



been available for competitive repopulation assay of human HSCs, but a major shortcoming of the NOD/SCID mouse is a lack of reproducible T cell differentiation from CD34⁺ cells [20–22]. We recently reported that the NOD/SCID/ γ c^{null} (NOG) mouse, which had been crossed with mice expressing a form of the IL-2R γ chain lacking the cytoplasmic region, reproducibly develops human T cells, in addition to myeloid, NK, and B-lymphoid cells when transplanted with cord blood CD34⁺ cells [23–25]. The repopulated T cells bear polyclonal $\alpha\beta$ TCR and respond not only to mitogenic stimuli, such as PHA and IL-2, but also to allogeneic human cells. These results indicate that functional human T lymphocytes can be reconstituted from CD34⁺ cells in NOG mice. These animals therefore provide a new system in which it is possible to analyze *in vivo* competitive repopulation of the full lymphopoietic system using human HSCs.

Here, we report a novel competitive repopulation system, in which two UCB units were labeled with recombinant lentiviral vectors carrying either an enhanced green fluorescent protein (EGFP)- or an enhanced yellow fluorescent protein (EYFP)-encoding gene to enable easy identification of progeny cells. Using this system, we evaluated competitive repopulation by purified CD34⁺ cells from two units of UCB with or without cotransplantation of mononuclear cells (MNC) and their purified populations.

RESULTS

Competitive Repopulation of Combined UCB CD34⁺ Cells Transplanted into the NOG Mouse

We transplanted 11 pairs of UCB CD34⁺ cells bearing distinct HLA alleles simultaneously into sublethally irradiated NOG mice (Table 1). To distinguish progeny derived from each UCB unit of CD34⁺ cells, we labeled one unit by transfection with a lentivirus vector containing the EGFP gene and the other unit with a lentivirus vector containing the EYFP gene. After transplantation, we collected peripheral blood (PB) cells retro-orbitally at various intervals and analyzed them by flow cytometry for the presence of human hematopoietic cells expressing the leukocyte common antigen CD45 (Figs. 1A and 1B). Individual progeny from each unit of UCB CD34⁺ cells were easily identified by the expression of these marker genes.

To evaluate the stability of any chimerism from two UCB donors in mice, we plotted the ratio of EGFP⁺ to EYFP⁺ cell number in PB over time after transplantation. Although the transduction efficiencies and numbers of CD34⁺ cells varied between each experiment, the chimeric ratio of EGFP⁺ to EYFP⁺ cells observed at 3 weeks after transplantation was fairly stable up to at least 18 weeks (Fig. 1C). In some experiments, the ratio was stable up to 6 months after transplantation ($n = 3$ analyzed, data not shown).

To evaluate the relative viability of the two UCB donors in mice, we plotted the ratio of EGFP⁺ to EYFP⁺

TABLE 1: Characteristics of the CB units

Expt	No. cells ($\times 10^4$)	CD34 ⁺ CD38 ⁻ (%)	Infection	Transduction efficiency (%)	HLA		
					A	B	DR
1	5.5	10.99	GFP	12.72	2/26	46/60	8/-
	11.4	8.7	YFP	12.11	2/-	51/75	15/-
2	11	5.86	GFP	17.04	1/24	37/52	14/15
	8	4.56	YFP	16.72	2/24	7/35	1/4
3	13.8	10.21	GFP	14.84	24/-	7/61	1/9
	16	4.8	YFP	15.13	2/24	46/-	8/-
4	15.8	4.35	GFP	7.56	24/33	44/52	9/13
	10.5	5.67	YFP	8.13	1/24	37/52	10/15
5	19	6.37	GFP	18.64	2/24	46/62	4/8
	13.9	4.1	YFP	17.87	24/26	7/61	9/13
6	17.5	8.39	GFP	18.76	N.D.	N.D.	N.D.
	24	6.22	YFP	17.81	2/-	59/61	4/-
7	8.75	13.14	GFP	10.35	2/24	35/62	4/11
	4	5.24	YFP	15.84	2/33	51/-	8/9
8	9	14.58	GFP	28.16	24/26	52/62	4/15
	35	5.4	YFP	25.58	2/24	46/61	8/9
9	98	7.57	GFP	25.28	24/33	7/44	1/13
	47	6.84	YFP	26.63	24/-	52/61	9/15
10	18.75	9.24	GFP	25.20	24/26	54/61	4/9
	15.75	4.55	YFP	12.55	11/24	51/62	11/15
11	10.5	17.6	GFP	27.01	24/33	44/52	9/13
	9.5	12.53	YFP	23.84	1/24	37/52	10/15

N.D., not determined. -, blank allele.

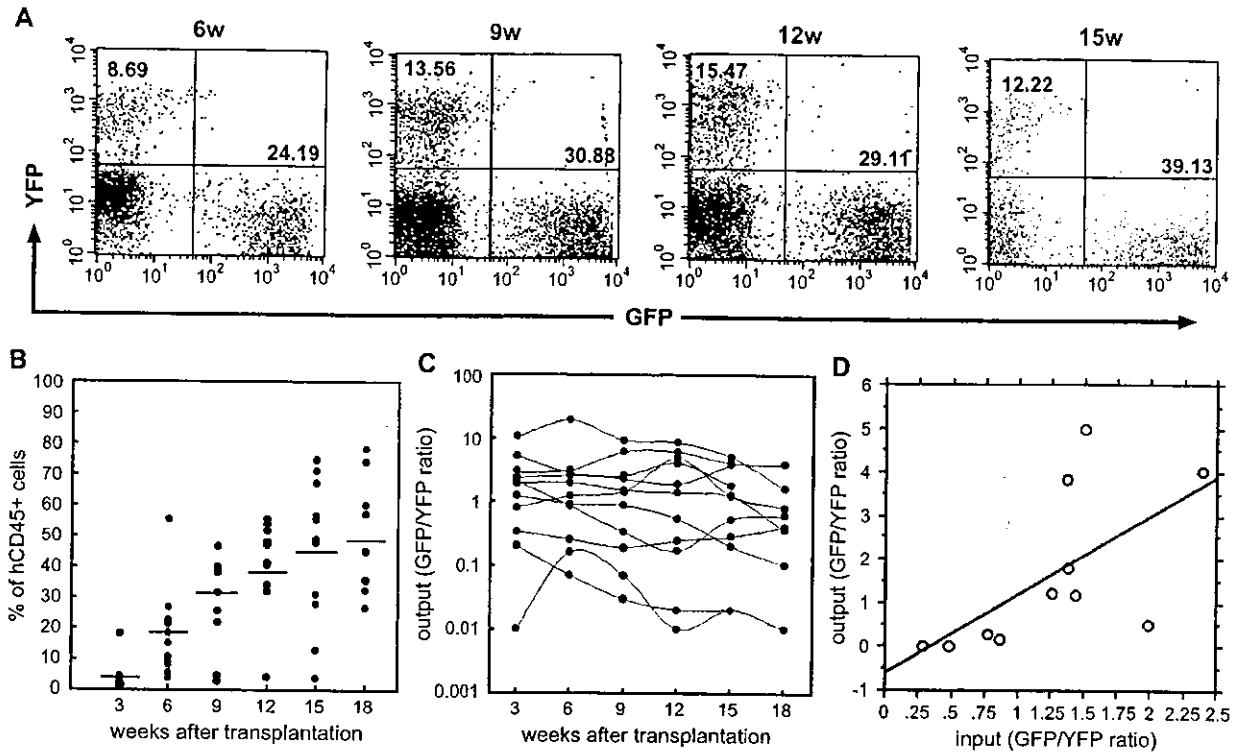


FIG. 1. (A) Representative FACS profiles of competitive repopulation of EGFP- or EYFP-transduced CB CD34⁺ cells in an individual NOG mouse recipient. PB cells were collected at specific intervals and stained with anti-human CD45 mAb. The stained cells were then analyzed by flow cytometry gated for the expression of CD45. Each donor-derived human hematopoietic cell was distinguished by the expression of the marker EGFP or EYFP. (B) Kinetics of human hematopoietic cells in NOG mice transplanted with two units of CB CD34⁺ cells. PB cells were collected at various intervals from transplanted NOG mice (*n* = 11) and stained with anti-human CD45 mAb. Each dot represents one mouse recipient, and bars indicate the average of engraftment. (C) The ratio of each donor's derived CD45⁺ hematopoietic cells detected in the NOG mouse PB was calculated. (D) A weak linear relationship between the ratio of injected CD34⁺ cell number (horizontal axis) and the ratio of human hematopoietic cells engrafted in the PB at 15 weeks after transplantation (vertical axis) (*r* = 0.623, *n* = 11, *P* = 0.039).

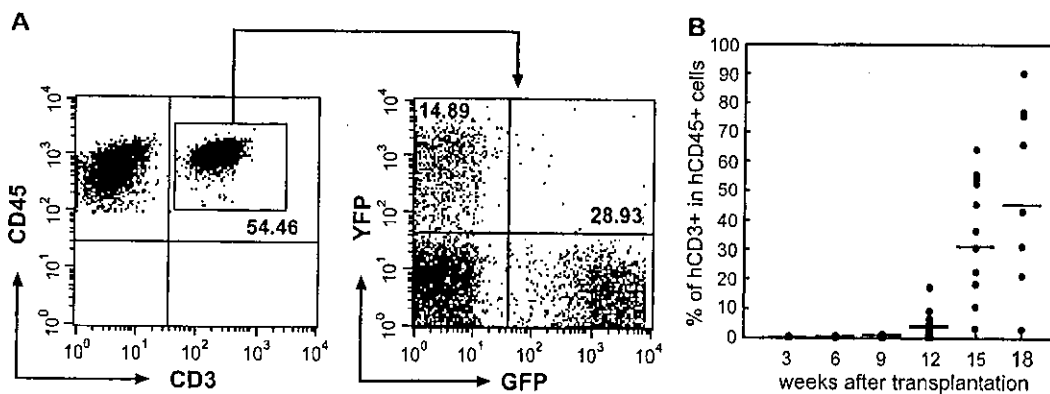


FIG. 2. (A) Representative FACS profiles of CD3⁺ T lymphocytes in competitive repopulation of EGFP- or EYFP-transduced CB CD34⁺ cells in an individual NOG mouse recipient. PB cells were collected at 15 weeks after transplantation. Anti-human CD3 mAb and anti-human CD45 mAb were used to detect human T lymphocytes in the PB of engrafted NOG mice. The CD3⁺CD45⁺ region was gated and marker gene expression was analyzed. (B) Kinetics of human CD3⁺ T lymphocytes in NOG mice transplanted with two units of CB CD34⁺ cells. PB cells were collected at various intervals from transplanted NOG mice (*n* = 11) and stained with anti-human CD3 mAb and anti-human CD45 mAb. The stained cells were then analyzed by flow cytometry gated for the expression of CD45. Each dot represents one mouse recipient, and bars indicate the average engraftment.

