

Murine Stromal Cell Line HESS-5 Maintains Reconstituting Ability of Ex Vivo-Generated Hematopoietic Stem Cells from Human Bone Marrow and Cytokine-Mobilized Peripheral Blood

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ABSTRACT

Human bone marrow (BM) or mobilized peripheral blood (mPB) CD34⁺ cells have been shown to lose their stem cell quality during culture period more easily than those from cord blood (CB). We previously reported that human umbilical CB stem cells could effectively be expanded in the presence of human recombinant cytokines and a newly established murine bone marrow stromal cell line HESS-5. In this study we assessed the efficacy of this xenogeneic coculture system using human BM and mPB CD34⁺ cells as materials. We measured the generation of CD34⁺CD38⁻ cells and colony-forming units, and assessed severe-combined immunodeficient mouse-repopulating cell (SRC) activity using cells five days after serum-free cytokine-containing culture in the presence or the absence of a direct contact with HESS-5

cells. As compared with the stroma-free culture, the xenogeneic coculture was significantly superior on expansion of CD34⁺CD38⁻ cells and colony-forming cells and on maintenance of SRC activity. The PKH26 study demonstrated that cell division was promoted faster in cells cocultured with HESS-5 cells than in cells cultured without HESS-5 cells. These results indicate that HESS-5 supports rapid generation of primitive progenitor cells (PPC) and maintains reconstituting ability of newly generated stem cells during ex vivo culture irrespective of the source of samples. This xenogeneic coculture system will be useful for ex vivo manipulation such as gene transduction to promote cell division and the generation of PPC and to prevent loss of stem cell quality. *Stem Cells* 2000;18:183-189

INTRODUCTION

Hematopoietic stem cells (HSCs) possess both multilineage differentiation and self-renewal ability, so that they sustain long-term hematopoiesis throughout their lifetime. Therefore, ex vivo manipulation of these cells such as gene introduction and amplification would open a new possibility of stem cell transplantation. However, many reports have indicated that the stem cell capability is easily lost during ex vivo manipulation; there is poor engraftment from cultured stem cells [1, 2]. Moreover, ontogeny-related differences in the ability to maintain stem cell quality during the culture period of human cord

blood (CB), bone marrow (BM), or mobilized peripheral blood (mPB) CD34⁺ cells have been pointed out and HSCs from BM lose their stem cell ability more easily than CB HSCs [3, 4]. Therefore, the difference between HSCs at different stages of development has to be taken into account in the design of therapeutic strategies, including ex vivo manipulation of HSCs. If stable methods to maintain stem cell activity during ex vivo culture irrespective of sample origins were obtained, it would contribute greatly to the field of stem cell biology.

We have recently established a hematopoietic-supportive murine stromal cell line HESS-5 that effectively supports

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proliferation of human CB primitive progenitor cells (PPC) by direct cell-to-cell interaction [5]; an over 100-fold increase in CB CD34⁺CD38⁻ cells was obtained after a very-short-term (five days) serum-free culture in the presence of human recombinant thrombopoietin (TPO), flk-2/flt-3 ligand (FL), interleukin 3 (IL-3), and direct contact with HESS-5. Colony-forming units in culture (CFU-C) and mixed colonies (CFU-Mix) were enhanced by 30-fold and 10-fold, respectively. The severe-combined immunodeficient (SCID) mouse-repopulating cell (SRC) assay confirmed extensive ability of the expanded PPC to reconstitute long-term hematopoiesis [6].

In this study, we further examined the efficacy of this xenogeneic coculture system on the generation of human BM- and PB-derived PPC. The results indicate that the system supports the proliferation of PPC and sustains high reconstituting ability of stem cells that was irrespective to the source of samples.

MATERIALS AND METHODS

CD34⁺ Cell Purification

Human BM ($n = 4$) and G-CSF-mPB samples ($n = 4$) were obtained from the harvests from normal donors after informed consent. There were no major differences in age or demographics between the donors of BM and PB. Mononuclear cells (MNC) were isolated from samples using Ficoll-Hypaque (Lymphoprep, 1.077 ± 0.001 g/ml; Nycomed; Oslo, Norway; <http://www.nycomed-pharma.no>) density gradient centrifugation. CD34⁺ cell purification utilized positive selection using the MACS immunomagnetic separation system (Miltenyi Biotec; Gladbach, Germany; <http://www.miltenyibiotec.com>) according to the manufacturer's instructions, as previously described [4]. Ninety percent or more of the enriched cells were CD34⁺ by flow cytometric analysis.

