synthesis. This precise quantitation was necessary because human $CD45^+$ cells in *bnx*/hu mice were present at a far lower frequency than in the human control samples. Therefore, a direct comparison of equal amounts of RNA from *bnx*/hu mouse marrow and human samples could have underestimated the human $CD34^+$ cell frequency in the mice, giving false-negative results in the analyses.

Following cDNA amplification from the standardized *bnx*/hu marrow, reisolated human $CD45^+$ cells from the *bnx*/hu marrow, and human KG-1a, UCB, or bone marrow control samples, polymerase chain reaction (PCR) was performed for CD34 and GAPDH (used as a loading control) as described.[19] Samples were loaded on 2% ethidium bromide–stained gels, electrophoresed, and read with an Eagle Eye reader (Stratagene, La Jolla, CA).

Statistical analyses

All analyses were done using the Microsoft Excel 5.0 software. Average values are listed with SDs. The significance of each set of values was assessed using the 2-tailed *t* test assuming equal variance.

Results

Engraftment of primary *bnx* mice with highly purified human $CD34^+/CD38^-$ cells and generation of $CD34^-$ cells

The FACS-purified human $CD34^+/CD38^-$ stem/progenitor cells from 12 different human marrow donors were transplanted into a total of 28 recipient *bnx* mice (Figure 1). The transplanted cells had high expression of CD34 and very low CD38 expression, as we have previously described (Figure 2A).[11,12] Following a period of long-term engraftment (8-12 months), the mice were harvested and FACS analysis was done on marrow, spleen, and blood cells to determine whether they were engrafted with human hematopoietic cells. Of the 28 mice receiving transplants, 23 mice in this study had more than 1% engraftment with human hematopoietic cells, as indicated by FACS analysis using an antihuman CD45 antibody. The 23 well-engrafted mice were further evaluated by a FACS immunophenotyping panel to identify the human T, B, myeloid

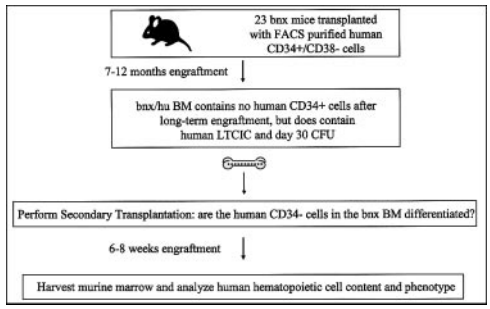

Figure 1. Experimental schema overview. $CD34^+/CD38^-$ cells were isolated from normal human bone marrow and transplanted into immunodeficient *bnx* mice. Marrow was harvested from the primary recipients 8 to 12 months after transplantation and analyzed by FACS, immunohistochemistry, RT-PCR, and CFU and LTCIC cell assays. No human $CD34^+$ cells were detected. Samples of marrow from the primary mice were then transplanted into secondary NOD/SCID recipients. The secondary recipients were killed 6 to 8 weeks later, and the marrow was subjected to FACS analysis for CD34 and human hematopoietic lineage development. Multilineage human hematopoietic development and human $CD34^+$ cells were detected in the secondary recipients.

lineages that had developed, as we have described.[11,13,20,21] In addition, the percentages of human $CD34^+$ and $CD38^+$ cells within the mouse marrow were evaluated by FACS.

The *bnx* mice had an average of 5.2 ± 1.1 (SEM) human $CD45^+$ cells in their marrow ($n = 23$). The percentage of the human graft that was composed of $CD33^+$ myeloid progenitors and cells averaged $47.3\% \pm 5.2\%$ of the human $CD45^+$ cells ($n = 23$). There were also significant percentages of $CD8^+$ (average, $23.8\% \pm 2.6\%$) and $CD4^+$ cells (average, $16.4\% \pm 2.0\%$), with fewer $CD19^+$ (average, $7.8\% \pm 1.5\%$) and lin^- cells (average, $5.4\% \pm 1.2\%$). In comparison, 11 NOD/SCID mice underwent transplantation, but were analyzed at 2 to 4 months after transplantation because this strain has a shorter lifespan. Human lineage distributions in the short-term engrafted NOD/SCID mice differed significantly from the long-term engrafted *bnx* mice. An average of 19.7 ± 3.7 (SEM) human $CD45^+$ cells was detected in the marrow, and 98% of the $CD45^+$ cells coexpressed CD38. The majority of the human $CD45^+$ cells in the NOD/SCID recipients were $CD19^+$ B cells (average, $56.4\% \pm 7.9\%$), with lower levels of $CD33^+$ and $CD4^+$ cells ($19.6\% \pm 3.8\%$ and $1.9\% \pm 0.5\%$, respectively). An average of $25.3\% \pm 4.2\%$ of the human $CD45^+$ cells expressed CD34. In sharp contrast, in all 23 *bnx* mice, the majority of the human $CD45^+$ cells expressed CD38 (97.1%), but all cells were absolutely devoid of human CD34 expression at the cell surface (Table 1 and Figure 2).

