ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 31 (2003) 413–420

Cotransplantation of human mesenchymal stem cells enhances human myelopoiesis and megakaryocytopoiesis in NOD/SCID mice

Maria Angelopoulou[a,*], Enrico Novelli[b,*], Joanna E. Grove[a], Henry M. Rinder[a], Curt Civin[b], Linzhao Cheng[b], and Diane S. Krause[a]

[a]*Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Conn., USA;*
[b]*Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Md., USA*

(Received 27 September 2002; revised 29 January 2003; accepted 31 January 2003)

***Objective*. For approximately 5% of autologous transplant recipients and a higher proportion of allogeneic transplant recipients, low level and delayed platelet engraftment is an ongoing problem. Mesenchymal stem cells (MSC), which can be derived from bone marrow as well as other organs, are capable of differentiation into multiple cell types and also support hematopoiesis in vitro. Because cotransplantation of marrow-derived stromal cells has been shown to enhance engraftment of human hematopoietic stem cells, we hypothesized that cotransplantation of MSC could enhance platelet and myeloid cell development.**

***Materials and Methods*. We tested this hypothesis by transplantation of CD34-selected mobilized human peripheral blood stem cells (PBSC) into sublethally irradiated NOD/SCID mice with or without culture-expanded human MSC and evaluated human myeloid, lymphoid, and megakaryocytic engraftment with flow cytometry and in vitro cultures.**

***Results*. We find that MSC cotransplantation enhances human cell engraftment when a limiting dose ($<1 \times 10^6$) of CD34 cells is administered. This enhancement is characterized by a shift in the differentiation of human cells from predominantly B lymphocytes to predominantly $CD13^+$, $CD14^+$, and $CD33^+$ myeloid cells with a corresponding increase in myeloid CFU in the marrow. Megakaryocytopoiesis is enhanced by MSC cotransplantation as assessed by an increase in both marrow CFU-MK and circulating human platelets. In contrast, MSC do not affect the percentage of human bone marrow cells that expresses $CD34^+$.**

***Conclusions*. Cotransplantation of human mesenchymal stem cells with $CD34^+$-selected hematopoietic stem cells enhances myelopoiesis and megakaryocytopoiesis. © 2003 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

Human mesenchymal stem cells (MSC) are adherent cells capable of self-renewal and multilineage differentiation [1,2]. Friedenstein and colleagues first demonstrated that adherent cells grown from bone marrow are capable of differentiation into osteoblasts and "mechanocytes" [3,4]. Since then, despite the use of different nomenclatures, common features among these cells have been identified [2]. MSC are adherent in culture, have a finite lifespan of 15 to 50 cell doublings, and have the capacity to differentiate to osteoblasts, chondroblasts, myocytes, and adipocytes when exposed to appropriate in vivo or in vitro stimuli [2,5–13]. Recent evidence suggests that if bone marrow–derived adherent cells are grown at very low density, a highly plastic subpopulation can grow in culture for at least 1 year, and perhaps indefinitely [14,15].

While there is not a clearly defined antigenic phenotype for MSC, there is agreement that they do not express typical hematopoietic antigens such as CD45, CD34, CD14, CD11b, CD43, or glycophorin A [5,16,17]. Surface antigens that have been identified on MSC include Stro-1 [5,17,18], Thy-1, c-kit [5,17,18], HOP26 [19], SH-2, SB-10, CD44, the β-1 integrin CD29, transferrin receptor CD71 [16,20], and osteogenic markers such as alkaline phosphatase, osteopontin, and collagen types I and III [21], as well as others [13].

Transplantation of mesenchymal stem cells is used clinically for bone and cartilage disorders. In preclinical animal

Offprint requests to: Diane Krause, M.D., Ph.D., Yale University School of Medicine, Department of Laboratory Medicine, 333 Cedar Street, PO Box 208035, New Haven, CT 06520-8035; E-mail: diane.krause@yale.edu

*Dr. Angelopoulou is currently affiliated with the Department of Hematology and Bone Marrow Transplantation, City of Hope Medical Center, Duarte, CA, USA. Dr. Novelli is currently affiliated with the Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA, USA.

0301-472X/03 $–see front matter. Copyright © 2003 International Society for Experimental Hematology. Published by Elsevier Science Inc.
doi: 10.1016/S0301-472X(03)00042-0

models, there are promising data for using MSC to treat central nervous system disorders [22], osteogenesis imperfecta [23], osteoporosis, and muscle damage [24]. MSC transplantation into patients with osteogenesis imperfecta [25] and infantile hypophosphatasia promotes bone formation and decreases disease severity [26]. The use of MSC in conjunction with hematopoietic stem cells for both autologous and allogeneic transplant has also been considered. In human autologous and allogeneic peripheral blood stem cell (PBSC) transplantation, most patients engraft quite rapidly (absolute neutrophil count [ANC] of 500 within 11 days and platelet count of 20,000 within 12 days post–autologous transplant). However, approximately 5% of autologous patients have delayed (>18 days) or defective platelet engraftment, and this occurs most often in patients who receive low doses of $CD34^+$ cells per kg [27,28]. Several lines of evidence suggest that cotransplantation of MSC could facilitate PBSC engraftment in patients who are at risk for poor or delayed engraftment: 1) MSC support the growth of hematopoietic stem and progenitor cells and dramatically enhance megakaryocyte and platelet formation in vitro [11,29,30]; 2) MSC produce several essential hematopoietic growth factors including IL6, IL11, LIF, SCF, and Flt3 ligand [11,12,31]; and 3) MSC express adhesion molecules and extracellular matrix proteins that are known to play a role in hematopoietic stem cell homing, including VCAM1, E-selectin, collagen I, and fibronectin [12,16,20].