TABLE 2: Lineage analysis of T lymphocytes derived from two UCB units in the thymus of NOG mice

Expt	Phenotype	Lineages (%)			
		DN	DP	CD4 ⁺ SP	CD8 ⁺ SP
1	GFP ⁺ /CD45 ⁺	N.D.	N.D.	N.D.	N.D.
	YFP ⁺ /CD45 ⁺	N.D.	N.D.	N.D.	N.D.
3	GFP ⁺ /CD45 ⁺	4.11	69.08	14.09	12.72
	YFP ⁺ /CD45 ⁺	3.86	66.80	16.22	13.13
4	GFP ⁺ /CD45 ⁺	8.00	50.50	26.00	15.50
	YFP ⁺ /CD45 ⁺	1.60	65.07	21.60	18.16
6	GFP ⁺ /CD45 ⁺	8.90	44.70	27.35	22.72
	YFP ⁺ /CD45 ⁺	1.96	67.99	18.22	11.83
7	GFP ⁺ /CD45 ⁺	15.17	45.07	18.16	21.6
	YFP ⁺ /CD45 ⁺	18.20	43.80	17.22	20.78
9	GFP ⁺ /CD45 ⁺	32.64	34.55	12.81	19.99
	YFP ⁺ /CD45 ⁺	22.32	20.06	16.47	41.15
10	GFP ⁺ /CD45 ⁺	2.03	35.29	28.26	34.42
	YFP ⁺ /CD45 ⁺	0.24	44.01	20.45	35.30
11	GFP ⁺ /CD45 ⁺	16.55	51.39	18.39	16.55
	YFP ⁺ /CD45 ⁺	0.29	38.07	24.08	37.56

N.D., not determined.

cell number in PB against the ratio of engrafted CD34⁺ cells. There was a linear relationship between the EGFP⁺ to EYFP⁺ cell ratio observed at 15 weeks after transplantation and the EGFP⁺ to EYFP⁺ CD34⁺ cell ratio at the time of transplantation ($r = 0.623$, $P = 0.039$, $n = 11$; Fig. 1D). Similarly, the EGFP⁺ to EYFP⁺ ratio in engrafted cells in bone marrow (BM) correlated with the EGFP⁺ to EYFP⁺ ratio at transplantation ($r = 0.713$, $P = 0.0458$, $n = 8$), although three mice died before the analysis.

These data demonstrate that one unit of UCB CD34⁺ cells will engraft with the same efficiency in NOG mice regardless of whether a second, different, unit of UCB CD34⁺ cells is simultaneously transplanted.

Multilineage Differentiation of Two Units of Cord Blood CD34⁺ cells

Consistent with our previous results [23], we began to detect human CD3⁺ T lymphocytes at 9 weeks posttransplantation of UCB CD34⁺ cells and their numbers steadily increased up to 18 weeks (Fig. 2B). Both EGFP⁺ and EYFP⁺ CD3⁺ T lymphocytes were detected in PB of NOG mice between 9 and 18 weeks posttransplantation (Fig. 2A).

Fifteen to 24 weeks after transplantation, we killed the mice and collected the thymus, BM, and spleen to analyze the progeny of the two units of UCB CD34⁺ cells. In the thymus, most thymocytes in the NOG mice were human CD3⁺ cells, which had differentiated from both EGFP⁺CD34⁺ cells and EYFP⁺CD34⁺ cells (Table 2 and Fig. 3). Thymocytes from both donors showed normal differentiation patterns, which comprised both double-positive and single-positive subsets of CD4/CD8.

In BM and spleen, we analyzed multilineage differentiation of EGFP⁺CD34⁺ cells and EYFP⁺CD34⁺ cells by gating EGFP⁺CD45⁺ and EYFP⁺CD45⁺ cells. In addition to CD3⁺ T cells, CD19⁺ B cells, CD56⁺ NK cells, CD14⁺

monocytes, CD33⁺ myeloid cells, and primitive CD34⁺ cells arose from both the EGFP⁺CD45⁺ and the EYFP⁺CD45⁺ units (Table 3, Fig. 4, and spleen data not shown).

These data demonstrate that the multilineage engraftment of one unit of UCB CD34⁺ cells in NOG mice is

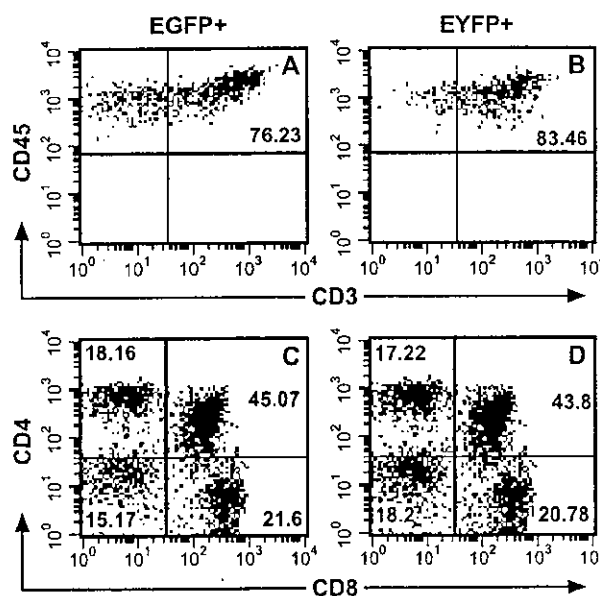


FIG. 3. Representative FACS analysis of human T cell engraftment in the thymus of a NOG mouse recipient transplanted with two combined units of CB CD34⁺ cells. At 18 weeks after transplantation, thymocytes were collected and stained with anti-human CD45 mAb. Each (A, C) CD45⁺EGFP⁺ and (B, D) CD45⁺EYFP⁺ region was gated, and the expression of (A, B) CD3 and (C, D) CD4/CD8 was analyzed. The relative frequencies of each population are indicated.

TABLE 3: Lineage analysis of two UCB units of CD34⁺-derived hematopoietic cells that had differentiated in BM of NOG mice

Expt	Phenotype	CD14 ⁺	CD19 ⁺	Lineages (%)		
				CD33 ⁺	CD34 ⁺	CD56 ⁺
1	GFP ⁺ /CD45 ⁺	11.76	38.46	37.27	15.91	4.00
	YFP ⁺ /CD45 ⁺	12.22	57.47	19.54	12.05	2.33
3	GFP ⁺ /CD45 ⁺	16.28	34.34	38.26	14.01	4.35
	YFP ⁺ /CD45 ⁺	18.24	25.07	49.22	16.47	8.13
4	GFP ⁺ /CD45 ⁺	9.21	21.37	10.16	10.29	0.88
	YFP ⁺ /CD45 ⁺	10.76	29.86	12.63	10.72	1.61
6	GFP ⁺ /CD45 ⁺	24.44	11.18	37.77	3.36	4.75
	YFP ⁺ /CD45 ⁺	10.97	46.70	13.52	5.86	8.04
7	GFP ⁺ /CD45 ⁺	17.17	62.97	28.72	18.91	1.32
	YFP ⁺ /CD45 ⁺	12.85	48.52	20.42	9.49	2.55
9	GFP ⁺ /CD45 ⁺	5.25	18.37	11.13	6.44	6.17
	YFP ⁺ /CD45 ⁺	17.19	37.23	31.25	6.60	7.23
10	GFP ⁺ /CD45 ⁺	5.33	24.63	10.65	3.22	2.98
	YFP ⁺ /CD45 ⁺	3.06	18.24	3.70	3.93	2.67
11	GFP ⁺ /CD45 ⁺	10.78	52.72	13.51	3.90	2.25
	YFP ⁺ /CD45 ⁺	26.24	29.46	7.44	9.76	1.06

stable and unaffected by lymphopoietic repopulation by another UCB unit.

Effects of Cotransplantation of CD34⁻ Mononuclear Cells on Competitive Repopulation in The NOG Mouse Unfractionated UCB cells containing mature immune-competent cells are commonly used in clinical UCBT. These cells can induce graft-versus-host, graft-versus-tumor, and graft-versus-graft reactions. The effects of the latter reaction on engraftment in particular are poorly understood in m-UCBT. To assess these effects, we performed three sets of competitive repopulation assay

by cotransplantation of pairs of CD34⁻ MNC with EGFP⁺CD34⁺ cells and EYFP⁺CD34⁺ cells (Table 4).

Four to 6 weeks posttransplantation, we sacrificed the mice and analyzed the ratio of engrafted EGFP⁺CD45⁺ and EYFP⁺CD45⁺ cells in BM MNC by flow cytometry. When only purified CD34⁺ cells were transplanted, both UCB units engrafted as expected. However, when CD34⁻ MNCs were cotransplanted with CD34⁺ cells, only CD45⁺ cells from one or the other UCB engrafted in all mice analyzed (Fig. 5). These results indicate that CD34⁻ MNC from one UCB somehow inhibited engraftment of cells from the other UCB.

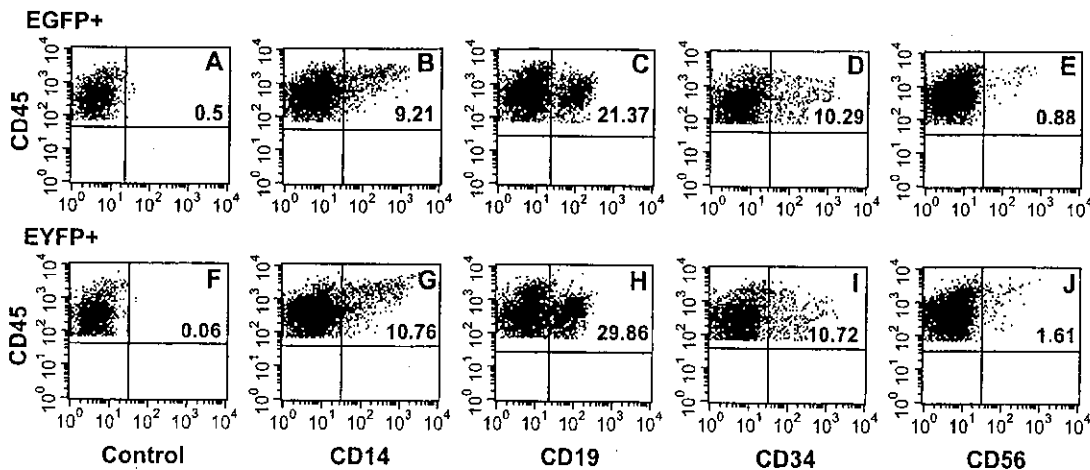


FIG. 4. Representative FACS profile of human multilineage engraftment in a NOG mouse recipient transplanted with two combined units of CB CD34⁺ cells. At 18 weeks after transplantation, BM cells were collected and analyzed for multilineage differentiation of engrafted human hematopoietic cells. To distinguish hematopoietic cells derived from each donor, each (A-E) CD45⁺EGFP⁺ and (F-J) CD45⁺EYFP⁺ region was gated, and the percentage of human CD45⁺ cells expressing the respective surface markers was measured. Human lineage-specific mAbs were used to detect (B, G) myeloid CD45⁺CD14⁺, (C, H) lymphoid CD45⁺CD19⁺, (E, J) CD45⁺CD56⁺, and (D, I) immature CD34⁺ progenitor cells in the marrow of engrafted NOG mice. The relative frequencies of each population are indicated.

TABLE 4: Characteristics of purified CD34⁺ cells and MNCs in each CB unit

Expt	Infection	CD34 ⁺ /mouse ($\times 10^4$)	MNC/mouse ($\times 10^6$)	HLA			
				A	B	DR	DRw
1	GFP	6.0	7.4	2/-	59/61	4/-	53/-
	YFP	6.0	8.8	2/24	51/67	9/12	52/53
2	GFP	12	1.0	2/24	7/51	1/9	53/-
	YFP	7.3	1.0	24/33	44/75	4/13	52/53
3	GFP	16	1.0	24/-	60/-	11/13	52/-
	YFP	11	1.0	2/24	7/61	1/9	53/-

-, blank allele.