Although there was no expression of CD34 on the surface of the human $CD45^+$ cells recovered from the long-term engrafted *bnx* mice, it was possible that there could have been RNA expressed, or CD34 protein that was lying beneath the membrane of the cells and not on the surface, as previously determined by Fackler et al.[19] To study this issue, mRNA was prepared and also immunohistochemistry was performed on cytospin preparations of FACS-isolated human $CD45^+$ cells from the *bnx* marrow, and also the total bone marrow suspensions. No human CD34 protein was detected in the immunohistochemical analyses of marrow cells from *bnx* mice, whereas human $CD34^+$ cells were readily detectable in the marrow of the control NOD/SCID mice ($n = 10$ each, data not shown).

To further confirm the FACS and immunohistochemical data that indicated a lack of CD34 protein on the long-term engrafted human hematopoietic cells, mRNA was prepared from both FACS-isolated human $CD45^+$ cells, recovered from the *bnx* marrow, and from the total *bnx*/hu bone marrow that contained the long-term engrafted cells. In the instances where human $CD45^+$

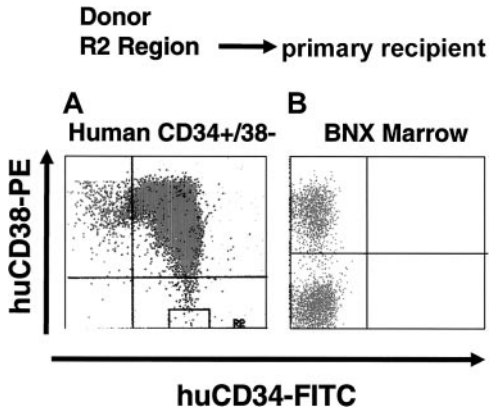

Figure 2. Lack of CD34 expression on long-term engrafted human cells. $CD34^+/CD38^-$ cells were purified from human marrow and isolated as shown in the R2 gate (A). The cells were transplanted into primary *bnx* recipients and harvested 12 months later. The total marrow from one well-engrafted primary *bnx* recipient of the sorted cells from panel A is shown in panel B, demonstrating that there is no human CD34 expression either in the human $CD38^+$ or in the total (human $CD38^-$ plus murine) fractions.

cells were not reisolated from the *bnx* bone marrow, the amount of RNA contributed by the human, as opposed to murine $CD45^+$ cells in the sample was first calculated from FACS analysis. The same amount of RNA was then used for the first-strand cDNA synthesis from KG-1A cells, human UCB, and marrow-positive controls. This precise quantitation was necessary because human $CD45^+$ cells in *bnx*/hu mice were present at a far lower frequency than in the human control samples. Therefore, a direct comparison of equal amounts of RNA from *bnx*/hu mouse marrow and human samples could have underestimated the human $CD34^+$ cell frequency in the mice, giving false-negative results in the analyses. No CD34 mRNA was detected in the human $CD45^+$ cells recovered from *bnx* mice, or in the *bnx*/hu marrow, in any of the reverse transcription-PCR (RT-PCR) assays. An example is shown in Figure 3.