Cotransplantation of human MSC together with human hematopoietic stem cells enhances long-term engraftment of human cells in fetal sheep and in immunodeficient mice [32,33]. To date, definitive data demonstrating specific enhancement of human megakaryocytic and myelocytic engraftment when MSC are cotransplanted with PBSC or bone marrow are lacking. Interactions between hematopoietic stem cells and stromal cells are essential for hematopoietic development and stromal cells are absent from purified $CD34^+$ PBSC [17,34,35]. In a Phase I clinical trial of autologous transplantation in patients with breast cancer, cotransplantation of MSC with CD34-selected PBSC has been performed [36]. Although the engraftment rates and stability of engraftment were not compared to a control group that received CD34 cells alone, infusion of MSC was proven to be safe, and all patients engrafted both white blood cells (WBC) and platelets rapidly, thus paving the way for Phase II randomized clinical trials [36].

We have used the human-to-mouse xenotransplantation model to test methods of enhancing human megakaryocytic engraftment from PBSC in human-mouse chimeras, and have proven that the NOD/SCID (nonobese diabetic/severe combined immunodeficient) model offers an excellent system to study those factors and conditions that contribute to defective posttransplant platelet engraftment [37,38]. In this report, we test directly whether cotransplantation of MSC enhances human myeloid and megakaryocytic engraftment of different doses of purified human $CD34^+$ PBSC into NOD/SCID mice. We also tested whether the effect of MSC is dependent on whether they are obtained from the autologous PBSC donor or an allogeneic human donor.

Materials and methods

Human cells

Human peripheral blood stem cells were collected by leukapheresis from cancer patients undergoing autologous stem cell transplantation after mobilization with chemotherapy and daily subcutaneous injections of 5 to 10 μg/kg granulocyte colony-stimulating factor (G-CSF). $CD34^+$ cells were isolated using the Isolex cell selection device (Baxter Immunotherapy, Irvine, CA, USA). The purity of the $CD34^+$-selected cells was 90 to 95%. Aliquots of the cells were stored frozen in 10% dimethylsulfoxide (DMSO) in liquid nitrogen under controlled conditions until ready for use. Patients were not selected with regard to age, race, or sex. Informed consent was obtained from the subjects, and the investigations had been approved by the Yale University Human Investigation Committee.

MSC from healthy volunteers and from PBSC donors were grown as previously described [13] and were either purchased from Poietics (Biowhittaker, Walkersville, MD, USA) or produced at Osiris Therapeutics (Baltimore, MD, USA) in precisely the same manner that they were produced for Poietics. In either case, MSC were confirmed to be negative for hematopoietic markers by flow cytometry and capable of differentiating into osteocytes in vitro. Cells were expanded according to the manufacturer's protocol by plating them at 3 to 5×10^3 cells/cm^2 in mesenchymal stem cell growth media (MSCGM, Biowhittaker). After approximately 5 days in culture, subconfluent cultures were trypsinized and split 1:3. MSC were stored in liquid nitrogen until the day of the experiment.

Xenotransplantation of PBSC into mice

NOD/SCID mice, purchased from Jackson Laboratories (Bar Harbor, ME, USA), were maintained in microisolator cages on laminar flow racks in the animal facilities of Yale University and Osiris Therapeutics. Mice were kept on sterile diets and were given autoclaved acidified water. All animal experiments were approved by the Animal Care Committee of Yale University School of Medicine. Six- to eight-week-old mice received sublethal total-body irradiation with 300 cGy from a ^{137}Cs source 4 to 6 hours before transplantation. $CD34^+$ cells and MSC were thawed in a 37ºC water bath and counted. Cell viability after thawing was 90 to 98% by trypan blue exclusion. $CD34^+$ cells were injected in a final volume of 100 μL IMDM containing 5% fetal bovine serum (FBS) per mouse and MSC were injected in MSCGM at a final volume of 400 μL per mouse. $CD34^+$ and MSC were infused simultaneously via tail vein injection. Thereafter, mice received trimethoprime/sulfomethoxazole for the duration of the experiment.

Experimental design

In seven separate experiments, mice were assigned to receive either CD34-selected PBSC alone ($CD34^+$ group) or PBSC plus MSC (CD34/MSC group). The effect on engraftment of MSCs from allogeneic vs autologous donors was compared in experiments 1 and 2. In subsequent experiments, only allogeneic MSC were used. In two experiments, a third group of mice received only MSC. A

one-tailed unpaired *t*-test was used to compare groups with $p \leq 0.05$ considered statistically significant.