To clarify the cell population responsible for this inhibitory effect, we cotransplanted purified CD4⁺ or CD8⁺ T lymphocytes or CD4/CD8 double-negative (DN) cells with EGFP⁺CD34⁺ cells and EYFP⁺CD34⁺ cells into NOG mice (Table 5). Although CD34⁻ MNCs eliminated either EGFP⁺CD45⁺ or EYFP⁺CD45⁺ cells as expected, there was no inhibition of engraftment of EGFP⁺CD45⁺ or EYFP⁺CD45⁺ cells by purified CD4⁺ or CD8⁺ T lymphocytes or CD4/CD8 double-negative cells in any mice (Fig. 6). These results indicate that a combination of two or more populations of CD4⁺, CD8⁺, and/or DN cells is required for this inhibitory effect.

To determine the effects of a combination of two populations on engraftment, we cotransplanted EGFP⁺CD34⁺ cells and EYFP⁺CD34⁺ cells with combined populations of CD4⁺ and CD8⁺ cells, CD4⁺ and DN cells, and CD8⁺ and DN cells into NOG mice (Table 6). Both CD4⁺ and CD8⁺ cells from one donor cooperated to inhibit engraftment of cells from the other donor, as we

observed with whole CD34⁻ MNC cotransplantation (Fig. 7A). There was no inhibition of engraftment of EGFP⁺CD45⁺ or EYFP⁺CD45⁺ cells by the combination of CD4⁺ and DN cells or CD8⁺ and DN cells (Fig. 7B).

DISCUSSION

The possibility of competitive reconstitution of the hematopoietic system by HSCs from multiple UCB units in clinical m-UCBT raises several important questions. Do immune-competent cells developed from two unrelated HSCs affect hematopoietic engraftment or reconstitution in a host? Do mature immune-competent cells derived from two UCB units affect hematopoietic engraftment or reconstitution in a host? To address these questions, we used a novel *in vivo* competitive repopulation system using NOG mice that allows complete reconstitution of human lymphocytes, including T cells, from CD34⁺ UCB that respond to both mitogenic stimuli, such as PHA and IL-2, and allogeneic human cells [23,25].

We demonstrated here that hematopoietic cells derived from two UCB units engrafted stably, even after CD3⁺ T cells were detected in recipient PB at 12 weeks after transplantation, and that the ratio of chimerism between cells derived from the two UCB units was correlated with the ratio of cells at transplantation (Fig. 1). Recently, Kim *et al.* [26] demonstrated that two lineage-depleted UCB units could coengraft in NOD/SCID mice. However, this assay could not assess the effects of an immune system derived from HSCs on engraftment by the other, since T cells did not differentiate from CD34⁺ cells in NOD/SCID mice. Therefore, this is the first demonstration that human immune-competent cells from two immunologically distinct CD34⁺ donor stem cells can develop normally in the same host environment without inhibiting each other. In recipient NOG mouse thymus, both EGFP⁺CD3⁺ and EYFP⁺CD3⁺ cells showed a normal pattern of T cell differentiation, which comprised both double-positive and single-positive CD4/CD8 subsets (Fig. 4 and Table 2). This suggests that there may have been negative selection in the clonal deletion of cells reactive to each other. Therefore, the content of HSCs may be a good predictor of the resultant chimerism in a mouse, as shown in Fig. 1D.

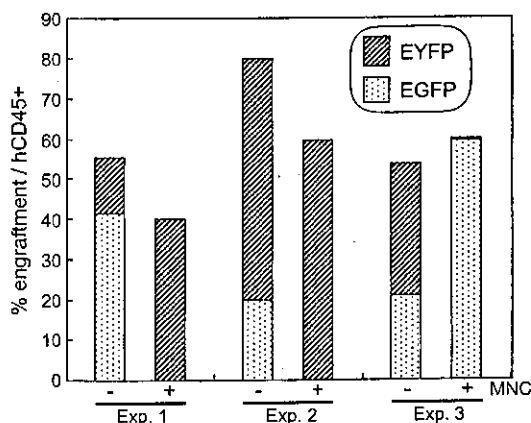


FIG. 5. The effects of CD34⁻ MNCs on competitive repopulation by CD34⁺ cells in the NOG mouse. EGFP- or EYFP-transduced CD34⁺ cells with or without a respective fraction of CD34⁻ MNCs were injected into the same NOG mouse (Table 4). At 4 to 6 weeks after transplantation, the ratio of engrafted donor-derived hematopoietic cells from each unit was measured using the expression of CD45 and the marker genes in flow cytometry. To exclude residual human T cells derived from CD34⁻ MNCs, CD3⁺ cells were gated out at FACS analysis. Data shown are the ratios of CD45⁺EGFP⁺ and CD45⁺EYFP⁺ cells in the BM MNCs of the same NOG recipient (three independent experiments).

TABLE 5: Characteristics of purified CD34⁺ cells and effector cells in each CB unit

Expt	Infection	CD34 ⁺ cells ^a	MNC fraction ^b	HLA			
				A	B	DR	DRw
1	GFP	+	+	2/31	51/54	4/15	51/53
	YFP	+	-	2/33	39/44	9/13	52/53
2	GFP	+	-	11/24	54/67	4/8	53/- ^c
	YFP	+	+	2/24	35/62	9/12	52/53
3	GFP	+	+	2/24	35/61	4/8	53/-
	YFP	+	-	2/26	35/61	9/15	51/53

^a EGFP- or EYFP-transduced CD34⁺ cells were transplanted into the same NOG mouse (range of transplanted cell dose 6.8-9 × 10⁴ cells).
^b Unfractionated MNCs (range 7-10 × 10⁵ cells), CD4⁺ cells (range 3.3-5 × 10⁵ cells), CD8⁺ cells (range 1.4-2 × 10⁵ cells), and CD4/CD8 double-negative fraction (range 2-3 × 10⁵ cells) were prepared from one CB CD34⁺ fraction (+) and injected into NOG mice together with CD34⁺ cells derived from the same donor and CD34⁺ cells derived from the second unit of CB (-).
^c -, blank allele.

In clinical m-UCBT, the transplanted whole MNC can induce a rapid graft-versus-graft reaction. One study reported successful engraftment of only 1 of 12 randomly selected units of UCB [7]. Another study demonstrated that only one of the two UCB donors was responsible for hematopoiesis in 16 of 18 patients with sustained engraftment [27]. However, another study found that both of the transplanted UCBs contributed to stable hematopoiesis, and this success was attributed to the grafts being closely matched, with only a single mismatch between them at one HLA-DRB1 allele [8]. In the present study, we observed engraftment of either of mismatched pairs of UCB units (Tables 4 and 5), confirming the utility of this animal model to analyze engraftment and hematopoiesis in m-UCBT. We also observed that graft-versus-graft reaction was associated with the CD34⁻ mononuclear cells in a cord blood unit. In future experiments it would be of interest to analyze the chimerism when an HLA-matched UCB pair is cotransplanted.

One possible drawback to our model is the potential antigenicity of the marker genes EGFP and EYFP used to identify the transplanted cell populations. These two proteins have very similar antigenic properties, since EYFP is simply a mutant form of EGFP [28]; therefore any specific immune reaction would be expected to have an equivalent effect on both cell populations. However, in every case shown in Figs. 5, 6, and 7 either EGFP⁺ or

EYFP⁺ cells were eliminated, suggesting that this was due to an allospecific reaction.

To identify further the cell population responsible for graft-versus-graft reaction, we cotransplanted purified populations of CD4⁺, CD8⁺, or DN cells from two UCBs together with CD34⁺ cells, but none of these cell populations alone was sufficient to generate a reaction against the other UCB unit (Fig. 6). This suggests that perhaps two populations of effector cells are needed to induce a graft-versus-graft reaction. Consistent with this, we could reproduce this inhibitory effect on engraftment by CD34⁻ mononuclear cells only when both CD4⁺ and CD8⁺ cells were transplanted simultaneously (Fig. 7). These results support the hypothesis that graft-reactive CD8⁺ CTL served as the terminal effector cell in the rejection, while CD4⁺ cells provided the signals required for CTL development and expansion [29]. As the strongest alloreaction is provoked by major histocompatibility complex (MHC) antigens, T cells recognize the alloantigens either "directly" as foreign antigens or "indirectly" as presented by self-MHC molecules [30,31]. Our results may suggest that CD4⁺ and CD8⁺ T cells from one graft recognized the MHC antigens from the other directly as nonself and then eliminated hematopoietic cells of the other graft, since DN cells were not required as antigen-presenting cells to elicit the reaction. However, Kim and colleagues demonstrated that the mixed transplantation

FIG. 6. The effects of fractionated effector cells contained in CD34⁻ MNCs on competitive repopulation by CD34⁺ cells in the NOG mouse. CD4⁺ or CD8⁺ T lymphocytes and the CD4/CD8 double-negative fraction were sorted from one CB CD34⁻ fraction and injected into NOG mice together with CD34⁺ cells derived from the same CB and CD34⁺ cells derived from the second unit of CB (Table 5). At 3 weeks after transplantation, the ratio of engraftment was analyzed by the same method as for Fig. 5. Data shown are the ratios of CD45⁺EGFP⁺ and CD45⁺EYFP⁺ cells in the BM MNCs of the same NOG recipient (three independent experiments). (-) Transplants without CD34⁻ MNCs; (W) transplants with whole CD34⁻ MNC.

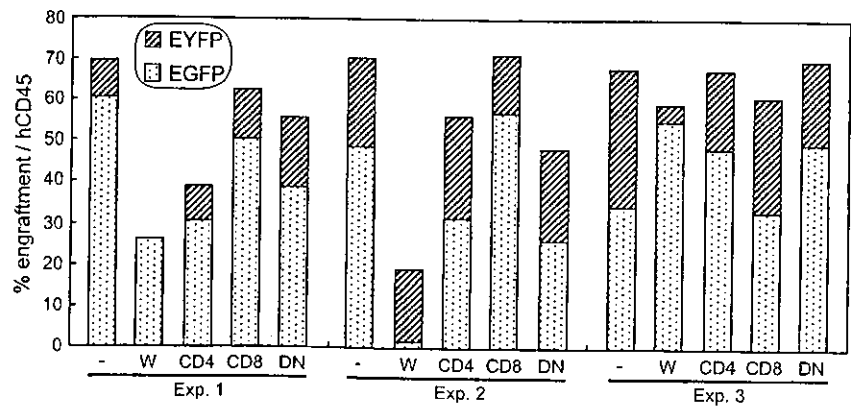


TABLE 6: Characteristics of purified CD34⁺ cells and effector cells in each CB unit

Expt	Infection	CD34 ⁺ cells ^a	MNC fraction ^b	HLA			
				A	B	DR	DRw
1	GFP	+	-	2/31	35/- ^c	12/15	51/52
	YFP	+	+	24/26	51/62	4/15	51/53
2	GFP	+	+	2/31	46/61	8/15	51/-
	YFP	+	-	2/24	35/55	4/9	53/-
3	GFP	+	+	24/33	44/75	8/9	53/-
	YFP	+	-	11/-	44/59	4/13	52/53

^a EGFP- or EYFP-transduced CD34⁺ cells (5×10^4 cells) were transplanted into the same NOG mouse.

