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ORIGINAL ARTICLE

Hypercalcemia in childhood acute lymphoblastic leukemia: frequent implication of parathyroid hormone-related peptide and *E2A-HLF* from translocation 17;19

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Hypercalcemia is relatively rare but clinically important complication in childhood leukemic patients. To clarify the clinical characteristics, mechanisms of hypercalcemia, response to management for hypercalcemia, incidence of t(17;19) and final outcome of childhood acute lymphoblastic leukemia (ALL) accompanied by hypercalcemia, clinical data of 22 cases of childhood ALL accompanied by hypercalcemia (>12 mg/dl) reported in Japan from 1990 to 2005 were retrospectively analyzed. Eleven patients were 10 years and older. Twenty patients had low white blood cell count ($<20 \times 10^9$/l), 15 showed hemoglobin ≥8 g/dl and 14 showed platelet count $\geqslant 100 \times 10^9$/l. Parathyroid hormone-related peptide (PTHrP)-mediated hypercalcemia was confirmed in 11 of the 16 patients in whom elevated-serum level or positive immunohistochemistry of PTHrP was observed. Hypercalcemia and accompanying renal insufficiency resolved quickly, particularly in patients treated with bisphosphonate. t(17;19) or add(19)(p13) was detected in five patients among 17 patients in whom karyotypic data were available, and the presence of *E2A-HLF* was confirmed in these five patients. All five patients with t(17;19)-ALL relapsed very early. Excluding the t(17;19)-ALL patients, the final outcome of ALL accompanied by hypercalcemia was similar to that of all childhood ALL patients, indicating that the development of hypercalcemia itself is not a poor prognostic factor.
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Introduction

Hypercalcemia is a frequent complication in adults with malignancy, and its incidence has been estimated as 5–20%.[1] In contrast, in the report from St Jude Children's Research Hospital, the incidence of hypercalcemia among children with malignancy who were treated from 1962 to 1991 was only 0.4%.[2] Among the 25 affected children reported from St Jude Children's Research Hospital, 10 had ALL including six cases of B-precursor ALL and three cases of mature B-ALL, and 14 had solid tumors including four cases of rhabdomyosarcoma. Therefore, hypercalcemia develops most commonly in ALL among childhood malignancies. Despite the importance in clinical management, owing to its relative rarity, the clinical characteristics of childhood ALL accompanied by hypercalcemia, mechanisms of hypercalcemia, response to current management for hypercalcemia and final outcome of ALL accompanied by hypercalcemia remain totally unclarified. There are two main mechanisms of hypercalcemia in malignancy: localized bone destruction by invasive cancer cells with the participation of various cytokines, and osteoclastic bone resorption mediated by humoral tumor-derived factors.[1,3,4] Hypercalcemia in malignancy is frequently mediated with parathyroid hormone-related peptide (PTHrP) by increasing osteoclastic bone resorption, renal resorption of calcium and renal phosphate loss.[5,6] Although several case reports showed the involvement of PTHrP in childhood ALL complicated with hypercalcemia,[7–9] its significance remains to be confirmed in a larger study.

t(17;19)(q21-q22;p13), which generates E2A-HLF fusion transcription factor,[10,11] is a rare translocation present in less than 1% of childhood ALL cases,[12] and its association with hypercalcemia, acquired coagulation abnormalities and extremely poor therapeutic outcome has been noticed.[13–16] The *E2A-HLF* fusion gene encodes a chimeric protein in which the transactivation domain of E2A links to the basic leucine zipper dimerization and DNA-binding domain of HLF.[10,11] E2A-HLF promotes anchorage-independent growth of murine fibroblasts[17,18] and protects cells from apoptosis owing to growth factor deprivation,[19–21] and *E2A-HLF* transgenic mice develop T-lineage lymphoid malignancies.[22,23] Although their significance in the clinical features has been controversial, there

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are two types of fusion: type 1 is generated by the fusion between exon 13 of *E2A* and exon 4 of *HLF* with an insertion of cryptic exon (joining region) maintaining an open-reading frame, whereas type 2 is generated by the fusion between exon 12 of *E2A* and exon 4 of *HLF* in the same reading frame.[24] Of note, despite its rarity, two of the six B-precursor ALL patients with hypercalcemia reported from St Jude Children's Research Hospital showed t(17;19) on cytogenetic analysis,[2] and the two patients were confirmed to have *E2A-HLF* fusion.[10] Although these observations suggest the frequent association of t(17;19) with the development of hypercalcemia in childhood ALL, more detailed analyses of additional cases are needed to adequately address this point.

In this study, we undertook a retrospective review of 22 patients with childhood ALL other than mature B-ALL, who developed hypercalcemia at onset or relapse of ALL. We found that childhood ALL accompanied by hypercalcemia was frequently associated with t(17;19) and *E2A-HLF* expression, and that PTHrP-mediated hypercalcemia was revealed in the half of the 22 patients and over two-thirds of the patients in whom conclusive data were available.

Materials and methods

Patients and diagnosis criteria for PTHrP-mediated hypercalcemia

Hypercalcemia was defined as total serum calcium concentration of greater than 12.0 mg/dl. From 1990 to 2005, 25 patients with childhood ALL with L1 and L2 subtypes in French–American–British (FAB) classification developed hypercalcemia during their clinical course and were reported at medical meetings (Japan Pediatric Society, Japanese Society of Pediatric Hematology, The Japanese Society of Hematology, and Japanese Society of Clinical Hematology) or in medical journals in Japan, where approximately 500 children are estimated to develop ALL per year. Three patients were excluded from the analysis owing to either loss of medical record or lack of response from the responsible physician, and as a result, 22 patients were enrolled in this study. The diagnosis of PTHrP-mediated hypercalcemia was made by the following criteria: (1) positive immunohistochemistry of PTHrP in leukemia cells, (2) elevated serum C-terminal PTHrP (C-PTHrP) level accompanying a low serum level of intact PTH (iPTH) (<10 pg/ml) owing to a negative feedback loop, (3) elevated serum C-PTHrP level accompanied by normal serum creatinine level. As the serum level of C-PTHrP, but not intact PTHrP (iPTHrP), was reported to be nonspecifically high in renal insufficiency,[5] the diagnosis of PTHrP-mediated hypercalcemia was not concluded in the cases with elevated serum C-PTHrP level, in whom the serum creatinine level was elevated or not monitored and the iPTH level was not downregulated or not monitored. The upper limit of normal range for serum C-terminal PTHrP level varied from 40 to 61 pmol/l in each institute, and elevation of serum C-terminal PTHrP level was determined depending on each institutional normal range.

Reverse transcription (RT)-polymerase chain reaction (PCR) for E2A-HLF

RT-PCR analysis for *E2A-HLF* using patients' samples was approved by the institutional review board of the University of Yamanashi. Informed consent was obtained from the patients or the parents. Total RNA was isolated from bone marrow cells with Trizol (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. RT was performed with 2 μg of total RNA, random hexamers and Superscript reverse transcriptase (Life Technologies) under conditions recommended by the manufacturer. PCR was performed using the following primers that were homologous to sequences in *E2A* exon 12 and exon 13, and *HLF* exon 4 (Figure 1a): *E2A* exon 12 (e12), 5′-gacatgcacacgctgctgcc-3′; E2A exon 13 (e13), 5′-gcctcatgcacaaccacgcg-3′; *HLF* exon 4 (e4), 5′-cccggatggcgatc tggttc-3′. Amplification was performed for 35 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. As a control, PCR for c-abl was performed under the same conditions using the following primers: *c-abl* sense, 5′-gtatcatctgactttgagcc-3′; *c-abl* antisense, 5′-gtaccaggagtgtttctcca-3′. The cell lines UOC-B1 and HAL-O1, which have type 1 fusion consisting of *E2A* exon 13 and *HLF* exon 4,[24] and Endo-kun, which has type 2 fusion consisting of *E2A* exon 12 and *HLF* exon 4[24] and was established from case 1, were used as positive controls.

Statistical analysis

All statistical analyses were performed with StatView (version 5.0.1) software. Event-free survival (EFS) was estimated according to Kaplan–Meier analysis. The starting point was the date of diagnosis of ALL, and the end point was relapse. Time was censored at last follow-up, and follow-up was updated in February 2006. Univariate comparison of EFS in different groups of patients was performed using the log-rank test, and χ^2-test and Student's *t*-test were used to assess the association between different characteristics.

Results

Characteristics of leukemia

The main clinical features of the 22 patients with ALL who developed hypercalcemia are summarized in Table 1. Although observation period was not completely identical, their clinical features were compared with those of the childhood ALL patients in the Tokyo Children's Cancer Study Group (TCCSG) treated with L89-12 (1989–1992, 418 patients) and L92-13 (1992–1995, 347 patients) protocols (Table 2),[25] in which approximately 20% of the patients in Japan were registered and Cases 3, 5 and 9 were enrolled. The incidence of age greater than 10 years at diagnosis of ALL in the present study (50%) was significantly higher ($P=0.005$) than that in the childhood ALL cases reported from TCCSG (23.8%). The male–female ratio was 54.5% and was identical to that in the childhood ALL cases reported from TCCSG (54.6%). Initial white blood cell count (WBC) of the cases with hypercalcemia ranged from 2 to 90×10^9/l (median, 6.2×10^9/l) and leukemic blasts were undetectable in the peripheral blood in eight patients. The rate of WBC $< 20 \times 10^9$/l (90.9%) was significantly higher ($P=0.010$) than the respective rate in childhood ALL reported from TCCSG (64.6%). The rates of severe anemia (hemoglobin < 8 g/dl) (27.3%) and thrombocytopenia (platelet count $< 100 \times 10^9$/l) (36.4%) were significantly lower ($P=0.025$ and 0.004, respectively) than those in childhood ALL in the TCCSG (51.1 and 66.1%, respectively). Intravenous coagulopathy was noticed in five of 20 patients. All of the patients except for Case 20 with dry tap marrow showed B-precursor immunophenotype. No patient showed T-cell immunophenotype. The incidence of T-cell immunophenotype (0%) tended to be low ($P=0.098$) compared with that in childhood ALL reported from the TCCSG (11.6%). Negative to low expression of CD19 (< 30%) was noted in two patients (Cases 19 and 21), and expression of CD13 and CD33

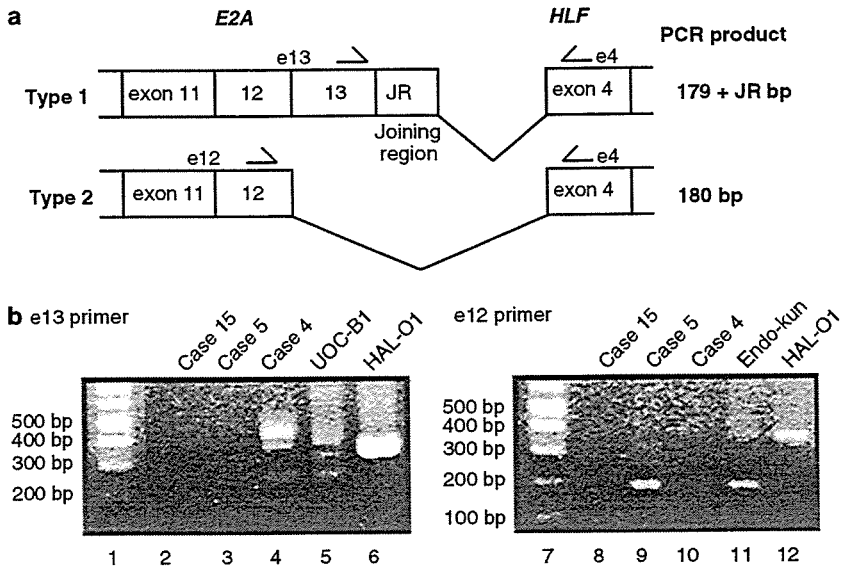

Figure 1 Representative analysis of RT-PCR amplification of *E2A-HLF*. (a) Schematic representation of two types of fusion. Type 1 is generated by fusion between exon 13 of *E2A* and exon 4 of *HLF* with an insertion of cryptic exon (joining region; JR) maintaining an open reading frame, whereas type 2 is generated by fusion between exon 12 of *E2A* and exon 4 of *HLF* in the same reading frame. The relative positions of the primers used to amplify the two types of *E2A-HLF* fusion complementary DNAs are shown. PCR using e13 and e4 primers is expected to generate a 179-bp + JR product from type 1 fusion but no product from type 2 fusion, whereas PCR using e12 and e4 primers generates a 180-bp product from type 2 fusion and 303-bp + JR product from type 1 fusion. (b) RT-PCR of *E2A-HLF* fusion. The left panel indicates the PCR products using e13 and e4 primers, and the right panel indicates the PCR products using e12 and e4 primers. Type 1 *E2A-HLF* was confirmed in Case 4 (lanes 4 and 10) as UOC-B1 (lane 5) and HAL-01 (lane 6), and type 2 *E2A-HLF* was confirmed in Case 5 (lanes 3 and 9) as Endo-kun (lane 11). None of the *E2A-HLF* transcripts was detectable in Case 15 (lanes 2 and 8). Lanes 1 and 7 demonstrate the molecular size marker. The RT-PCR product of *c-abl* was confirmed in each of the samples (data not shown).