Recovery of clonogenic human progenitors that lack CD34 expression from the marrow of long-term engrafted *bnx* mice

For years we have puzzled over the presence of clonogenic human progenitors and LTCIC in *bnx*/hu mouse marrow, without expression of CD34 at the cell surface. In the current studies we plated human-specific CFUs and LTCICs from the marrow of 23 long-term engrafted *bnx* mice, in comparison to the marrow of 11

Table 1. Comparison of total human $CD45^+$ cell engraftment levels, $CD34^+$ cell levels, and human-specific CFU content in *bnx* and NOD/SCID mice

	Total hu $CD45^+$ cells/femur + tibia	Total hu $CD34^+$ cells/femur + tibia	Hu CFU/100 000 hu $CD45^+$ cells	Hu LTCIC/100 000 hu $CD45^+$ cells
bnx/hu				
1	7.6×10^5	0	290	ND
2	8.2×10^5	0	820	87
3	5.0×10^5	0	590	60
4	1.1×10^6	0	760	115
5	6.4×10^5	0	480	69
6	7.2×10^5	0	630	33
7	1.7×10^6	0	470	45
8	1.6×10^6	0	400	ND
9	1.0×10^6	0	490	ND
10	5.0×10^5	0	670	ND
11	2.4×10^5	0	830	104
12	1.3×10^6	0	600	28
13	6.8×10^5	0	530	61
14	1.6×10^6	0	170	ND
15	9.2×10^5	0	300	ND
16	1.0×10^6	0	570	ND
17	4.2×10^5	0	560	ND
18	4.4×10^5	0	1090	132
19	6.0×10^5	0	990	96
20	1.5×10^6	0	120	ND
21	2.3×10^6	0	130	ND
22	8.0×10^5	0	880	73
23	1.0×10^6	0	440	35
Average	$9.6 \times 10^5 \pm 1 \times 10^5$	0	550 ± 50	72.2 ± 9.2
NOD/SCID				
1	3.8×10^6	5.0×10^5	190	21
2	1.1×10^6	4.4×10^5	400	13
3	7.0×10^6	1.3×10^6	100	ND
4	1.1×10^7	3.0×10^6	110	9
5	1.5×10^7	3.9×10^6	90	ND
6	1.5×10^7	1.3×10^6	80	ND
7	6.7×10^6	9.4×10^5	130	15
8	2.9×10^6	5.6×10^5	190	17
9	3.6×10^6	8.8×10^5	160	12
10	8.2×10^6	1.0×10^6	80	ND
11	1.3×10^7	1.4×10^6	80	ND
Average	$7.9 \times 10^6 \pm 1.5 \times 10^6$	2.2×10^6	145 ± 30	14.5 ± 1.7

ND indicates not determined.

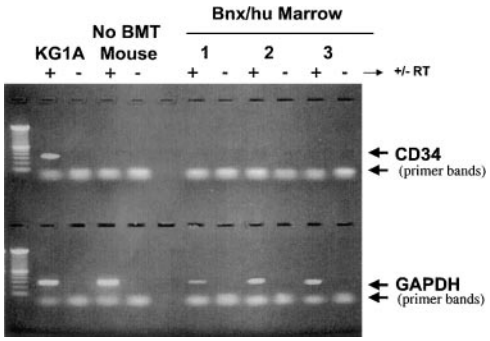

Figure 3. RNA analysis. RT-PCR analysis was done to analyze CD34 expression in the marrow of long-term engrafted primary *bnx* recipients of human $CD34^+/CD38^-$ cells. The KG-1A cell line was used as a positive control, and nontransplanted *bnx* mouse marrow as a negative control. Marrow samples from 3 *bnx* mice that had no cell surface CD34 expression were tested. Eight months after engraftment, mouse 1 had 22.1% human $CD45^+$ cells in its marrow. Twelve months after engraftment, mouse 2 had 13.4% human $CD45^+$ cells in its marrow and mouse 3 had 35.6% human $CD45^+$ cells in its marrow.

NOD/SCID mice that had undergone transplantation with similar marrow-derived and purified human hematopoietic cell populations, but harvested after only 2 to 4 months, due to the abbreviated lifespan of the strain.

Approximately one fourth of the human graft in the NOD/SCID mice was composed of $CD34^+$ cells, whereas none were detected in the *bnx* marrow, as determined by FACS analysis of 100 000 cells/sample (Table 1). However, although the human cells engrafted in the marrow of *bnx* mice lacked CD34 expression, the number of clonogenic cells (day 30 CFUs) and LTCICs per human $CD45^+$ cell engrafted in the marrow of the *bnx* mice was significantly higher than the number detected in the NOD/SCID marrow ($P < .005$; Table 1).