Analysis of human WBC engraftment

Blood was analyzed weekly for human WBC with the combination of FITC-mouse anti-human CD45 and PE-rat anti-mouse CD45. Mice were sacrificed 6 to 7 weeks posttransplant and cells from the bone marrow (BM) and spleens were counted and then analyzed by 3-color-flow cytometric analysis. Erythrocytes were lysed using PharM Lyse (Pharmingen, San Diego, CA, USA). Cells were then incubated with rat anti-mouse CD16 (Mouse Fc Block, Pharmingen) to block FcgII/IIIR-mediated nonspecific binding and were subsequently incubated with the appropriate monoclonal antibodies for 30 minutes at 4ºC, washed, resuspended in 1% paraformaldehyde in phosphate-buffered saline, and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). In addition to isotypic controls, cells from a nontransplanted mouse were stained with the same antibody combinations. All antibodies were purchased from Pharmingen (San Diego, CA, USA). The combination of FITC-anti-human CD45 and PE-anti-mouse CD45 was used to determine the ratio of human to mouse leukocytes. For further clarification of human subpopulations, three antibody panels were used: PeCy5-anti-human CD45, FITC-CD14, and PE-CD13; PeCy5-anti-human CD45, FITC-CD3, and PE-CD19; and PeCy5-anti-human CD45, FITC-CD33, and PE-CD34. PE-Cy5 is a tandem conjugate which combines R-phycoerythrin (PE) and a cyanine dye to boost (increase) the emitted fluorescence wavelength to about 670 nm, thereby giving a signal distinct from fluorescein isothiocyanate (FITC) or PE. The PE-Cy5 fluorochrome, like FITC, is excited at 488 nm, so we used this conjugate in our FACScan with these other compatible fluorochromes to give 3-color analysis with a single laser system. A live gate was used, excluding debris, red cells, and dead cells, and 10,000 to 15,000 gated events were collected for each sample. Mixtures of increasing percentages of human cells with murine cells were used as positive controls. A sample was counted as positive if more than 5 events showed fluorescence greater than the 99% of the fluorescence of the isotypic control. For a 1:1000 dilution of human cells in a population of murine cells, the standard deviation was equal to 12% of the mean.

Analysis of human platelet engraftment

Blood was collected from the retroorbital venous plexus 3, 4, and 5 weeks posttransplant. We have shown previously that the highest circulating human platelet levels are detected 3 weeks after transplantation [37]. An aliquot of the blood was used for complete blood counts and the numbers of human vs mouse platelets were analyzed by FACS using FITC-mouse anti-human CD41a (Pharmingen, San Diego, CA, USA) and PE-rat anti-mouse CD61 (Pharmingen) as previously described [37]. Nontransplanted animals were used at each analysis point as an additional negative control. To insure adequate sensitivity, 50,000 events were collected by flow cytometry for each sample. Only $FITC^+$ events that had a fluorescence intensity at least one log above background and consisting of a well-defined population were considered positive for human platelets.

Colony-forming unit megakaryocyte (CFU-Mk) assay

Bone marrow samples from each mouse were assessed for their potential to grow human megakaryocytic colonies using the MegaCult kit (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. The CFU-Mk assay was performed in experiments #2, 5, and 7. CFU-Mk were not detectable in bone marrow from mice that had been sublethally irradiated without subsequent administration of human PBSC.

Colony-forming cell (CFC) assay

Bone marrow cells from each mouse in experiments 3 through 7 were resuspended in IMDM/2% FBS, and, after lysing of the red cells, were plated in methylcellulose-based media MethoCult GF H4434 (Stem Cell Technologies, Vancouver, BC, Canada). Cells were plated at a final concentration of 2×10^5 nucleated cells/30-cm^2 dish, which was optimal for the detection of human colonies while the growth of background mouse colonies was minimal. Colonies (primarily human-derived, but with rare murine fibroblastoid colonies) were scored at day 16 under an inverted microscope. Although the murine-derived colonies were fibroblastoid in appearance and relatively diffuse (i.e., not hematopoietic), for each experiment the average number of colonies from nontransplanted mice was subtracted from the number of colonies found in the experimental mice in order to negate the chance of false-positive results.

Results

Comparison of allogeneic and autologous MSC

If cotransplantation of MSC is found to be advantageous for patients undergoing autologous transplantation, then it will be important to know whether the MSC must be derived from the patient's own marrow or could be derived from a cell population that has been grown in vitro after donation from a normal donor, which would allow for extensive testing for quality control. In two separate experiments, donor CD34-selected PBSC were cotransplanted with either allogeneic or autologous MSC prepared in an identical manner. In this direct comparison, both populations of MSC equivalently enhanced human cell engraftment (Fig. 1). The

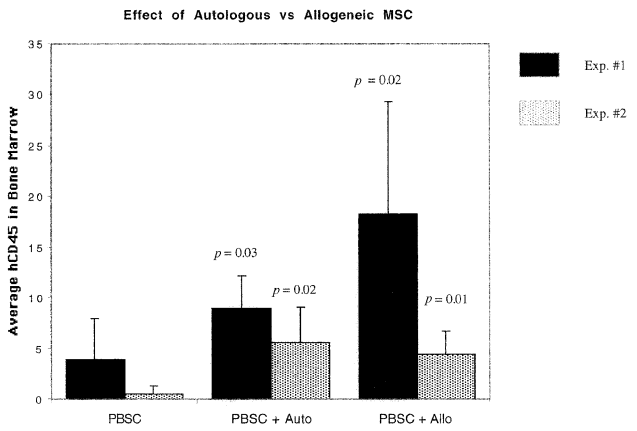

Figure 1. Both allogeneic and autologous MSC enhance human cell engraftment in NOD/SCID mice. Sublethally irradiated mice were transplanted with 0.4×10^6 CD34 + 1.0×10^6 autologous (auto) or allogeneic (allo) MSC as indicated. Data shown in solid and dotted bars are from two separate experiments. Shown is the mean ± SD of the percentage of bone marrow cells that expressed CD45 at the time of sacrifice 6–7 weeks posttransplant. $p = 0.03$ for PBSC + auto MSC vs PBSC in exp. #1, $p = 0.02$ for PBSC + auto MSC vs PBSC in exp. #2, $p = 0.02$ for PBSC + allo MSC vs PBSC in exp. #1, $p = 0.01$ for PBSC + allo MSC vs PBSC in exp. #2.