($\geqslant$10%) was confirmed in 6 (Cases 1, 2, 5, 6, 10 and 22) and eight patients (Cases 1, 2, 3, 4, 5, 9, 18 and 22), respectively.

Incidence of t(17;19)

On karyotypic analysis (Table 1), metaphase was not obtained in five patients, and normal karyotype was revealed in nine patients. Cases 1 and 4 harbored t(17;19)(q21;p13) and add(19)(p13) at disease onset, respectively. Cases 2, 3 and 5 who showed normal karyotype at onset, harbored t(17;19)(q21;p13) at relapse. RT-PCR analysis of *E2A-HLF* was performed in nine patients in whom frozen marrow samples were available (Table 1 and Figure 1). Type 1 *E2A-HLF* was detected in Cases 2 and 4, whereas type 2 *E2A-HLF* was detected in Cases 1, 3 and 5. In contrast, *E2A-HLF* transcripts were not detectable in Cases 7, 8, 15 and 18, who showed neither add(19)(p13) nor t(17;19)(q21;p13) in the cytogenetic study. Of note, all five patients with t(17;19)-ALL expressed CD33 (Table 1) and the incidence of CD33 expression among patients with t(17;19)-ALL was significantly higher than that among the other 16 patients ($P=0.002$ by χ^2-test). Three patients with t(17;19)-ALL had L2 phenotype (Table 1) and the incidence of L2 phenotype among patients with t(17;19)-ALL was significantly higher than that among the other patients ($P=0.008$ by χ^2-test). Four patients with t(17;19)-ALL developed the disease at age greater than 10 years, and two patients had coagulopathy.

Clinical characteristics of hypercalcemia

Hypercalcemia was present at the time of original diagnosis in 18 patients and at disease recurrence in four patients (Cases 1, 2, 5 and 7), and a diagnosis of hypercalcemia was made before treatment. Table 3 summarizes the clinical symptoms and laboratory data at diagnosis except for serum levels of blood–urea–nitrogen and creatinine, which were shown as the highest values before resolution of hypercalcemia. The total serum calcium concentration was 15 mg/dl or greater in 16 patients (72.7%). At least one clinical symptom associated with hypercalcemia was observed in all patients and the incidence of each symptom was as follows: emesis in 11 patients (50%), bone pain in 13 patients (59.1%), osteolytic lesion in 14 patients (63.6%), fracture of the vertebral bone in four patients (18.2%), and renal insufficiency with elevated serum creatinine level to 1.0 mg/dl or higher in 14 (66.7%) of 21 patients. Elevated serum creatinine was associated with older children ($\geqslant$10 years old) ($P=0.0002$ by χ^2-test) and infrequently associated with hypophosphatemia ($\leqslant$4 mg/dl) ($P=0.02$ by χ^2-test).

Mechanisms of the development of hypercalcemia

The serum C-terminal PTHrP (C-PTHrP) level was elevated in 15 of the 16 patients assayed (Table 3). The diagnosis of PTHrP-mediated hypercalcemia was made by positive immunohistochemistry of PTHrP in leukemia cells in the two patients assayed (Cases 6 and 7). The seven patients with an elevated serum C-PTHrP level (Cases 1, 2, 3, 8, 9, 10 and 11) accompanying a low serum level of iPTH owing to a negative feedback loop were diagnosed to have typical PTHrP-mediated hypercalcemia. Cases 12 and 13 with elevated serum C-PTHrP level accompanied by normal serum creatinine level were concluded to have PTHrP-mediated hypercalcemia. In contrast, Cases 4, 14, 15, 16 and 17 with elevated serum C-PTHrP level, in whom the serum creatinine level was elevated or missing and the iPTH

Table 1 Clinical and laboratory characteristics of the patients with ALL with hypercalcemia

	Age/sex (year)	Initial WBC ($\times 10^9$/l)	Blast (%)	Hb (g/dl)	Plt ($\times 10^9$/l)	FAB	Coagulopathy	Cell surface antigen CD (%)				Cytogenetics	E2A-HLF mRNA	EFS(m)	Relapse	Ref.
								10	19	13	33					
t(17;19) positive																
1	14/F	24.1	63	8.4	51	L2	–	85	90	22	32	46,XX,t(17;19)(q21;p13)	Type 2	9	+	16
2	10/M	3.2	4	8.1	127	L2	+	92	92	79	60	46,XY,t(17;19)(q21;p13)[a]	Type 1	14	+	
3	4/M	7.3	4	7.3	144	L1	–	81	87	2	10	46,XY,t(17;19)(q21;p13)[a]	Type 2	5	+	15
4	14/F	4.2	0	8.7	128	L1	–	97	98	8	86	46,XX,13,del(13)(q14q22),add(19)(p13)	Type 1	3	+	9
5	12/F	90.0	88	8.4	32	L2	+	63	72	13	62	51,XX, +8,t(17;19),(q21;p13), +18,+21,+21,+22[a]	Type 2	2	+	16
t(17;19) negative or not confirmed																
6	14/M	4.9	12	12.0	164	L1	–	93	92	10	1	No metaphase	NA	+49	–	
7	12/M	13.1	52	11.8	23	L2	–	29	63	NT	4	46,XY, −21,+13,-9, t(8;9)(q13;p22)	Neg.	8	+	7
8	8/F	19.8	7	11.1	142	L1	–	60	92	0	1	46,XX,t(15;17)(q13;q21)	Neg.	+154	–	
9	10/F	5.1	0	10.7	138	NA	–	8	96	2	6	46,XY	NA	+51	–	
10	10/M	5.7	0	12.2	176	L1	+	91	100	60	18	No metaphase	NA	+66	–	
11	10/M	4.7	2	8.0	92	L1	+	58	69	4	2	46,XY	NA	32	+	
12	2/F	10.0	12	6.9	206	L1	–	55	NT	1	NT	46,XX	NA	+236	–	8
13	8/M	5.2	0	9.1	324	L1	NA	60	54	2	4	46,XY	NA	58	+	8
14	11/M	6.6	19	14.2	193	L1	–	37	95	5	3	46,XY	NA	19	+[b]	
15	5/M	8.9	26	7.3	76	L1	+	50	100	1	1	No metaphase	Neg.	+35	–	
16	7/F	6.2	40	9.4	30	L1	–	55	99	1	0	46,XX	NA	+147	–	8
17	3/F	12.1	0	9.1	399	L1	NA	35	68	8	5	46,XX	NA	+49	–	8
18	10/M	12.7	28	11.2	248	L1	–	35	37	5	14	44,add(X)(q22),del(Y)(q12),−4,−8,−9,+mar	Neg.	11	+	
19	2/M	5.9	0	7.1	184	L1	–	98	3	5	3	46,XY	NA	35	+	30
20	6/F	3.3	0	5.9	55	L1	–		NT(dry tap)			NT(dry tap)	NA	+127	–	
21	2/F	14.8	0	11.0	200	L1	–	89	25	4	4	46,XX	NA	+19	–	30
22	7/F	2.0	3	5.9	54	L1	–	97	96	74	33	No metaphase	NA	+83	–	

Abbreviations: ALL, acute lymphoblastic leukemia; EFS, event-free survival; F, female; FAB, French–American–British; Hb, hemoglobin; M, male; m, month; Ref, reference; NA, data or samples not available; NT, not tested; Neg, negative; Plt, platelet count.
[a]Karyotype at relapse is indicated, but normal karyotype was obtained at diagnosis.
[b]Relapsed as AML.

level was not downregulated or missing, were not concluded to have PTHrP-mediated hypercalcemia. iPTHrP was undetectable in serum in all four patients assayed (Cases 18, 19, 20 and 21), indicating that the involvement of PTHrP in their hypercalcemia is excluded. Among these four patients, the serum phosphorus level was low and serum 1,25-$(OH)_2$ vitamin D (calcitriol) level was normal in the three patients assayed, indicating that involvement of calcitriol-mediated hypercalcemia is unlikely because it is characterized by elevated calcitriol level accompanied by normal serum phosphorus level.[4] In Case 22, iPTH was markedly elevated but C-PTHrP was normal in the serum, suggesting ectopic production of PTH by leukemia cells. Collectively, among the 21 patients whose PTHrP data were available, involvement of PTHrP-mediated hypercalcemia was confirmed in 11 patients (Cases 1, 2, 3, 6, 7, 8, 9, 10, 11, 12 and 13) but ruled out in five patients (Cases 18, 19, 20, 21 and 22). Of importance, hypercalcemia was definitely mediated by PTHrP in all three patients with t(17;19)-ALL in whom informative data were available (Cases 1, 2 and 3).

Table 2 Comparison of characteristics of patients in present study and patients with childhood ALL in the TCCSG

	Present study		*TCCSG*[a]		*χ^2-test*
	Incidence	*%*	*Incidence*	*%*	*P-value*
Age ⩾10 years	11/22	50.0	182/765	23.8	$P=0.005$
Male	12/22	54.5	418/765	54.6	$P=0.993$
WBC $<20\times10^9$ /l	20/22	90.9	490/764	64.6	$P=0.010$
Hb <8 g/dl	6/22	27.3	387/757	51.1	$P=0.025$
Plt $<100\times10^9$/l	8/22	36.4	501/758	66.1	$P=0.004$
T-ALL	0/21	0.0	84/725	11.6	$P=0.098$

Abbreviations: ALL, acute lymphoblastic leukemia; Hb, hemoglobin; Plt, platelet count; TCCSG, Tokyo Children's Cancer Study Group; WBC, white blood cell count.
[a]The data of 765 childhood ALL patients excluding B-ALL and infants in the L89-12 and L92-13 study.