^b Unfractionated MNCs (10×10^5 cells), CD4⁺ cells (5×10^5 cells), CD8⁺ cells (2×10^5 cells), and CD4/CD8 double-negative fraction (range $2-3 \times 10^5$ cells) were prepared from one CB CD34⁺ fraction (+). Each fraction were combined as CD4⁺ and CD8⁺ cells, CD4⁺ and DN cells, and CD8⁺ and DN cells and transplanted into NOG mice together with CD34⁺ cells derived from the same donor and CD34⁺ cells derived from the second unit of CB (-).

^c -, blank allele.

of two allogeneic UCB grafts led to single-donor predominance independent of the degree of HLA compatibility between the two grafts [26]. Further investigation is necessary to determine how CD4⁺ and CD8⁺ cells contribute to predominant engraftment of HSCs, through either direct recognition of the MHC antigens or other non-specific effects.

We have established a novel *in vivo* competitive repopulation assay using a new NOG mouse system that may facilitate study of the behavior of different units of stem cells after transplantation. Furthermore, we clearly demonstrated that the lentiviral gene marking strategy can easily distinguish the two distinct donor cells by flow cytometric analysis, independent of

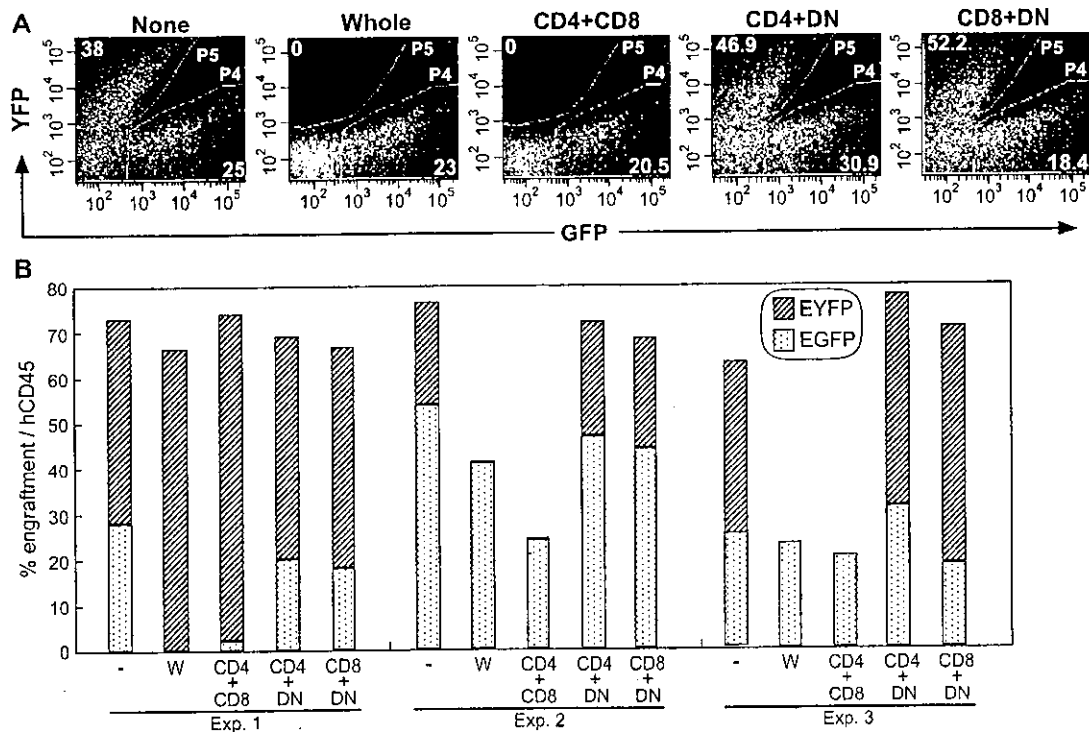


FIG. 7. The effects of combined cell populations contained in CD34⁺ MNCs on competitive repopulation by CD34⁺ cells in the NOG mouse. CD4⁺ and CD8⁺ T lymphocytes and the CD4/CD8 double-negative fraction were sorted from one CB CD34⁺ fraction. Each sorted fraction was combined to give CD4⁺ and CD8⁺ cells, CD4⁺ and DN cells, or CD8⁺ and DN cells and injected into NOG mice together with CD34⁺ cells derived from the same CB and CD34⁺ cells derived from the second unit of CB (Table 6). At 3 weeks after transplantation, the ratio of engraftment was analyzed by the same method as for Fig. 5. (A) Representative FACS profiles of competitive repopulation of EGFP- or EYFP-transduced CB CD34⁺ cells in an individual NOG mouse recipient. (B) Summary of three independent experiments. Data shown are the ratios of CD45⁺EGFP⁺ and CD45⁺EYFP⁺ cells in the BM MNCs of the same NOG recipient. (-) Transplants without CD34⁺ MNCs; (W) transplants with whole CD34⁺ MNC.

cell surface antigens, expression of intracellular isoenzymes, or hemoglobin subtypes. This strategy is also useful for the examination of the stem cell competitive repopulation activity of variety cell sources such as BM cells and cord blood cells, *ex vivo* expanded cells, and nonexpanded cells. Gene transduction by lentiviral infection did not have any apparent untoward effects on the behavior of HSCs, such as multilineage differentiation and long-term repopulation.

We hope that the *in vivo* assay system described here will be useful, not only for examining the competitive hematopoietic repopulation from multiple UCB units, but also for predicting the effects of MNCs cotransplanted in clinical m-UCBT. To evaluate the relevance of this system as a surrogate human HSC assay, it will be important to correlate the results of engraftment in this model with engraftment in clinical m-UCBT.

MATERIALS AND METHODS

Cells. CB samples were obtained from full-term deliveries following institutional guidelines approved by the Tokai University Committee on Clinical Investigation. MNCs were isolated by Ficoll-Hypaque (Lymphoprep, 1.077 ± 0.001 g/ml; Nycomed, Oslo, Norway) density gradient centrifugation. Cells were washed and suspended in phosphate-buffered saline (PBS) containing 0.1% of human serum globulin (Sigma, St. Louis, MO, USA). CD34⁺ cell fractions were prepared from Ficoll-separated MNCs using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Sunnyvale, CA, USA) according to the manufacturer's directions. The enriched CD34⁺ cell fraction was stained either with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 mAb (581; Coulter/Immunotech, Marseille, France) or with allophycocyanin (APC)-conjugated anti-CD3 mAb (UCHT1; Coulter/Immunotech). CD34⁺CD3⁻ cells were sorted using a FACSVantage flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with HeNe and argon lasers. This resulted in a highly purified CD34⁺ cell fraction (more than 99%) in which no CD3⁺ cells were detected on FACS analysis. Sorted CD34⁺CD3⁻ cells were cryopreserved in liquid nitrogen until use. Column passthrough CD34⁺ MNC fractions were also cryopreserved. On the day of transplantation, cryopreserved CD34⁺ MNC fractions were thawed and stained with PE-conjugated anti-CD4 (SK3) and FITC-conjugated anti-CD8 (SK1) mAbs (all BD Biosciences). Stained cells were sorted using the FACSVantageSE Diva option (BD Biosciences) gated on each CD4⁺CD8⁻, CD4⁻CD8⁺, and CD4⁻CD8⁻ region. Purified populations were >98% pure. Dead cells stained with PI were excluded from the analysis. The HLA of each CB unit was determined by molecular typing using a Micro SSP HLA Classes I and II ABDR DNA Typing Tray (One Lambda, Canoga Park, CA, USA).

Lentivirus infection. Transduction of the EGFP or its yellow variant EYFP gene into CD34⁺ cells by recombinant lentivirus infection was performed as described previously [32,33]. Briefly, cryopreserved CB CD34⁺ cells were thawed and prestimulated by incubation in StemPro-34 medium (Invitrogen, Carlsbad, CA, USA) containing cytokines at 37°C in 5% CO₂ for 24 h. Recombinant human thrombopoietin (50 ng/ml; kindly donated by Kirin Brewery, Tokyo, Japan), stem cell factor (50 ng/ml; donated by Kirin Brewery), and Flk-2/Flt-3 ligand (50 ng/ml; R&D Systems, Minneapolis, MN, USA) were used. Prestimulated CD34⁺ cells were cultured for 12 h under the same conditions in the presence of highly concentrated lentivirus supernatant at an m.o.i. of 50. Lentivirus-infected CD34⁺ cells were transplanted into irradiated NOG mice. The efficiency of infection was determined by FACS analysis on the day of transplantation.

Mice. NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan) and were maintained in the animal facility of the Tokai University School of Medicine in microisolator cages with autoclaved food and water. Seven- to nine-week-old NOG mice were irradiated with 250 cGy of X-rays and thereafter received acidified water containing 1.1 g/L neomycin sulfate and 131 mg/L polymyxin B sulfate (Sigma). The following day, two units of CB CD34⁺CD3⁻ cells were simultaneously injected intravenously into NOG mice. In some experiments, CD34⁻ MNCs were cotransplanted with CD34⁺ cells. All experiments were approved by the animal care committee of Tokai University.

Flow cytometric analysis. For kinetic analysis, mice were anesthetized with ethyl ether and PB samples were aspirated from the retro-orbital sinus. Samples were prepared as single-cell suspensions in PBS containing 0.1% human serum globulin and heparin. Human hematopoietic cells were distinguished from mouse cells by expression of human CD45. At the time of sacrifice, BM, spleen, and PB were collected for analysis of the presence of human cells by flow cytometry. BM cells were suspended in PBS using a 27-gauge needle. Spleen was teased apart, and PB was aspirated from the retro-orbital sinus. Samples were prepared as single-cell suspensions in PBS containing 0.1% human serum globulin and passed through a nylon filter to remove debris. Cells were stained with mAbs to human leukocyte differentiation antigens. APC-conjugated anti-human CD3, CD4, CD14, CD19, CD33, CD34, and CD56 mAbs (all Coulter/Immunotech) and ECD-conjugated anti-human CD8 and CD45 mAbs (all Coulter/Immunotech) were used. Four-color flow cytometric analysis was conducted using FACSVantage (BD Biosciences). Quadrants were set to include at least 97% of the isotype-negative cells. The proportion of each lineage was calculated from 20,000 events acquired using CELLQuest or FACSDiva software (BD Biosciences).

Statistical analysis. Results are expressed as individual data. Input (transplanted EGFP/EYFP ratio) and output (engrafted EGFP/EYFP ratio) were calculated by the following calculation: input (EGFP/EYFP ratio) = (EGFP transplanted cell number × infection efficiency)/(EYFP transplanted cell number × infection efficiency). Output (EGFP/EYFP ratio) = (percentage of CD45⁺EGFP⁺ cells in the recipient)/(percentage of CD45⁺EYFP⁺ cells in the recipient). The correlation of input values and output values was analyzed using StatView-J4.02 software (Abacus Concepts, Berkeley, CA, USA). P values <0.05 were considered to be significant.

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Potential and origin of the hematopoietic population in human skeletal muscle

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Abstract

While mononuclear cells isolated from murine skeletal muscle were shown to be capable of hematopoietic activity, similar hematopoietic cells (HC) recently were reported to exist in primate muscle. We investigated muscle-derived HC from young and adult human subjects. Although hematopoietic stem cells were rare in muscle, their frequency nonetheless was approximately four times greater than in peripheral blood. These cells in muscle appeared to originate from CD45⁺ bone marrow cells. Our results suggested an additional function of human skeletal muscle as a reservoir of HC.