Table 1. Summary of human cell engraftment

	Conditions		Percent hCD45 in bone marrow	
	CD34 dose	MSC dose	CD34 only	CD34 + allo MSC
Exp 1*	0.4×10^6	1×10^6	0.7, 1.1, 4.4, 9.5 (4/4)	5.2, 11.1, 16.8, 25.9, 32.5 (5/5)
Exp 2*	0.4×10^6	1×10^6	0, 0.1, 0.3, 1.8, (3/4)	1.3, 4.4, 5.0, 6.9, (4/4)
Exp 3*	0.4×10^6	1×10^6	0.9, 0.8, 2.2 (3/3)	1.7, 2.9, 3.1, 3.5, 5.1 (5/5)
Exp 4†	0.5×10^6	1×10^6	0, 0, 0, 0, 0, 0.67, 1.2 (2/7)	0, 0, 0.2, 0.3, 1.8, 2.7 (4/6)
Exp 5*†	0.5×10^6	2×10^6	0, 0, 0, 0, 0, 0 (0/6)	0, 0.2, 0.45, 0.71, 1.0 (4/5)
Exp 6*	0.5×10^6	2×10^6	0.1, 0.4, 0.7 (3/3)	6, 6, 13 (3/3)
Exp 7	1.5×10^6	2×10^6	0, 0.8, 1.5, 2.0, 2.1, 3.0, 3.4 (6/7)	0.2, 0.4, 0.4, 0.6, 1.4, 1.8, 2.2, 15.8 (8/8)

*Indicates experiments in which the MSC gave a statistically significant increase in human cell engraftment ($p < 0.05$).

†In experiments 4 and 5, a third cohort of animals received MSC only and none of the animals showed any human engraftment. Data shown are for each individual mouse. In parentheses is the number of mice with detectable human engraftment in the marrow over the number of animals transplanted in each group.

increase in engraftment with autologous and allogeneic MSC was significant compared with $CD34^+$ PBSC alone (p values indicated in Fig. 1). There was not a significant difference in enhancement of engraftment between autologous and allogeneic MSC in either experiment. These data suggest that allogeneic MSC can be used to enhance engraftment if the patient's own MSC are believed to be contaminated with tumor cells or if collection of these marrow cells is otherwise contraindicated. For subsequent studies in this report, allogeneic MSC were used.

Effect of MSC on human white blood cell engraftment in NOD/SCID chimeras

In a total of seven experiments including the two above, the effect of MSC on human PBSC engraftment was assessed. Different numbers of CD34-selected PBSC and MSC were infused as indicated in Table 1. In experiments 1, 3, and 6, all of the animals in both groups (with and without MSC) engrafted, while in the other four experiments, the percentage of animals that had detectable levels of human WBC engraftment was higher when MSC were cotransplanted (Table 1). Human $CD45^+$ WBC were not detectable in sublethally irradiated mice that were transplanted with MSC only (n = 6 from 2 separate MSC donors), nor in control mice that received no human cells.

MSC significantly increased the level of human cell engraftment in the bone marrow (Table 1, Fig. 2). Enhancement was most dramatic when a lower number of $CD34^+$ cells was infused along with a higher number of MSC. In experiments 1, 2, and 3, 0.4×10^6 $CD34^+$ PBSC were transplanted with or without 1×10^6 MSC. With this ratio, the mice that received MSC showed significantly enhanced engraftment in all three experiments ($p < 0.04$, n = 3 to 5 per condition as shown in Table 1). However, in experiment 4, in which 0.5×10^6 $CD34^+$ PBSC were cotransplanted with 1×10^6 MSC, there was a small but not statistically significant increase in engraftment. In experiments 5 and 6, 0.5×10^6 PBSC were again transplanted, but with a higher dose of MSC (2×10^6 MSC), and a statistically significant increase ($p \leqslant 0.01$ in each experiment) in engraftment occurred in both experiments. Finally, when 2×10^6 MSC were cotransplanted with a higher dose of $CD34^+$ PBSC (1.5×10^6), no statistically significant enhancement occurred. These data suggest that cotransplantation of MSC can enhance engraftment when only a low dose of CD34 cells is available.

Human WBC engraftment in the murine peripheral blood was assessed in experiments 2, 5, and 7. As was observed for the marrow engraftment, there was a statistically significant increase in engraftment in the CD34/MSC group in experiments 1 ($p = 0.04$) and 5 ($p = 0.02$) but not in experiment 7 (data not shown). The percentage of human $CD45^+$ cells in blood was consistently lower than in bone marrow and there was a strong correlation between the two parameters (correlation coefficient = 0.81).

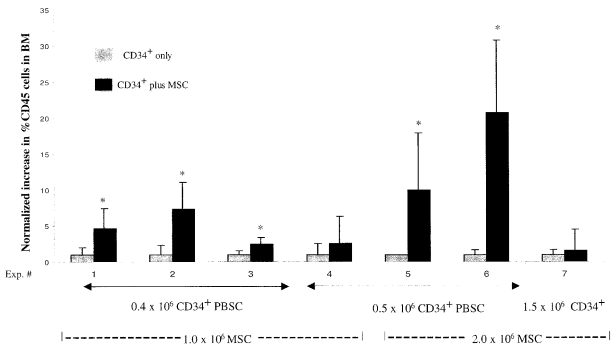

Figure 2. Allogeneic MSC enhance human cell engraftment in the bone marrow. Shown is the mean ± SD of the relative increase in the average percentage human CD45 cells in the bone marrow at the time of sacrifice. For each experiment, the mean engraftment in the CD34-only group was normalized to 1. The mean percentage engraftment in the CD34-only group for experiments 1–7, respectively, were 3.9 ± 4.0, 0.55 ± 0.8, 1.3 ± 0.8, 0.27 ± 0.5, 0.0 ± 0.0, 0.4 ± 0.3, and 1.8 ± 1.2. *indicates statistically significant difference in engraftment between animals transplanted with $CD34^+$ cells only and those transplanted with $CD34^+$ cells + MSC.