Management of hypercalcemia

Intravenous hydration with or without furosemide was administered to all patients. In Case 5, the hypercalcemia resolved without additional therapy. Fifteen patients received calcitonin (Cases 1, 2, 3, 4, 6, 7, 9, 10, 12, 13, 14, 16, 17, 18 and 22), 11 patients received bisphosphonate (Cases 2, 4, 6, 9, 10, 11, 15, 18, 19, 21 and 22), and seven of those patients received both calcitonin and bisphosphonate. Ten of the 12 patients who were diagnosed in or after 1997 received bisphosphonate, whereas only one of the 10 patients diagnosed before 1997 did so. Among the 17 patients who received chemotherapy before resolution of hypercalcemia (Cases 1, 2, 3, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20 and 22), chemotherapy was started by oral predonisolone (PSL) alone in seven patients (Cases 2, 9, 10, 11, 18, 19 and 22). Hypercalcemia and renal insufficiency ultimately resolved in all patients treated with or without bisphosphonate (Figure 2). As summarized in Table 4, although the levels of serum calcium before treatment and the incidences of concomitant use of chemotherapy and calcitonin were equivalent between the patients treated with and without bisphosphonate, rapid reduction of serum calcium level (<10 mg/dl within 4 days) was observed significantly more often in patients who were treated with bisphosphonate than in patients who were not treated with bisphosphonate. Consistently, the serum creatinine level tended to decrease

Table 3 Clinical and laboratory characteristics of hypercalcemia complicated in the patients with ALL

Case	*Ca (mg/dl)*	*Pi (mg/dl)*	*PTHrP (pmol/l)*		*iPTH (pg/ml)*	*1,25 $(OH)_2D$ (pg/ml)*	*BUN (mg/dl)*	*Cr (mg/dl)*	*Bone Pain*	*Osteolytic lesion*	*Emesis*
			C	*Intact*							
t(17;19) positive											
1	15.7	5.4	72		<1	NT	81	2.9	+	+	−
2	16.5	5.7	124		<3	<5	39	1.9	−	−	+
3	12.7	3.3	110		<3	NT	31	0.6	+	−	−
4	16.6	4.0	92		15.0	NT	28	2.6	+	+	−
5	12.9	2.6	NT		7.0	NT	76	3.4	−	−	−
t(17;19) negative or not confirmed											
6	16.3	4.9	76[a]		NT	5.5	49	2.3	+	−	−
7	14.6	4.2	NT[a]		<5	NT	44	1.45	+	+	+
8	19.2	3.9	107		7.0	26.0	36	0.9	−	+	−
9	20.0	5.0	114		<10	NT	62	1.2	−	−	+
10	14.3	4.6	46		9.0	2.8	34	1.8	+	+[b]	−
11	12.2	6.2	57		<1	<10	52	2.4	+	+[b]	+
12	20.8	3.2	240		NT	NT	39	0.6	−	+	+
13	15.0	4.5	75		NT	<5	19	0.6	+	+[b]	+
14	15.0	5.0	108		18.3	NT	58	2.4	−	+	+
15	15.0	4.5	134		NT	NT	33	1.6	−	−	−
16	15.8	4.9	112		NT	14.3	55	1.4	−	+	+
17	14.6	NA	100		NT	14.0	NA	NA	+	+	−
18	17.4	2.7		<1.1	12.7	3.8	24	1.3	+	+[b]	+
19	15.8	3.6		<1.1	7.0	8.0	8	0.3	+	+	−
20	15.4	2.8		<1.1	13.4	NT	16	0.6	+	−	−
21	16.6	3.5		<1.1	4.2	12.0	15	0.3	+	+	+
22	19.1	7.6	<1		86.0	NT	NA	0.9	−	−	+

Abbreviations: ALL, acute lymphoblastic leukemia; BUN, blood–urea–nitrogen; C, C-terminal; Cr, creatinine; iPTH, intact PTH; NA, data or sample not available; NT, not tested; PTHrP, parathyroid hormone-related peptide.
[a]Positive for immunohistochemistry of PTHrP in leukemia cells.
[b]Fracture of the vertebral bone was disclosed by X-ray.

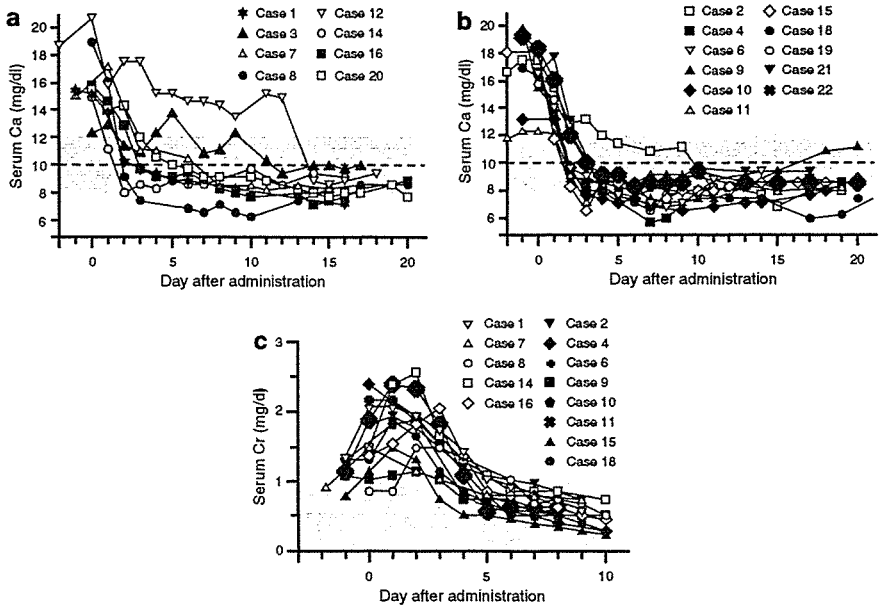

Figure 2 Changes in serum calcium (Ca) and creatinine (Cr) levels. Changes in the serum Ca level of the eight patients who were not treated with bisphosphonate (**a**) and the 11 patients who were treated with bisphosphonate (**b**) and changes in the serum Cr level of the patients who were (closed symbols) or were not (open symbols) treated with bisphosphonate, whose maximum level of serum Cr exceeded 1 mg/dl (**c**). The day of administration of the first dose of bisphosphonate was defined as day 0.

Table 4 Comparison of clinical course of hypercalcemia between patients who were or were not treated with bisphosphonate

	Bisphosphonate (n=11)	*Others (n=8)*	*P-value (t-test/χ^2-test)*
Serum Ca level			
Pre-therapy	16.0±0.6 mg/dl	16.0±1.0 mg/dl	$P=0.973$[a]
Maximum	16.8±0.7 mg/dl	16.4±0.9 mg/dl	$P=0.708$[a]
Minimum	6.8±0.2 mg/dl	7.7±0.3 mg/dl	$P=0.024$[a]
Chemotherapy			
Started	8	7	$P=0.436$
Not started	3	1	
Calcitonin			
Administered	7	6	$P=0.599$
Not administered	4	2	
Ca <12 mg/dl on day 2			
Yes	7	3	$P=0.260$
No	4	5	
Ca <10 mg/dl on day 4			
Yes	10	4	$P=0.046$
No	1	4	
Minimum Ca <8 mg/dl			
Yes	9	3	$P=0.048$
No	2	5	
Ca supplementation			
Yes	5	0	$P=0.026$
No	6	8	
Cr <1 mg/dl on day 5			
Yes	7	2	$P=0.071$
No	1	3	

Abbreviations: Ca, serum calcium level; Cr, serum creatinine level.
[a]The *P*-value was analyzed by *t*-test.

more rapidly in patients who were treated with bisphosphonate compared with those who were not treated with bisphosphonate. The minimum serum calcium level of the patients treated with bisphosphonate was significantly lower than that of the patients treated without bisphosphonate, and hypocalcemia (<8 mg/dl) developed more frequently in the

patients who were treated with bisphosphonate. Although no clinical symptoms owing to hypocalcemia were noted, five of 11 patients treated with bisphosphonate and none of those treated without bisphosphonate required calcium supplementation. During the therapies for hypercalcemia, precipitation of calcium phosphate in urine was noticed in Cases 9, 10 and 14, and renal calculus developed in Case 10.

Treatment outcome of leukemia

All patients achieved complete remission (CR), but leukemia relapsed in 11 patients including Case 14 who developed AML at relapse (Table 1). The EFS rate at 5 years from diagnosis of ALL was estimated as 46.2±11.7%. Of note, all five patients with t(17;19)-ALL relapsed very early and their 5-year EFS rate (0%) was significantly lower ($p<0.0001$ in log-rank test) than that of the other 17 patients (59.7±13.4%). Among the 18 patients who developed hypercalcemia at disease onset (excluding Cases 1, 2, 5 and 7), the 5-year EFS rate of two patients with t(17;19)-ALL (0%) was still significantly lower ($P<0.0001$ in log-rank test) than that of the other 16 patients (63.5±13.7%). Thus, excluding the patients with t(17;19)-ALL, the 5-year EFS rate of ALL patients accompanied by hypercalcemia is almost similar to that of childhood ALL patients in the TCCSG treated with L89-12, L92-13,[25] L95-14 (1995–1999; 596 patients), and L99-15 (1999–2003; 623 patients) protocols, in which the 5-year EFS rate was 67.8±2.3, 63.4±2.7, 76.0±1.9 and 76.4±2.5%, respectively.

Discussion

This is the first cohort study of hypercalcemia associated with childhood ALL, and we retrospectively analyzed 22 Japanese patients reported in the last 15 years. Despite several limitations in a retrospective study, the present study newly demonstrated some characteristics of childhood ALL accompanied by hypercalcemia. The incidence of onset at age 10 years and older among the patients with hypercalcemia in the present study was significantly higher than that among all childhood ALL patients. Most of our patients had a low initial WBC and two-thirds of our patients had mild anemia and normal to mildly low platelet count, suggesting that symptoms associated with hypercalcemia rather than hematological abnormalities might lead to the diagnosis of leukemia in these patients. Importantly, we identified five patients with t(17;19)-ALL, and its incidence was estimated to be over 20%, indicating the frequent association of t(17;19) with the development of hypercalcemia in childhood ALL. In all of five t(17;19)-ALL patients in the present study, the leukemia relapsed very early. However, excluding the patients with t(17;19)-ALL, the 5-year EFS rate of ALL accompanied by hypercalcemia was almost similar to that of childhood ALL patients enrolled in TCCSG, indicating that the development of hypercalcemia itself is not a poor prognostic factor in childhood ALL. Thus, identification of t(17;19) is very critical to predict the prognosis of ALL with hypercalcemia.

Among the 21 patients whose PTHrP data were available, the involvement of PTHrP-mediated hypercalcemia was confirmed in 11 patients but ruled out in five patients, indicating that hypercalcemia in childhood ALL is most frequently mediated by PTHrP. The *PTH* and *PTHrP* genes are localized on 11p15.3–15.1 and 12p12.1–11.2, respectively, and none of the patients had detectable chromosomal abnormalities involving these regions in their cytogenetic analysis (Table 1), suggesting that amplification of the regions encoding the *PTH* and *PTHrP* genes might be unlikely as a mechanism for hypercalcemia. Of note, we confirmed the contribution of PTHrP in all three patients with t(17;19)-ALL in whom informative data were available, suggesting that E2A-HLF might induce the production of PTHrP as one of the downstream targets. PTHrP is a potent humoral factor of hypercalcemia, but it was reported that asymptomatic carriers of human T-cell leukemia virus-1 have an elevated serum level of PTHrP without hypercalcemia,[26] suggesting that elevated PTHrP alone might not induce hypercalcemia. We previously reported that E2A-HLF induced the expression of SRPUL, which may play a role in bone invasion as an adhesion molecule.[27] Therefore, frequent association of t(17;19)-ALL with hypercalcemia might result from a synergistic action of PTHrP and SRPUL as downstream targets of E2A-HLF. In contrast, the involvement of PTHrP was specifically ruled out in five patients. Among these five patients, the involvement of PTH in hypercalcemia was strongly suggested in one patient (Case 22) but not in the other four patients (Cases 18–21). Moreover, calcitriol-mediated hypercalcemia, the most frequent cause of hypercalcemia in lymphoma,[4] was unlikely in these four patients. Of note, two patients (Cases 19 and 21) had B-precursor ALL with negative- or low-level expression of CD19 and their serum levels of tumor necrosis factor-α and interleukin-6, which are known to promote osteoclastic bone resorption and involve in hypercalcemia in malignancies,[28,29] were elevated at the onset of hypercalcemia,[30] representing an independent subgroup of ALL with PTHrP-independent hypercalcemia.