Cryopreservation and Thawing of Samples

Purified CD34⁺ cells were frozen in RPMI medium (GIBCO/BRL; Grand Island, NY; <http://www.lifetech.com>) supplemented with 10% dimethylsulfoxide and 20% fetal calf serum (FCS) using a step-down freezing procedure and placed in liquid nitrogen. Aliquots of frozen samples were thawed before use. The thawed cells were washed twice and trypan blue staining was performed. When trypan-blue-negative cell was more than 95%, the samples were subjected to further studies.

Murine Stromal Cell Line

The murine hematopoietic-supportive stromal cell line, HESS-5, was previously established from the C3H/HeN-Crj

strain murine bone marrow [5]. HESS-5 cells were maintained in minimal essential medium α ([MEM- α]; Nikken Bio Medical Laboratory; Kyoto, Japan) supplemented with 10% (v/v) horse serum (GIBCO/BRL) at 37°C under 5% CO₂ humidified air.

Human Cytokines

Recombinant human TPO, IL-3, and interleukin 6 (IL-6) were generously provided by Kirin Brewery (Tokyo, Japan; <http://www.kirin.com/home.html>). Recombinant human stem cell factor (SCF) was a gift from Amgen Biologicals (Thousand Oaks, CA; <http://www.amgen.com>). Recombinant human FL was purchased from R&D Systems (Minneapolis, MN; <http://www.rndsystems.com>). The final concentrations of cytokines used in liquid culture were as follows: TPO, 100 ng/ml; FL, 100 ng/ml; IL-3, 20 ng/ml; SCF, 100 ng/ml; and IL-6, 100 ng/ml.

Culture Systems

For stroma-contact culture, a novel culture system that was devised by modifying the cell culture insert system (Becton Dickinson Labware; Franklin Lakes, NJ; <http://www.bd.com>) was used, as previously described [6]. Briefly, HESS-5 cells were cultured on the reverse (back) side of the track-etched membrane of the insert for 12-well microplates in MEM- α supplemented with 10% horse serum. After obtaining a confluent feeder layer, stromal cells were irradiated with 15 Gy using a ¹³⁷Cs γ -irradiator. Then, cells were washed five times and the medium was changed for coculture. Isolated CD34⁺ cells were seeded on the upper side of the membrane of the insert where the cytoplasmic villi of HESS-5 cells passed through the etched 0.45 μ m pores (pore density $1.0 \times 10^8/\text{cm}^2$). Therefore, while HESS-5 cells directly adhered to human hematopoietic cells during culture, expanded cells could easily be harvested without contamination with HESS-5 cells. Stroma-free culture was performed in the same condition as stroma-contact culture other than the absence of HESS-5 cells.

Short-Term Ex Vivo Expansion of Hematopoietic Progenitors

Serum-free liquid culture was carried out using StemProTM-34SFM (GIBCO/BRL) supplemented with StemProTM-34 Nutrient Supplement (GIBCO/BRL), 2 mM L-glutamine (GIBCO/BRL), and penicillin/streptomycin with designated cytokines. Ten thousand to 2×10^4 and 5×10^4 CD34⁺ cells were subjected to cytokine-containing and cytokine-free culture, respectively. Culture plates were incubated at 37°C in a humidified atmosphere consisting of air enriched with 5% CO₂. On day 5 of culture, aliquots of cultured cells were harvested and subjected to cell count and further analyses.

Immunophenotyping by Flow Cytometry

Aliquots of cells were suspended in EDTA-bovine serum albumin (BSA)-phosphate-buffered saline (PBS) and incubated with mouse IgG (Inter-Cell Technologies; Hopewell, NJ) to block nonspecific binding. Cells were then reacted for 15 min with fluorescein isothiocyanate (FITC)-conjugated CD34 and phycoerythrin (PE)-conjugated CD38 monoclonal antibodies (Becton Dickinson; San Jose, CA; <http://www.bd.com>) at 4°C. Unbound antibodies were removed by two washes, and cells were resuspended in EDTA-BSA-PBS. Stained cells were then passed through a nylon mesh filter and subjected to two-color flow cytometric analysis. Cells labeled with FITC- and PE-conjugated mouse isotype-matched antibodies were used as negative controls. The analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest software (Becton Dickinson). At least 10^4 events were acquired for each analysis.