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Keywords: Human skeletal muscle; Hematopoietic progenitors; Hematopoietic stem cells

1. Introduction

A variety of tissue-specific stem cells, including those in hematopoietic, nervous, and muscular systems, recently have been reported to have potential to differentiate into cells other than those specific to their origin. For example, hematopoietic stem cells were found capable of regenerating hepatocytes [1]; while neuronal stem cells reportedly can differentiate into hematopoietic cells [2].

Previously, investigators in several laboratories observed that cells isolated from murine skeletal muscle could carry out hematopoietic reconstitution in lethally irradiated mice [3–6].

Accordingly, we assessed the nature and origin of hematopoietic progenitors and stem cells in murine muscles using clonal cell culture and transplantation techniques [7,8]. The frequency of hematopoietic progenitors among mononuclear cells (MNC) derived from murine muscle proved 10 times greater than among MNC derived from peripheral blood (PB) [7]. Furthermore, we demonstrated that 10⁵ MNC derived from murine muscle could accomplish hematopoietic engraftment in lethally irradiated recipient mice [8].

Recently a hematopoietic population also was demonstrated in primate skeletal muscle [9,10]; Mahmud et al. [9] and Jay et al. [10] reported that muscle cells isolated from human fetuses contained detectable numbers of hematopoietic progenitors. Human skeletal muscle therefore was suspected to be third source of HC in adults, in addition to BM and PB. In this study, we evaluated the nature and potential of

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hematopoietic populations derived from muscles from young and adult human subjects.

2. Materials and methods

2.1. Cell preparation

After the patients had given informed consent, skeletal muscle tissues were obtained under general anesthesia at the time of surgery to correct musculoskeletal defects (Table 1). The study protocol had been approved by the Human Research Committee of Tokai University School of Medicine. After removal of fat, epimysium, tendons, and internal connective tissue, the muscle samples were minced with scissors and incubated at 37 °C in 0.1% trypsin under constant agitation for 45 min. Cells were released from the tissue fragments by vigorous trituration. Pooled cells were filtered through a 40 µm nylon mesh, washed, and resuspended in α -modification of Eagle's medium (α -MEM; Life Technologies, Grand Island, NY). Samples were purified further by discontinuous Percoll density-gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ). Here, the samples were centrifuged at 15 000 g for 5 min at 8 °C in a fixed-angle rotor, and MNC were collected from the 20–60% interface. PB and BM cells also were collected from the patients during the operation. MNC were prepared from PB and BM cells by Percoll density-gradient centrifugation, as described above. In some experiments, we separated the muscle MNC into CD45⁺ and CD45⁻ cells using a FACS Vantage fluorescence-activated cell sorter (FACS; Becton Dickinson, San Jose, CA). For transplantation experiments, to avoid graft-versus-host disease, CD3⁺ T-lymphocytes were depleted from the samples before transplantation using a MACS immunomagnetic separation system (Miltenyi Biotec, Glodbach, Germany).

2.2. Clonal cell culture

Methylcellulose culture was performed in 35 mm Petri dishes (Iwaki Glass, Chiba, Japan), as described previously [11]. One milliliter of culture mixture contained designated numbers of MNC, α -MEM, 1.2% 1500 cP methylcellulose (Shin-etsu Chemical, Tokyo, Japan), 1% deionized fraction V bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 30% fetal calf serum (StemCell Technologies, Vancouver, Canada), 100 ng/mL recombinant human thrombopoietin, 100 ng/mL recombinant human stem cell factor, 3 U/mL recombinant human erythropoietin, 10 ng/mL recombinant human granulocyte-colony stimulating factor, 10 ng/mL recombinant human granulocyte/macrophage-colony stimulating factor, and 20 ng/mL recombinant human interleukin-3. All cytokines were provided by the Kirin Brewery (Tokyo, Japan). Dishes were incubated at 37 °C in a humidified atmosphere consisting of 5% CO₂ in air. Hematopoietic colonies

consisting of 50 or more cells were scored using an inverted microscope after 14 days of culture. Abbreviations of colony types used here are as follows: colony-forming units in culture (CFU-C), erythroid burst-forming units (BFU-E); granulocyte and/or macrophage colonies (CFU-GM); and mixed colonies containing erythroid and myeloid cells (CFU-Mix).

2.3. Immunofluorescence photography

Cryostat sections of fresh frozen samples were fixed for 10 min at room temperature with 4% paraformaldehyde, washed in phosphate buffered saline solution (PBS), and incubated for 45 min with anti-laminin (Sigma-Aldrich). After washing in PBS sections, then were exposed for 45 min to secondary antibodies conjugated with tetramethylrhodamine isothiocyanate isomer R (Dako, Kyoto, Japan). Sections next were incubated overnight at 4 °C with biotin conjugated anti-human CD45 (BD Pharmingen, San Diego, CA), washed, and allowed to react for 4 h at 4 °C with streptavidin Alexa 488 (Molecular Probes, Eugene, OR). Then the sections were incubated for 1 h with anti-CD31 (TECHNE Corporation, Minneapolis, MN), washed, and exposed for 45 min to secondary antibodies conjugated with Alexa 594 (Molecular Probes). Nuclei were stained with TOTO-3 (Molecular Probes). Slides were examined under a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany).

2.4. Immunophenotyping by flow cytometry

MNC isolated from human muscle tissues were stained with FITC- or PE-conjugated monoclonal antibodies, as described previously [11]. Samples were analyzed using a FACSCalibur instrument (Becton Dickinson).

2.5. Transplantation

NOD/SCID/ γ c^{-/-} (NOG) mice 7–9 weeks old were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All animals were handled under sterile conditions and maintained under isolation at the animal facility of Tokai University School of Medicine. Mice were sublethally X-irradiated with 250 cGy, after which they received acidified water containing 1.1 g/L neomycin sulfate and 131 mg/L polymyxin B sulfate (Sigma-Aldrich). Two hours after irradiation, the mice were transplanted with human muscle-derived cells, BM cells, or PB cells by intra-BM injection (IBM). IBM was carried out as described previously [12]. In brief, a 29-gauge needle was inserted into the joint surface of the right tibia of anesthetized mice, and human cells suspended in 30 µL of PBS were injected into the BM cavity. Mice were killed 8 weeks after transplantation, and BM cells were harvested from the femora and tibiae. The population of human hematopoietic cells was determined by detecting cells stained by FITC-conjugated anti-human CD45, using flow cytometry.

Table 1
Patients from whom samples of muscle were obtained

Patient no.	Age (years)	Gender	Underlying disease	Muscle separated (g)	MNC collected ($\times 10^5$)	Experiment
1	14	Female	Idiopathic scoliosis	Iliopsoas (5.2)	0.73	Clonal cell culture
2	55	Female	Lumbar spinal canal stenosis	Iliopsoas (2.5)	0.75	Clonal cell culture
3	34	Male	Lumbar disk herniation	Iliopsoas (5.4)	0.64	Clonal cell culture
4	65	Male	Lumbar spinal canal stenosis	Iliopsoas (4.6)	25.8	Clonal cell culture transplantation
5	7	Female	Cartilage-hair hypoplasia	External abdominal oblique (not weighed)	1.0	STR analysis
6	49	Male	Right lower leg necrosis due to diabetes mellitus	Gastrocnemius (33.0)	25.4	Clonal cell culture transplantation
7	65	Female	Chondrosarcoma at right first rib	Pectoralis major (not weighed)	Not isolated	Immunohistochemistry
8	57	Male	Leiomyosarcoma at right lower leg	Quadriceps (4.9)	1.9	FCM
9	69	Male	Lumbar spinal canal stenosis	Iliopsoas (2.4)	0.54	FCM
10	59	Female	Degenerative scoliosis	Iliopsoas (6.0)	7.77	FCM
11	32	Male	Lumbar burst fracture	Iliopsoas (7.0)	21.7	Clonal cell culture transplantation

Human muscle samples were obtained from patients after they gave their informed consent. MNC, mononuclear cells; STR, short tandem repeat; FCM, immunophenotyping by flow cytometry.

2.6. Detection of human cell engraftment in mice and donor cells in the recipient by polymerase chain reaction (PCR)

Engraftment of human cells in the BM of recipient mice also was assessed by PCR. Genomic DNA was isolated from the BM cells using standard extraction protocols. DNA (100 ng) was subjected to PCR to detect a 1171-base pair (bp) fragment of human chromosome 17-specific α -satellite using specific primers previously reported [12]. PCR was performed using an RNA PCR kit (Takara Shuzo, Tokyo, Japan). PCR products were separated on 1.0% agarose gels and visualized by ethidium bromide staining. To determine the origin of CD45⁺ and CD45⁻ cells in the muscle of the BM transplantation recipient, a short tandem repeat (STR) locus (ACTBP2) was amplified by PCR using specific primers previously reported [13]. PCR products were separated using the 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.7. Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences.

3. Results

3.1. Hematopoietic populations in human muscle

We first assessed the nature of hematopoietic populations in human muscle tissue. We cultured MNC isolated from muscle in methylcellulose, and compared these cells

with MNC isolated from PB. The frequency of CFU-C in muscle-derived MNC was approximately four times greater than in PB-derived MNC ($P < 0.05$, Fig. 1A). The majority of colonies grown from the muscles were CFU-GM or BFU-E. However, CFU-Mix also were observed (Fig. 1A and B). The number of CFU-GM in muscle was significantly higher than in PB ($P < 0.05$). These results indicated that a distinct hematopoietic population indeed exists in human muscle.

We further separated muscle-derived MNC into CD45⁺ cells and CD45⁻ cells by FACS, and aliquots were cultured separately in methylcellulose, since CD45⁻ muscle cells isolated from human fetuses were reported to contain assayable hematopoietic progenitors [9,10]. Fourteen days later, hematopoietic colonies were detected in dishes in which CD45⁺ cells were cultured (Table 2). On the other hand, the CD45⁻ fraction contained no cells capable of forming hematopoietic activity *in vitro*.

We then set out to localize the hematopoietic population in the muscle tissue. Although some CD45⁺ cells were found in vessels, the majority of CD45⁺ cells were located outside of vessels (Fig. 2a). Extravascular CD45⁺ cells were 24 times as numerous as CD45⁺ cells in the vessels. Most CD45⁺ cells, including CD45⁺CD34⁺ cells, were observed in the interstitial space between muscle fibers in the skeletal muscle specimens (Fig. 2a and b).

The phenotype of muscle MNC also was analyzed by flow cytometry. Approximately half of the cells were positive for CD45, and most CD45⁺ cells expressed lineage-specific markers (Table 3). However, CD45⁺CD34⁺ cells were also found in muscle, and approximately half of these lacked lineage-specific markers (data not shown).