In the present study, for the treatment of hypercalcemia, 17 patients received chemotherapy before complete resolution of hypercalcemia, and seven of them received PSL alone. Fifteen patients received calcitonin, 11 patients received bisphosphonates and seven of them received both. Bisphosphonates have been reported to be highly effective for the treatment of hypercalcemia complicated in malignancies with long duration of action by reducing osteoclast viability and inhibiting osteoclast-mediated resorption of bone.[3,31–33] As a first cohort study, we retrospectively confirmed that hypercalcemia resolved quickly in the patients treated with bisphosphonate compared with the patients who were not treated with bisphosphonate. Most of the patients treated with bisphosphonate developed hypocalcemia and almost the half of them required calcium supplementation. Elevated serum creatinine level was observed in two-thirds of our patients, particularly among patients with older age of onset, but was less common among patients with hypophosphatemia. Of importance, treatment of hypercalcemia resolved renal insufficiency and chemotherapy could be started safely. In particular, renal insufficiency rapidly resolved in patients who were treated with bisphosphonate. However, as there was no difference in the final outcome of survival or renal insufficiency between the patients treated with and without bisphosphonate, the usefulness of bisphosphonate in treating hypercalcemia that develops in ALL patients must be confirmed in a future large prospective study.

Table 5 summarizes the characteristics of 12 previously reported cases of t(17;19)-ALL[9–11,14–16,34,35] in addition to five cases identified in the present study. Hypercalcemia developed in 10 of 14 cases: four patients at their original diagnosis and six patients at disease recurrence. There was no association between hypercalcemia and the type of *E2A-HLF* fusion. Twelve of 15 t(17;19)-ALL patients were older than 10 years and eight of 16 patients had accompanying coagulopathy, which is a relatively rare complication in childhood ALL.[13,24] Although no available data in the previously reported cases, it should be noted that all t(17;19)-ALL cases in the present study expressed

Table 5 Characteristics of t(17;19)-ALL

Case	*Age*	*Sex*	*Coagulopathy*	*Hyper calcemia*	*Type of fusion*	*Karyotype at diagnosis*	*Allo BMT in 1stCR*	*Therapeutic outcome*	*Cell line*	*Reference*	*Identification*
1	16	M	+D	+D	Type 1	t(17;19)	ND	Death on 21m	UOC-B1	10	Pt.1
2	15	F	+D	+R	Type 1	t(17;19)	ND	Death on 23m		10	Pt.2
3	17	F	ND	ND	Type 1v	ND	ND	Death on 2.5m	HAL-01	11	
4	17	F	–	–	Type 2	t(17;19)	Yes	Relapse at 42m		14	Pt.1
5	11	M	–	+D	Type 2	t(17;19)	No	Relapse at 5m		14	Pt.2
6	13	M	–	+R	Type 1+2	ND	No	Relapse at 16m		14	Pt.3
7	ND	ND	–	ND	Type 2	ND	ND	ND		24	DEN-R
8	ND	ND	–	ND	Type 2	ND	ND	ND		24	RFH-N
9	12	F	+D	–	Type 1	Normal	No	Relapse at 5m		34	
10	5	F	+R	+R	Type 2	t(17;19)	No	Relapse at 18m		35	Pt.1
11	5	F	+D	–	Type 2	NA	No	Relapse at 15m		35	Pt.2
12	14	M	+D	–	ND	t(17;19)	No	1stCR untill 12m		35	Pt.3
13	14	F	–	+R	Type 2	t(17;19)	No	Relapse at 9m	Endo-kun	Present Study,[16]	Case 1
14	10	M	+R	+R	Type 1	Normal	No	Relapse at 14m		Present Study	Case 2
15	4	M	–	+D	Type 2	Normal	No	Relapse at 5m	YCU-B2	Present Study,[15]	Case 3
16	14	F	–	+D	Type 1	Add(19)(p13)	No	Relapse at 3m		Present Study,[9]	Case 4
17	12	F	+R	+R	Type 2	Normal	No	Relapse at 2m		Present Study,[16]	Case 5

Abbreviations: ALL, acute lymphoblastic leukemia; Allo BMT, allogeneic bone marrow transplantation, CR, complete remission; D, at diagnosis; F, female; m, month; M, male; ND, not described; R, at relapse; v, variant.

CD33 and three cases had L2 phenotype. Accordingly, older age of onset, coagulopathy, CD33 expression and L2 phenotype in FAB classification in childhood ALL accompanied by hypercalcemia strongly suggest t(17;19)-ALL. In addition to five patients in the present study, almost all of the patients with t(17;19)-ALL relapsed very early. The prognosis of leukemia in the patients who did not develop hypercalcemia (cases 4, 9, 11 and 12 in Table 5) was as poor as that in the patients who developed hypercalcemia at disease onset (cases 1, 5, 15 and 16 in Table 5), indicating that hypercalcemia did not affect prognosis of the patients with t(17;19)-ALL. Of note, one previously reported patient (Case 4 in Table 5) who exceptionally underwent allogeneic bone marrow transplantation (allo-BMT) in the first CR (13 weeks after diagnosis) maintained CR for 42 months,[14] suggesting that allo-BMT performed early in the first CR might prolong the disease-free survival of t(17;19)-ALL patients even if not cured.

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STEM CELLS
TISSUE-SPECIFIC STEM CELLS

Highly Efficient Ex Vivo Expansion of Human Hematopoietic Stem Cells Using Delta1-Fc Chimeric Protein

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Key Words. AC133 antigen • Hematopoietic stem cells • Notch • Stem cell expansion

ABSTRACT

Ex vivo expansion of hematopoietic stem cells (HSCs) has been explored in the fields of stem cell biology, gene therapy, and clinical transplantation. Here, we demonstrate efficient ex vivo expansion of HSCs measured by long-term severe combined immunodeficient (SCID) repopulating cells (SRCs) from human cord blood CD133-sorted cells using a soluble form of Delta1. After a 3-week culture on immobilized Delta1 supplemented with stem cell factor, thrombopoietin, Flt-3 ligand, interleukin (IL)-3, and IL-6/soluble IL-6 receptor chimeric protein (FP6) in a serum- and stromal cell-free condition, we achieved approximately sixfold expansion of SRCs when evaluated by limiting dilution/transplantation assays. The maintenance of full multipotency and self-renewal capacity during culture was confirmed by transplantation to nonobese diabetic/SCID/γc^{null} mice, which showed myeloid, B, T, and natural killer cells as well as $CD133^+CD34^+$ cells, and hematopoietic reconstitution in the secondary recipients. Interestingly, the CD133-sorted cells contained approximately 4.5 times more SRCs than the CD34-sorted cells. The present study provides a promising method to expand HSCs and encourages future trials on clinical transplantation. STEM CELLS *2006;24: 2456–2465*

INTRODUCTION

Umbilical cord blood (CB) is an established stem cell source for hematopoietic stem cell (HSC) transplantation. In many cases, however, CB transplantation is unavailable to patients with relatively high body weight because of the insufficient number of HSCs obtained from a single CB unit [1–3]. Recently, transplantation of multiple units of CB in adult patients was reported in an experimental attempt to infuse a higher number of HSCs or hematopoietic progenitor cells (HPCs), but the effectiveness of this novel trial needs further investigation [4–7].

Another possibility to acquire a higher number of stem cells is ex vivo expansion of HSCs. Although many reports have described potential methods to increase HSCs ex vivo, only a few of them have clearly demonstrated the expansion of long-term severe combined immunodeficient (SCID) repopulating cells (SRCs), currently the only reliable measure of HSCs [8–10]. According to one of these reports, combined use of soluble interleukin (IL)-6 receptor (sIL-6R) and IL-6 together with stem cell factor (SCF), thrombopoietin (TPO), and flt-3 ligand (FL) appeared to be helpful for the successful expansion

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of HSCs, probably because gp130-mediated signals play a critical role in stem cell proliferation and combined use of sIL-6R and IL-6 can transmit signals through gp130 in HSCs, which express gp130 but lack IL-6R [10]. Based on these findings, an artificially generated IL-6/sIL-6R fusion protein, named FP6, which could more efficiently transmit gp130 signals in hematopoietic cells [11], might be a promising agent for ex vivo expansion of HSCs.

Another method that is potentially useful for stem cell expansion is the use of Notch signaling. It is mediated by interactions between transmembrane receptors (Notch1, -2, -3, and -4) and their membrane-bound ligands (Delta and Jagged family molecules). The signaling pathway is known to have differentiation-inhibitory effects in different stem cell systems, including hematopoiesis [12–14]. It has been reported that Notch signaling might play a role in the bone marrow niche, in which Notch ligands are presented by osteoblasts, main components of the niche [15]. In fact, soluble forms of the Notch ligands have been shown to increase immature hematopoietic cells [16–18]. These findings strongly prompt us to use Notch ligands in combination with FP6, for stem cell expansion.

The initial stem cell source is also an important issue for obtaining the maximum efficiency of stem cell expansion. Whereas many investigators use the CD34-sorted cells as a source of stem cell expansion, recent reports suggested that CD133 sorting can concentrate SRCs more efficiently than CD34 sorting [19, 20], and it is still open to question which population is more suitable for stem cell expansion.

In this study, we first addressed the issue of stem cell sources, demonstrating that the CB CD133-sorted cells contained an approximately 4.5-fold greater absolute number of SRCs than CD34-sorted cells. We next evaluated the integrated effect of Notch and gp130 signalings using soluble Delta1 and FP6 in combination with SCF, TPO, FL, and IL-3 and found that this combination could expand human CB CD133-sorted SRCs by 5.8-fold in a serum- and stromal cell-free condition.

MATERIALS AND METHODS

Separation of CD133- and CD34-Enriched Cells from Human CB

Human CB samples were collected from normal full-term deliveries after informed consent was obtained. Mononuclear cells (MNCs) were separated by density gradient centrifugation (Lymphoprep; Axis-shield, Oslo, Norway, http://www.axis-shield.com) after depletion of phagocytes with Silica (Immuno-Biological Laboratories Co., Takasaki, Gunma, Japan, http://www.ibl-japan.co.jp). CD133- and CD34-enriched cells were separated from MNCs by using magnetic cell sorting (MACS) CD133 MicroBead Kit or MACS Direct CD34 Progenitor Cell Isolation Kit (hereafter CD133-MACS and CD34-MACS, respectively; Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com), respectively. In some experiments, separated cells were examined by flow-cytometric analyses using FcR Blocking Reagent, fluorescein isothiocyanate (FITC)-conjugated anti-human CD34, allophycocyanin (APC)-conjugated anti-human CD133 (clone 293C3) (Miltenyi Biotec), phycoerythrin (PE)-conjugated anti-human CD38 antibodies (BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen), and 7-amino-actinomycin D (7-AAD) (Via-Probe; BD Pharmingen). The yield of the target cells was calculated as follows: [(number of cells after separation) × (purity of the target cells after separation defined by flow-cytometric analysis)] / [(number of MNCs before separation) × (frequency of the target cells among MNCs before separation defined by flow-cytometric analysis)] × 100 (%).

Cytokines

Recombinant human SCF, TPO, IL-3, and IL-6/sIL-6R chimeric protein FP6 were generated by Kirin Brewery Co., Ltd. (Tokyo, http://www.kirin.co.jp/english), and the recombinant Delta1-Fc chimeric protein was generated as previously described [17]. These reagents were certified as free from endotoxin (<0.28 EU/mg protein). Recombinant human IL-6 and FL were purchased from Wako Pure Chemicals (Osaka, Japan, http://www.wako-chem.co.jp/english) and R&D Systems Inc. (Minneapolis, http://www.rndsystems.com), respectively.