We observed that the human CFUs plated from the *bnx* marrow did not begin to develop for the first 10 to 14 days in methylcellulose culture, whereas the human CFUs from the NOD/SCID marrow were already dividing 4 days after plating, at the time of addition of Epo. Because the early forming cells from the NOD/SCID mice were still intact at day 30, we counted all colonies at that point. We have previously observed that cells from 4-hydroperoxycyclophosphamide–treated human marrow do not form day 14 CFUs, but do form day 30 CFUs,[18] and that these cells are synchronized in the G_0 phase of the cell cycle,[20] and remain capable of multilineage reconstitution in humans.[18] Shah et al have demonstrated that extended LTCICs are primitive, quiescent cells with late, but not early, colony-forming capacity.[17] The observation of late, but not early, colony formation from the *bnx* marrow suggested that the human cells engrafted in the *bnx* mice at the 8- to 12-month time point might be more primitive than those engrafted in the NOD/SCID mice at the 2- to 4-month time point, or that, at the late time point, the *bnx* marrow no longer contained committed human progenitors that were readily triggered to divide by the cytokines in the methylcellulose medium.

Secondary transplantation to establish the regenerative capacity of the human $CD34^-$ cells recovered from the *bnx* marrow

The capacity for LTCIC and for late-forming CFU generation from the human $CD34^-$ cells in *bnx* mice suggests that the human stem and progenitor cells had lost expression of CD34 over time, but had retained hematopoietic generative capacity. We next tested this theory further in secondary reconstitution studies. Sublethally irradiated (300 rads) NOD/SCID mice were used as the recipients for secondary transplantation of the human hematopoietic cells from the primary *bnx* bone marrow.

Our initial secondary transplantation attempts (n = 16 mice receiving transplants from 8 primary transplant donors) did not result in engraftment when the human cells from the *bnx* marrow were injected into NOD/SCID mice directly after thawing and removal of the dimethyl sulfoxide (DMSO) cryoprotectant. We then learned that an overnight preincubation of the cells from the primary recipients in IL-6 plus SCF prior to injection would greatly enhance the homing and engraftment of the human cells (J. Dick, personal oral communication, February 25, 2000). This may be due to the fact that the combination of IL-6 and SCF up-regulates CXCR4, which enhances stem cell homing.[22,23]

In subsequent experiments we preincubated the thawed, cryopreserved samples in SCF plus IL-6 (50 ng/mL) overnight, prior to the secondary transplantation, and met with much better success. Two mice each received transplants of cells from 6 primary recipients. One fourth of the marrow from each primary recipient had been used for FACS analysis, RNA preparation, and CFU and LTCIC assays, as described above, at the time of harvest. The remaining (cryopreserved) three fourths of the marrow cells from each primary recipient, averaging 2.8×10^7 total marrow cells with 9.2% human $CD45^+$ cell content, was then thawed and divided between 2 secondary recipients per primary *bnx* mouse. We did not attempt to reisolate the human cells from the thawed marrow, in case the manipulation lost or altered the content. We used the murine cells from the primary *bnx* recipients as "carriers" for the human cells, rather than using human mesenchymal stem cell carriers as we have described in our previous studies.[13-15,20] We were worried that, due to the recent reports of stem cell plasticity, transplantation of human MSCs might give rise to $CD34^+$ cells and invalidate the secondary assays. In the primary recipients, no human MSCs homed into the murine marrow, as we have previously reported,[15] and neither *bnx* nor NOD/SCID mice given transplants of MSCs alone ever had human $CD45^+$ or $CD34^+$ cells in their blood or marrow. The murine carrier cells from the primary *bnx* mice were optimal for the current studies because they provided no background in our end-point analyses because all antibodies, primers, and culture assays are strictly optimized for the specific detection of human cells.

The transplanted human cells from the *bnx* marrow generated multilineage reconstitution in secondary NOD/SCID recipients. Human myeloid, B, and T cells were detected (n = 12, example shown in Figure 4). The most significant information that we obtained from the secondary transplants, however, was that $CD34^+$ cells were easily identified in the secondary recipients. In 6 mice that had more than 5% total human $CD45^+$ cell engraftment, an average of 14.6% ± 2.5% of those cells clearly expressed CD34 (Figure 4). These data demonstrate that CD34 expression is reversible: $CD34^+/CD38^-$ cells can generate $CD45^+/CD34^-$ cells, which can then generate $CD45^+/CD34^+$ cells. This is the first demonstration, to our knowledge, that human $CD34^+$ cells can generate $CD34^-$ cells in vivo, which retain clonogenicity and reconstitution capacity, and can regenerate a pool of $CD34^+$ cells.