Clonal Cell Culture

Aliquots from initial samples or cultured cells were incubated in methylcellulose media (MethoCult H4434V; StemCell Technologies; Vancouver, Canada; <http://www.stemcell.com>) at concentrations of $1-2 \times 10^2$ cells/ml for purified CD34⁺ cells and $5-10 \times 10^2$ cells/ml for cultured cells in 35-mm tissue culture dishes (Iwaki Glass; Tokyo, Japan). Culture mixture contained 0.9% methylcellulose, 10^{-4} M/1 2-mercaptoethanol, 2 mM L-glutamine, 30% fetal bovine serum, 1% BSA, recombinant human erythropoietin (3 units/ml), IL-3 (10 ng/ml), SCF (50 ng/ml), GM-CSF (10 ng/ml), and cells. Dishes were incubated at 37°C in a humidified atmosphere with 5% CO₂ in air. All cultures were done in triplicate. Total CFU-C and CFU-Mix consisting of 50 or more cells were scored on an inverted microscope at 14 days of culture.

SRC Assay

SRC assay was performed as previously described [6]. Briefly, 8- to 12-week-old NOD/Shi-scid (NOD/SCID) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All animals were handled under sterile conditions and maintained under microisolators in the animal facility located at the Tokai University School of Medicine. Human hematopoietic cells at the indicated doses were transplanted by tail-vein injection into sublethally irradiated mice (350 cGy using a 4×10^6 V linear accelerator). Cells were cotransplanted with 1×10^6 of irradiated (15 Gy using a ¹³⁷Cs γ -irradiator) peripheral blood MNCs from normal volunteers as accessory cells. Mice were killed six weeks after transplantation, and the BM was harvested from the femurs and tibiae. The population of human hematopoietic cells was determined by Southern blot analysis using a human chromosome 17-specific α -satellite probe, as previously described [7, 8].

PKH26 Study

Purified CD34⁺ cells were stained with PKH26 (Sigma ImmunoChemicals; St Louis, MO; <http://www.sigma-aldrich.com>), the red fluorescent cell linker, before cultivation according to the manufacturer's instruction with slight modifications. Briefly, aliquots of cells suspended in 1 ml of Diluent C (Sigma ImmunoChemicals) were transferred into a polypropylene tube containing 1 ml of 4×10^{-6} M PKH26 dye in Diluent C. After incubation for 5 min at room temperature, 2 ml of 1% BSA-PBS were added. After 1 min, the total volume was brought up to 8 ml using the serum-free medium, and the cells were washed three times. Then, cells were subjected to serum-free culture. Before and after five days of cultivation, the labeled cells were subjected to flow cytometry, and the cell division history was assessed based on the fact that fluorescence intensity of PKH26 is reduced by one half with each cell division [9-11].

Statistical Analysis

Data were compared using analysis of variance. Where significant differences were inferred, sample means were compared using the *t*-test.

RESULTS

Ex Vivo Expansion of PPC in a Very-Short-Term (Five Days) Serum-Free Culture

The cytokine combination of TPO, FL, and IL-3 (T/F/3) is known to be the most effective in expanding CB-PPC [6]. Therefore, we first studied the effects of this combination on the ex vivo expansion of BM and mPB PPC in five-day serum-free culture. In stroma-free condition, the number of CD34⁺CD38⁻ cells from mPB and BM was expanded by 40-fold and 6-fold, respectively (Table 1). The addition of SCF and IL-6 to T/F/3 (T/F/3/S/6), enhanced the expansion and promoted colony formation in both BM and mPB. We then assessed supportive effects of HESS-5 cells. As a result, in the presence of T/F/3/S/6 and HESS-5 cells, the number of CD34⁺CD38⁻ cells from mPB and BM was expanded up to 90-fold and 40-fold, respectively; cells of both CFU-C and CFU-Mix were enhanced by 20-fold and 10-fold, respectively. Fold expansion of PB CD34⁺ and CD34⁺CD38⁻ cells was significantly superior to that of BM CD34⁺ cells in either combination of cytokines irrespective of the presence of HESS-5 cells ($p < 0.01$).