Cell Culture

Plates or dishes not treated with tissue culture were precoated with 10 μg/ml Delta1-Fc or control Fc fragment of human IgG (IgG-Fc) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, http://www.jacksonimmuno.com) followed by 10 μg/ml human fibronectin (Boehringer Ingelheim GmbH, Ingelheim, Germany, http://www.boehringer-ingelheim.de). Cells were cultured in serum-free medium composed of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10 mg/ml bovine serum albumin, 10 μg/ml human insulin, 200 μg/ml human transferrin, 2 mM L-glutamine, 0.1 mM 2-Mercaptoethanol, 4.7 μg/ml linoleic acid, 4.7 μg/ml oleic acid, and 8 μg/ml cholesterol (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, http://www.kyokutoseiyaku.co.jp) at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Cytokines were added at concentrations of 100 ng/ml for SCF, 10 ng/ml for TPO, 100 ng/ml for FL, 100 ng/ml for FP6, 100 ng/ml for IL-6, and 10 ng/ml for IL-3. Cell culture was initiated in 24-well plates and serially transferred to six-well plates and 10-cm dishes to avoid overgrowth of the cells. Half of the culture medium was changed every 2 or 3 days.

Colony Assays

At the indicated time points, cultured cells were harvested and plated in a semisolid medium, Methocult GF H4434, containing IMDM with 30% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml human SCF, 10 ng/ml human granulocyte-macrophage colony stimulating factor, 10 ng/ml human IL-3, and 3 units/ml human erythropoietin (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) and incubated at 37°C. Colony-forming ability was assessed after 15–16 days of culture.

Transplantation to Nonobese Diabetic/SCID or Nonobese Diabetic/SCID/γc^{null} Mice

To assess the in vivo repopulating capacity of isolated cells and their cultured progeny, we used nonobese diabetic (NOD)/SCID (NOD/Shi-scid; CLEA Japan, Inc., Tokyo, http://www.clea-japan.com) and NOD/SCID/γc^{null} (NOG) mice [21] (Central Institute for Experimental Animals, Kanagawa, Japan, http://www.ciea.or.jp/English/eindex.htm) as xenotransplantation recipients. Cells separated by CD133-MACS and their cultured

progeny were transplanted intravenously into sublethally irradiated (2.5 Gy using an x-ray irradiator), 8–10-week-old NOD/SCID or NOG mice. When transplanting cells into NOD/SCID mice, we injected intraperitoneally 20 μl of anti-asialo GM1 antibody (Wako Pure Chemicals) diluted in phosphate-buffered saline (PBS) to a total volume of 420 μl immediately before transplantation and on days 11, 22, and 33 after transplantation to reduce the natural killer (NK) cell activity in NOD/SCID mice [10]. Because NOG mice lack intrinsic NK cell activities, administration of anti-asialo GM1 antibody to NOG mice was not needed [21]. Mice were fed with autoclaved acidified water and sterilized food. At 10–13 weeks after transplantation, mice were sacrificed, and cells were harvested from both femurs, peripheral blood, spleen, and thymus. In the indicated experiments, analyses were performed 24 weeks after transplantation. In the limiting dilution transplantation analyses, we transplanted cells into six to 12 recipient mice in each limiting dose for reliable estimation.

In the serial transplantation experiment, we isolated bone marrow cells from the primary NOG recipient mouse 24 weeks after the first transplantation, and MNCs were separated by density gradient centrifugation (Histopaque-1083; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). The MNCs were divided into three aliquots and injected intravenously into secondary NOG recipients. Ten weeks after the second transplantation, bone marrow cells were harvested and analyzed.

Flow-Cytometric Analysis of Transplanted NOD/SCID and NOG Mice

Engraftment of human cells was examined by analyzing human surface antigens using BD LSR2 (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Cells harvested from the bone marrow, peripheral blood, spleen, and thymus of recipient mice were treated with ammonium chloride red blood cell lysis buffer (Sigma-Aldrich) and blocked with PBS containing 2% FBS, anti-mouse CD16/32 antibody (BD Pharmingen), and FcR Blocking Reagent (Miltenyi Biotec). Then, they were stained with FITC-conjugated anti-human CD45 (clone HI30; BD Pharmingen) and anti-human CD3 (Beckman Coulter, Inc., Fullerton, CA, http://www.beckmancoulter.com), PE-conjugated anti-human CD13, CD33, CD56, CD4 (Beckman Coulter, Inc.), and CD133 (clone 293C3; Miltenyi Biotec), APC-conjugated anti-murine CD45 (clone 30-F11; BD Pharmingen), anti-human CD3, CD19, CD8 (Beckman Coulter, Inc.), and CD34 (Miltenyi Biotec), and 7-AAD (Via-Probe; BD Pharmingen). Successful engraftment of human hematopoietic cells was determined by detection of greater than 0.1% of human $CD45^+$ cells in recipient bone marrow cells.

Limiting Dilution Analysis

The frequencies of SRCs capable of repopulating in NOD/SCID mice were quantified by a limiting dilution analysis by applying Poisson statistics to the single-hit model as described previously [8, 22, 23]. The frequencies of SRCs and statistical comparison between individual populations were calculated by using L-Calc software (StemCell Technologies).

Statistical Analysis

Data are presented as mean ± SEM. Analysis of statistical significance was determined by paired *t* test.

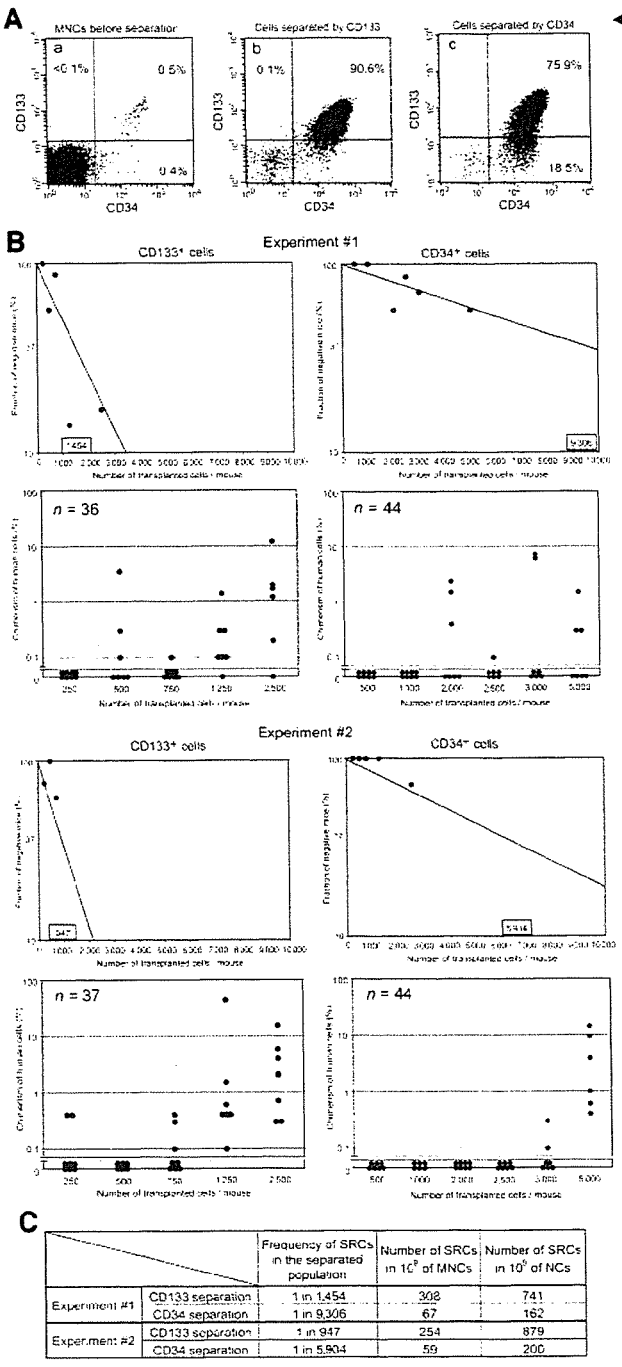
RESULTS

Stem Cell Isolation by the CD133-MACS Recovers a Higher Number of SRCs than by CD34

Because several investigators have suggested that SRCs are more concentrated in $CD133^+$ cells than in $CD34^+$ cells [19, 20], we directly compared the frequency of SRCs contained in the populations sorted by CD133-MACS and CD34-MACS. Flow-cytometric analyses of four CB samples showed that 0.2%–1.4% (mean 0.8%) and 0.8%–3.0% (mean 1.9%) of MNCs were positive for CD133 and CD34, respectively. More than 98% of $CD133^+$ cells were $CD34^+$, and approximately 43% (25%–56%) of $CD34^+$ cells were $CD133^+$ (Fig. 1A, a).

Then, we prepared two identical CB MNC aliquots and isolated CD133- and CD34-enriched cells by CD133- and CD34-MACS. Flow-cytometric analyses after isolation showed that the purities of separated cells were variable among samples (53.1%–93.5% for CD133 and 53.9%–96.3% for CD34), but there was no significant difference between the two separation methods ($p = .12$). Calculated recovery rates of the target cells (see Materials and Methods) were 66% ± 10% for CD133 and 46% ± 10% for CD34, showing a tendency of better recovery of CD133 cells by CD133-MACS than recovery of CD34 cells by CD34-MACS, but the difference was not significant ($p = .06$). After separation, approximately 75% of the CD34-sorted cells were $CD133^+$, whereas virtually all of the CD133-sorted cells were $CD34^+$ with only rare (0.1%) $CD34^-$ cells in most of the samples (Fig. 1A, b and c). Based on the comprehensive calculation, the recovery rates of $CD133^+CD34^+$ cells (i.e., the major SRC-containing population) in the individual samples were 66% ± 10% and 83% ± 8% by CD133- and CD34-MACS separation, respectively. The recovery efficiency of this most immature fraction by CD34-MACS tended to be superior to the one by CD133-MACS, but again it was not significantly different ($p = .14$).

Then, to compare the number of SRCs contained in the populations separated by CD133- and CD34-MACS, we transplanted cells of each population into irradiated NOD/SCID mice intravenously and examined their in vivo hematopoietic repopulating capacity. To evaluate the number of SRCs quantitatively, we transplanted serially reduced numbers of cells. Frequencies of SRCs in the CD133-sorted population were one of 1,454 and 947 in samples 1 and 2, respectively. In contrast, those in the CD34-sorted population were one of 9,306 and 5,904 in samples 1 and 2, respectively (Fig. 1B, 1C). This means that SRCs were sixfold more concentrated in the CD133-sorted population than in the CD34-sorted one. Converting this frequency into the absolute number of SRCs obtained from the same number of primary MNCs, CD133 sorting recovered 308 (sample 1) and 254 (sample 2) SRCs, and CD34 sorting recovered 67 (sample 1) and 59 (sample 2) SRCs from 10^8 of total MNCs (Fig. 1C). Therefore, despite the similar recovery rate of $CD133^+CD34^+$ cells by CD133- and CD34-sorting procedures, CD133 sorting provides 4.3–4.6-fold greater absolute numbers of SRCs than CD34 sorting. Thus, for our subsequent SRC expansion experiments, we used the CD133-sorted population as the culture-initiating cells.



C

		Frequency of SRCs in the separated population	Number of SRCs in 10^5 of MNCs	Number of SRCs in 10^5 of NCs
Experiment #1	CD133 separation	1 in 1,454	308	741
	CD34 separation	1 in 9,306	67	162
Experiment #2	CD133 separation	1 in 947	254	879
	CD34 separation	1 in 5,904	59	200

Figure 1. Separation of CD133- or CD34-enriched cells from CB MNCs and comparison of their in vivo repopulating capacity. **(A):** Expression profiles of CD133 and CD34 on CB MNCs (a) and cells separated by CD133- (b) and CD34-MACS (c). Representative data among several samples are shown. **(B):** The repopulating ability of CD133- and CD34-sorted cells isolated from the same CB samples (1 and 2). Frequencies of SRCs estimated by limiting dilution analyses are shown. The lower panels show chimeric proportion of human $CD45^+$ cells in the bone marrow of recipient mice, and the number of transplanted mice is shown in the upper-left margin of the panels. **(C):** Estimated frequencies and numbers of SRCs in the transplanted samples. In both experiments, CD133-sorted cells contain higher frequencies of SRCs than CD34-sorted cells, and CD133 sorting provides higher numbers of SRCs from the same volume of original MNCs or NCs than CD34 sorting. Abbreviations: CB, cord blood; MACS, magnetic cell sorting; MNC, mononuclear cell; NC, nucleated cell; SRC, severe combined immunodeficient repopulating cell.