Effect of MSC on human cell differentiation

In experiments 2, 5, and 7, flow cytometry was used to assess whether cotransplantation of MSC affected the differentiation profile of human leukocyte subpopulations after transplantation (Fig. 3A–C). As in previous experiments [37], when purified $CD34^+$ PBSC engraft in NOD/SCID mice, the majority of the human cells are B lymphocytes (Fig. 3C). The remaining human cells are $CD34^+$ progenitor cells, early $CD33^+$ myeloid cells (Fig. 3B), more mature $CD13^+$ myelocytic cells, and $CD14^+$ monocytic cells. T lymphocytes are rarely detected (data not shown).

In contrast to engrafted mice that received only $CD34^+$ cells, mice that received CD34/MSC showed a statistically significant shift of hematopoiesis towards the myelocytic lineage ($p < 0.05$, Table 2). Taking all three experiments together, in animals transplanted with $CD34^+$ cells only, 7 of 9 engrafted mice had a higher percentage of B lymphocytes than myeloid cells. The percentage of B and myeloid cells in the CD34-only group was 51.5 ± 18.5 and 33.0 ± 11.7, respectively ($n = 9$, $p = 0.04$). In contrast, in animals from the CD34/MSC group, 14 of 16 engrafted mice had a myeloid cell predominance with an average percentage of B and myeloid cells of 24.4 ± 15.2 and 59.7 ± 13.5, respectively ($n = 16$, $p < 0.01$). Representative data are shown for the CD34 only and CD34/MSC groups (Fig. 3A–C). The relative increase in myeloid cells was primarily due to the presence of more early myeloid $CD33^+$ and monocytic $CD14^+$ cells, and mature granulocytes did not differ significantly between the groups. The increase in myeloid cell engraftment when MSC were cotransplanted was accompanied by an increase in the number of myeloid colony-forming units (CFU-GM, CFU-G, and CFU-M) in the bone marrow (data not shown). This difference was statistically significant for experiments 3, 5, and 6, but not for experiment 4 or 7, consistent with the other parameters tested. (CFU analysis was not performed for experiments 1 or 2.) For example, in experiment 5, the number of myeloid CFU increased from 3.1 ± 5.6 in the CD34 group to 11.5 ± 8.2 ($p = 0.04$) in the CD34/MSC group. The relative percentage of human $CD34^+$ progenitors did not differ between animals transplanted with $CD34^+$ cells alone and those that received CD34/MSC (mean percentage: 6.6 ± 7.2, $n = 17$ vs 9.3 ± 5.5, $n = 17$, respectively, $p = 0.11$).

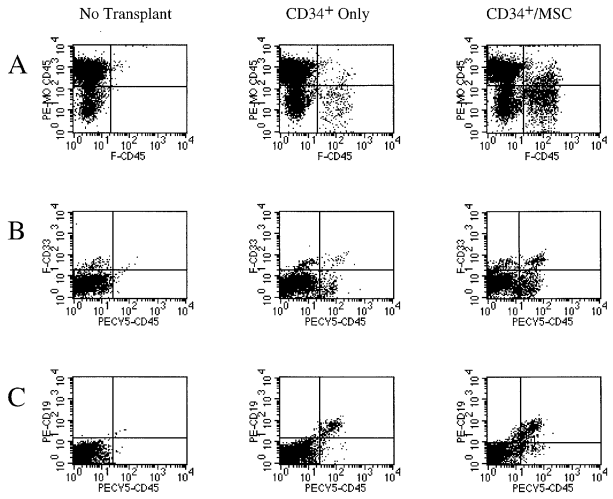

Figure 3. MSC cotransplantation enhances myeloid differentiation. Shown in A through C are representative FACS data from three mice in experiment 7, a control untransplanted mouse (left), a mouse with 2.1% human $CD45^+$ cell engraftment from the CD34-only group (center) and a mouse with 15.8% human cell engraftment in the marrow from the CD34/MSC group (right). (**A**): dotplots show human FITC-CD45 on the x-axis and mouse PE-CD45 on the y-axis. Red blood cells in the lower left quadrant do not express CD45. (**B**): dotplots show human PeCy5-CD45 on the x-axis vs human FITC-CD33 on the y-axis. (**C**): human PeCy5-CD45 is on the x-axis and human PE-CD19 is on the y-axis.

Effect of MSC on human megakaryocytic engraftment in the NOD/SCID chimeras

Evaluation of human megakaryocytic engraftment was performed in experiments 2, 5, and 7 (Table 3). The peak level of human platelets occurred 3 weeks posttransplant for all positive mice and the highest percentage among all experiments was 0.27%, which translated into 1.2×10^9/L circulating human platelets. In experiments 2 and 5, human megakaryocytopoiesis was enhanced by the cotransplantation of MSC as assessed by both production of mature platelets in the peripheral blood and growth of human CFU-Mk from the marrow. In experiment 5, none of the 7 mice from the $CD34^+$ group had human platelets in blood vs 3 of 5 animals from the CD34/MSC group. Similarly, none of the animals transplanted with $CD34^+$ cells alone, and 3 of 5 mice in the CD34/MSC group, had human CFU-Mk. The difference in absolute number of human platelets between the two groups in experiments 2 and 5 was statistically significant (Table 3), and there was a statistically significant increase in the number of CFU-Mk in experiment 2. In contrast, when a higher dose of $CD34^+$ cells was administered (experiment 7), no statistically significant difference occurred between the two groups. Both the percentage of human platelets and the number of human megakaryocytic colonies correlated with the level of human WBC engraftment in the bone marrow ($r = 0.88$ and 0.98, respectively).