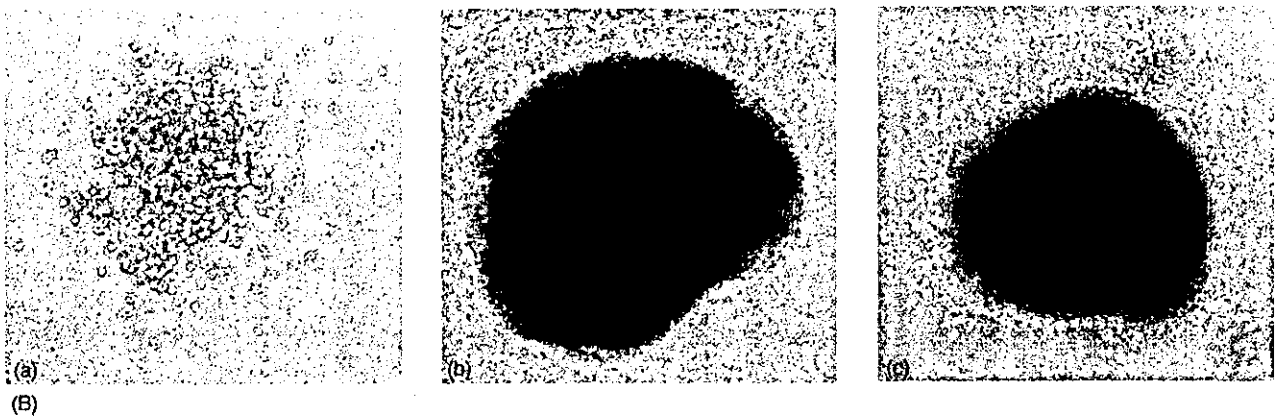
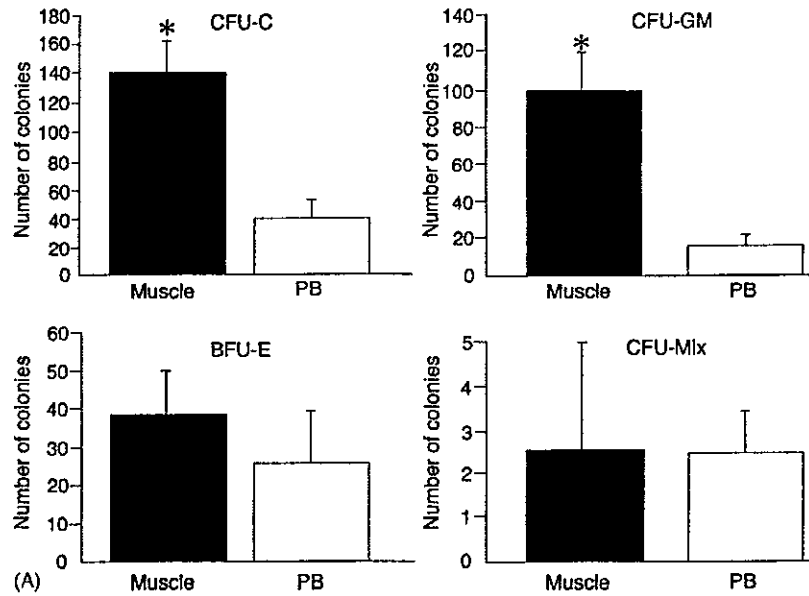


Fig. 1. Formation of hematopoietic colonies by mononuclear cells (MNC) derived from human muscles and peripheral blood (PB). (A) Comparison of colony formation by MNC derived from muscles and PB. From six patients (patients 1–4, 6, and 11 in Table 1), MNC were isolated from muscle and PB, and 2×10^5 muscle MNC and 8×10^5 PB MNC were plated in quadruplicate in the presence of hematopoietic cytokines. On day 14 of culture, colonies were counted. Bars represent the mean and standard deviation for the results obtained from 8×10^5 MNC in the six samples. * $P < 0.05$ compared with PB. No significant differences were evident in BFU-E or CFU-Mix between muscle and PB. (B) Representative hematopoietic colonies from human muscle. (a) CFU-GM, (b) BFU-E, (c) CFU-Mix.

Table 2
Comparison of colony formation between CD45⁺ cells and CD45⁻ cells separated from muscle mononuclear cells (MNC)

Patient no.	Cells	Hematopoietic colony			
		GM	BFU-E	GEMM	Total ^a
4	CD45 ⁺ (4×10^4 /dish)	5 ± 1	2 ± 1	0	7 ± 1
	CD45 ⁻ (6×10^4 /dish)	0	0	0	0
6	CD45 ⁺ (5×10^4 /dish)	5 ± 2	2 ± 1	1 ± 1	8 ± 2
	CD45 ⁻ (5×10^4 /dish)	0	0	0	0
11	CD45 ⁺ (2×10^4 /dish)	2 ± 1	1 ± 1	0	3 ± 1
	CD45 ⁻ (2×10^4 /dish)	0	0	0	0

CD45⁺ cells and CD45⁻ cells were separated by FACS sorting from muscle MNC of the patients (numbers 4, 6, and 11 in Table 1). Fractionated cells were plated in quadruplicate in the presence of hematopoietic cytokines. The number of cells plated per dish was proportional to the number of CD45⁺ and CD45⁻ cells among the MNC. On day 14 of culture, colonies were assessed by observation under an inverted microscope. Data represent the mean and standard deviation of values obtained from four dishes.

^a Total number of colonies.

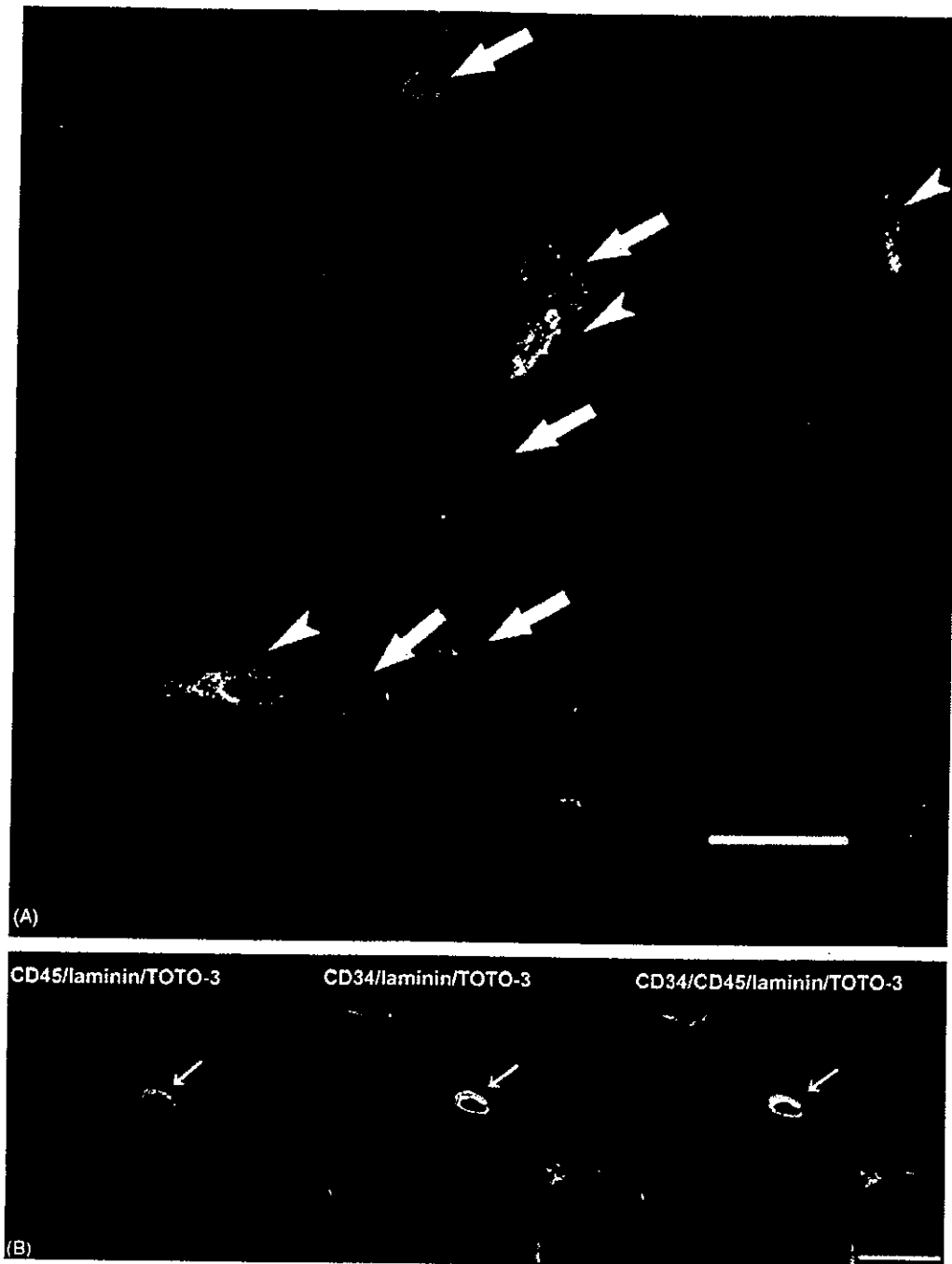


Fig. 2. Localization of hematopoietic cells in human muscle. (a) In muscle, $CD45^+$ cells were observed in the interstitial space between muscle fibers. Green, red, orange, and blue signals indicate CD45, laminin, CD31, and TOTO-3, respectively. Arrows indicate $CD45^+$ cells, and arrowheads indicate vessels in the interstitium of the muscle. Most $CD45^+$ cells were extravascular. Bar indicates $20\ \mu\text{m}$. (b) $CD45^+CD34^+$ cells also were observed in the interstitial space between muscle fibers. The representative result is shown. Green, orange, red, and blue signals indicate CD45, CD34, laminin, TOTO-3, respectively. Bar indicates $20\ \mu\text{m}$.

Table 3
Phenotypes of human muscle cells

Phenotype	Positive cells (%) ^a
CD45 ⁺	49 ± 11
CD45 ⁺ CD34 ⁺	3 ± 2
CD45 ⁺ CD31 ⁺	2 ± 1
CD45 ⁺ CD38 ⁺	14 ± 7
CD45 ⁺ CD3 ⁺	28 ± 16
CD45 ⁺ CD19 ⁺	7 ± 3
CD45 ⁺ CD13/14/33 ^{ab}	11 ± 7
CD45 ⁻ CD34 ⁺	14 ± 4
CD45 ⁻ CD31 ⁺	6 ± 2
CD45 ⁻ CD38 ⁺	3 ± 1
CD45 ⁻ CD3/19/13/14/33 ^{ac}	0

Phenotypes of mononuclear cells derived from human muscles were assessed by flow cytometry. Approximately, half of the muscle cells were positive for CD45.

^a Data are the mean ± standard deviation for three different experiments.

^b CD45⁺ cells expressing CD13, CD14, or CD33.

^c CD45⁻ cells expressing CD3, CD19, CD13, CD14, or CD33.

3.2. Origin of the hematopoietic population in human muscle

Next we examined the origin of the hematopoietic population in the muscle. MNC were isolated from muscle of a patient previously diagnosed with cartilage-hair hypoplasia, a congenital malformation associated with immunodeficiency, and had undergone allogeneic BM transplantation at the age of 4 years from an unrelated healthy donor selected through the Japan Marrow Donor Program, 3 years previously (patient no. 5 in Table 1). The preconditioning regimen for transplantation had been thoraco-abdominal irradiation followed by cyclophosphamide and anti-thymocyte globulin. We divided the muscle MNC into CD45⁺ cells and CD45⁻ cells by FACS, and subjected them to analysis of STR polymorphism. If CD45⁻ recipient muscle cells could differentiate into hematopoietic cells, some of the CD45⁺ cells in the muscles should show the recipient genotype. However, all CD45⁺ cells in the muscle showed the donor genotype, while all CD45⁻ cells showed the recipient genotype (Fig. 3b). These results indicated that the hematopoietic cells in the muscles were derived from BM, and that CD45⁻ cells did not differentiate into hematopoietic cells *in vivo*.