Discussion

Human $CD34^+/CD38^-$ cells engraft in *bnx* mice, and their progeny can be recovered from the murine bone marrow for as long as 18 months after transplantation.[11,20] Using the *bnx*/hu xenograft model,

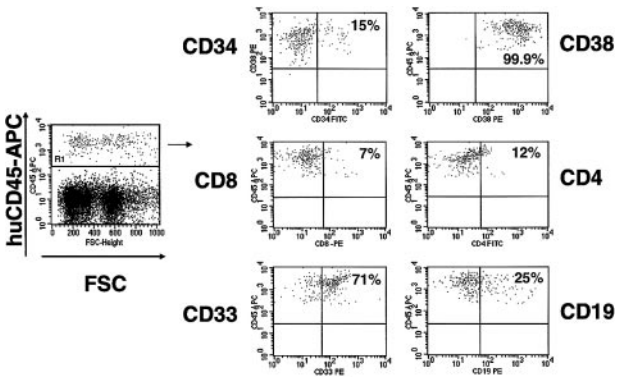

Figure 4. Human hematopoietic lineage development in secondary NOD/SCID recipients. Marrow from a primary *bnx* mouse, engrafted with human CD34$^+$/CD38$^-$ cells for 1 year, was transplanted into a secondary NOD/SCID recipient. Marrow from the secondary mouse was harvested 6 weeks after transplantation and analyzed by FACS. Multilineage human hematopoietic lineage engraftment was detected, including expression of CD34, which was not detectable in the primary recipient at the time of harvest (data shown in Figures 2 and 3).

we found that there are human cells with extensive LTCIC and 30-day CFU capacity that are recovered from the marrow of long-term engrafted animals. The human cells recovered from the marrow of the *bnx* mice express human CD45, but are devoid of expression of CD34 at the cell surface and mRNA levels. Therefore, their CFU and LTCIC-forming capacity had appeared to be a paradox. When calculated on a "per human CD45$^+$ cell" basis, the in vitro generative capacity from human cells recovered from *bnx* mice, which had no human CD34$^+$ cells, was significantly higher than that from human cells recovered from NOD/SCID mice (Table 1). These data were very interesting because the cells recovered from the NOD/SCID marrow had a relatively high percentage of CD34 expression, whereas the human cells recovered from *bnx* mice had none (Table 1).

Until 1996, when the first report on CD34$^-$ stem cells was published,[1] we had hypothesized that the cells isolated from the long-term *bnx* marrow may have lacked expression of CD34 because they were terminally differentiating and were no longer primitive. Because there are high levels of human CD34$^-$/CD33$^+$ cells recovered from the marrow of *bnx* mice, we had surmised that they might be the cells responsible for the in vitro generative capacity that we had observed for several years, and were some type of committed progenitor cell that still retained LTCIC and day 30 CFU capacity. This hypothesis turned out to be wrong. In the current studies, we tested the secondary generative capacity of the cells by transplanting them into NOD/SCID mice. In the secondary recipients, the human CD45$^+$/CD34$^-$ cells recovered from the *bnx* mice were capable of multilineage differentiation capacity with regeneration of a pool of human CD34$^+$ cells, in addition to both lymphoid and myeloid lineages.