Assessment of Cell Kinetics During Culture

We then assessed cell kinetics during cultivation by using PKH26 staining. On day 0, all purified CD34⁺ cells labeled with PKH26 dye were PKH26^{high} (Fig. 1). Aliquots of the PKH26^{high} cells were subjected to the serum-free

Cell population or colony		Stroma-free			With HESS-5 cells		
		Cytokine (-)			Cytokine (-)		
		TPO/FL/IL-3	TPO/FL/IL-3	TPO/FL/IL-3/IL-6/SCF	TPO/FL/IL-3	TPO/FL/IL-3	TPO/FL/IL-3/IL-6/SCF
Total number of cells	PB	0.8 ± 0.1	5.0 ± 0.3	18.9 ± 1.2 ^b	0.8 ± 0.1	8.8 ± 0.5	25.7 ± 0.3 ^d
	BM	0.5 ± 0.0	3.6 ± 0.2	8.1 ± 1.7 ^a	0.6 ± 0.0	5.6 ± 0.3	12.6 ± 0.6 ^c
CD34 ⁺ cells	PB	0.4 ± 0.0	2.2 ± 0.1	4.3 ± 0.3 ^b	0.6 ± 0.0	4.9 ± 0.3	9.6 ± 0.1 ^d
	BM	0.4 ± 0.0	0.9 ± 0.0	2.3 ± 0.5 ^b	0.4 ± 0.0	1.6 ± 0.1	4.4 ± 0.2 ^d
CD34 ⁺ /CD38 ⁺ cells	PB	0.4 ± 0.0	1.5 ± 0.1	3.5 ± 0.2 ^b	0.6 ± 0.0	4.0 ± 0.2	8.4 ± 0.1 ^d
	BM	0.3 ± 0.0	0.6 ± 0.0	1.3 ± 0.3 ^a	0.3 ± 0.0	1.1 ± 0.1	2.6 ± 0.1 ^d
CD34 ⁺ /CD38 ⁻ cells	PB	1.1 ± 0.1	44.6 ± 2.7	60.2 ± 3.9 ^b	4.0 ± 0.3	59.6 ± 3.7	87.4 ± 1.1 ^d
	BM	0.8 ± 0.1	6.5 ± 0.3	20.7 ± 4.3 ^b	1.3 ± 0.1	10.6 ± 0.6	37.7 ± 1.9 ^d
CFU-C	PB	0.1 ± 0.0	3.6 ± 0.0	13.9 ± 0.1 ^b	0.1 ± 0.0	6.3 ± 0.1	19.6 ± 0.6 ^d
	BM	0.2 ± 0.0	3.1 ± 0.3	11.7 ± 0.2 ^b	0.3 ± 0.0	5.3 ± 0.2	13.5 ± 0.1 ^d
CFU-Mix	PB	0.0 ± 0.0	3.6 ± 0.4	12.1 ± 0.0 ^b	0.3 ± 0.1	7.2 ± 1.3	21.0 ± 3.0 ^d
	BM	0.1 ± 0.1	1.2 ± 0.0	5.7 ± 1.1 ^b	0.3 ± 0.1	3.7 ± 0.0	11.0 ± 1.4 ^d

Fold expansion was shown by mean ± SE (n = 4). ^ap < .01; ^bp < .001 versus T/F/3; ^cp < .01; ^dp < .001 versus T/F/3.

