was administered to Patient No. 12 because CMV antigenemia persisted despite ganciclovir therapy.

VZV. Seven of 14 patients developed VZV infection at a median of 409 days (range 351–1263 days) after PBSCT. All patients were treated with either oral valacyclovir or intravenous acyclovir promptly after diagnosis. All patients had dermatomal disease, and no dissemination was observed.

AdV. Patients No. 1 and 6 developed AdV HC on days 64 and 33, respectively. Cidofovir was administered in both patients.

Fungal

No fungal infection was observed in this case series.

Discussion

We present a long-term analysis of infectious complications in AD patients who had undergone CD34-selected autologous PBSCT.

Bacterial infections were observed in 3 patients. Despite the increased frequency of isolation of viridans streptococci from the blood of neutropenic patients (19, 20), bacteremia from *S. mitis* was observed in only 2 patients in the present study. The incidence of streptococcal bacteremia (2/14; 14%) was the same as that reported in other studies (16–31%) (21, 22). Although antibacterial prophylaxis was undertaken with ciprofloxacin (23), the susceptibility of the pathogen to penicillin was intermediate in both cases; hence, vancomycin was added to the treatment regimen. *L. monocytogenes* infection in HSCT is rare (24) because TMP-SMX, traditionally used for prophylaxis, is active against the microbe. However, the patient did not take TMP-SMX when she developed *L. monocytogenes* bacteremia. As TMP-SMX is effective not only in prophylaxis for *Pneumocystis jirovecii* pneumonia but also against Listeria, Nocardia, and Toxoplasma, its prophylactic administration is mandatory.

CMV reactivation has been reported to be uncommon in unselected autologous PBSCT (25–27). However, 64% of our patients (9/14) became positive for CMV antigenemia after PBSCT and were treated with ganciclovir. Although no patient developed CMV disease, the level of antigenemia in 2 patients was so high that they might have developed CMV disease without monitoring and preemptive therapy. Although it is still not clear whether CD34 selection of autograft itself is a risk for CMV infection (7, 28), this proportion of CMV reactivation is comparable to that of CD34-selected autologous HSCT for hematological malignancies, or allogeneic HSCT (13). Thus, we consider CMV monitoring necessary in AD patients undergoing CD34-selected autologous PBSCT.

Infectious complications in autoimmune patients receiving CD34-selected transplant

Patient number	FN (days)	Bacterial infection (days)	CMV Ab	CMV (days)	VZV (days)	Others (days)
1	5	–	+	38	1263	AdV cystitis (64)
2	–	–	+	–	–	–
3	–	–	+	10	351	–
4	–	–	+	24	418	–
5	–	–	+	–	409	–
6	7	*Streptococcus mitis* (8)	+	33	–	AdV cystitis (31)
7	0	–	–	–	–	–
8	1	–	+	–	–	–
9	5	–	+	22	374	–
10	–	–	+	60	427	–
11	8	*Streptococcus mitis* (9)	+	28	–	–
12	5	*Lysteria monocytogenes* (119)	+	22	358	–
13	9	–	+	–	–	–
14	9	–	+	–	–	–

FN, febrile neutropenia; days, days after hematopoietic stem cell transplantation; CMV, cytomegalovirus infection; Ab, antibody VZV, varicella zoster virus infection; AdV, adenovirus.

Table 2

AdV HC developed in 2 of 14 patients (14%). The incidence of AdV infection in the autologous HSCT is reported to be 1% (29), and HC is a rare development. T-cell depletion and lymphopenia are the risk factors (30, 31) for AdV disease in adults, and severe lymphopenia was seen in both the patients (139 and 318/μL, respectively). They were successfully treated with cidofovir (16).

Regarding late infectious complications, 50% of the patients (7/14) developed VZV infection at a median of 409 days (range 351–1263 days) after PBSCT. Delayed recovery of CD4 + T cell (32, 33) and increased incidences of the VZV infections (8) have been reported in CD34-selected transplantation. In addition to CD34 selection of auto-graft, low-dose steroids were continued after PBSCT in our study. Delayed CD4 + T-cell recovery along with high incidence of late infection were both observed. We used prophylactic acyclovir from days 1 to 35 and did not use long-term low-dose acyclovir prophylaxis (25, 34, 35).

Data are conflicting whether CD34-selected PBSCT for hematologic malignancies and breast cancer cause increased incidence of infections compared with non-CD34-selected PBSCT. In this study, underlying AD itself, prolonged immunosuppressive therapy before PBSCT, CD34 selection of the auto-graft, and low-dose systemic steroid administration after the PBSCT might contribute to the high incidence of infectious complications.

In conclusion, our findings confirm a very high incidence of infectious complications after CD34-selected autologous PBSCT for AD. Because the risk for infections approaches that seen in allogeneic transplant recipients, infection surveillance, diagnostic workup, and prevention strategies similar to those applicable to allogeneic recipients are recommended.

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ORIGINAL ARTICLE

Rituximab for the treatment of corticosteroid-refractory chronic graft-versus-host disease

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Abstract We prospectively evaluated the safety and efficacy of the anti-CD20 chimeric monoclonal antibody rituximab for the treatment of corticosteroid-refractory chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation. Seven patients were treated with 375 mg/m^2 rituximab weekly for 4 consecutive weeks. Rituximab was well tolerated with no severe toxicity observed during treatment. At 1 year, 3 patients showed a partial response to rituximab therapy, 3 had stable disease, and 1 had progressive disease. Rituximab allowed a reduction in the dose of steroids in 4 patients. Responsive manifestations included mild to moderate skin and oral lesions, and immune hemolytic anemia, and thrombocytopenia. Severe manifestations involving the skin, fascia, and eye did not respond to treatment. These observations suggest that rituximab therapy may be effective for select patients with corticosteroid-refractory chronic GVHD that is not advanced.

Keywords Rituximab · Chronic GVHD · Corticosteroids · Allogeneic transplantation

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1 Introduction

Chronic graft-versus-host disease (GVHD) remains to be the major cause of late morbidity and mortality, and has a significant effect on the functional status and quality of life in long-term survivors after allogeneic hematopoietic cell transplantation (HSCT). Chronic GVHD is a pleiomorphic syndrome with highly variable clinical manifestations, involving the skin, liver, eyes, mouth, esophagus, lung, serosal surfaces, lower gastrointestinal tract, female genitalia, and fascia [1, 2]. Corticosteroids in addition to the continuous administration of a calcineurin inhibitor are the standard treatment for chronic GVHD. The prognosis of patients with corticosteroid-refractory chronic GVHD is extremely poor, and there is no standard treatment for these patients [1, 3].

Although the biological mechanisms leading to chronic GVHD are not well understood compared with those leading to acute GVHD, multiple cellular and humoral mechanisms are likely to be involved in chronic GVHD [4, 5]. Much evidence suggest that B cells and humoral immunity are likely to play a role in the pathogenesis of chronic GVHD; the B cell compartment paradoxically shows simultaneous B lymphocytopenia and B cell hyperreactivity manifested by the production of autoantibodies.

CD86 expression is highly upregulated in B cells upon stimulation with toll-like receptor 9 in patients with chronic GVHD, as compared to that in controls [6]. Alloantibodies specific for recipient minor histocompatibility antigens have been detected in patients with chronic GVHD, usually 4–6 months after transplantation [7, 8]. Patients with antibodies to recipient minor histocompatibility antigens also have T cells specific for the same antigens [9]. A more direct role of B cells has been suggested by experiments showing that the depletion of donor B cells can protect mice from chronic GVHD [10].

Rituximab is a chimeric mouse/human anti-CD20 monoclonal antibody. It binds with high affinity to $CD20^+$ cells and specifically depletes B cells in vivo. Several phase II studies and case series studies have suggested that rituximab may be effective in the treatment of chronic GVHD [11–17]. Such beneficial effects of B cell depletion by rituximab further emphasize a potential pathogenic role of B cells in the development of chronic GVHD. However, the organ-specific responses observed between studies are substantially different, possible, in part, because previous retrospective studies involved patients who were heavily treated with different types of immunosuppressive therapy.

Ethnicity is associated with the incidence and severity of GVHD [18]. Japanese that have remained geographically isolated for significant periods of time are likely to have less genetic diversity than other ethnic populations experiencing recent and multiple immigrations. Japanese patients receiving allogeneic HSCT have a lower incidence of acute and chronic GVHD compared with patients in Western countries [19–22]. Furthermore, immunosuppressants other than calcineurin inhibitors and corticosteroids are rarely used to prevent and treat GVHD in Japan because they have not been approved for use. Thus, Japanese patients with chronic GVHD might represent a more homogeneous population in terms of genetic background and prior therapies. Here, we prospectively evaluated the safety and efficacy of rituximab in the treatment of corticosteroid-refractory chronic GVHD in Japanese patients undergoing allogeneic HSCT.

2 Patients and methods

2.1 Patients

An open-labeled and early phase II study of rituximab therapy for corticosteroid-refractory chronic GVHD was conducted. The primary objective was to determine the safety, toxicity, and efficacy of 4 courses of rituximab therapy. Eligible subjects had extensive chronic GVHD, which had shown resistance to prednisolone (PSL) at doses greater than 0.5 mg/kg for 30 days within the previous 12 months, who were receiving a stable dose of cyclosporine (CSP) or tacrolimus (TAC). The patients excluded from the study had a previous history of HSCT, an uncontrolled infection, were carriers of hepatitis B or C viruses, and younger than 18 years. This study was approved by the Institutional Review Board of each participating institute, according to the Declaration of Helsinki, and written informed consent was obtained from each participating patient.

2.2 Rituximab therapy

The patients were premedicated with acetaminophen and diphenhydramine, and then 375 mg/m^2 rituximab was intravenously administered weekly for 4 weeks. The initial rate of infusion was 25 mg/h, which was increased to 100 mg/h if there was no reaction to the infusion. During 4 courses of treatment, all patients were required to receive a stable dose of immunosuppressive agents. Following 4 courses of rituximab therapy, decisions regarding the tapering of the dose of immunosuppressive medications were prepared by the transplant physician. The recommended sequence was the withdrawal of corticosteroids and then the withdrawal of the calcineurin inhibitors based on the resolution of chronic GVHD.