Immobilized Delta1-Fc Chimeric Protein Can Expand Immature CB Hematopoietic Precursors in the Presence of Cytokines

Before evaluating methods for HSC expansion ex vivo, we first explored optimal culture conditions to expand immature hematopoietic precursors by using various combinations of hematopoietic cytokines and soluble Notch ligands. Because immobilization of Notch ligands has been demonstrated to be important for their efficient activity [24, 25], and it has been suggested that immobilized fibronectin fragment CH-296 along with Notch ligands augmented the effect of Notch ligands [24], we immobilized Delta1-Fc chimeric protein on the bottom of the culture plates along with human fibronectin prior to starting culture. We included SCF, TPO, and FL in the culture system as a basal cytokine combination (designated hereafter three growth factors, 3GFs) because these cytokines have been repeatedly shown to be effective for immature HSC/HPC expansion [26].

We cultured CB CD133-sorted cells in four cytokine combinations of (a) 3GFs + IL-6, (b) 3GFs + IL-6 + IL-3, (c) 3GFs + FP6, and (d) 3GFs + FP6 + IL-3, plus additional conditions with Delta1-Fc or IgG-Fc in each cytokine combination, and compared the expansion rate of total cells, $CD133^+$ $CD34^+CD38^-$ immature hematopoietic cells, and mixed colony-forming cells (CFU-Mix). All culture conditions increased the number of total cells and $CD133^+CD34^+CD38^-$ cells during 3-week culture (Fig. 2A, 2B). Addition of IL-3 or replacement of IL-6 with FP6 gave greater expansion of total cells and $CD133^+CD34^+CD38^-$ cells. However, Delta1-Fc had very little effect on the expansion of these cells (Fig. 2A, 2B).

In contrast, addition of IL-3 was always required for the consistent expansion of CFU-Mix until 3 weeks (Fig. 2C). In the presence of IL-3, addition of FP6 increased the number of CFU-Mix significantly better than IL-6 ($p < .01$), recapitulating the previous findings of the lack of IL-6R on immature HPCs [10, 22] and of the requirement of gp130 signaling for the optimal expansion of these immature cells [10, 11]. Regarding the effect of soluble Notch ligands, Delta1-Fc remarkably increased the number of CFU-Mix for a period of 3 weeks, particularly when combined with IL-3 and FP6. Ultimately, the maximum expansion of CFU-Mix was achieved when cells were cultured with 3GFs + FP6 + IL-3 + Delta1-Fc for 3 weeks ($p < .05$) (Fig. 2C).

Effects of IL-6-gp130, IL-3, and Notch Signalings on SRC Expansion in the Serum-Free Culture

We have found that the number of CFU-Mix was continuously increased until 3 weeks in several conditions (Fig. 2C) and declined thereafter (data not shown). And although a previous report demonstrated that serum-containing culture with 3GFs and IL-6/sIL-6R for 1 week increased the number of SRCs by fourfold, no increase of human blood cell chimerism in recipient mice was observed when we cultured cells for 1 week in the serum-free conditions with either 3GFs + FP6 or 3GFs + FP6 + IL-3 + Delta1-Fc (data not shown). Based on these obser-

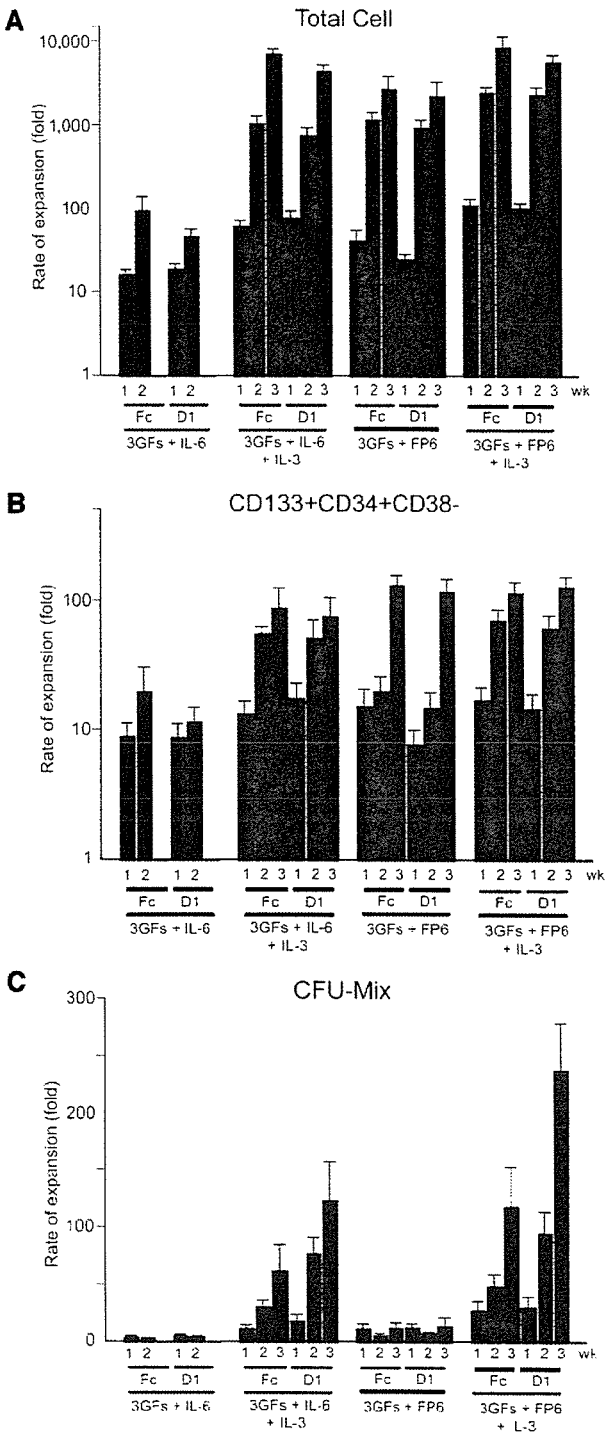


Figure 2. The expansion rates of total cells, $CD133^+CD34^+CD38^-$ immature cells, and CFU-Mix. CB CD133-sorted cells were cultured in the presence of indicated cytokines and Notch ligands for 1, 2, and 3 weeks. **(A, B):** The numbers of total cells **(A)** and $CD133^+CD34^+CD38^-$ immature hematopoietic cells **(B)** were counted, and the expansion rates are shown. **(C):** After indicated periods of culture, cells were replated in a semisolid medium and the number of CFU-Mix was evaluated. The expansion rate of the number of CFU-Mix is shown. Abbreviations: 3GFs, three growth factors; CFU-Mix, mixed colony-forming cells; D1, Delta1-Fc; Fc, IgG-Fc; FP6, interleukin-6/soluble interleukin-6 receptor chimeric protein; IL, interleukin; wk, week.

vations, we determined to culture cells for 3 weeks to evaluate SRC expansion.

As shown in Figure 3A, all the mice transplanted with more than 5,000 fresh CD133-sorted cells showed engraftment, but fewer than 2,500 cells failed to engraft in some of the mice. The frequency of SRCs in this sample was calculated as one of 1,020 (95% confidence interval [CI], 1/548–1/1,899) CD133-sorted cells. Progeny of the CD133-sorted cells grown for 3 weeks with 3GFs + FP6 contained SRCs at a frequency of equivalent to one of 640 (95% CI, 1/414–1/988) culture-initiating cells, and there was no statistical difference between the frequencies of SRCs in these populations, indicating that the addition of FP6–3GFs does not expand SRCs in the serum-free condition ($p = .11$; Fig. 3B), unlike in the serum-containing condition [10].

In contrast, when immobilized Delta1-Fc was present in the same cytokine combination (i.e., 3GFs + FP6), the frequency of SRCs increased to the equivalent to one of 361 (95% CI, 1/218–1/596) culture-initiating cells, indicating 2.8-fold SRC expansion compared with the SRC number before culture ($p = .005$, Fig. 3C). The addition of IL-3 to this condition further augmented the expansion efficiency, achieving the SRC frequency of equivalent to one of 175 (95% CI, 1/109–1/279) culture-initiating cells, indicating 5.8-fold expansion ($p = .0001$, Fig. 3D). To our knowledge, this ranks with the highest human SRC expansion efficiency ever reported. It is of note that two of six mice transplanted with cultured progeny equivalent to 60 culture-initiating cells showed human blood cell chimerism. To further compare the effects of IL-6 and FP6, we replaced FP6 with IL-6. In this condition, SRC frequency was equivalent to one of 266 (95% CI, 1/159–1/446) culture-initiating cells. The expansion rate was reduced from 5.8-fold to 3.8-fold, although significant expansion was still achieved ($p = .0006$; Fig. 3E).

Taken together, significant SRC expansion was realized in all three conditions with immobilized Delta1-Fc chimeric protein. Among these, combination of Delta1-Fc, IL-3, and IL-6/sIL-6R chimeric protein, FP6, in addition to 3GFs, provided the most significant expansion in the serum-free condition. It is noteworthy that IL-3 showed a positive effect in this condition, in contrast to the negative impact in the serum-containing condition without Notch signaling [10].

SRCs Cultured for 3 Weeks in the Serum- and Stromal Cell-Free Condition with 3GFs, FP6, IL-3, and Delta1-Fc Normally Contribute to Myeloid, B, T, and NK Cell Lineages in NOG Recipient Mice and Repopulate Recipients of Secondary Transplantation

To examine the long-term in vivo myeloid and lymphoid repopulating capacity of the cells cultured with 3GFs, FP6, IL-3, and Delta1-Fc, we transplanted these cells into NOG mice, which were generated by intercrossing NOD/SCID mice with IL-2 receptor common γ chain-knockout (γc^{null}) mice. These mice, unlike NOD/SCID mice, are known to allow transplanted human HSCs/HPCs to differentiate even into the T-cell lineage [21], and therefore we could examine the in vivo differentiation capacity of the ex vivo expanded HSCs most efficiently. These mice also have the advantage of higher engraftment of transplanted human cells. We cultured 10,000 CB CD133-sorted cells for 3 weeks and transplanted them into NOG mice. After 12 weeks, we observed 53%–67% human $CD45^+$ cells in the