Our data agree with that of Sato's group, who hypothesized that up-regulation of CD34 expression in the murine system is an "activation event."[10] The "activation" to which Sato and colleagues refer is not likely to be simply cell cycle induction, which could be induced by recruiting primitive hematopoietic stem cells into cycle to reconstitute hematopoiesis in a 5-FU–conditioned animal. Human hematopoietic cells can be identified that express high levels of CD34, yet are very highly quiescent,[17,24] cannot be readily induced into cell cycle by cytokines, and lack expression of the marker Ki67, the absence of which indicates that they are in the G_0 phase of the cell cycle.[20,25-27] In the studies where our group induced highly quiescent CD34$^+$/CD38$^-$ and CD34$^+$/4- hydroperoxycyclophosphamide–resistant cells into cycle by reduction of cyclin-dependent kinase (CDK) inhibitors,[20] there were no commensurate alterations in the levels of CD34 expression (M.A.D. and J.A.N., unpublished data, March-June 1997). The molecular nature of the "activating" factors that regulate induction of CD34 expression in the 5-FU–treated mice described by Sato et al[10] remain unknown at the current time. Whether CD34 is up-regulated by an increase in metabolic activity as the quiescent cells progress toward cell cycle by down-regulation of CDK inhibitors and induction of cyclins and cell cycle regulatory proteins,[28] or whether one or both progeny from the initial divisions of a CD34$^-$ cell acquire CD34 expression, is not yet clearly defined.

The murine and human CD34 promoters have been characterized to determine which transcription factors act as positive and negative regulators.[29-32] Okuno et al[35] have introduced human artificial chromosomes that carry the entire human CD34 genomic locus into transgenic mice. Human CD34 was expressed in murine stem cells that were CD34$^-$.[35] These types of interesting studies will give us clues as to which signaling pathways induce CD34 expression, under both steady-state and transplantation/reconstitution conditions. Fackler et al demonstrated that phosphorylation of preformed CD34 protein determines whether it is expressed on the cell surface or retained inside the cell.[19,33] These studies show the importance of not relying on FACS data for cell surface CD34 expression to determine whether populations are truly CD34$^+$ versus CD34$^-$. Analyses to assess levels of CD34 mRNA or intracellular protein should also be done to determine whether CD34 is present, but not yet localized to the cell surface. In the current studies, no human CD34 mRNA was detected in the marrow of the long-term engrafted *bnx* mice.

Fackler et al hypothesized that small numbers of CD34$^+$ cells contaminating the CD34$^-$ populations could account for the previous results that had been reported.[34] Very low levels of engraftment were obtained in Fackler's study when highly purified CD34$^-$/lin$^-$ populations were transplanted, agreeing with previous reports in which freshly isolated CD34$^-$ cells had low engraftment potential.[2,3] Then, a report from Nakamura et al confirmed that freshly isolated human CD34$^-$ cells *do* have very low engraftment capacity, as measured in the NOD/SCID xenograft system.[4] However, Nakamura et al used the stromal cell line HESS-5 to demonstrate that the human CD34$^-$ cells could give rise to CD34$^+$ cells in vitro that had improved engraftment capacity.[4] Highly purified human CD34$^-$/lin$^-$ cells cultured with cytokines for 7 days on the murine stromal cell line HESS-5 expanded and up-regulated expression of CD34.[4] The expanded cells had increased levels of engrafting capacity for NOD/SCID mice. These studies suggest that the human CD34$^-$ population may be a reservoir that can generate CD34$^+$ cells. The number of human hematopoietic cells generated from the expanded CD34$^-$ cells ("CD34-induced" cells) in the study by Nakamura et al was far superior to what can be achieved by transplanting the freshly isolated CD34$^-$ cells into NOD/SCID mice, as shown in the earlier engraftment studies.[2,3]

The current studies demonstrate that human CD34$^+$ stem/progenitor cells can give rise to a population of human CD34$^-$ stem/progenitor cells in vivo, in immunodeficient mice. Although this may be an artifact of our long-term xenograft system, it is also possible that the same phenomenon may be observed when CD34$^+$ cells are transplanted into human recipients. It has been proposed that the human CD34$^-$/lin$^-$ cell population could be a precursor to the CD34$^+$ cell, and therefore should be included in stem cell transplantation. In the current studies, we show that the expression

of CD34 on primitive, engrafting human stem cells can be reversible. However, whether the $CD34^-$ pool that is regenerated from $CD34^+$ cells after long-term transplantation, as we have shown here, differs in any way from the native, or endogenous $CD34^-$ cells remains to be determined. It is possible that there could be differences in telomere length, telomerase activity, alternate cell surface proteins and receptors, or possibly even pluripotency and the types of lineages or tissues that each population could generate. More research to define the differences that exist between the $CD34^-$, $CD34^+$, and "CD34 reinduced" human hematopoietic stem cells will determine whether there are any differences in reconstitution capacity, at the level of homing, survival, or expansion.