2.3 Study evaluation

The diagnosis of chronic GVHD required the presence of at least one diagnostic clinical sign of chronic GVHD or diagnostic manifestation confirmed histologically or by other relevant tests in the absence of acute characteristics of GVHD [2]. The disease was classified as limited or extensive and as de novo, quiescent, or progressive GVHD [1, 23]. Chronic GVHD was staged and graded according to National Institute of Health consensus criteria [2]. The global assessment of the severity of chronic GVHD was derived by combining organ- and site-specific scores. Each organ or site was scored according to a 4-point scale (0–3), with 0 representing no involvement and 3 representing severe impairment. In addition, performance status (PS) was evaluated on this 4-point scale. For thrombocytopenia, a score of 0 was defined as platelets $\geq 140 \times 10^9/l$, 1 as platelets $\geq 100 \times 10^9/l$, 2 as platelets $\geq 50 \times 10^9/l$, and 3 as platelets $< 50 \times 10^9/l$. For autoimmune hemolytic anemia (AIHA), a score of 0 was defined as hemoglobin ≥ 12 g/dl and a negative Coombs test result. Scores of 1, 2, and 3 were defined as hemoglobin ≥ 10, ≥ 7, and < 7 g/dl, respectively. A post-treatment evaluation was performed every week until 6 weeks and then 2, 3, 4, 6, and 12 months thereafter, which included an assessment of the severity of chronic GVHD in each organ or tissue and a safety analysis. The analysis included the monitoring of

blood counts and liver and renal function test results and documenting unexpected side effects. The severity of adverse events attributable to rituximab was evaluated on the basis of the Common Terminology Criteria for Adverse Events, version 3.0. The therapeutic response was assessed 1 year after the initiation of the study, and was defined as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). CR was defined as the resolution of all symptoms and signs of chronic GVHD. PR was defined as a partial improvement in scores of ≥ 2 for at least one organ with no progression in any other organs and no requirement of additional systemic immunosuppressive therapy for chronic GVHD. SD was defined as no change in score and no requirement of additional systemic therapy. PD was defined as the objective worsening of the disease or the need for dose escalation of immunosuppressive agents or additional systemic treatment. Statistical analysis was performed using an unpaired 2-tailed t test.

3 Results

3.1 Patient characteristics

Seven patients (5 men and 2 women; median age 48 years, age range 24–55 years) were enrolled in this study between April 2006 and March 2007. The patients' characteristics are summarized in Table 1. All patients had extensive and corticosteroid-refractory chronic GVHD after allogeneic HSCT. The diseases for which transplantation was performed were as follows: acute myelogenous leukemia (AML, $n = 3$), chronic myelogenous leukemia (CML, $n = 2$), acute lymphoblastic leukemia (ALL, $n = 1$), and myelodysplastic syndrome (MDS, $n = 1$). Four patients underwent bone marrow transplantation (BMT) from a human leukocyte antigen (HLA)-matched or HLA-DR-mismatched unrelated donor, and 3 underwent peripheral blood stem cell transplantation (PBSCT) from an HLA-matched sibling donor. Myeloablative conditioning regimens were used in 5 patients, whereas fludarabine-based reduced-intensity conditioning regimens were used in 2. GVHD prophylaxis consisted of CSP and short-term methotrexate (MTX) ($n = 4$), TAC and short-term MTX ($n = 2$), or TAC alone ($n = 1$). All patients developed acute GVHD (grade II in 6 patients and grade I in 1 patient), which was successfully treated with 1–2 mg/kg of methylprednisolone (mPSL) or PSL and subsequently developed into quiescent and extensive chronic GVHD. On the basis of the global staging system [2], 4 patients had "severe" chronic GVHD, and 3 had "moderate" disease. The median time from transplantation to study enrollment was 42 months (range 19–112 months). The median time

Table 1 Patients' characteristics

UPN	Age/sex	Diagnosis	Donors	HLA	Stem cell source	GVHD prophylaxis	Type of onset	Prior therapy	Interval from transplantation to rituximab (months)	Interval from onset of chronic GVHD to rituximab (months)
1	24/F	CML	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, CSP	19	8
2	39/M	MDS	Unrelated	Identical	BM	TAC+MTX	Quiescent	PSL, pulse mPSL, CSP, TAC	42	39
3	48/M	AML	Unrelated	Identical	BM	TAC	Quiescent	PSL, TAC	46	43
4	51/M	CML	Unrelated	DR mismatch	BM	TAC+MTX	Quiescent	PSL, CSP, TAC	112	109
5	55/F	AML	Unrelated	Identical	BM	CSP+MTX	Quiescent	PSL, CSP	34	30
6	55/M	AML	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, CSP	47	37
7	29/M	ALL	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, mPSL, CSP	27	25

from the onset of chronic GVHD to study enrollment was 37 months (range 8–109 months). In all patients, prior therapy for chronic GVHD was a combination of corticosteroid and CSP or TAC. None of the patients received other immunosuppressive medications. The intervals between dose escalations of corticosteroids and rituximab administration were at a minimum of 1 month. All subjects were followed for 1 year after the initiation of rituximab therapy.

3.2 Toxicity

All patients completed a 4-week course of rituximab treatment. Only one patient developed grade 2 allergic toxicity, i.e., an infusion reaction after the first dose of rituximab. None of the patients developed grade 3 or 4 adverse events attributable to rituximab during the 4-week treatment. Later adverse events, occurring within 1 year of the initiation of therapy, included the following: grade 3 bacterial infection that required intravenous administration of cephepim in 1 patient at 2 months, grade 2 herpes simplex virus infection that required treatment with valaciclovir in 1 patient at 4 months, grade 1 hepatic injury in 1, and grade 2 renal damage in 1. These adverse events were likely related to other drugs that were used or to pronounced immune suppression related to transplantation and chronic GVHD.

3.3 Efficacy

All patients were evaluable for their response to rituximab therapy at 1 year after the study initiation (Table 2). Unique patient number (UPN) 1 developed skin sclerosis, which was initially treated with 0.5 mg/kg of PSL. Six month later, her chronic GVHD progressed to "severe" skin sclerosis and contracture. Chronic GVHD initially responded to rituximab with an improvement of symptoms, leading to successful tapering of PSL by 67% over 6 weeks. However, sclerosis progressed thereafter, and the PSL dose was increased. The PSL dose was subsequently reduced again by 67% of the initial dose at 1 year, at which time the global staging and organ-specific scores were unchanged as compared to those before rituximab therapy. The overall response at 1 year was classified with PD because of the need for an escalation in the dose of PSL. UPN 2 developed chronic GVHD in the skin and mouth, which was initially responded to 250 mg of mPSL. Skin and oral lesions were exacerbated 10 months before enrollment to this study. CSP was replaced with TAC and PSL dose was increased to 0.5 mg/kg, but chronic GVHD progressed to "moderate" cutaneous and oral disease. Rituximab therapy was started, but was not effective. However, the disease was stable during the study period without the need for an escalation in dose of CSP and PSL.

UPN 3 developed extensive chronic GVHD, including cutaneous, oral, and hepatic lesions, and autoimmune hemolytic anemia (AIHA) and immune thrombocytopenia. This patient had steroid-induced diabetes mellitus and a history of tuberculosis. The patient was initially treated with 1 mg/kg of PSL. Three months before study enrollment, PSL was increased to 0.8 mg/kg, which was maintained until study entry according to the past history of exacerbation with less doses of PSL. Rituximab therapy improved "severe" GVHD to "moderate" GVHD, and allowed an 82% reduction in the dose of PSL within 1 year of the study.

UPN 4 had a 9-year history of chronic GVHD. The most severe manifestation was slowly progressive sclerodermatous lesions in the cervical and lower facial skin and fascia, which resulted in severe flexion and rotation contracture and difficulty in mouth opening and swallowing. Rituximab therapy failed to improve these manifestations, but the disease did not progress during the study period with stable doses of CSP and PSL. However, the patient required additional immunosuppressive therapy with high-dose cyclophosphamide 17 months after rituximab therapy and died of bacterial pneumonia, which developed during cyclophosphamide-induced neutropenia.

UPN 5 had "severe" sclerodermatous skin lesions in both the upper and lower extremities. The patient also had recurrent pleural effusion and ascites and a motility disorder of the intestine. The patient was initially treated with 0.5 mg/kg of PSL. Nine months before study enrollment, the disease was deteriorated and PSL dose was increased to 0.5 mg/kg, which was discontinued before rituximab therapy because of a lack of improvement and steroid intolerance. Rituximab therapy temporally improved serositis and diarrhea, but global staging and organ-specific scores were unchanged at 1 year. The patient died of bacterial pneumonia 19 months after the initiation of rituximab therapy.

UPN 6 developed corticosteroid-refractory chronic GVHD in the skin, mouth, eyes, and muscles. Rituximab improved these symptoms, and the patient was able to discontinue PSL by 1 year. Interestingly, the patient developed conductive hearing loss due to inflammation in the bilateral middle ear at the onset of chronic GVHD. The patient recovered dramatically from deafness after the fourth dose of rituximab therapy. UPN 7 developed cutaneous chronic GVHD and treated with PSL. The disease was progressed to sclerodermatous skin disease and the patient was started on 2 mg/kg of mPSL, which was reduced due to a lack of improvement and the patient entered to this study. Sclerodermatous skin lesion improved slowly after rituximab therapy and disappeared

Table 2 Response to rituximab therapy

UPN	Pretreatment			2 months			1 year				
	Global staging	Organ/ manifestation	Score	Global staging	Score	% PSL reduction	Global staging	Score	% PSL reduction	Global response	Follow-up
1	Severe	PS	1	Severe	1	67	Severe	1	67	PD	Alive at 36 months
		Skin	2		2			2			
		Mouth	1		1			1			
		Joints and fascia	3		3			3			
2	Moderate	PS	1	Moderate	1	0	Moderate	1	0	SD	Alive at 35 months
		Skin	2		2			2			
		Mouth	2		2			2			
3	Severe	PS	1	Moderate	1	40	Moderate	1	72	PR	Alive at 34 months
		Skin	1		1			1			
		Mouth	1		1			1			
		Liver	3		2			2			
		Thrombocytopenia	2		1			1			
		AIHA	1		0			0			
4	Severe	PS	1	Severe	1	0	Severe	1	0	SD	Died of infection at 20 months
		Skin	3		3			3			
		Eye	1		1			1			
		Joints and fascia	3		3			3			
5	Severe	PS	2	Severe	2	–	Severe	2	–	SD	Died of infection at 19 months
		Skin	3		3			3			
		Eye	1		1			1			
		Intestine	1		1			1			
		Joints and fascia	1		1			1			
		Serositis	2		2			2			
		Thrombocytopenia	2		2			1			
6	Moderate	PS	2	Moderate	1	0	Moderate	1	100	PR	Alive at 30 months
		Skin	2		1			1			
		Mouth	2		1			1			
		Eye	2		1			1			
		Muscle	1		0			0			
7	Moderate	PS	1	Moderate	1	0	Moderate	1	25	PR	Alive at 23 months
		Skin	2		2			0			
		Mouth	1		1			1			
		Eye	2		2			2			
		Joints and fascia	1		1			0			

at 1 year, although dry eye and oral mucositis did not improve.