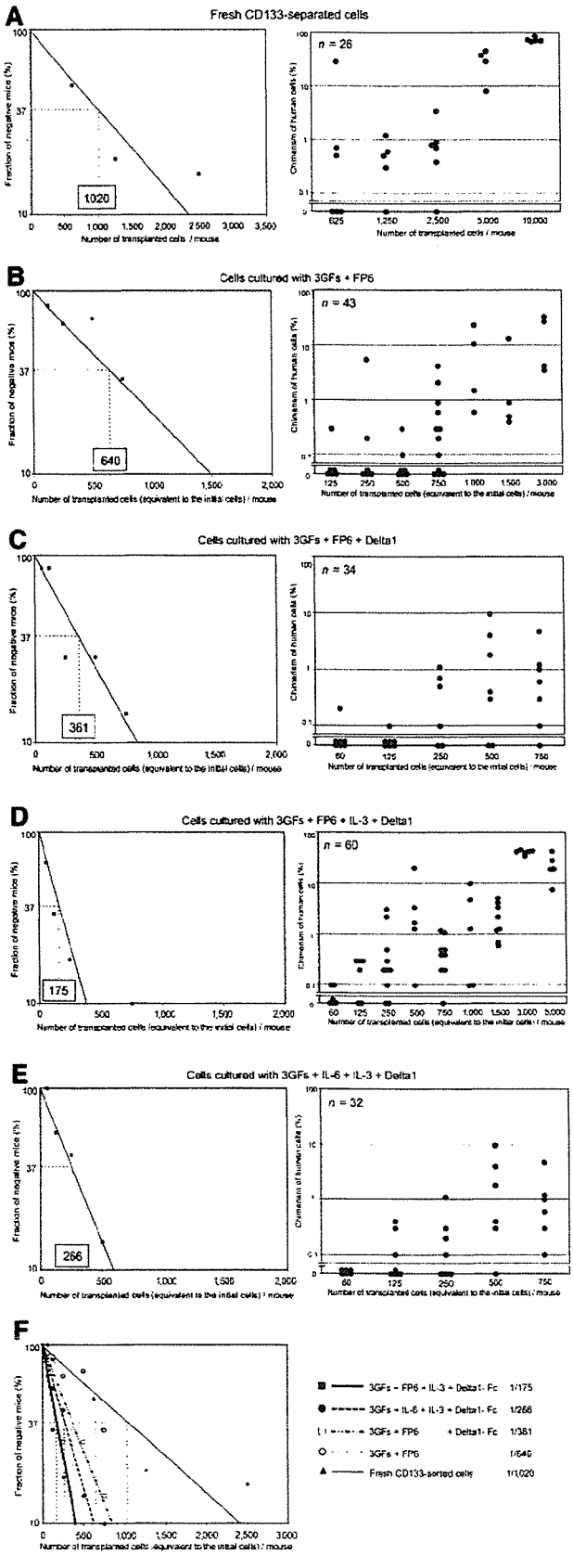


Figure 3. The repopulating ability of fresh CB CD133-sorted cells and their progenies after the culture with various combinations of cytokines for 3 weeks. **(A–E):** The frequencies of SRCs in fresh CD133-sorted cells **(A)**, cells cultured with SCF + TPO + FL (3GFs) + FP6 **(B)**, 3GFs + FP6 + Delta1-Fc **(C)**, 3GFs + FP6 + IL-3 + Delta1-Fc **(D)**, and 3GFs + IL-6 + IL-3 + Delta1-Fc **(E)**. They were estimated as 1/1,020 **(A)**, 1/640 **(B)**, 1/361 **(C)**, 1/175 **(D)**, and 1/266 **(E)**, respectively, by limiting dilution analyses. The right panels show chimeric proportion of human $CD45^+$ cells in the bone marrow of recipient mice, and the number of transplanted mice is shown in the upper-left margin of the panels. **(F):** Integrated representation of **(A–E)**. Correspondence of the symbols and lines is noted in the right. Abbreviations: 3GFs, three growth factors; FL, flt-3 ligand; FP6, interleukin-6/soluble interleukin-6 receptor chimeric protein; IL, interleukin; SCF, stem cell factor; TPO, thrombopoietin.

recipient bone marrow. Further analyses of the bone marrow, peripheral blood, spleen, and thymus of recipient mice revealed that human hematopoietic cells differentiated into myeloid ($CD13^+$ or $CD33^+$), B ($CD19^+$), T ($CD3^+$), and NK ($CD56^+$) cell lineages (Fig. 4A). In addition, in the bone marrow of recipient mice, we detected $CD133^+CD34^+$ immature hematopoietic cells at frequencies of 0.5%–1.1% of human cells. In the thymus, human cells represented virtually all the $CD3^+$ cells (data not shown), and among the $CD3^+$ cells, the patterns of differentiation to CD4/CD8 double-positive, CD4 single-positive, and CD8 single-positive cells were very similar to that of normal thymocytes (Fig. 4A). Robust human hematopoietic repopulation was confirmed in another recipient mouse 24 weeks after transplantation (Fig. 4B). In this mouse, more definite reconstitution of $CD3^+$ mature T cells was observed in the peripheral blood and spleen.

To confirm that transplanted HSCs still retain their self-renewal capacity after primary transplantation, we collected bone marrow cells 24 weeks after the transplantation from a primary recipient, which had been transplanted with the progeny of 1×10^4 CB CD133-sorted cells ex vivo expanded, and injected them into three secondary NOG mice. Ten weeks after the secondary transplantation, we observed engraftment of human $CD45^+$ cells (0.1%) in the bone marrow of two recipient mice (Fig. 5A), and human hematopoietic cells differentiated into myeloid ($CD13^+$ or $CD33^+$) and lymphoid ($CD19^+$) cells (Fig. 5B). These findings strongly indicate that cells cultured with 3GFs, FP6, IL-3, and Delta1-Fc for 3 weeks retain long-term repopulating capacity and normal differentiation capacity in vivo.

DISCUSSION

Efficient Ex Vivo Expansion of SRCs

In this study, we demonstrated successful expansion of SRCs by approximately sixfold, by culturing human CB CD133-enriched cells with SCF, TPO, FL, FP6, IL-3, and Delta1-Fc. SRCs have now been widely accepted as the most immature human hematopoietic cells and are regarded as surrogates for HSCs [26]. In many reports, expansion of SRCs has been discussed by comparison of human blood cell chimerism in recipient mice [16–18, 27–31]. However, to quantify the number of SRCs, limiting dilution/transplantation analyses are essential. Moreover, because the human blood cell chimerism in the bone marrow of recipient NOD/SCID mice typically stabilizes at 10–12 weeks

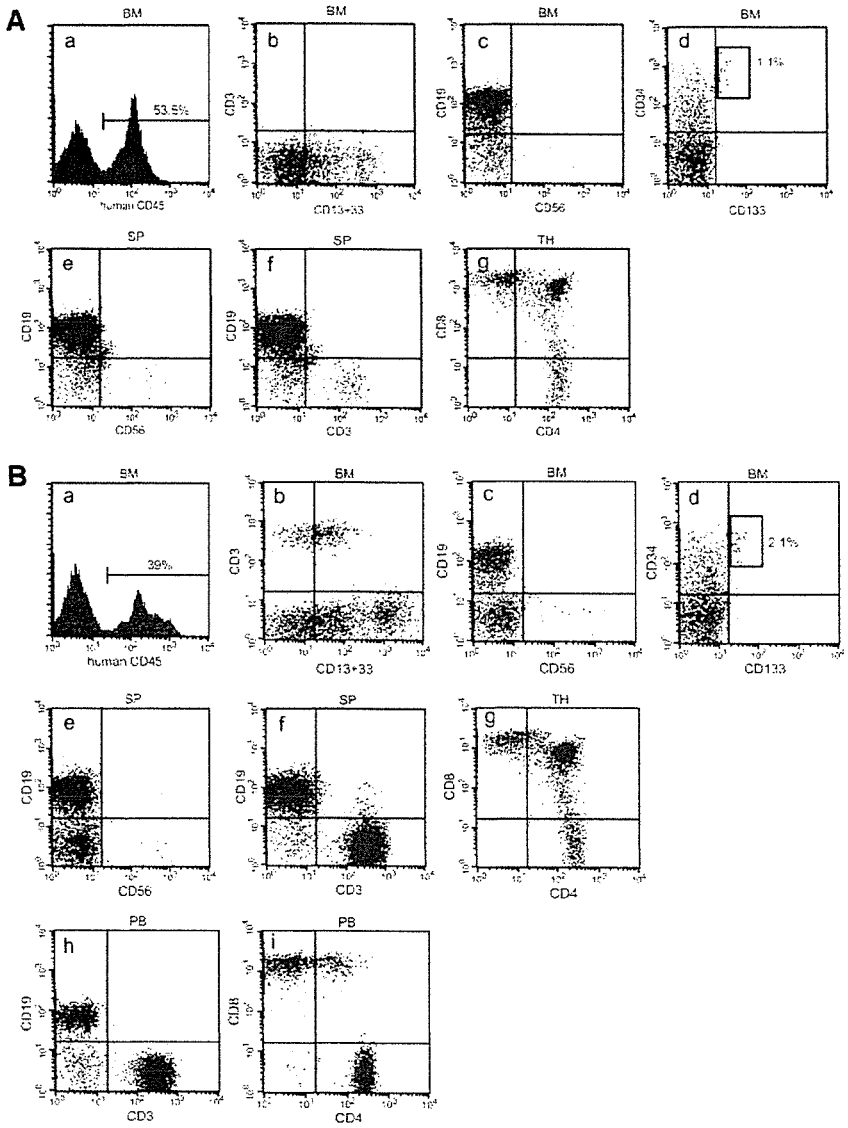


Figure 4. In vivo repopulating and differentiation capacity of the cells cultured with 3GFs + FP6 + IL-3 + Delta1-Fc. Cord blood CD133-sorted cells were cultured with 3GFs + FP6 + IL-3 + Delta1-Fc for 3 weeks and were transplanted into NOG mice. The BM, PB, SP, and TH of recipient mice were collected 12 weeks **(A)** and 24 weeks **(B)** after transplantation, and contribution of human cells to various hematopoietic lineages was examined by flow-cytometric analyses. **(A):** Representative data of recipient mice examined 12 weeks after transplantation. Human $CD45^+$ cells accounted for 53.5% of total BM cells (a), and a substantial number of human $CD3^+$ (b, f), $CD13^+$ (b), $CD33^+$ (b), $CD19^+$ (c, e), and $CD56^+$ (c, e) cells were detected in the BM and spleen. $CD133^+CD34^+$ immature hematopoietic cells were also clearly identified (1.1%) in the BM (d). In the thymus, $CD3^+$ cells expressed CD4 and/or CD8 (g) showing a solid development of human T cells. (b–g) represent data gated by human $CD45^+$ cells. **(B):** Flow-cytometric data from a mouse examined 24 weeks after transplantation. A high level of engraftment (a) (39%), reconstitution of $CD133^+CD34^+$ immature cells (d) (2.1%), and contribution to myeloid (b), B-cell (c, e, f, h), T-cell (b, f–i) and NK-cell (c, e) lineages were confirmed in the BM, spleen, thymus, and peripheral blood. (b–i) represent data gated by human $CD45^+$ cells. Abbreviations: 3GFs, three growth factors; BM, bone marrow; FP6, interleukin-6/soluble interleukin-6 receptor chimeric protein; IL, interleukin; NK, natural killer; PB, peripheral blood; SP, spleen; TH, thymus.

after transplantation [32–34], observation for at least 8–10 weeks is optimal to evaluate SRC numbers accurately, and only a few reports have fulfilled these conditions [8–10]. Our analysis, satisfying these criteria, revealed expansion of SRCs, which ranks as the most efficient one. Our method also enabled a 240-fold expansion of CFU-Mix, demonstrating its surprisingly strong effect on expanding immature progenitors.

We also demonstrated that the cultured cells can differentiate in vivo into myeloid, B, T, and NK cell lineages in the bone marrow, peripheral blood, spleen, and thymus in NOG mice. Human cells transplanted into NOG mice can engraft at significantly higher levels than NOD/SCID mice, and transplanted cells can differentiate even to the T-cell lineage. Based on these features, NOG mice are increasingly used as recipients of human stem cells as well as NOD/SCID/β2-microglobulin null mice [21, 35, 36]. We found immature $CD133^+CD34^+$ human cells in the bone marrow of recipient NOG mice at a substantial frequency, and after serial transplantation, progeny of the cultured cells engrafted most of the secondary recipients. These findings suggest that the culture system preserves normal stem cell functions.