In summary, we have demonstrated that a portion of the human stem/progenitor cell compartment loses CD34 expression in the immunodeficient mouse marrow following the primary transplantation. When harvested 8 to 12 months later, the human cells are very quiescent and are no longer producing progeny into the bloodstream of the mice. At this point the human $CD45^+$ cells are not expressing human CD34 protein or mRNA. However, when transplanted into sublethally irradiated secondary immunodeficient mouse recipients, "activation" occurred, and the cells were stimulated to produce multilineage progeny, and either to up-regulate CD34 expression, or to produce progeny that were $CD34^+$. The current studies provide the first evidence that expression of CD34 is reversible on engrafting human hematopoietic stem/progenitor cells that retain the capacity for multilineage secondary reconstitution. To understand the processes involved in greater detail, further studies are required to define the cell cycle characteristics, biology, transduction, and hematopoietic reconstitution of the $CD34^-/lin^-$ stem cell, compared with the $CD34^+/lin^-$ stem cells.

Acknowledgments

The authors would like to thank Renee Traub-Workman and Miriam Figueroa for talented and devoted care of our immunodeficient mice. Sally Worttman heads our excellent animal facility and Dr Janet Baer provided highly useful veterinary advice.

References

1. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science. 1996;273:242-245.
2. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity [see comments]. Nat Med. 1998;4:1038-1045.
3. Fujisaki T, Berger MG, Rose-John S, Eaves CJ. Rapid differentiation of a rare subset of adult human lin(−)CD34(−)CD38(−) cells stimulated by multiple growth factors in vitro. Blood. 1999;94: 1926-1932.
4. Nakamura YAK, Chargui J, Kawada H, et al. Ex vivo generation of $CD34^+$ cells from $CD34^-$ hematopoietic cells. Blood. 1999;94:4053-4059.
5. Zanjani ED, Almeida-Porada G, Livingston AG, Flake AW, Ogawa M. Human bone marrow $CD34^-$ cells engraft in vivo and undergo multilineage expression that includes giving rise to $CD34^+$ cells. Exp Hematol. 1998;26:353-360.
6. Zanjani ED, Almeida-Porada G, Livingston AG, Porada CD, Ogawa M. Engraftment and multilineage expression of human bone marrow $CD34^-$ cells in vivo. Ann N Y Acad Sci.1999;872:220-231; discussion 231-232.
7. Verfaillie CM, Almeida-Porada G, Wissink S, Zanjani ED. Kinetics of engraftment of CD34(−) and CD34(+) cells from mobilized blood differs from that of CD34(−) and CD34(+) cells from bone marrow. Exp Hematol. 2000;28:1071-1079.
8. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996;183:1797-1806.
9. Goodell MA, Rosenzweig M, Kim H, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med. 1997;3:1337-1345.
10. Sato T, Laver JH, Ogawa M. Reversible expression of CD34 by murine hematopoietic stem cells [see comments]. Blood. 1999;94:2548-2554.
11. Dao MA, Shah AJ, Crooks GM, Nolta JA. Engraftment and retroviral marking of $CD34^+$ and $CD34^+CD38^-$ human hematopoietic progenitors assessed in immune-deficient mice. Blood. 1998; 91:1243-1255.
12. Hao QL, Shah AJ, Thiemann FT, Smogorzewska EM, Crooks GM. A functional comparison of $CD34^+$ $CD38^-$ cells in cord blood and bone marrow. Blood. 1995;86:3745-3753.
13. Nolta JA, Hanley MB, Kohn DB. Sustained human hematopoiesis in immunodeficient mice by cotransplantation of marrow stroma expressing human interleukin-3: analysis of gene transduction of long-lived progenitors. Blood. 1994;83: 3041-3051.
14. Nolta JA, Smogorzewska EM, Kohn DB. Analysis of optimal conditions for retroviral-mediated transduction of primitive human hematopoietic cells. Blood. 1995;86:101-110.
15. Dao MA, Pepper KA, Nolta JA. Long-term cytokine production from engineered primary human stromal cells influences human hematopoiesis in an in vivo xenograft model. Stem Cells. 1997;15:443-454.