Overall, none of the patients achieved a CR, whereas a PR was noted in 3 patients. SD was noted in 3 patients and PD in 1. One year after rituximab therapy began, PSL was discontinued or reduced in 4 of 6 patients; the median reduction rate was 67% (range 0–100%). None of the 7 patients required additional immunosuppressive therapy within 1 year after the initiation of the study. At a median follow-up of 30 months, 5 patients were alive with active and continuing chronic GVHD, and 2 had died of infection after the study period.

On the basis of global staging, only 1 patient with "severe" disease improved to "moderate" disease at 1 year, whereas 3 others with "severe" disease experienced no change. Patients with severe (score 3) skin sclerosis and joint contracture related to sclerodermatous skin GVHD and fascitis did not respond to rituximab therapy. One patient with severe (score 3) hepatic GVHD responded partially to rituximab therapy. Clinical responses were

observed primarily in patients with moderate (score 2) to mild (score 1) manifestations. It is noteworthy that 6 of 11 manifestations with a score 2 responded to rituximab therapy. Improvement in the skin, mouth, eye, liver, joints and fascia, intestine, and serous membrane was observed in 2 of 7, 1 of 5, 1 of 4, 1 of 1, 1 of 4, 0 of 1, and 0 of 1 cases, respectively. Notably, all cases of immune thrombocytopenia and anemia were responded well to rituximab. However, PS improved only in 1 patient who achieved a PR.

3.4 Immunological monitoring

B cell numbers were monitored after rituximab therapy using a flowcytometric analysis. $CD19^+$ B cells were quickly eliminated within 2 weeks after the first treatment and did not repopulate at least by 12 weeks (Fig. 1). Serum levels of IgG and IgA were unchanged by 6 weeks, but gradually declined thereafter. Serum IgM levels decreased much earlier and more profound compared with those of IgG and IgA.

4 Discussion

The prognosis of corticosteroid-refractory chronic GVHD is poor, and no standard therapy for corticosteroid-refractory chronic GVHD is available [1, 3]. In the present study, we evaluated the efficacy and safety of rituximab therapy in patients with quiescent-type chronic GVHD. This condition may have been related to the ethnicity of the transplant patients. The incidence of progressive-type chronic GVHD is high, reportedly 10–70% in Western countries [24, 25]. In contrast, progressive-type GVHD is rare and quiescent-type GVHD is common in Japanese patients [22]. Rituximab therapy was well tolerated, and no severe adverse events were attributed to rituximab therapy. A 4-week course of rituximab treatment produced an overall response rate of 43% at 1 year, which is slightly lower than

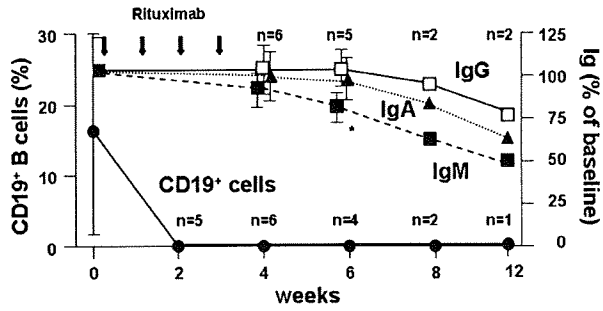

Fig. 1 Laboratory parameters over time after rituximab therapy. IgG, IgA and IgM levels are shown as percentage of baseline levels.*$P < 0.01$ compared with IgG or IgA

the overall response rate of 50–83% reported in previous studies testing the efficacy of rituximab [11–13, 15–17]. CR rates ranged from 0 to 20% in previous studies [12, 13, 16, 17]. In the present study, none of the patients achieved CR. The steroid-sparing effect is an important indicator of efficacy assessments of GVHD [26]. Rituximab therapy resulted in a median reduction in the dose of corticosteroids of 67%, which was slightly lower than the 75–86% reduction in dose observed in 3 previous studies that addressed the steroid-sparing effect of rituximab in this setting [13, 15, 16]. These results were surprising because we initially hypothesized that rituximab would be more effective in Japanese patients who tend to develop less severe chronic GVHD than Caucasians [22].

Previous studies of the efficacy of rituximab therapy for steroid-resistant chronic GVHD highlight the potential activity of rituximab against skin involvement, including scleroderma, whereas the responses to rituximab appear to be less pronounced in other organs or tissues [11–17]. These studies also suggested that the steroid-sparing effect might be more pronounced in the skin and oral lesions than others in chronic GVHD [15, 16]. In addition, hematologic abnormalities associated with chronic GVHD also respond well to rituximab therapy [11, 15, 27]. In our study, rituximab was most effective against immune thrombocytopenia and AIHA, and less effective against skin sclerosis and joint contracture related to sclerodermatous skin lesions and fascitis. This discrepancy between the current study and previous studies might have resulted because more patients with advanced sclerodermatous chronic GVHD were enrolled in our study than in the previous studies. The interval between the time of the onset of chronic GVHD and the time of study enrollment was longer in the present study (median duration 37 months) than in most of the previous studies (median duration 14–37 months) [11–13, 15, 17]. Nonetheless, our patients had undergone less immunosuppressive therapy before study enrollment than did the patients in the previous studies, most of whom had received multiple courses of immunosuppressive therapy [11, 12, 15]. Thus, the long-term duration of disease without sufficient intervention might have resulted in the development of irreversible damage in our patients.

Many advanced manifestations in chronic GVHD are potentially irreversible, including skin and joint contracture, chronic dry eye, esophageal and vaginal stricture, and bronchiolitis obliterans in the lung. The enrollment of patients with advanced chronic GVHD may not be appropriate when the endpoint of the study is the response to treatment. Alternatively, irreversible lesions could be excluded from consideration in the assessment of response [28, 29]. Such considerations were not specified in our protocol. The results of our study suggest that rituximab

may be more effective against mild to moderate manifestations than against severe manifestations of chronic GVHD. Thus, earlier treatment with rituximab or with other investigational agents for corticosteroid-refractory chronic GVHD may increase the chances of a good response. Another possible explanation for the poorer response to rituximab in our study than in previous studies, although unlikely, is that dominant immunological mechanisms associated with chronic GVHD and treatment outcomes may differ by ethnicity, because the prognostic scoring system [25], which was developed on the basis of clinical findings in Western patients, is not prognostic in Japanese patients [22].

We confirmed complete depletion of B cells after rituximab therapy. B cells were still absent 2 months after the last infusion of rituximab. In the initial multi-institutional trial evaluating a single four dose course of rituximab in patients with follicular lymphoma, the median B cell count did decline to almost undetectable levels after the first dose in the majority of patients, with recovery beginning from 6 to 9 months post-treatment, and return to normal levels between 9 and 12 months [30]. Similarly, B cells were undetectable in patients with chronic GVHD until 1 year after rituximab therapy [13]. Such a profound and prolonged B cell depletion may explain why rituximab treatment is effective in several antibody-mediated autoimmune diseases with some responses ongoing for more than 1–2 years [31]. On the other hand, rituximab therapy could results in impaired humoral immune responsiveness [32]. We also found that serum immunoglobulin levels decrease after rituximab therapy. Of note, IgM fell much more than IgG and IgA. This phenomenon was observed in patients with rheumatoid arthritis and chronic GVHD [13, 33]. This may be due to higher sensitivity of IgD^+ memory B cell subset, which produces natural mutated IgM antibodies as a first-line of defense against blood-borne antigens [33, 34], to rituximab than plasma cells.

In conclusion, the current study suggests that rituximab therapy may be effective for selective patients with corticosteroid-refractory chronic GVHD that is not advanced. A recent study indicated that that low-dose rituximab therapy is also effective [17]. However, the optimal schedule and dosing regimens for rituximab need to be determined. Furthermore, a well-designed, large-scale, prospective study is needed to conclusively address the efficacy of rituximab in the treatment of corticosteroid-refractory chronic GVHD.

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Human Flt3 Is Expressed at the Hematopoietic Stem Cell and the Granulocyte/Macrophage Progenitor Stages to Maintain Cell Survival[1]

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FLT3/FLK2, a member of the receptor tyrosine kinase family, plays a critical role in maintenance of hematopoietic homeostasis, and the constitutively active form of the FLT3 mutation is one of the most common genetic abnormalities in acute myelogenous leukemia. In murine hematopoiesis, Flt3 is not expressed in self-renewing hematopoietic stem cells, but its expression is restricted to the multipotent and the lymphoid progenitor stages at which cells are incapable of self-renewal. We extensively analyzed the expression of Flt3 in human (h) hematopoiesis. Strikingly, in both the bone marrow and the cord blood, the human hematopoietic stem cell population capable of long-term reconstitution in xenogeneic hosts uniformly expressed Flt3. Furthermore, human Flt3 is expressed not only in early lymphoid progenitors, but also in progenitors continuously along the granulocyte/macrophage pathway, including the common myeloid progenitor and the granulocyte/macrophage progenitor. We further found that human Flt3 signaling prevents stem and progenitors from spontaneous apoptotic cell death at least through up-regulating Mcl-1, an indispensable survival factor for hematopoiesis. Thus, the distribution of Flt3 expression is considerably different in human and mouse hematopoiesis, and human FLT3 signaling might play an important role in cell survival, especially at stem and progenitor cells that are critical cellular targets for acute myelogenous leukemia transformation. *The Journal of Immunology*, 2008, 180: 7358–7367.