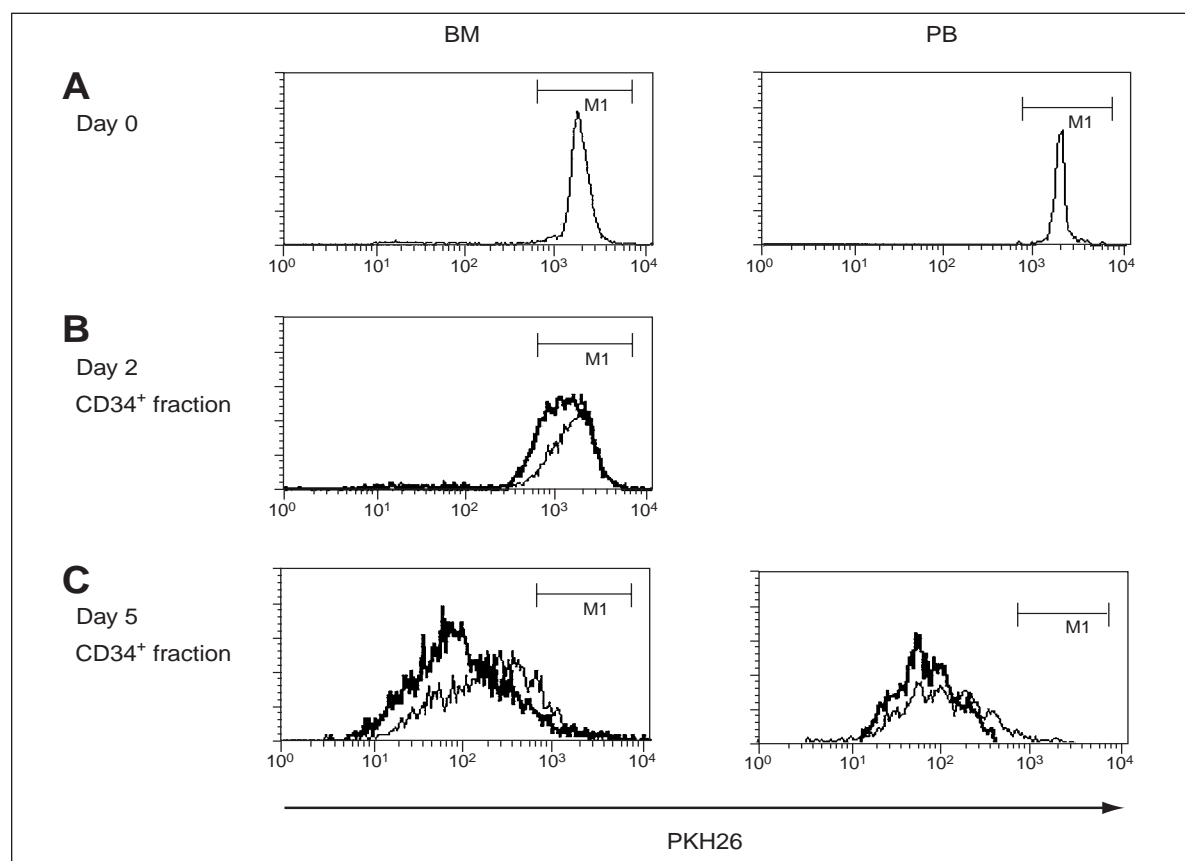


Figure 1. Flow cytometric analysis of cells tracked with PKH26. CD34⁺ cells isolated from human peripheral blood (PB) and bone marrow (BM) were stained with PKH26 and then cultured for five days in the presence of thrombopoietin, flk-2/flt-3 ligand, IL-3, stem-cell factor, and IL-6 in serum-free condition. (A) PKH26 fluorescence of initial CD34⁺ cells. M1 fraction in BM and PB indicated 98.4% and 99.9%, respectively. (B) PKH26 fluorescence of the CD34⁺ fraction of cells after two days of cultivation with (thick line) or without (thin line) HESS-5 cells. M1 fraction in BM indicated 83.8% with HESS-5 and 93.2% without HESS-5. (C) PKH26 fluorescence of the CD34⁺ fraction of cells after five days of cultivation with (thick line) or without (thin line) HESS-5 cells. M1 fraction in BM indicated 3.6% with HESS-5 and 12.2% without HESS-5. M1 fraction in PB indicated 0.0% with HESS-5 and 2.2% without HESS-5.

culture in the presence of T/F/3/S/6 with or without direct contact with HESS-5 cells. After two days of culture, PKH26^{high} proportion (M1 fractions in Fig. 1) was significantly higher in stroma-free condition than in stroma-dependent condition ($93.6 \pm 2.5\%$ versus $83.3 \pm 3.7\%$, $p < 0.001$). After five days of culture, the majority of the BM and PB cells became PKH26^{low}, indicating that they experienced cell division. Therefore, cell division and generation from CD34⁺ cells were promoted faster in cells cocultured with HESS-5 cells than in cells cultured without HESS-5 cells. Furthermore, a small fraction of CD34⁺ cells still stayed as PKH26^{high} (M1 fractions in Fig. 1) even after five days of culture in stroma-free conditions— $12.8 \pm 2.0\%$ in BM ($n = 4$), $2.5 \pm 0.8\%$ in mPB ($n = 4$)—whereas no such fraction was detected in stroma-dependent cultures— $2.9 \pm 2.1\%$ in BM ($n = 4$), $0.4 \pm 0.5\%$ in mPB ($n = 4$). These results indicate that the cell cycle progression of CD34⁺ cells was accelerated in both BM and mPB by the presence of HESS-5 cells.