16. Dao MA, Nolta JA. Retroviral-mediated transduction of human hematopoietic stem and progenitor cells. In: Jordan C and Klug C. Hematopoietic Stem Cell Protocols. Totowa, NJ: Humana Press; 2002:253-274.
17. Shah AJ, Smogorzewska EM, Hannum C, Crooks GM. Flt3 ligand induces proliferation of quiescent human bone marrow $CD34^+CD38^-$ cells and maintains progenitor cells in vitro. Blood. 1996; 87:3563-3570.
18. Lenarsky C, Weinberg K, Petersen J, et al. Autologous bone marrow transplantation with 4-hydroperoxycyclophosphamide purged marrows for children with acute non-lymphoblastic leukemia in second remission. Bone Marrow Transplant. 1990;6:425-429.
19. Fackler MJ, Civin CI, May WS. Up-regulation of surface CD34 is associated with protein kinase C-mediated hyperphosphorylation of CD34. J Biol Chem. 1992;267:17540-17546.
20. Dao MA, Taylor N, Nolta JA. Reduction in levels of the cyclin-dependent kinase inhibitor p27(kip-1) coupled with transforming growth factor beta neutralization induces cell-cycle entry and increases retroviral transduction of primitive human hematopoietic cells. Proc Natl Acad Sci U S A. 1998;95:13006-13011.
21. Nolta JA, Dao MA, Wells S, Smogorzewska EM, Kohn DB. Transduction of pluripotent human hematopoietic stem cells demonstrated by clonal analysis after engraftment in immune-deficient mice. Proc Natl Acad Sci U S A. 1996;93:2414-2419.
22. Lapidot T. Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice: the role of SDF-1/CXCR4 interactions. Ann N Y Acad Sci. 2001;938:83-95.
23. Peled A, Petit I, Kollet O, et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science. 1999;283: 845-848.
24. Hao QL, Thiemann FT, Petersen D, Smogorzewska EM, Crooks GM. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. Blood. 1996;88:3306-3313.
25. Jordan CT, Yamasaki G, Minamoto D. High-resolution cell cycle analysis of defined phenotypic subsets within primitive human hematopoietic cell populations. Exp Hematol. 1996;24:1347-1355.
26. Landberg G, Roos G. Proliferating cell nuclear antigen and Ki-67 antigen expression in human haematopoietic cells during growth stimulation and differentiation. Cell Prolif. 1993;26:427-437.
27. Gothot A, Pyatt R, McMahel J, Rice S, Srour EF. Functional heterogeneity of human CD34(+) cells isolated in subcompartments of the G0 /G1 phase of the cell cycle. Blood. 1997;90:4384-4393.
28. Dao MA, Hwa J, and Nolta JA. Molecular mechanism of TGFB-mediated quiescence in human $CD34^+$ progenitors: modulation of the cyclin-dependent kinase inhibitor $p15^{ink4}$. Blood. 2002;99: 499-506.
29. Radomska HS, Satterthwaite AB, Taranenko N, Narravula S, Krause DS, Tenen DG. A nuclear factor Y (NFY) site positively regulates the human CD34 stem cell gene. Blood. 1999;94:3772-3780.
30. Radomska HS, Satterthwaite AB, Burn TC, Oliff IA, Huettner CS, Tenen DG. Multiple control elements are required for expression of the human CD34 gene. Gene. 1998;222:305-318.
31. Krause DS, Ito T, Fackler MJ, et al. Characterization of murine CD34, a marker for hematopoietic progenitor and stem cells. Blood. 1994;84:691-701.
32. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility [see comments]. Blood. 1996;87:1-13.
33. Fackler MJ, Civin CI, Sutherland DR, Baker MA, May WS. Activated protein kinase C directly phosphorylates the CD34 antigen on hematopoietic cells. J Biol Chem. 1990;265:11056-11061.
34. Gao Z, Fackler MJ, Leung W. Human $CD34^+$ cell preparations contain over 100-fold greater NOD/SCID mouse engrafting capacity than do $CD34^-$ cell preparations. Exp Hematol. 2001;29:910-921.
35. Okuno Y, Iwasaki H, Huettner CS, et al. Differential regulation of the human and murine CD34 genes in hematopoietic stem cells. Proc Natl Acad Sci U S A. 2002;99:6246-6251.