Hematopoiesis is one of the most intensely studied stem cell systems where hematopoietic stem cells (HSCs)[3] self-renew, generate a variety of lineage-restricted progenitors, and continuously supply all types of mature blood cells. The technical advances of the multicolor FACS and the use of mAbs have enabled the prospective isolation of hematopoietic stem and progenitor cells according to the surface marker expression. In mice, multipotent hematopoietic activity resides in a small fraction of bone marrow (BM) cells lacking the expression of lineage-associated surface marker (Lin) but expressing high levels of Sca-1 and c-Kit (1, 2). Within the c-Kit$^+$Lin$^-$Sca-1$^+$ (KLS) fraction, the most primitive self-renewing HSCs with long-term reconstituting activity (LT-HSCs) do not express murine (m) CD34, but they do express mCD38 and a low level of mCD90 (Thy1), whereas mCD34$^+$, mCD38$^-$, or mThy1$^-$ KLS cells are short-term HSCs (ST-HSCs) or multipotent progenitors that do not self-renew (3–5). Downstream of the mCD34$^+$ ST-HSC stage, common lymphoid progenitors (CLPs) (6) and common myeloid progenitors (CMPs) (7) that can differentiate into all lymphoid cells and myelo-erythroid cells, respectively, have been purified. CMPs differentiate into granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs), both of which are also prospectively isolatable by FACS (7).

Interestingly, the expression pattern of these surface markers in early stem and progenitor populations are considerably different in human (h) hematopoiesis. In humans, LT-HSCs express hCD34 (8). The hLT-HSC resides in the hCD34$^+$hCD38$^-$ (9, 10) or the hCD34$^+$hCD90$^+$ (11–13) fractions in both human BM and cord blood (CB). It is still unclear what percent of hCD34$^+$hCD38$^-$ or hCD34$^+$hCD90$^+$ cells are LT-HSCs in human hematopoiesis. The human counterpart for mCMPs, mGMPs, mMEPs, or mCLPs is also isolatable in the BM and the CB within the hCD34$^+$hCD38$^+$ progenitor fraction (14, 15). It has thus been suggested that, despite the difference in the expression patterns of key Ags in human and mouse hematopoiesis, lineage commitment processes from HSCs to mature blood cells might be generally preserved in both species. For example, the existence of prospectively isolatable CMPs and CLPs suggests that lineage commitment from HSCs involves myeloid vs lymphoid bifurcation in both mouse and human.

Recently, two independent groups have reported that in murine hematopoiesis, Flt3/Flk2, a tyrosine kinase receptor, is expressed in ST-HSCs but not in LT-HSCs. One group showed that mCD34$^-$

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[3] Abbreviations used in this paper: HSC, hematopoietic stem cell; AML, acute myelogenous leukemia; BM, bone marrow; KLS, c-Kit$^+$Lin$^-$Sca-1$^+$; LT-HSC, HSC with long-term reconstituting activity; ST-HSC, short-term HCS; m, murine; h, human; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GM, granulocyte/macrophage; GMP, GM progenitor; MEP, megakaryocyte/erythrocyte progenitor; CB, cord blood; MegE, megakaryocyte/erythrocyte; FL, Flt3 ligand; PI, propidium iodide; SCF, stem cell factor; Tpo, thrombopoietin; Epo, erythropoietin; CFU-GEMM, CFU-granulocyte/erythroid/macrophage/megakaryocyte; RTK, receptor tyrosine kinase; ITD, internal tandem duplication.

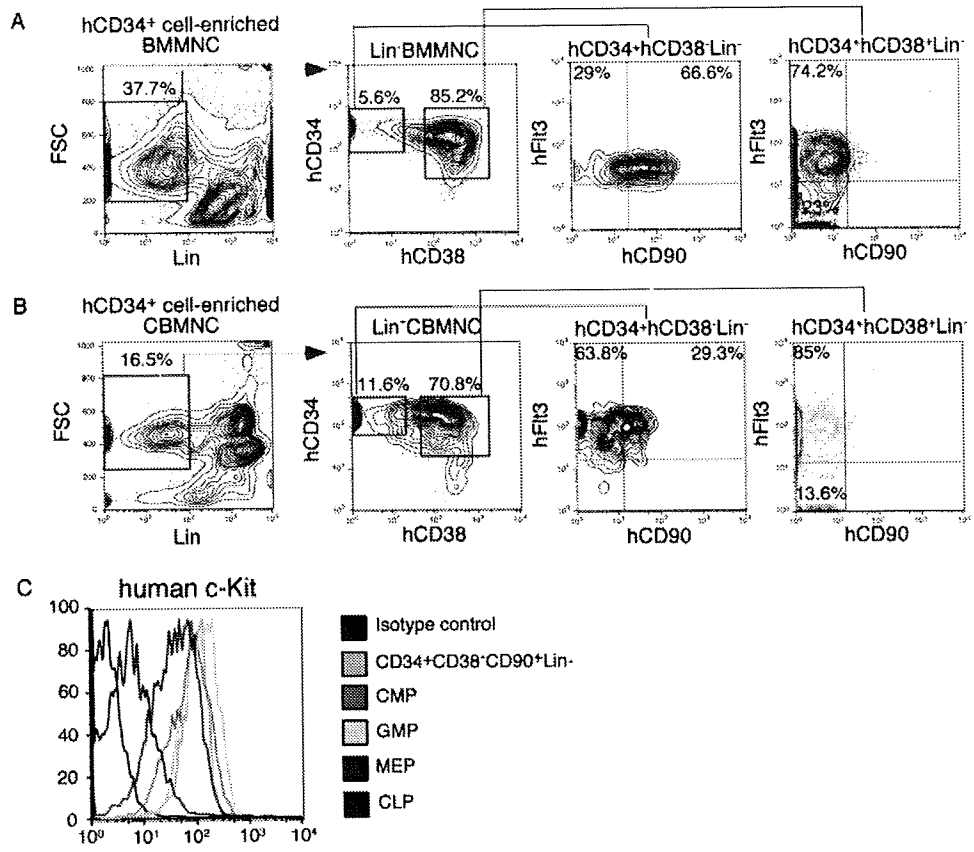

FIGURE 1. A flow cytometric analyses of human early hematopoietic populations in the BM and the CB. In $hCD34^+hCD38^-$ immature BM (*A*) and CB (*B*) cells, hFlt3 was expressed at a low level in both hCD90 (Thy1) positive and negative fractions. In contrast, the $hCD34^+hCD38^+$ BM and CB progenitor populations did not express hCD90, and hFlt3 was expressed in only a fraction of these populations. *C*, hHSCs and myeloid progenitors expressed c-Kit at high levels, and CLPs at a low level. Representative data of independent five experiments are shown here.

KLS cells (LT-HSCs) are $mFlt3^-$ (16), and the other showed that only the $mFlt3^-$ fraction of $mCD90^{low}$ KLS cells possesses LT-HSC activity (17). Each group further studied the detailed differentiation activity of $mFlt3^+$ KLS cells, but drew different conclusions. Adolfsson et al. (18) reported that the $mFlt3^+mCD34^+$ KLS population maintains the granulocyte/macrophage (GM) and the T/B lymphoid, but not the megakaryocyte/erythrocyte (MegE) potential, if any. This result suggests that, in addition to the lymphoid vs myeloid developmental pathway represented by CLPs and CMPs, respectively, there is a critical stage common to GM, T, and B lymphoid cells. The other group, however, showed that $mFlt3^+mCD90^-$ KLS cells are multipotent, thus claiming that the stage common to GM/lymphoid lineages proposed by Adolfsson et al. (18) does not constitute a major pathway for hematopoietic development (19). In contrast, downstream of the mST-HSC stage, there is a general agreement that mFlt3 is expressed in progenitors with lymphoid potential, such as the majority of CLPs and a minor fraction of CMPs, that retain a weak B cell potential (20), whereas it is down-regulated in late myeloid stages, such as GMPs and MEPs (20, 21). The Flt3 ligand (FL) is required for development of CLPs from $mFlt3^+$ KLS cells, whereas mFlt3 is dispensable for HSC maintenance and myeloid development (22). These results suggest that in mouse hematopoiesis, Flt3 signaling plays an important role in lymphoid, but not in HSC or myeloid, development.

The precise expression and the role of hFlt3 in human hematopoiesis, however, remain unclear. Around 40–80% of $hCD34^+$ BM and CB cells express hFlt3 (23, 24). Although a fraction of both the $hFlt3^+$ and the $hFlt3^-$ populations gave rise to multilineage "mixed" colonies containing all myelo-erythroid components, the $hFlt3^+hCD34^+$ and $hFlt3^-hCD34^+$ populations predominantly formed GM and erythroid colonies, respectively (23–25). It has also been shown that cells with NOD/SCID reconstitution activity reside in the $hCD34^+hFlt3^+$ fraction (24). These data collectively suggest that LT-HSCs and GMPs may reside mainly in the $hFlt3^+hCD34^+$ fraction, whereas MEPs may be contained in the $hFlt3^-hCD34^+$ fraction. Therefore, the expression pattern of Flt3 could be quite different in mouse and human hematopoiesis. Flt3 expression has also been implicated in development of human acute myelogeneous leukemia (AML). Flt3 is expressed in leukemic blasts in most cases with AML (26, 27). Furthermore, *FLT3* is one of the most frequently mutated genes in AML (28, 29), and the FLT3 mutants transduce the constitutively active FLT3 signaling, that could be the cause of poor prognosis in AML with *FLT3* mutations (30–32).

In this study, we extensively analyzed the expression and function of hFlt3 in steady-state human BM and CB hematopoiesis. Interestingly, hFlt3 was expressed in the entire human BM and CB HSC population, and purified $hFlt3^+$ HSCs could reconstitute multilineage cells for a long-term in our xenogeneic transplantation system (33). Therefore, unlike mouse hematopoiesis, the negative expression of Flt3 does not mark LT-HSCs in human. Furthermore, in striking contrast to mouse hematopoiesis where mFlt3 is expressed in CLPs but not GMPs (20, 21), hFlt3 was expressed in GMPs as well as in CLPs at a high level. The hFlt3 signaling did not affect the lineage fate decision of hHSCs, but supported cell survival of $hFlt3^+$ stem and progenitor cells, at least through the up-regulation of Mcl-1, a survival promoting Bcl-2 homologue (34). These data collectively suggest that Flt3 signaling plays a critical role in maintenance of self-renewing LT-HSCs, and of GM and lymphoid progenitors in human hematopoiesis.