Discussion

We report that human MSC enhance human engraftment in NOD/SCID mice after cotransplantation with human $CD34^+$-selected PBSC. This is the first report that human MSC enhance human megakaryopoiesis and skew the differentiation of the human engraftment cells toward the myeloid series. The most striking enhancement of human engraftment occurred when $CD34^+$ cells were given at a limiting dose ($0.4 - 0.5 \times 10^6$/animal). When the $CD34^+$ cell dose was increased to 1.5×10^6, the enhancing effect of MSC was

Table 2. Differentiation profile of human cells derived from murine bone marrow

		CD34$^+$ only							CD34 + MSC							
Expt. 2	BM % hCD45, $p = 0.01$	0	0.1	0.3	1.8				1.3	4.4	5.0	6.9				
	Blymphocytes		50%	45%	72%				18%	15%	10%	51%				
	Myelocytic		43%	13%	21%				47%	42%	62%	29%				
	CD34$^+$ cells		10%	8%	6%				7%	12%	8%	11%				
Expt. 5	BM % hCD45, $p = 0.01$	0	0	0	0	0	0		0	0.2	0.45	0.71	1.0			
	Blymphocytes									14%	29%	14%	17%			
	Myelocytic									60%	49%	78%	67%			
	CD34$^+$ cells									10%	9%	10%	10%			
Expt. 7	BM % hCD45, $p = 0.01$	0	0.8	1.5	2.0	2.1	3.0	3.4	0.2	0.4	0.4	0.6	1.4	1.8	2.2	15.8
	Blymphocytes		69%	51%	10%	46%	64%	56%	18%	26%	6%	30%	22%	22%	65%	33%
	Myelocytic		27%	34%	62%	35%	33%	29%	70%	64%	81%	50%	56%	59%	37%	49%
	CD34$^+$ cells		19%	14%	16%	10%	19%	10%	10%	7%	13%	20%	9%	12%	13%	17%

blunted. The enhancement of human hematopoiesis was more pronounced when MSC were given at a dose of 2×10^6 cells/animal compared to 1×10^6. Although absolute engraftment levels varied from experiment to experiment, they were always higher in the CD34/MSC group within each experiment. The difference in WBC engraftment was statistically significant in favor of the CD34 + MSC group in 5 of 6 experiments where CD34 cells were given at a limited dose. Even in experiment #4, where the difference was not statistically significant, engraftment was higher in the CD34 + MSC group, with 4 of 6 animals engrafting in this latter group and only 2 of 7 mice engrafted in the CD34-only group. The variability of engraftment between experiments is a known characteristic of the NOD/SCID model. The implication of this finding for the clinical transplantation setting is that cotransplantation of MSC might provide enhancement of engraftment and more efficient hematologic recovery in patients for whom the dose of CD34$^+$cells is low or marginal.

Although it would mean exposure to allogeneic donor antigens, allogeneic MSC cells can provide equal enhancement of engraftment as autologous cells. Theoretically, allogeneic MSC could be produced and stored from healthy donors for transplantation into any patient undergoing autologous or allogeneic transplantation. These cells could be manufactured under optimal Good Manufacturing Practices and quality control assays could be performed to assure the safety and optimize the efficacy of the cells. In a clinical study, promising results were obtained when autologous MSC were given to breast cancer patients undergoing autologous PBSC transplantation [36]. Engraftment in these patients was rapid and sustained, indicating the safety of coadministering culture-expanded MSC. Further studies are ongoing to assess whether administration of MSC results in clinical benefit.

This is the first report to date showing that cotransplanted MSC shift the differentiation pattern from a lymphoid to a myeloid predominance and enhance megakaryocytic engraftment. The myeloid-to-lymphoid ratio was enhanced when MSC were cotransplanted even at low levels of engraftment, suggesting that this is an effect on the differentiation profile of the human cells rather than on the absolute number of human myeloid cells. The percentage of human CD34$^+$ cells in the bone marrow was not affected, suggesting that the shift in differentiation toward myeloid cells does not deplete the pool of more immature hematopoietic progenitors. These data contrast with those of Fibbe et al. [39], who cotransplanted human fetal lung MSC with cord blood and achieved an increase in the level and efficiency of engraftment, but not in the myeloid:lymphoid ratio. This difference could be due to their use of a different MSC source or of a different hematopoietic cell source; cord blood is known to have a different differentiation profile from PBSC in the NOD/SCID transplantation model [39].

Because platelet recovery is delayed more often than WBC recovery after stem cell transplantation, the finding that both mature human circulating platelets and the maintenance of megakaryocytic progenitors (CFU-Mk) in the bone marrow were enhanced in the animals that received MSC is likely to be clinically significant. In experiments where megakaryocytic engraftment was assessed, animals that received limiting doses of CD34$^+$ cells + MSC had statistically higher human platelet and/or megakaryocytic colonies than the animals that received CD34$^+$ cells only. In contrast, this difference was not established when the CD34$^+$ cell dose was increased to 1.5×10^6 CD34$^+$ cells, in accordance with the white blood cell engraftment. These data also confirmed that human WBC and megakaryocytic engraftment correlate closely. From our previous studies and ongoing work (unpublished data), we have found that the level of human myeloid/megakaryocytic engraftment in the animals correlates with the time to engraftment in the patients. Thus, we believe that the level of human engraftment in the chimeras reflects the capacity of the human graft for rapid reconstitution, whereas the kinetics of human engraftment in the NOD/SCID model are relatively constant and do not reflect the rate of engraftment in the