Effects of Ex Vivo Culture on Human Reconstituting Hematopoietic Progenitors

We studied the SRC assay to determine whether those ex vivo-manipulated PPC had reconstituting ability. Twenty thousand or 8×10^4 CD34⁺ cells from PB and BM were initially cultured in the presence of T/F/3/S/6 with or without HESS-5 cells for five days. Harvested cells were then transplanted into NOD/SCID mice. As controls, uncultured 2 and/or 8×10^4 CD34⁺ cells obtained from the same sample sources were also transplanted into other mice. Six weeks after transplantation, the population of human hematopoietic cells in the murine BM was assessed by both fluorescence-activated cell sorting and Southern blot analysis.

When 2×10^4 mPB cells were used as the sample, engraftment was found only when cells were cocultured initially with HESS-5 cells (Table 2). All mice transplanted with 8×10^4

mPB CD34⁺ cells cocultured with HESS-5 cells showed reconstitution of human hematopoiesis as well as those transplanted with uncultured cells. BM samples cocultured with HESS-5 cells showed higher SRC activity than both BM cells cultured without HESS-5 cells and uncultured cells. A representative result of Southern blot analysis of human mPB cell engraftment in the BM of NOD/SCID mice is as shown in Figure 2. These results indicate that HESS-5 cells at least prevent loss of SRC activity in BM and mPB during five days of culture, and it may expand SRC activity in the case of mPB.

DISCUSSION

Ontogeny-related changes in the proliferative potential of human hematopoietic cells have been reported. It is important to take into account the difference between HSC at different stages of development for the procedure of their ex vivo manipulation [12]. Therefore, in this study, we evaluated supportive effects of a novel murine BM stromal cell line HESS-5 on the proliferation of PPC from human BM and mPB. In the presence of HESS-5 cells, PPC production was enhanced significantly in serum-free cytokine (T/F/3)-containing liquid culture, which is concurrent with our previous study in which CB cells were used as material [6]. However, compared with

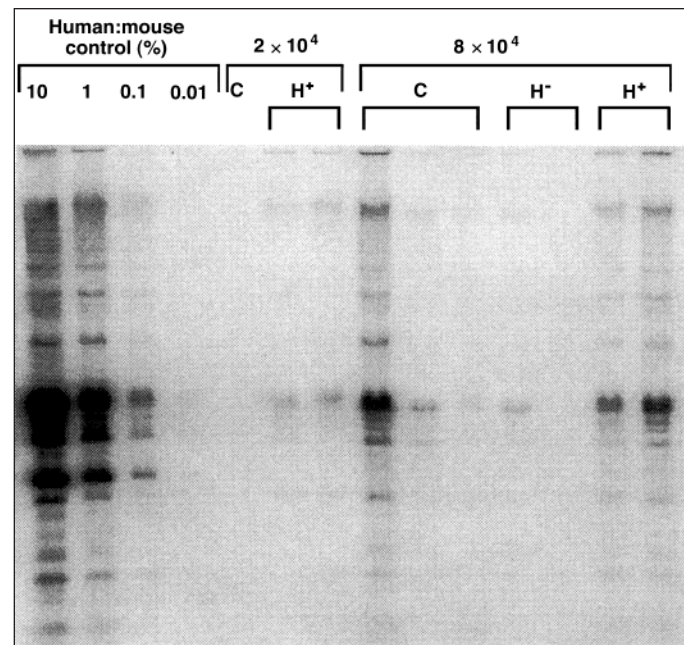


Figure 2. Representative Southern blot analysis of individual NOD/SCID mice transplanted with cultured or uncultured human peripheral blood (PB) cells. Twenty thousand or 8×10^4 PB cells were transplanted into NOD/SCID mice before (C) or after five days of cultivation with (H⁺) or without HESS-5 cells (H⁻) in the presence of thrombopoietin, flk-2/flt-3 ligand, IL-3, stem-cell factor, and IL-6. DNA was extracted from the murine bone marrow six weeks after the transplant and hybridized with a human chromosome 17-specific α -satellite probe. Human:mouse DNA controls are given as percent human DNA.