Materials and Methods

BM and CB samples

Fresh human steady-state BM and CB samples were collected from healthy adults and newborns after normal deliveries. Informed consent was obtained from all subjects. The Institutional Review Board of each institution participating in this project approved all research on human subjects.

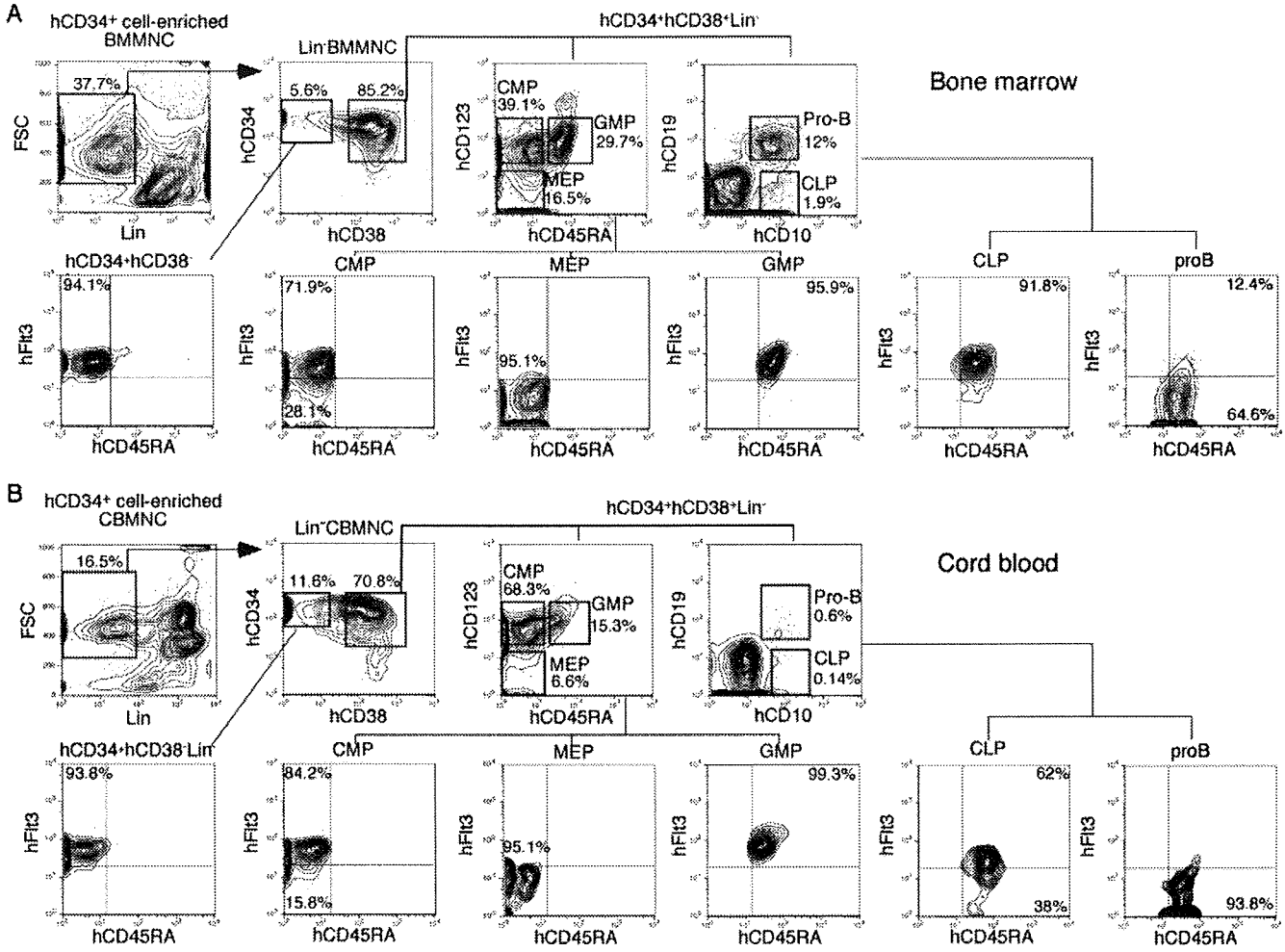

FIGURE 2. The expression patterns of hFlt3 are similar in early human BM (*A*) and CB (*B*) hematopoiesis. In the myeloid pathway in both the BM and CB, hFlt3 was up-regulated into the GM pathway, but was down-regulated in the MegE pathway; GMPs expressed hFlt3 at a high level, whereas MEPs did not express hFlt3. CMPs contained both $hFlt3^+$ and $hFlt3^-$ fractions. In the lymphoid pathway, CLPs expressed hFlt3 at a high level in BM (*A*) and a low level in CB (*B*), whereas $hCD10^+hCD19^+$ proB cells did not express hFlt3 in either the BM or the CB. Representative data of independent five experiments are shown here.

Cell preparation, flow cytometric analysis, and cell sorting

The BM and CB mononuclear cells were prepared by gradient centrifugation and the $CD34^+$ cells were enriched from mononuclear cells by using the Indirect CD34 MicroBead kit (Miltenyi Biotec) as described previously (14). For the analyses and sorting of myeloid progenitors, cells were stained with a Cy5-PE- or PC5-conjugated lineage mixture, including anti-hCD3 (HIT3a), hCD4 (RPA-T4), hCD7, hCD8 (RPA-T8), hCD10 (HI10a), hCD19 (HIB19), hCD20 (2H7), hCD11b (ICFR44), hCD14 (RMO52), hCD56 (NKH-1), and hGPA (GA-R2), FITC-conjugated anti-hCD34 (8G12), or anti-hCD45RA (HI100), PE-conjugated anti-hFlt3 (CD135) (4G8), or anti-hCD123 (6H6), allophycocyanin-conjugated anti-hCD34 (8G12), or anti-hCD38 (HIT2), Pacific Blue-conjugated anti-hCD45RA (HI100), and biotinylated anti-hCD38 (HIT2), or anti-hCD123 (9F5). The lymphoid progenitors were stained with the same lineage mixture except for the omission of anti-hCD10 and hCD19 followed by FITC-conjugated anti-hCD10 (SS2/36), PE-Cy7-conjugated anti-hCD19 (SJ25C1), and anti-hFlt3, hCD34, hCD38, and hCD45RA as described above. For additional analyses, PE-Cy7-conjugated anti-hCD34 (8G12), FITC-conjugated anti-hCD90 (5E10), PE-conjugated anti- hCD117 (YB5.B8), biotinylated anti-hFlt3 (BV10A4H2), and PE-conjugated anti-hCD127 (R34.34) mAbs were used. Streptavidin-conjugated allophycocyanin-Cy7 or PE-Cy7 was used for visualization of the biotinylated Abs (BD Pharmingen). The dead cells were excluded by propidium iodide (PI) staining. Appropriate isotype-matched, irrelevant control mAbs were used to determine the level of background staining. The cells were sorted and analyzed by FACS Aria (BD Biosciences). The sorted cells were subjected to an additional round of sorting using the same gate to eliminate contaminating cells and doublets. For single-cell assays, the re-sort was performed by using an automatic cell-deposition unit system (BD Biosciences).

In vitro assays to determine the differentiation potential of progenitors

Clonogenic CFU assays were performed using a methylcellulose culture system that was set up to detect all possible outcomes of myeloid differentiation as reported previously (14, 35). For myeloid colony formation, cells were cultured in IMDM-based methylcellulose medium (Methocult H4100; StemCell Technologies) supplemented with 20% FCS, 1% BSA, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics in the presence of human cytokines such as IL-3 (20 ng/ml), stem cell factor (SCF) (20 ng/ml), FL (20 ng/ml), IL-11 (10 ng/ml), thrombopoietin (Tpo) (50 ng/ml), erythropoietin (Epo) (4 U/ml), and GM-CSF (50 ng/ml). All cytokines were obtained from R&D Systems. Colony numbers were enumerated on day 14 of culture. For the short-term liquid cell culture, cells were cultured in IMDM with 10% FCS in the presence of the cytokines described above. All of the cultures were incubated at 37°C in a humidified chamber under 5% CO_2.

Apoptosis assay and cytokine stimulation assays in the serum-free medium

To exclude the unexpected effects of FCS and to evaluate the effects of cytokine stimulation precisely, the cells were prepared in the FCS-free condition. The anti-apoptotic effect of FL and SCF was evaluated after 24 h