Table 3. Summary of megakaryocytic engraftment

		CD34$^+$ only							CD34/MSC						
Expt. 2*	BM % hCD45, $p = 0.01$	0	0.1	0.3	1.8				1.3	4.4	5.0	0.9			
	PB % Hu Plts, $p = 0.12$	0	0.03	0.02	0.03				0.02	0.04	0.07	0.04			
	PB, Plts/uL $p = 0.03$	0	3.85	257	584				648	733	1440	857			
	CFU-Mk, $p = 0.04$	0	0	0	1				2	1	11	7			
Expt. 5*	BM % hCD45. $p = 0.01$	0	0	0	0	0	0		0	0.2	0.45	0.71	1.0		
	PB % Hu Plts, $p = 0.06$	0	0	0	0	0	0		0	0.27	0	0.80	0.15		
	PB, Plts/uL, $p = 0.04$	0	0	0	0	0	0		0	1185	0	453	945		
	CFU-Mk	0	0	0	0	0	0		0	NE	NE	NE	1		
Expt. 7	BM % hCD45, $p = 0.31$	0	0.8	1.5	2.0	2.1	3.0	3.4	0.2	0.4	0.4	0.6	1.4	1.8	2.2	15.8
	PB % Hu Plts, $p = 0.43$	0	0.01	0.11	0.06	0.03	0.06	0.14	0.06	0.04	0.02	0.06	0.05	0.07	0.07	0.25
	PB, Plts/uL, $p = 0.75$	0	331	1349	810	388	688	1634	718	292	287	681	645	827	736	2665
	CFU-Mk, $p = 0.40$	0	1		8	9	NE	3	0	2	2	6	10	7	2	143

Each number represents an individual value of engraftment in one mouse.

*There is a statistically significant ($p < 0.05$) difference between the CD34-only and CD34/MSC groups. The p values are indicated for individual parameters. BM, bone marrow; PB, peripheral blood; Hu Plts, human platelets; hCD45, human CD45; NE, not evaluable. The CFU-Mk data are the number of human CFU-Mk per 10^6 marrow cells at the time of sacrifice.

clinical setting. Enhancement of platelet engraftment could benefit patients by decreasing the risk of bleeding and avoiding exposure to allogeneic platelet transfusions, which can lead to platelet refractoriness and/or transfusion-transmitted diseases.

The mechanisms responsible for enhancement of hematopoiesis by MSC are unknown. We speculate that MSC might provide critical growth factors and/or adhesion receptors for human cell development. Growth factors that are known to be produced by human MSC include interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), stem cell factor (SCF), and Flt3-ligand [11,12,31]. Alternatively, MSC may act nonspecifically to prevent human cell loss through the reticuloendothelial system. Others have shown that human bone marrow engraftment in mice can be enhanced by different accessory cell (AC) populations including irradiated mononuclear cells [40]. Although these AC may produce cytokines, the theory that their effect is nonspecific is strengthened by the findings of Bittencourt et al., who show that cotransplantation of apoptotic irradiated leukocytes significantly enhances engraftment of human cells in mice [41]. Although we did not assess the survival and location of the MSC posttransplant, others have shown that MSC derived from human fetal lung can be found in the lung for the first 48 hours after administration, but not in the bone marrow. Several months following transplantation into NOD/SCID mice, human fetal lung–derived MSC were not detectable [42].

In conclusion, MSC capable of differentiating in vitro to different mesenchymal tissues enhance human myelopoiesis and megakaryocytopoiesis in vivo in NOD/SCID mice when cotransplanted with CD34$^+$ PBSC. These findings suggest that cotransplantation of MSC could enhance engraftment in poorly mobilized patients who undergo autologous stem cell transplantation.

Acknowledgments

This research was supported by NIH grants HK69207 and CA70970, and a Leukemia Lymphoma Society Translational Research Award.

The Johns Hopkins University holds patents on CD34 monoclonal antibodies and related inventions. Dr. Civin is entitled to a share of the sales royalty received by the University under licensing agreements between the University, Becton-Dickinson Corporation, and Baxter HealthCare Corporation. The terms of these arrangements have been reviewed and approved by the University in accordance with its conflict of interest policies.

References

1. Gronthos S, Simmons PJ. The biology and application of human bone marrow stromal cell precursors. J Hematother. 1996;5:15–23.
2. Prockop D. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276:71–74.
3. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 1970;3:393–403.