3.3. Engrafting capabilities of MNC isolated from human muscle

We tested the hematopoietic engrafting capabilities of MNC derived from human muscle by transplantation into immunodeficient mice. In previous studies, all (5/5) congenic recipient mice transplanted with 10⁵ MNC derived from murine muscles showed hematopoietic engraftment, while only one-fourth of the mice transplanted with 10⁴ of the same cells showed hematopoietic engraftment [8]. Therefore, for this study, we decided to use more than 10⁵ human MNC for trans-

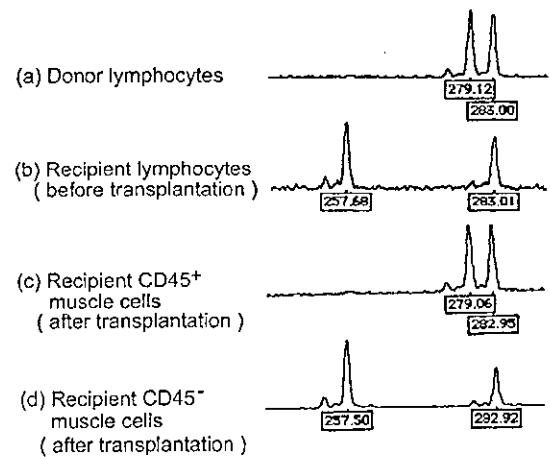


Fig. 3. Origin of the hematopoietic population in muscles from a patient treated with bone marrow (BM) transplantation. DNA collected from donor and recipient cells was amplified using the ACTBP2 locus-specific primer, and automatically analyzed by an ABI 373A sequencer using Genescan software 672, following electrophoresis on a 6% denaturing polyacrylamide gel. Numbers indicate base pairs (bp). All CD45⁺ cells from recipient muscle showed the donor genotype, indicating BM origin.

plantation experiments. Furthermore, we chose NOG mice as the recipients and used IBM injection for transplantation, because these mice were previously demonstrated to be 10 times more sensitive for SCID-repopulating cell assay than NOD/SCID mice, while IBM injection also was considered to be a strategy 15 times more sensitive for the SRC assay than intravenous injection [12,14].

MNC (5 × 10⁵ cells) derived from muscle, BM, or PB were transplanted separately into irradiated NOG mice by IBM injection. Additionally, we separated 5 × 10⁵ muscle-derived MNC into CD45⁺ cells and CD45⁻ cells by FACS; and these cells also were transplanted into recipient mice. Eight weeks later, BM cells were collected from the recipient mice, and levels of engraftment were analyzed by flow cytometry. As expected, human CD45⁺ cells were detected in mice transplanted with BM-derived MNC. However, we did not detect human CD45⁺ cells in mice transplanted with muscle-derived MNC, CD45⁺ cells, or CD45⁻ cells, or PB-derived MNC (data not shown).

We also examined BM samples collected from recipient mice by PCR. Here again, engraftment of human cells was recognized in mice transplanted with PB-derived MNC (Fig. 4), but not in mice transplanted with muscle-derived MNC or CD45⁺ cells. Interestingly, engraftment was detected at a very low percentage in one mouse transplanted with muscle-derived CD45⁻ cells. Since no human CD45⁺ cells were detected in the BM of recipient mice by flow cytometry (data not shown), human CD45⁻ cells were considered to have engrafted in this mouse as a non-hematopoietic population, although we could not determine the phenotype of the engrafted cells.

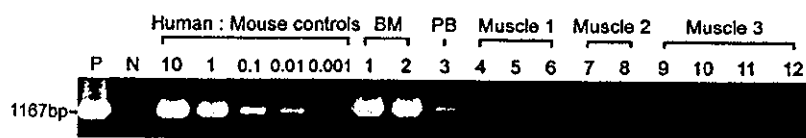


Fig. 4. Polymerase chain reaction (PCR) analysis of bone marrow (BM) samples from individual NOD/SCID/ $\gamma c^{-/-}$ (NOG) mice transplanted with human muscle-, peripheral blood (PB)-, or BM-derived mononuclear cells (MNC). Five hundred thousand MNC derived from human muscle, PB, or BM were transplanted separately into irradiated NOG mice by intra-BM injection. PB, BM, and Muscle 1-derived cells were isolated from patient 4 in Table 1. Muscle 2- and Muscle 3-derived cells were from patients 6 and no. 11, respectively. Lanes 1–12 represent results obtained from individual recipient mice. Muscle 3-derived cells (5×10^5 MNC) were separated further into CD45⁺ (lanes 9 and 10) and CD45⁻ cells (lanes 11 and 12), and these cell fractions then were transplanted separately into mice. Eight weeks after transplantation, DNA was extracted from recipients BM cells, and engraftment was assessed by PCR. Human: mouse DNA controls are designated by the percentage of human DNA. P, positive control; N, negative control.

4. Discussion

In this study, we sought to characterize the hematopoietic population in muscle of young and adult human subjects. We first demonstrated that the frequency of hematopoietic progenitors in muscle tissue differed from that in PB. We clarified that these hematopoietic cells were derived from CD45⁺ cells. The presence of enriched numbers of hematopoietic progenitors in the muscle cannot be explained as contamination by blood cells. In fact, most hematopoietic cells had an extravascular location in the muscle. However, they appeared to have originally arisen from BM. These results are essentially compatible with those of previous studies in mice [7,8,15,16], but HSC activity was not detected in adult human muscle by IBM injection of 5×10^5 MNC into NOG mice. Taking the present results together with those of clonal cell culture, we considered most of the hematopoietic population in the muscle to be at the progenitor level and that HSC, if present at all, were much rarer in human than mouse muscle [8]. Dose escalation studies or further enrichment of HSC might provide further information regarding HSC activity in human muscle, although obtaining sufficiently large samples would be difficult.

Although little is known about differentiation potential in human stem cells, several reports demonstrated that plasticity phenomena also could be recognized in human adults [17,18]. Clinical applications of stem cell plasticity therefore have been expected in the field of regenerative medicine. However, in this study, we demonstrated that muscle-derived CD45⁻ cells from young or adult human subjects were not capable of differentiating into hematopoietic cells *in vitro* and *in vivo*, in contrast to the reported result that CD45⁻ cells from fetal human muscle could differentiate into hematopoietic cells [10]. These findings were compatible with the results of Mahmud et al. [9], who showed that CD45⁻ cells isolated from the muscles of adult baboons did not show hematopoietic activity; the same result also was obtained in mice [8]. The hematopoietic potential of muscle-derived CD45⁻ cells might be limited to the fetal and neonatal periods [9,10,19]. However, Howell et al. [19] reported that hematopoietic colony-forming activity and engraftment potential of muscle-derived CD45⁻ cells from neonatal mice were induced or increased by cell culture. Furthermore, Dell'Agnola et al. [20] recently reported that cultured human stem cells residing in

adult muscle showed hematopoietic engraftment in immunodeficient mice, while noncultured human stem cells did not. Since *ex vivo* expansion of human BM-derived HSC has been difficult to attain so far, *in vitro* culture might recruit extrafunctions of cells residing in adult muscular tissues.

In conclusion, we demonstrated a unique hematopoietic population in human skeletal muscle. Our results indicated that while human muscle is not expected to be an HSC source in the clinical settings, skeletal muscles collectively constitute the largest organ in the human body and contain hematopoietic progenitors at higher frequency than peripheral blood. Therefore, human skeletal muscle, an organ specializing in movement, also could function as a reservoir of hematopoietic cells in addition to BM and spleen.

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転移性乳癌に合併した骨髓異形成症候群症例に対し 施行した体外増幅を併用した臍帯血移植

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臍帯血移植の成人例への適用は移入細胞数不足により限定されている。われわれはマウス骨髓ストローマ細胞株 HESS-5 細胞を利用して臍帯血 CD34 陽性細胞を体外増幅するシステムを開発し、その安全性と有効性を確認するための臨床 I/II 相試験を開始した。転移性乳癌に骨髓異形成症候群を合併した 52 歳女性に対し、骨髓非破壊的前処置後に体外増幅細胞を併用した臍帯血移植を施行した。臍帯血細胞の 75% は未処置のまま第 0 日に輸注した。移植片の 25% は体外で CD34 陽性細胞を純化し、TPO, SCF, Flk2Flt3 ligand (FL) を含む無血清培地を用いて HESS-5 細胞上で 5 日間培養した後、第 5 日に輸注した。患者は移植後 37 日目に肺炎、移植後 48 日目に脳出血を合併し、移植後 50 日目に死亡した。体外増幅細胞輸注に伴う急性の有害事象は認められなかった。染色体検査でドナー造血が認められたが、血液学的生着は確認されなかった。

(臨床血液 45 (9): 1048 ~ 1052, 2004)

Key words : *ex vivo* expansion, Cord blood transplantation, Nonmyeloablative transplantation

緒 言

臍帯血は近年造血幹細胞移植のソースとして利用され、小児例のみならず成人例へも適応されるようになってきた¹⁻⁷。骨髓、末梢血幹細胞移植と比較して移植片対宿主病の頻度が低く HLA2 抗原不一致まで利用可能であり、凍結保存されているので迅速に移植できる利点がある。一方、生着の遅延と成人例には移植細胞数が不足することが欠点である^{1,5,7}。また移植後の免疫能低下、移植片対白血病効果減弱が懸念されている⁸。前者の問題を解決するため、臍帯血前駆細胞・幹細胞を体外で培養し増幅する試みが行われている⁹。われわれはマウス骨髓ストローマ細胞株である HESS-5 細胞を利用して臍帯血 CD34 陽性 (CD34⁺) 細胞を体外増幅するシステムを開発した (Fig. 1)^{9,10}。この系では Thrombopoietin (TPO), Stem cell factor (SCF), Flk2Flt3 ligand (FL) 存在下に無血清培地で培養すると 5 日間に CD34⁺ 細胞で 15 倍, CFC (colony-forming cell) で 30 倍, 定量的 SCID mouse-Repopulating Cells アッセイで 10 倍に増幅された。HESS-5 細胞は CD34⁺ 細胞と膜で隔離され互いに混入すること

はないが、0.45 μ m の小孔を介して接触することは可能になっている。既に海外では培養皮膚細胞移植、遺伝子治療などでマウス細胞と接触したヒト細胞が利用されており、HESS-5 細胞の安全性もこれらと同様に米国 FDA 基準に準拠して確認された。そこで本システムによる培養臍帯血細胞を用いた「体外増幅幹細胞の安全性と有効性に関する臨床試験」を計画し、プロトコールが 2000 年 2 月学内倫理委員会の承認を得た。体外増幅臍帯血移植の概略は Fig. 2 に示す。今回われわれは転移性乳癌を合併した骨髓異形成症候群 (MDS) 症例に対し本プロトコールを施行したので臨床経過を報告する。