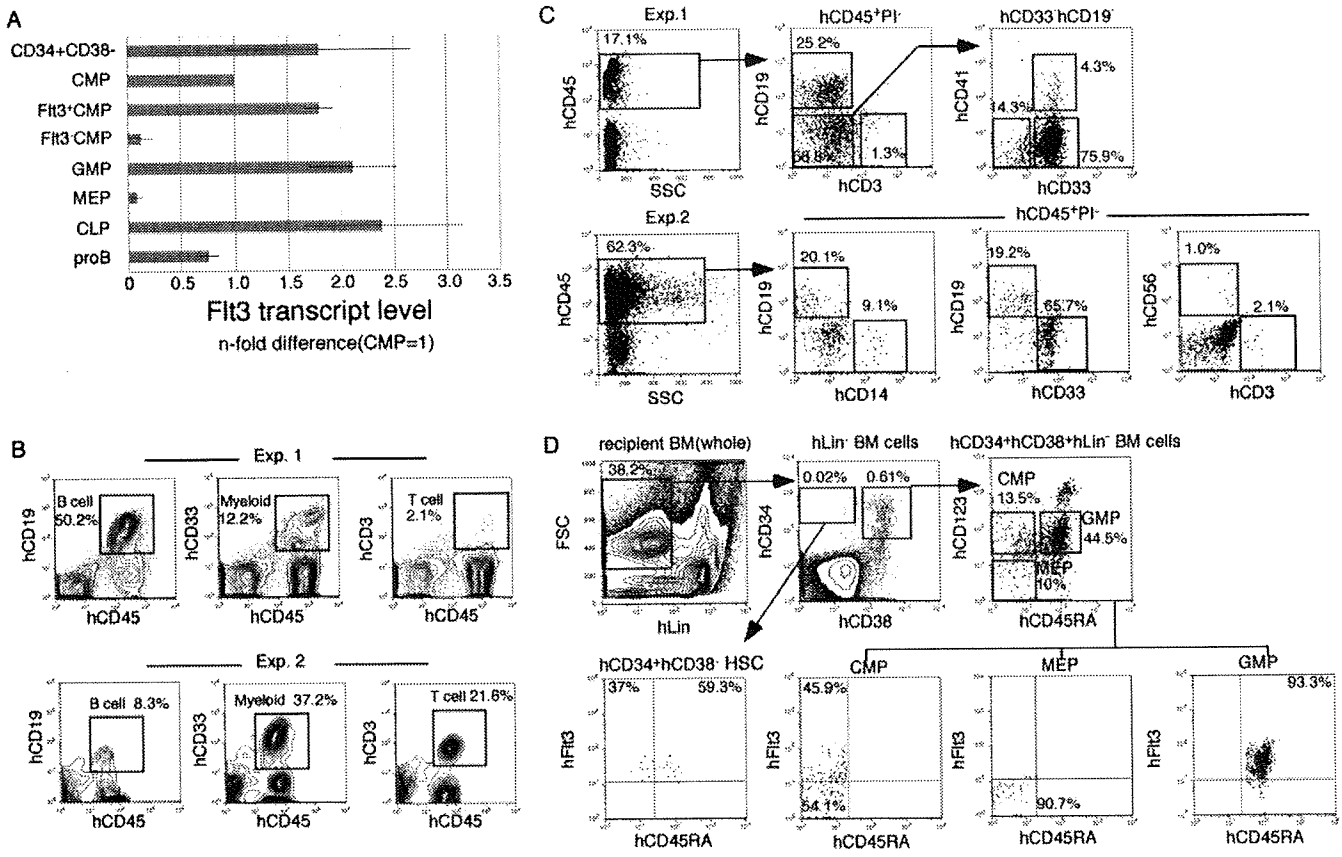

FIGURE 3. Long-term reconstitution potential of hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$Lin$^-$ cells in NOD/SCID/IL2rγ^{null} newborn mice. *A*, Analyses of the relative expression levels of hFlt3 transcript by real-time PCR. Each bar shows the *n*-fold difference of the level of hFlt3 mRNA in comparison to that of the whole CMP. The mean value and SD of BM samples from three independent normal donors are shown. Note that the levels of hFlt3 transcripts are well correlated with those of surface hFlt3 expression determined by FACS (Fig. 2*A*). *B*, The long-term and multilineage reconstitution of human cells in mice injected with 1×10^3 hFlt3$^+$hCD34$^+$hCD38$^-$Lin$^-$ CB cells 4 (*upper panels*) or 6 (*lower panels*) mo after transplantation. Representative two results out of five experiments are shown. *C*, Multilineage reconstitution 6 (*upper panels*) and 15 wk (*lower panels*) after i.v. injection of 5×10^3 hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$Lin$^-$ BM HSCs into NOD/SCID/IL2rγ^{null} newborns. Donor-derived viable human cells were evaluated as hCD45$^+$PI$^-$ cells. hCD33$^+$ granulocytes, hCD14$^+$ monocytes, hCD41$^+$ megakaryocytes, hCD19$^+$ B cells, hCD3$^+$ T cells, and hCD56$^+$ NK cells were detected in the BM of recipient mice. *D*, Stem and progenitor analyses of BM from mice reconstituted with hFlt3$^+$ HSCs. The BM contained hFlt3$^+$hCD34$^+$hCD38$^-$ HSCs, and all types of myeloid progenitors within the hCD34$^+$hCD38$^+$ population, including hCD45RA$^-$hCD123^{low} CMPs, hCD45RA$^+$hCD123^{low} GMPs, and hCD45RA$^-$hCD123$^-$ MEPs. The expression patterns of hFlt3 in each population were identical with those of freshly isolated stem and progenitor cells. A representative experiment by using BM samples from three independent normal donors is shown.

serum-free liquid culture, using Annexin V and PI staining (BD Pharmingen). The sorted cells were cultured in the serum-free medium (STEMPRO-34 SFM; Invitrogen) with or without FL (20 ng/ml) and/or SCF (20 ng/ml) for 24 h. The living cells were defined as Annexin V$^-$/PI$^-$ among the live-gated cells (as shown in Fig. 5*B*). For the cytokine stimulation assays, cells were sorted in the IMDM and then the cytokines were added.

In vivo assays to determine the differentiation potential and reconstitution capacity

The NOD.Cg-Prkdc^{scid}IL-2rg^{tm1Wjl}/Sz (NOD/SCID/IL2rγ^{null}) mice were developed at The Jackson Laboratory. The NOD/SCID/IL2rγ^{null} strain was established by backcrossing a complete null mutation at γc locus (36) onto the NOD.Cg-Prkdc^{scid} strain. The establishment of this mouse line has been reported elsewhere (37). For the reconstitution assays, the sorted cells were transplanted into irradiated (100cGy) NOD/SCID/IL2rγ^{null} newborns via a facial vein within 48 h of birth. To confirm the long-term reconstitution by hHSCs, the chimerism of circulating human blood cells were analyzed until at least 24 wk after transplantation, as previously reported (33). In addition to the Abs described above, the following mAbs were used: allophycocyanin-conjugated anti-hCD45 (J33), PE-Cy7-conjugated anti-hCD123 (6H6), FITC-conjugated anti-hCD33 (HIM3–4) or hCD14 (M5E2), and PE-conjugated anti-hCD41 (VIPL3), hCD56 (B159), anti-hGlycophorin A (GPA) (GA-R2), or anti-hCD3 (HIT3a).

Quantitative real-time PCR

To examine the gene expression profile of each population, RNA was isolated from 2,000-sorted cells using Isogen reagent (Nippon gene) according to the manufacturer's instructions. The total RNA was reverse transcribed to cDNA using a TaKaRa RNA PCR kit (Takara Shuzo). The mRNA levels were quantified in triplicate using a real-time PCR (7500 Real-Time PCR system; Applied Biosystems). hβ2-microglobulin mRNA was separately amplified in the same plate to be used for internal control. The primer and probes were designed by Primer Express software (Applied Biosystems).

Results

The hCD34$^+$hCD38$^-$ HSC fraction express hFlt3 at a low level in both BM and CB

The hCD34$^+$Lin$^-$ population was divided into hCD38$^+$ and hCD38$^-$ populations (Fig. 1, *A* and *B*). It has been shown that HSCs with long-term reconstitution activity reside in the hCD38$^-$ fraction within the hCD34$^+$ BM and CB populations (9, 10). As shown in Fig. 1*A*, in the BM, hCD38$^-$ cells constituted only ~5% of the Lin$^-$hCD34$^+$ population. This population uniformly expressed hFlt3 at a low level. More than

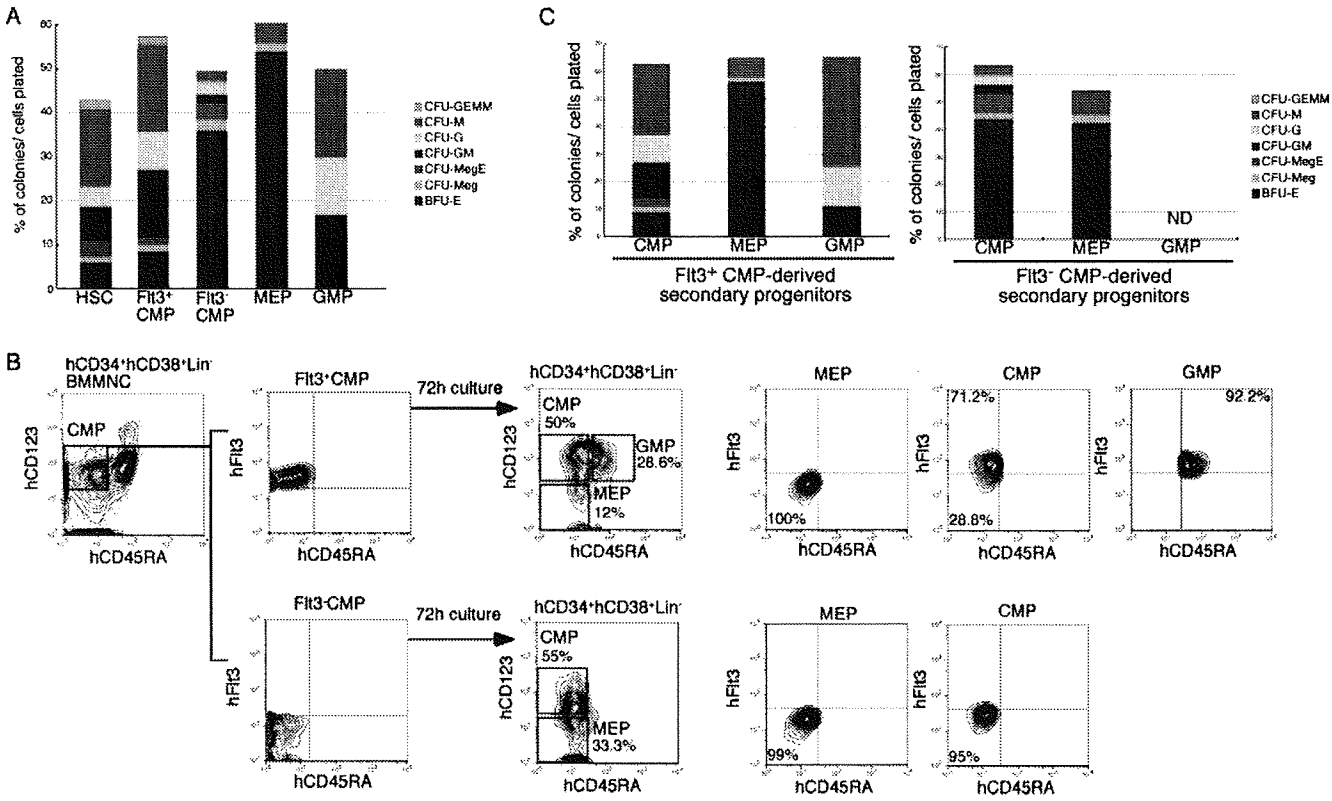

FIGURE 4. The lineage potential and the relationship of myeloid progenitor populations. *A*, Clonogenic colony formation of purified populations on methylcellulose in the presence of cytokine mixture. The $hCD34^+hCD38^-$ HSCs and $hFlt3^+$CMPs gave rise to various myeloid colonies including CFU-GEMM, whereas GMPs and MEPs formed exclusively GM and MegE lineage-related colonies, respectively. In contrast, $hFlt3^-$CMPs predominantly gave rise to MegE lineage-related colonies but failed to form CFU-GEMM. The mean value of eight independent experiments is shown. CFU-M: CFU-macrophage, CFU-G: CFU-granulocyte, CFU-GM: CFU-granulocyte/macrophage, CFU-MegE: CFU-megakaryocyte/erythroid, CFU-Meg: CFU-megakaryocyte, and BFU-E: burst-forming units-erythroid. *B*, The lineal relationship between $hFlt3^+$CMPs and $hFlt3^-$CMPs. After 72 h of culturing, $hFlt3^+$CMPs gave rise to $hFlt3^-$CMPs, GMPs, and MEPs. In contrast, $hFlt3^-$CMPs differentiated into only MEPs, thus suggesting $hFlt3^-$CMP to be a transitional intermediate population from $hFlt3^+$CMPs to $hFlt3^-$MEPs. *C*, The colony formation activity of phenotypically defined secondary CMPs, GMPs, and MEPs purified from the primary culture of $hFlt3^+$CMPs or $hFlt3^-$CMPs. Each population displayed the colony formation activity consistent with their phenotypic definition. The mean value of four independent experiments is shown.