Positive Effects of Notch Signaling on SRC Expansion

A positive effect of soluble Notch ligands on human SRCs was previously suggested by two groups, although they did not confirm the increase of SRCs quantitatively [16–18]. In the current study, we have provided clear data that demonstrate that the soluble Notch ligand can truly increase the number of human SRCs ex vivo. There are increasing lines of evidence suggesting that the Notch signaling pathway physiologically plays an important role in maintaining HSCs in the bone marrow niche [15, 37], where the Notch signal might inhibit differentiation of HSCs [15, 37]. Recently, the negative effects of reactive oxygen species for HSC maintenance were discovered [38], and the importance of the low oxygen environment in the HSC niche has been highlighted [39]. Interestingly, maintenance of the undifferentiated state by hypoxia-induced hypoxia-inducible

A

Primary transplant		Secondary transplants			
Cell dose	Human engraftment (%)	Mouse	Transplanted Cell dose	Transplanted Human Cell dose	Human engraftment (%)
cultured cells from 1×10^4 $CD133^+$ cells	24.2	S1	1.1×10^6	2.6×10^5	0.1
		S2	1.1×10^6	2.6×10^5	0
		S3	1.1×10^6	2.6×10^5	0.1

B

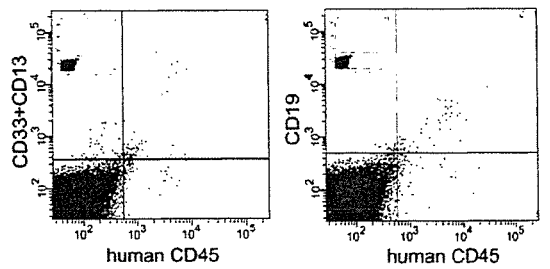


Figure 5. Cells cultured with three growth factors (3GFs) + interleukin (IL)-6/soluble receptor chimeric protein (FP6) + IL-3 + Delta1-Fc retain long-term repopulating capacity after serial transplantation into secondary nonobese diabetic/severe combined immunodeficient/γc^{null} (NOG) recipients. Ten-thousand cord blood CD133-sorted cells were cultured with 3GFs + FP6 + IL-3 + Delta1-Fc for 3 weeks and were transplanted into a primary NOG mouse. Twenty-four weeks after transplantation, bone marrow (BM) cells were harvested and serially transplanted into three secondary NOG recipients. **(A):** Ten weeks after secondary transplantation, BM cells of recipient mice were harvested and chimerism of human cells was analyzed. Two of the three secondary recipients showed substantial human engraftment. **(B):** Representative flow-cytometric data of BM cells in a secondary recipient (mouse S1). Human myeloid ($CD13^+$ or $CD33^+$) and lymphoid ($CD19^+$) cells can be identified. Data with isotype controls are shown as insets in the upper-left margin of the figures.

factor 1α (HIF1α) activation requires Notch signaling, and conversely, Notch signaling is enhanced by activation of HIF1α [39]. It is thus interesting to combine our system with hypoxic conditions for further better efficiency of ex vivo HSC expansion.

Effects of IL-3 and gp130 Signaling Pathways on SRC Expansion

We found that IL-3 exerts positive effects on amplifying SRCs at least in the presence of SCF, TPO, FL, FP6, and Delta1-Fc in a serum-free condition. To date, many researchers have examined the effects of IL-3 on HSCs, but the results have been controversial: some reports showed maintenance of HSCs, whereas others showed negative effects [40]. This discrepancy may depend on the addition of serum, the difference of coexisting cytokines, and the culture-initiating cells. Our result may suggest that IL-3 has additive or synergistic effects with Delta1-Fc on HSCs in the absence of serum.

We also found that replacement of IL-6 with FP6 had some superior effects on SRC expansion. Unlike the addition of IL-3, however, the effects of FP6 were marginal in the presence of Delta1-Fc and IL-3. This could be because the combination of Delta1-Fc and IL-3 could transmit nearly optimal growth signals in HSCs. Or the difference of the cell source (i.e., CD133- vs. CD34-sorted cells) might explain the results [10, 11].

Stem Cell Source for Transplantation and Ex Vivo Culture

To obtain the maximum efficiency of stem cell expansion, the isolation method for culture-initiating cells is also very important. CD34 sorting has been most widely used for positive selection of HSCs in the clinical practice. Recently, feasibility of the CD133-sorted cell transplantation has been evaluated in several clinical trials [41, 42]. There has been, however, no direct comparison of the SRC numbers obtained by these two methods. To the best of our knowledge, most of the CB SRCs are present in the $CD133^+CD34^+$ population [19, 20], and thus, the isolation methods are expected to provide a similar number of SRCs if the separation efficiencies are the same. Surprisingly, however, we found that the absolute SRC numbers were approximately 4.5-fold greater in the CD133-sorted population than in the CD34-sorted one, despite the fact that the recovery efficiency of $CD133^+CD34^+$ cells was very similar. One explanation to this apparently unexpected result could be that CD34, a cell-surface sialomucin protein, might be interfered with by the anti-CD34 antibody used for isolation. Given that accumulating evidence suggests that CD34 regulates homing of the cells to the proper microenvironment after i.v. injection by inhibiting inappropriate cell adhesion [43–45], the anti-CD34 antibody might interfere with the proper homing of the cells by modulating the adhesion capacity. Specifically, we used MACS Direct CD34 Progenitor Cell Isolation Kit for enrichment of $CD34^+$ cells, and this system uses monoclonal antibody QBend10, which recognizes the Class II epitope of the CD34 antigen. The QBend10 and other ClassII antibodies have been shown to induce actin polymerization and enhance cytoadhesiveness of KG-1 cells and primary bone marrow $CD34^+$ cells [46, 47], and this biological property may have reduced the SCID repopulating capacity of the CD34-sorted cells.

Recently, a small population of $CD133^+CD34^-$(Lineage$^-$ [Lin^-]) hematopoietic cells was identified. Because these cells give rise to $CD34^+$ SRCs during culture, $CD133^+CD34^-$ cells might represent precursors of SRCs [48–50]. Therefore, although these cells account for no more than 0.1% of the CD133-sorted cells, the use of CD133-sorted cells as the culture-initiating cells may help increase the absolute number of HSCs after culture.

All these considerations imply that CD133-sorted cells are more advantageous as a direct source for HSC transplantation and as a culture-initiating source for ex vivo HSC expansion than CD34-sorted cells to obtain a greater number of HSCs. According to our results, we can estimate that culturing CD133-sorted cells with Delta1-Fc yields as many as approximately 25-fold greater numbers of HSCs compared with the fresh CD34-sorted cells. Currently, clinical devices for CD34 sorting which use QBend10 or other Class II anti-CD34 antibodies are widely used. Our findings suggest that CD133 sorting might be a better way to collect and enrich HSCs than CD34 sorting by QBend10 or other ClassII antibodies. Future studies that directly compare the clinical outcome of CD133 and CD34 sortings may deepen our understandings for effective enrichment of HSCs.

CONCLUSION

In this report, we have demonstrated that serum-free culture of the CD133-sorted human CB cells in the presence of SCF, TPO, FL, FP6, IL-3, and Delta1-Fc is an optimized condition to obtain

the highest number of ex vivo expanded HSCs. Based on this condition, some additional explorations should be considered. Increase of differentiated cells surpasses that of immature cells, which might interfere with the SRC expansion because the differentiated cells might secrete various substances that inhibit SRC expansion. Indeed, removal of differentiated cells during culture of CB Lin^- cells has been shown to have strongly positive effect on the efficient SRC expansion in a serum-free culture with 3GFs [51]. Therefore, a much higher level of SRC expansion might be possible if we apply similar differentiated cell-removal protocols in our culture condition. Culture under the hypoxic condition may also improve the expansion efficiency. In this study, we expanded HSCs by a 3-week culture system. In general, shorter ex vivo culture periods are preferable in clinical settings from the viewpoint of safety or costs. Further studies based on our results and above ideas may provide improved methods with shorter culture periods and higher expansion efficiency, which could be the most efficient ex vivo HSC expansion system for clinical applications in the future.

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AML1/Runx1 rescues Notch1-null mutation-induced deficiency of para-aortic splanchnopleural hematopoiesis

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The Notch1-RBP-Jκ and the transcription factor Runx1 pathways have been independently shown to be indispensable for the establishment of definitive hematopoiesis. Importantly, expression of Runx1 is down-regulated in the para-aortic splanchnopleural (P-Sp) region of *Notch1*- and *Rbpsuh*-null mice. Here we demonstrate that Notch1 up-regulates Runx1 expression and that the defective hematopoietic potential of *Notch1*-null P-Sp cells is successfully rescued in the OP9 culture system by retroviral transfer of Runx1. We also show that Hes1, a known effector of Notch signaling, potentiates Runx1-mediated transactivation. Together with the recent findings in zebrafish, Runx1 is postulated to be a cardinal downstream mediator of Notch signaling in hematopoietic development throughout vertebrates. Our findings also suggest that Notch signaling may modulate both expression and transcriptional activity of Runx1. (Blood. 2006;108:3329-3334)

Introduction

Mammalian hematopoietic development is believed to arise from 2 distinct cellular origins. In mice, primitive hematopoiesis arises in the yolk sac (YS) blood island at embryonic day (E) 7.5, while definitive hematopoiesis starts at the ventral region of the aorta-gonad-mesonephros (AGM) around E10.5, which shifts to the liver, spleen, and bone marrow, in this order. Progenitors for definitive hematopoiesis are first detected in the para-aortic splanchnopleural (P-Sp) region at E7.5 to E9.5,[1,2] where the *Notch1* gene has a nonredundant role in hematopoietic stem cell (HSC) development.[3] *Notch1* encodes a 300-kDa heterodimeric single-span transmembrane receptor consisting of a 180-kDa extracellular and a 120-kDa transmembrane subunit. Together with 3 other paralogs, it belongs to the evolutionarily conserved Notch family receptors that mediate cell-fate determination in multiple species. The Notch signaling is initiated by the binding of the Jagged and Delta families of ligands expressed on the neighboring cells, which induces the cleavage of the Notch transmembrane subunit and the release of the Notch intracellular domain. The latter in turn translocates to the nucleus and forms a transactivation complex by interacting with the DNA-binding protein RBP-Jκ and induces the expression of their target genes, such as those for the hairy/enhancer of split (*Hes*) family of basic helix-loop-helix transcription factors.[4] Molecular channels downstream of these, however, are largely unknown.

Mice deficient in *Runx1* (also known as *AML1*, *CBFA2*, or *PEBP2αB*), *Scl*, and *Gata2* genes are lethal during the embryonic stage and show failure in the establishment of definitive hematopoiesis.[5-7] A connection between Notch signaling and these transcription factors has been shown by the analyses of *Notch1*- and RBP-Jκ–encoding *Rbpsuh*-null mice. In the E9.5 P-Sp cells from *Notch1*-null mice, expression levels of SCL, GATA2, and Runx1 mRNA are significantly reduced.[3] *Rbpsuh*-null mice also show markedly reduced levels of SCL, GATA2, and Runx1 mRNA in the endothelial-cell layer of the E9.5 P-Sp region,[8] supporting the notion that the Notch1-RBP-Jκ pathway up-regulates the expression of these key transcription factors. Among these, Runx1, which has close homology to a *Drosophila* protein, Runt, functions as a transcriptional activator or repressor for its target genes in concert with several specific coactivators or corepressors, depending on the context.[9] Importantly, presence of the Notch-Runx pathway has been proposed in *Drosophila* embryonic hemocytogenesis[10] and zebrafish hematopoiesis during both developmental and postnatal periods.[11] Similarly reported has been transcriptional regulation by Notch of the *Gata2* gene in mouse AGM hematopoiesis[8] and of the *Gata* homolog *Serpent* gene in *Drosophila* embryonic hemocytogenesis.[12] In mammals, the existence of Notch-Runx pathway has been unclear.

In this study, we show that Notch1 up-regulates Runx1 mRNA expression in NIH3T3 cells. When introduced to the defective prehematopoietic precursor cells derived from the P-Sp region of *Notch1*-null embryos using retroviruses, Runx1, but neither SCL nor GATA2, restores the definitive hematopoiesis. We also demonstrate that Hes1, one of the Notch signal effectors, augments the transcriptional activity of Runx1 protein. These findings indicate that Runx1 is a key molecule in Notch1-RBP-Jκ–mediated mammalian hematopoiesis.

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