症 例

患者: 52 歳, 女性。

主訴: 血小板減少。

現病歴: 1986 年右乳癌発症し右乳房切除術および計 44 Gy の放射線療法を右腋窩リンパ節に対し施行され、以後無治療で経過していた。2001 年 8 月血小板減少を認め当院に紹介された。CTにて脾臓、腸骨への転移再発が疑われた。骨髓検査にて乳癌細胞の浸潤と血液細胞の 3 系統の形態異常、骨髓芽球の増加 (7.3%) を認め、乳癌の骨髓転移および MDS と診断された。骨髓細胞の染色体検査で +8 を認めた。2001 年 10 月 31 日当科に入院した。

既往歴: 37 歳時乳癌。44 歳時糖尿病、48 歳時よりインスリン導入。

受付: 2004 年 2 月 18 日

受理: 2004 年 7 月 15 日

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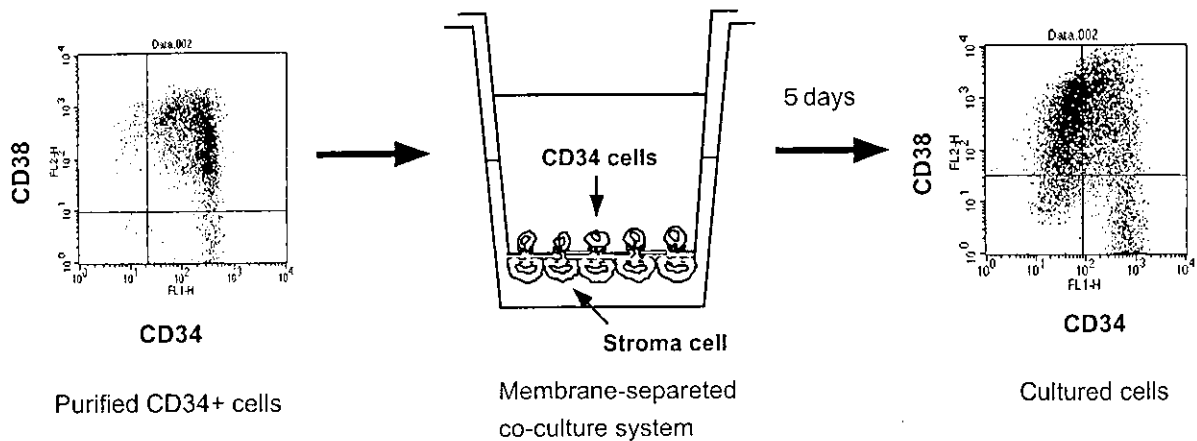


Fig. 1 System for ex-vivo expansion of cord blood CD34+ cells

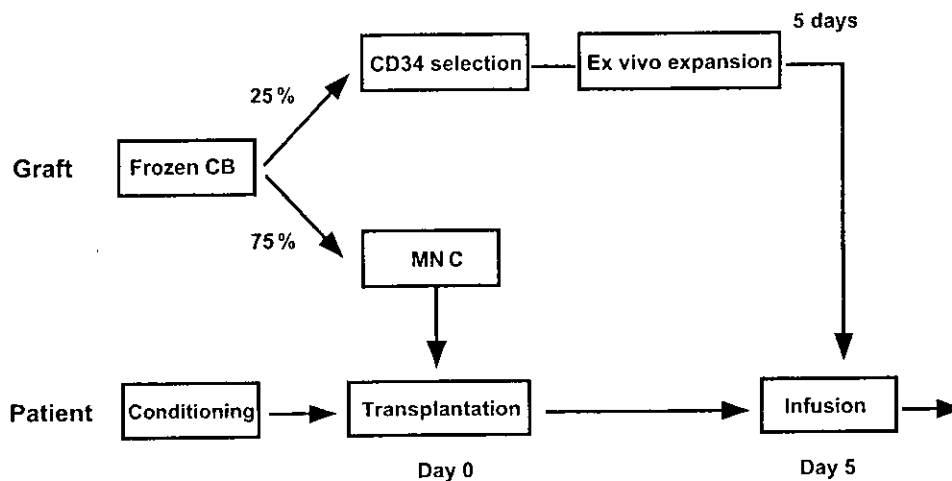


Fig. 2 Protocol for ex vivo expanded CBT

家族歴：特記事項なし。

入院時現症：身長 155cm, 体重 39kg, 意識清明, 血圧 94/58 mmHg, 脈拍 114 /分整, 体温 36.2℃, 貧血黄疸なし, 心肺雑音なし, 肝脾腫なし, 下腿浮腫なし, 神経学的異常所見なし。

入院時検査所見 (Table 1)：末梢血で軽度の白血球減少, 貧血, および著明な血小板減少を認め, 凝固時間の延長は認めなかった。生化学検査では高血糖, 胆道系酵素の上昇, LDH, CA15-3, CEA 高値を認めた。末梢血スミアでは Howell-Jolly 小体, Pappenheimer 小体, 巨大血小板を認め, 骨髓検査は低形成で外来で認められた芽球の増加はなかったが, 多核赤芽球, 巨赤芽球様変化を認め, MDS (不応性貧血) と診断された。VitB12, 葉酸は正常値で, 鉄染色では環状鉄芽球は認めなかった。

入院後経過：ホルモン受容体陽性の転移性乳癌に対しアロマターゼ阻害薬を開始した。MDS と転移性乳癌をと

もに加療し得る造血幹細胞移植を検討した。血縁およびバンクにドナー候補がいなかったため臍帯血移植を選択した。HLA 抗原が 4/6 以上一致し, 細胞数 8.44×10^8 ($2.16 \times 10^7/\text{kg}$) を含む臍帯血が利用可能であった。本例は「体外増幅幹細胞の安全性と有効性に関する臨床試験」の対象として適格基準を満たすため試験について説明し, 患者および家族の同意を得た。

移植細胞プロセッシングと培養：有核細胞数 8.44×10^8 のうち, 25% に相当する 2.11×10^8 より CD34+ 細胞を純化し体外増幅に用いた。すなわち, 抗 CD34 抗体と反応後, 免疫磁気ビーズカラム処理 (MACS system, Miltenyi Biotec) を行い 9.00×10^5 の細胞を得た。CD34+ 細胞の純度は 29.75% であり, 回収率は解凍後細胞の 43.9% であった。この細胞をカルチャーインサートを利用して HESS-5 細胞と接触を保ちつつ膜隔離培養した。無血清培地中で TPO50ng/ml, SCF50ng/ml, FL50ng/ml 存在

Table 1 Laboratory findings on admission

Complete blood count		Blood Chemistry		Coagulation	
WBC	2700/ μ l	TP	6.9 g/dl	PT	11.7 sec
Seg	54.0 %	Alb	3.9 g/dl	APTT	34.9 sec
Stab	3.0	AST	36 IU/l	Fib	417 mg/dl
Ly	18.0	ALT	37 IU/l	FDP	6.84 μ g/ml
Mon	20.0	LDH	628 IU/l	Tumor marker	
Eos	1.0	Alp	866 IU/l	CA15-3	255.0 μ g/ml
Bas	1.0	GGT	279 IU/l	CEA	26.5 μ g/ml
Blasts	1.0	T. Bil	0.7 mg/dl	HbA1c	7.0 %
RBC	2.62×10^6 / μ l	Glu	267 mg/dl		
Hb	8.7 g/dl	BUN	17 mg/dl		
Ht	26.6 %	Cr	0.7 mg/dl		
Plt	0.4×10^4 / μ l	Na	135 mEq/l		
MCV	101.5	K	4.6 mEq/l		
MCH	33.2	Cl	97 mEq/l		
MCHC	32.7	Ca	9.1 mg/dl		
		CRP	1.58 mg/dl		
Howell-Jolly bodies (+), Pappenheimer bodies (+)					
Giant platelets (+)					
Bone marrow					
: hypocellular marrow, polyerythroblasts (+), megaloblastic change(+), M/E=0.15, malignant cells (+), myeloblast 0.8%					
Karyotype					
: 47, XX, +8 (20/20)					

下に 5 日間培養し CD34⁺細胞で 4.6 倍, CFC で 53.4 倍の増幅を得た。(Table 2)

移植後経過 (Fig. 3) : Busulfan を 4mg/kg/day で 2 日間, Fludarabine を 30mg/m²/day で 5 日間, TBI 2Gy を 1 日による骨髄非破壊の前処置後 2002 年 1 月 29 日臍帯血有核細胞数の 75% に相当する 6.33×10^8 (1.62×10^7 /kg, CD34⁺細胞として 4.60×10^4 /kg, CFC として 1.25×10^4 /kg) を移植し, 培養細胞 1.23×10^7 (CD34⁺細胞として 3.09×10^4 /kg, CFC として 1.17×10^5 /kg) を 2 月 4 日移植した。末梢血による生着は確認されなかったが, 骨髄細胞の染色体検査では移植後 27 日, 37 日でそれぞれ 20/20, 1/1 で XY のドナー由来細胞を認めた。初診時に認められた 47,XX,+8 の核型異常は認めなかった。骨髄所見はいずれも線維化と乳癌細胞の存在を認め, 造血細胞は低形成であった。移植後 37 日目に右肺炎, 胸水を合併し, 移植後 48 日目に意識障害, 右下肢麻痺, 左共同偏視が出現し, 移植後 50 日に永眠された。剖検所見にて肺アスペルギルス症が確認され, 乳癌細胞の浸潤は骨髄, 骨, 肝臓, 胃, 脾臓, 胆嚢, 心筋, 肺, 腎臓, 膀胱, 副腎, 甲状腺, 子宮頸部, 卵巣に及んだ。骨髄は線維化を認め低形成であった。

考 察

本症例は 2 つの悪性疾患を合併し他に有効な治療法がない状況の中で, 「体外増幅幹細胞の安全性と有効性に関

する臨床試験」を施行した一例である。臍帯血移植では移入細胞数, 特に CD34⁺細胞が $1.2 \sim 1.7 \times 10^5$ /kg^{2,7)} あるいは CFC が 5.0×10^4 /kg⁶⁾ 以上の場合良好な生着が期待されるが, 成人の場合この条件を満たすことは困難であり造血前駆・幹細胞の体外増幅が試みられる⁸⁾。われわれのマウス骨髄ストローマ細胞を利用する方法は, 既存の方法と比較して効率の良い CD34⁺細胞の増幅が得られる^{9,10)}。一方, 安全性に関しては FDA 基準に準拠してマウス由来病原微生物の検査済みではあるが, 未知の微生物感染の可能性を除くことは不可能である。従来異種移植の定義は「ヒト以外の動物に由来する生きた細胞・組織・臓器を, ヒトに移植, 移入または体外灌流に用いること」(第 26 回厚生科学審議会先端医療技術評価部会議事録, 2000 年 9 月 22 日)とされていたが, 本例の移植施行後 2002 年 7 月 9 日に上記に加えて「体外において, ヒト以外の動物に由来する生きた細胞, 組織または臓器に接触したヒトの体液, 細胞, 組織または臓器をヒトに移植, 埋め込みまたは注入すること」(異種移植の実施に伴う公衆衛生上の感染症問題に関する指針)と変更された。この指針により本臨床試験は中止した。最近ストローマ細胞の重要性を裏付けるような遺伝子 mKirre が同定されており¹¹⁾, 今後幹細胞増幅環境の分子基盤が明らかにされ無細胞系の造血幹細胞増幅システムが構築されることが期待される。

本例では CD34⁺細胞の回収率が 43.9% で増幅率が 4.6