60% of the $hCD34^+hCD38^-$ BM cells also expressed hCD90, another critical marker for hHSCs (11–13), whereas the $hCD34^+hCD38^+Lin^-$ fraction was constituted of $hCD90^-$ lineage-committed progenitors.

In the CB, only ~30% of $hCD34^+hCD38^-$ cells expressed hCD90 (Fig. 1*B*). In the NOD/SCID/IL2rγ^{null} newborn system, the $hCD34^+hCD38^-hCD90^+$ population was highly enriched for HSCs capable of long-term reconstitution as compared with the $hCD34^+hCD38^-hCD90^-$ CB fraction (F. Ishikawa, unpublished data). The vast majority of $hCD34^+hCD38^-$ cells expressed hFlt3 at a low level as previously reported (38). Furthermore, the $hCD34^+hCD38^-hCD90^+$ CB population expressed hFlt3.

These data clearly show that hFlt3 is expressed in all cells with the hHSC phenotype in both the BM and the CB, and suggest that Flt3 expression does not discriminate ST-HSCs from LT-HSCs in human as it does in mouse (16, 17). In contrast, the BM and the CB $hCD34^+hCD38^+$ progenitor fraction expressed negative to high levels of hFlt3. We thus further subfractionated the $hCD34^+hCD38^+$ population to evaluate the hFlt3 expression in a variety of lineage-restricted progenitors.

The expression of hFlt3 within the $hCD34^+hCD38^+$ progenitor fraction

In mouse hematopoiesis, the expression of mFlt3 is associated with early lymphoid progenitor activities; it is expressed in the majority of CLPs, and in the minority of CMPs with weak B cell potential (20), but not in MEPs or GMPs (20) (21). Fig. 2 shows the expression of hFlt3 in the myeloid and lymphoid progenitor populations. According to the phenotypic definition of human myeloid and lymphoid progenitors (14, 15, 39, 40), $hCD34^+hCD38^+$ cells were subfractionated into myeloid and lymphoid progenitors, including the $hCD45RA^-hCD123$ $(IL\text{-}3R\alpha)^{low}$ CMP, the $hCD45RA^-hCD123^-$ MEP, the $hCD45RA^+hCD123^{low}$ GMP, the $hCD10^+hCD19^-$ CLP, and the $hCD10^+hCD19^+$proB populations. Interestingly, in both the human BM and CB, ~70–80% of CMPs expressed hFlt3, whose level was progressively up-regulated at the GMP stage. In contrast, hFlt3 expression was completely shut down in MEPs. In the lymphoid lineage, the $hCD34^+hCD38^+hCD10^+$ CLP (15) strongly expressed hFlt3, whereas hFlt3 was down-regulated in the proB cells. The expression level of hFlt3 in GMPs and CLPs appears to be higher than that in $hCD34^+hCD38^-hCD90^+$ HSCs (Fig. 2). We also tested the level of hFlt3 transcripts in purified hBM HSCs and progenitor populations (Fig. 3*A*). The pattern of hFlt3 mRNA expression was generally consistent with that in hFlt3 protein, as evaluated by using anti-hFlt3 Abs on FACS (Figs. 1 and 2). Consistent with a previous report (41), MEPs and $hFlt3^-$ CMPs had the lowest levels, GMPs and CLPs had the highest levels, and the $hCD34^+hCD38^-$ HSC population had a medium level of hFlt3 mRNA. Collectively, functional hLT-HSCs express hFlt3 mRNA

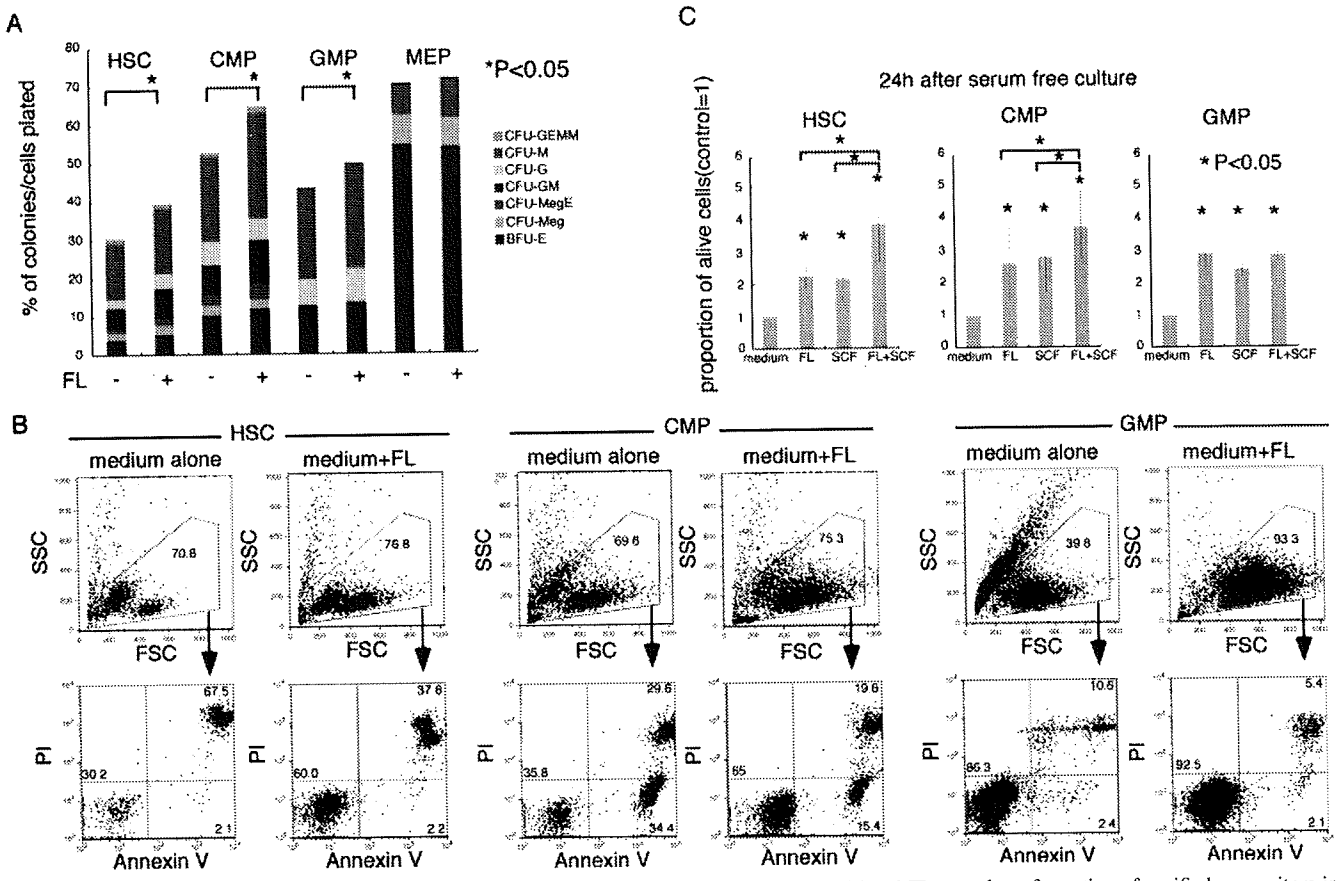

FIGURE 5. Effect of FL and SCF on the survival of purified progenitors. *A*, The effect of additional FL on colony formation of purified progenitors in methylcellulose in the presence of SCF, IL-3, IL-11, GM-CSF, Epo, and Tpo. Results from five independent experiments are shown here. Note that colony numbers are increased by the addition of FL into cultures in all hFlt3-expressing subsets including HSCs, CMPs, and GMPs but not in hFlt3$^-$ MEPs. *B*, An evaluation of apoptotic cell death in cultures of stem and progenitor cells. HSCs, hFlt3$^+$ CMPs, and GMPs were cultured in the serum-free media, with or without FL, and analyzed at 12, 18, 24, 30, 48, and 72 h after initiation of culture. A representative data obtained after 24-h culture is shown. *C*, Anti-apoptotic effects of FL and/or SCF on HSCs and Flt3$^+$ CMPs. Annexin$^-$ PI$^-$ live cells were enumerated after 24-h culture in a serum-free media. Each graph shows n-fold differences in the percentage of live cells relative to the ones without cytokine. Each bar represents the mean value and the SD of five independent samples.

and surface protein, and the distribution of Flt3 is quite different between human and mouse in early hematopoiesis.