4. Friedenstein AJ, Ivanov-Smolenski AA, Chajlakjan RK, et al. Origin of bone marrow stromal mechanocytes in radiochimeras and heterotopic transplants. Exp Hematol. 1978;6:440–444.
5. Colter DC, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci USA. 2000;97:3213–3218.
6. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991;9:641–650.
7. Keating A, Horsfall W, Hawley RG, Toneguzzo F. Effect of different promoters on expression of genes introduced into hematopoietic and marrow stromal cells by electroporation. Exp Hematol. 1990;18:99–102.
8. Mardon HJ, Bee J, von der Mark K, Owen ME. Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. Cell Tissue Res. 1987;250:157–165.
9. Piersma AH, Brockbank KG, Ploemacher RE, van Vliet E, Brakel-van Peer KM, Visser PJ. Characterization of fibroblastic stromal cells from murine bone marrow. Exp Hematol. 1985;13:237–243.
10. Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve. 1995;18:1417–1426.
11. Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol. 1998;176:57–66.
12. Haynesworth SE, Baber MA, Caplan AI. Cell surface antigens on human marrow–derived mesenchymal cells are detected by monoclonal antibodies. Bone. 1992;13:69–80.
13. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–147.
14. Reyes M, Verfaillie CM. Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann NY Acad Sci. 2001;938:231–233; discussion 233–235.
15. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood. 2001;98:2615–2625.
16. Bruder SP, Horowitz MC, Mosca JD, Haynesworth SE. Monoclonal antibodies reactive with human osteogenic cell surface antigens. Bone. 1997;21:225–235.
17. Simmons P, Torok-Storb B. CD34 expression by stromal precursors in normal human adult bone marrow. Blood. 1991;78:2848–2853.
18. Stewart K, Walsh S, Screen J, et al. Further characterization of cells expressing STRO-1 in cultures of adult human bone marrow stromal cells. J Bone Miner Res. 1999;14:1345–1356.
19. Joyner CJ, Bennett A, Triffitt JT. Identification and enrichment of human osteoprogenitor cells by using differentiation stage–specific monoclonal antibodies. Bone. 1997;21:1–6.
20. Bruder SP, Jaiswal N, Ricalton NS, Mosca JD, Kraus KH, Kadiyala S. Mesenchymal stem cells in osteobiology and applied bone regeneration. Clin Orthop. 1998:S247–256.
21. Rickard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC, Riggs BL. Isolation and characterization of osteoblast precursor cells from human bone marrow. J Bone Miner Res. 1996;11:312–324.
22. Schwarz EJ, Alexander GM, Prockop DJ, Azizi SA. Multipotential marrow stromal cells transduced to produce L-DOPA: engraftment in a rat model of Parkinson disease. Hum Gene Ther. 1999;10:2539–2549.
23. Pereira R, Halford K, O'Hara M, et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci. 1995;92:4857–4861.
24. Ferrari G, Cusella-DeAngelis G, Coletta M, et al. Muscle regeneration by bone marrow–derived myogenic progenitors. Science. 1998; 279:1528–1530.
25. Horwitz EM, Prockop DJ, Fitzpatrick LA, et al. Transplantability and therapeutic effects of bone marrow–derived mesenchymal cells in children with osteogenesis imperfecta [see comments]. Nat Med. 1999;5:309–313.
26. Moore CA, Curry CJ, Henthorn PS, et al. Mild autosomal dominant hypophosphatasia: in utero presentation in two families. Am J Med Genet. 1999;86:410–415.
27. Bielski M, Yomtovian R, Lazarus HM, Rosenthal N. Prolonged isolated thrombocytopenia after hematopoietic stem cell transplantation: morphologic correlation. Bone Marrow Transplant. 1998;22:1071–1076.
28. Dercksen MW, Rodenhuis S, Dirkson MKA, et al. Subsets of $CD34^+$ cells and rapid hematopoietic recovery after peripheral-blood-stem-cell transplantation. J Clin Oncol. 1995;13:1922–1932.
29. Schuening FG, Storb R, Meyer J, Goehle S. Long-term culture of canine bone marrow cells. Exp Hematol. 1989;17:411–417.
30. Cheng L, Qasba P, Vanguri P, Thiede MA. Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from $CD34^+$ hematopoietic progenitor cells. J Cell Physiol. 2000;184:58–69.
31. Mbalaviele G, Jaiswal N, Meng A, Cheng L, Van Den Bos C, Thiede M. Human mesenchymal stem cells promote human osteoclast differentiation from $CD34^+$ bone marrow hematopoietic progenitors. Endocrinology. 1999;140:3736–3743.
32. Almeida-Porada G, Flake AW, Glimp HA, Zanjani ED. Cotransplantation of stroma results in enhancement of engraftment and early expression of donor hematopoietic stem cells in utero. Exp Hematol. 1999;27:1569–1575.
33. 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.
34. Eaves CJ, Cashman JD, Kay RJ, et al. Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. II. Analysis of positive and negative regulators produced by stromal cells within the adherent layer. Blood. 1991; 78:110–117.
35. Dexter TM, Allen TD, Lajtha LG. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol. 1977; 91:335–344.
36. Koc ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol. 2000;18:307–316.
37. Perez LE, Rinder HM, Wang C, Tracey JB, Maun N, Krause DS. Xenotransplantation of immunodeficient mice with mobilized human blood $CD34^+$ cells provides an in vivo model for human megakaryocytopoiesis and platelet production. Blood. 2001;97:1635–1643.
38. van der Loo JC, Hanenberg H, Cooper RJ, Luo FY, Lazaridis EN, Williams DA. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse as a model system to study the engraftment and mobilization of human peripheral blood stem cells. Blood. 1998;92:2556–2570.
39. Fibbe WE, Noort WA, Schipper F, Willemze R. Ex vivo expansion and engraftment potential of cord blood–derived $CD34^+$ cells in NOD/SCID mice. Ann NY Acad Sci. 2001;938:9–17.
40. Bonnet D, Bhatia M, Wang JC, Kapp U, Dick JE. Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive human hematopoietic cells transplanted at limiting doses into NOD/SCID mice. Bone Marrow Transplant. 1999;23:203–209.
41. Bittencourt MC, Perruche S, Contassot E, et al. Intravenous injection of apoptotic leukocytes enhances bone marrow engraftment across major histocompatibility barriers. Blood. 2001;98:224–230.
42. Noort WA, Kruisselbrink AB, in't Anker PS, et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood–derived $CD34^+$ cells in NOD/SCID mice. Exp Hematol. 2002;30:870–878.