Table 2. Determination of human hematopoietic reconstitution in NOD/SCID mice six weeks after transplantation

Cell dose	Cell source	Culture condition		
		Control	HESS ⁻	HESS ⁺
2×10^4	Peripheral blood	0/3 (0)	0/4 (0)	2/4 (50.0)
	bone marrow	ND	ND	ND
8×10^4	Peripheral blood	3/3 (100)	2/3 (66.7)	3/3 (100)
	bone marrow	1/6 (16.7)	3/6 (50.0)	5/6 (83.3)

Twenty thousand and/or 8×10^4 CD34⁺ cells were transplanted into NOD/SCID mice before (control) and after five days of culture in the presence of T/F/3/S/6 with (HESS⁺) or without HESS-5 cells (HESS⁻). The ratios indicate the number of engrafted mice/number of transplanted mice. The numbers in the parentheses denote percentages of engrafted mice. ND = not determined.

CB, the fold expansion of BM and PB PPC was relatively low in the presence of T/F/3. Thus, we then added IL-6 and SCF to T/F/3, and the expansion of CFU and CD34⁺CD38⁻, which is considered to contain very immature stem cells [13-15], was further enhanced to almost the same level as CB. On the other hand, when IL-6 and SCF were added to the T/F/3 in CB, this enhancement was not observed (data not shown). Expansion of mPB CD34⁺ cells was significantly superior to that of BM CD34⁺ cells in either combination of cytokines irrespective of the presence of HESS-5 cells ($p < 0.01$). These data suggest differences in cytokine requirement on proliferation among developmentally different sample sources. Recently *Zandstra et al.* reported that CD34⁺CD38⁻ cells from BM required FL, SCF, and IL-3 for their optimal amplification of long-term culture-initiating cells (LTC-ICs) whereas those from CB required FL and IL-6/sIL-6R [16]. The difference between the cytokine combinations in the results of *Zandstra et al.* and in our results may depend on the differences between target populations and culture conditions. They amplified LTC-ICs and we amplified CD34⁺CD38⁻ cells. They used stroma-free culture system whereas we used murine BM stroma-dependent culture condition.

Although both LTC-ICs and SRCs are used to assess the human stem cell activity, LTC does not always reflect results obtained from in vivo studies. Actually, various reports indicate that SRCs are biologically distinct from and more primitive than most LTC-ICs; the efficiency of gene transfer into SRCs is low whereas LTC-ICs are easily transduced [17], LTC-ICs are found in the CD34⁺CD38⁺ fraction while SRCs are exclusively CD34⁺CD38⁻ [15], and SRC and LTC-ICs were differentially maintained after incubation on human BM stromal cells [4]. We thus assessed whether those expanded PPC possess the reconstituting ability because the well-enhanced CD34⁺CD38⁻ fraction can often result in the exhaustion of the stem cell quality during ex vivo culture periods [1, 2]. Furthermore, the ability of maintaining primitive human SRCs during culture periods was also reported to be different between BM and CB. *Gan et al.* found that the frequency of SRCs in BM declined sixfold after one week of culture whereas SRC was maintained in the case of CB [4]. As a result, the SRC assay clearly indicated that coculture with HESS-5 cells

maintained the reconstituting ability of PPC from BM and mPB during cultivation and was superior over stroma-free culture. The possibility of the expansion of SRCs has to be confirmed in the future by a quantitative SRC assay by using graded doses of cells before and after culture. Our data presented here supported the previous study using CB, and we clarified the HESS-5 cells could highly maintain reconstituting ability of ex vivo-generated PPC irrespective of the source of samples. Some reports suggested that culture in IL-3 led to decrease and/or loss of primitive stem cells with reconstitution potential [18-21]. We did not observe loss of SRCs by IL-3 in our system. The difference could be the use of HESS-5 cells.

PKH26, a fluorescent dye that stably inserts in the lipid bilayer cell membrane, was used for cell tracking and allowed us to identify a group of cells that failed to respond to cytokine stimulation. Such CD34⁺PKH26^{high} cells have been previously reported in studies examining ex vivo expansion of BM and mPB cells [9, 22, 23]. In our system, both mPB and BM CD34⁺ cells were promoted into cell cycle more rapidly when cocultured with HESS-5 cells. The absence of a CD34⁺PKF26^{high} population and the maintenance of SRCs support the possibility of self-renewal of stem cells in the stroma-dependent culture.

Although several researchers including ourselves confirmed that stroma-contact prevents loss of stem cell activity during ex vivo manipulation [6, 24-27], the mechanism for the prevention is still unknown. Candidate molecules for that include cytokines, adhesion molecules, and Notch ligand family such as delta-like/preadipocyte factor-1 (dlk) on murine stromal cell line AFT024 [28]. The elucidation of such molecules will make these xeno-coculture systems more clinically relevant in the near future.

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