In contrast, c-Kit was expressed at high levels in human HSCs and myelo-erythroid progenitors, while at a low level in CLPs (Fig. 1*C*). The expression pattern of c-Kit in human hematopoietic stem and progenitor cells is generally consistent with that in mouse hematopoiesis (4, 6, 7), suggesting that the c-Kit expression program is preserved in mouse and human hematopoiesis.

hFlt3 is expressed in functional hHSCs capable of reconstituting normal hematopoiesis in the NOD/SCID/IL-2 receptor γ-chain null (NOD/SCID/IL2rγ^{null}) mouse model

In the NOD/SCID/IL2rγ^{null} newborn system, hCD34$^+$hCD38$^-$ BM and CB cells are capable of reconstitution of all hematopoietic lineages for a long term (33). The entire hCD34$^+$hCD38$^-$ BM population expressed hFlt3 (Fig. 1*A*), suggesting that functional hBM HSCs possess hFlt3 on their surface. In contrast, hCD34$^+$hCD38$^-$ CB cells contained some hCD90$^-$ cells that did not express hFlt3. To formally test whether Flt3-expressing hCD34$^+$hCD38$^-$ CB cells possess LT-HSC activity, we transplanted hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ CB cells in to NOD/SCID/IL2rγ^{null} newborns. As shown in Fig. 3*B*, NOD/SCID/IL2rγ^{null} mice transplanted with 1 × 10^3 hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ CB cells reconstituted all hematolymphoid lineages for >6 mo, indicating that hFlt3 is expressed in functional hHSCs in CB as well as in BM.

Fig. 3*C* shows the phenotypic analysis of human progeny from 5 × 10^3 hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ BM cells 6 (*upper panels*) or 15 wk (*lower panels*) after transplantation into NOD/SCID/IL2rγ^{null} newborns (33). hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ BM cells differentiated into all hematopoietic lineage cells, including hCD33$^+$ granulocytes, hCD14$^+$ monocytes, hCD41$^+$ megakaryocytes, hCD19$^+$ B cells, hCD3$^+$ T cells, hCD56$^+$ NK cells (Fig. 3*C*), and hGPA$^+$ erythrocytes (not shown). Furthermore, transplanted hFlt3$^+$hCD34$^+$hCD38$^-$ HSCs purified from primary recipients developed secondary hFlt3$^+$ HSCs and hFlt3$^{-/+}$ CMPs, hFlt3$^-$ MEPs, and hFlt3$^+$ GMPs recapitulating normal human hematopoietic development. Thus, the hCD34$^+$hCD38$^-$hCD90$^+$ BM population contains cells with long-term SCID reconstitution potential as reported (33, 42), and all cells within this population express hFlt3 on their surface (Fig. 3*D*).

The up- or down-regulation of hFlt3 in the myeloid pathway is associated with GM or MegE differentiation activity, respectively

Fig. 4*A* shows the differentiation potential of purified BM progenitors in vitro in the presence of the myeloid cytokine mixture containing SCF, FL, IL-3, IL-11, Tpo, Epo, and GM-CSF. hFlt3$^+$

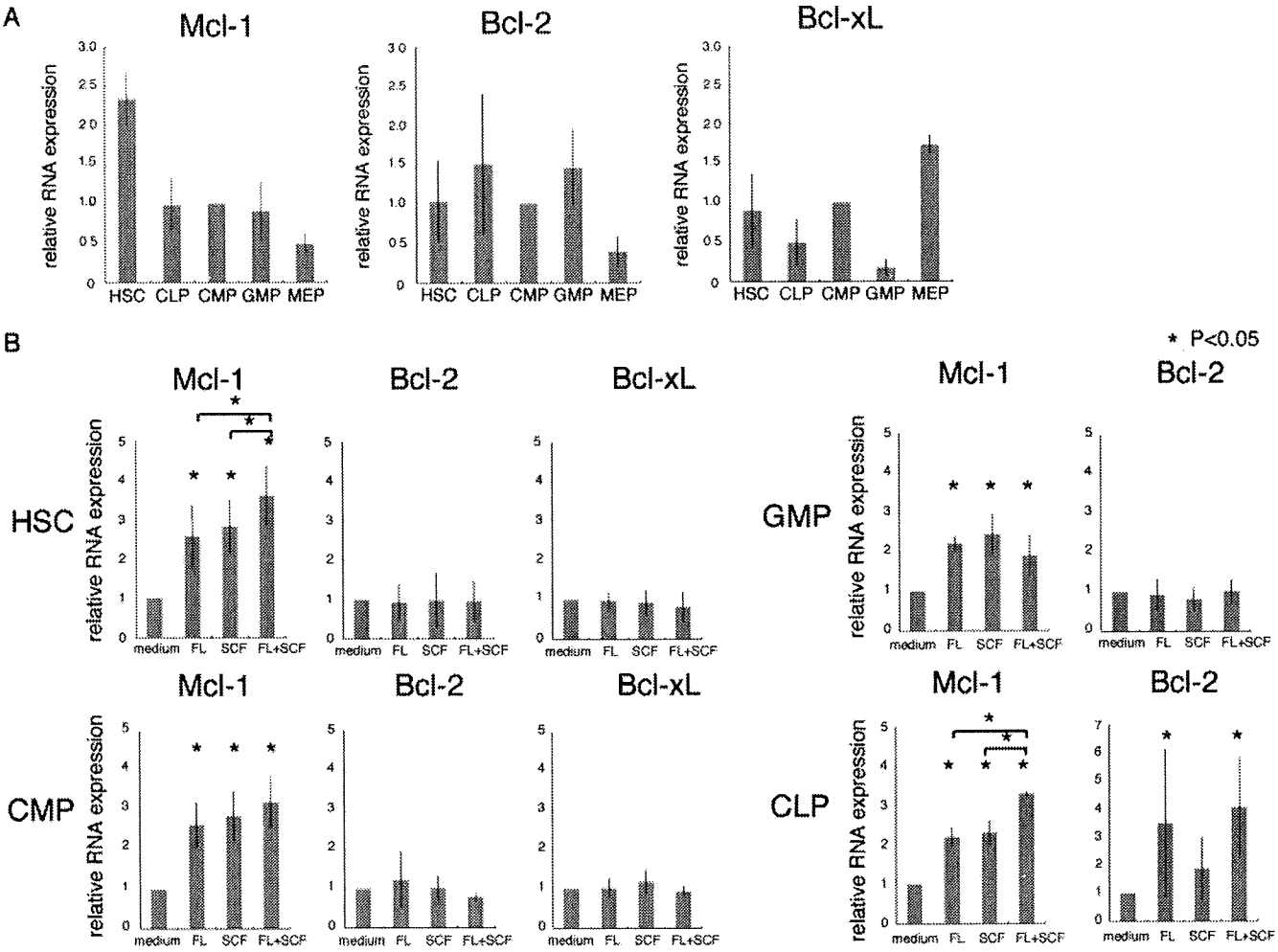

FIGURE 6. *A*, Quantitative RT-PCR assays for human anti-apoptotic genes such as Mcl-1, Bcl-2, and Bcl-x_L in purified HSCs and each progenitor population. Each bar represents an *n*-fold difference in the amount of anti-apoptotic gene expression relative to that in Flt3$^+$ CMPs. Note that Mcl-1 expression level is highest in HSCs, whereas Bcl-2 and Bcl-x_L expression is most pronounced in GMPs and MEPs, respectively. *B*, Changes in anti-apoptotic gene expression in each progenitor after incubation with FL and/or SCF. Significant up-regulation of Mcl-1 mRNA was seen in HSCs, Flt3$^+$ CMPs, GMPs, and CLPs after incubation with FL and/or SCF. Each bar represents the mean value and the SD of sixe independent samples.

CMPs formed a variety of myelo-erythroid colonies including clonogenic CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM), whereas hFlt3$^-$ CMPs did not form CFU-GEMM, but preferentially differentiated into the MegE lineage. Since GMPs (hFlt3$^+$) and MEPs (hFlt3$^-$) exclusively gave rise to GM- and MegE-related colonies, respectively, hFlt3 expression could be associated with GM lineage development. These results suggested that hFlt3$^+$ CMPs might differentiate into MEPs via hFlt3$^-$ CMPs. We thus directly tested the lineage relationship of these purified myelo-erythroid progenitor populations (Fig. 4*B*). hFlt3$^+$ and hFlt3$^-$ CMPs were purified and cultured in vitro. Then, 72 h after the initiation of culture, hFlt3$^+$ CMPs gave rise to hFlt3$^-$ CMPs, hFlt3$^+$ GMPs and hFlt3$^-$ MEPs, whereas hFlt3$^-$ CMPs did not up-regulate hFlt3, differentiating only into hFlt3$^-$ MEPs. Such phenotypically defined secondary myeloid progenitors displayed differentiation activity consistent with their phenotypic definition (Fig. 4*C*). These data suggest that multipotent hFlt3$^+$ CMPs can differentiate into both GMPs and MEPs, whereas hFlt3$^-$ CMPs represent a transitional stage into MEPs.

Flt3 signaling protects human hematopoietic stem and progenitor cells from apoptotic cell death

We wished to elucidate the role of Flt3 signaling in human hematopoiesis. We first tested the effect of Flt3 signaling on the differentiation of HSCs, CMPs, and GMPs. Purified hFlt3$^+$ HSCs, CMPs, and GMPs were cultured in methylcellulose in the presence of the myeloid cytokine mixture, with or without hFL. As shown in Fig. 5*A*, the addition of FL in the culture did not affect the percentage of GM, MegE, or mix colonies in any of these populations. Interestingly, however, the colony numbers significantly increased in all cases when FL was added to the culture. This effect was dose-dependent, and the stimulatory activity of FL reached its peak at a concentration of 5 ng/ml (not shown). The plating efficiencies of hFlt3$^+$ HSCs, CMPs, and GMPs cultured with the cytokine mixture containing FL (20 ng/ml) were significantly higher than those cultured without FL, suggesting that FL signaling may enhance the viability of cells (Fig. 5*A*). We then directly tested the viability of HSCs, CMPs, and GMPs 24 h after the initiation of culture in serum-free media, with or without FL. The live, apoptotic, and dead cells after culture were enumerated by the Annexin/PI staining (43). In this staining, live cells are Annexin$^-$/PI$^-$, whereas Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells are apoptotic and dead cells, respectively (Fig. 5*B*). Without FL, a considerable proportion of purified HSCs, CMPs, and GMPs rapidly became Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells undergoing apoptotic cell death. The addition of FL significantly blocked apoptotic cell death in all of these populations, indicating that FL plays a critical role in human hematopoietic stem and progenitor cell survival (Fig.

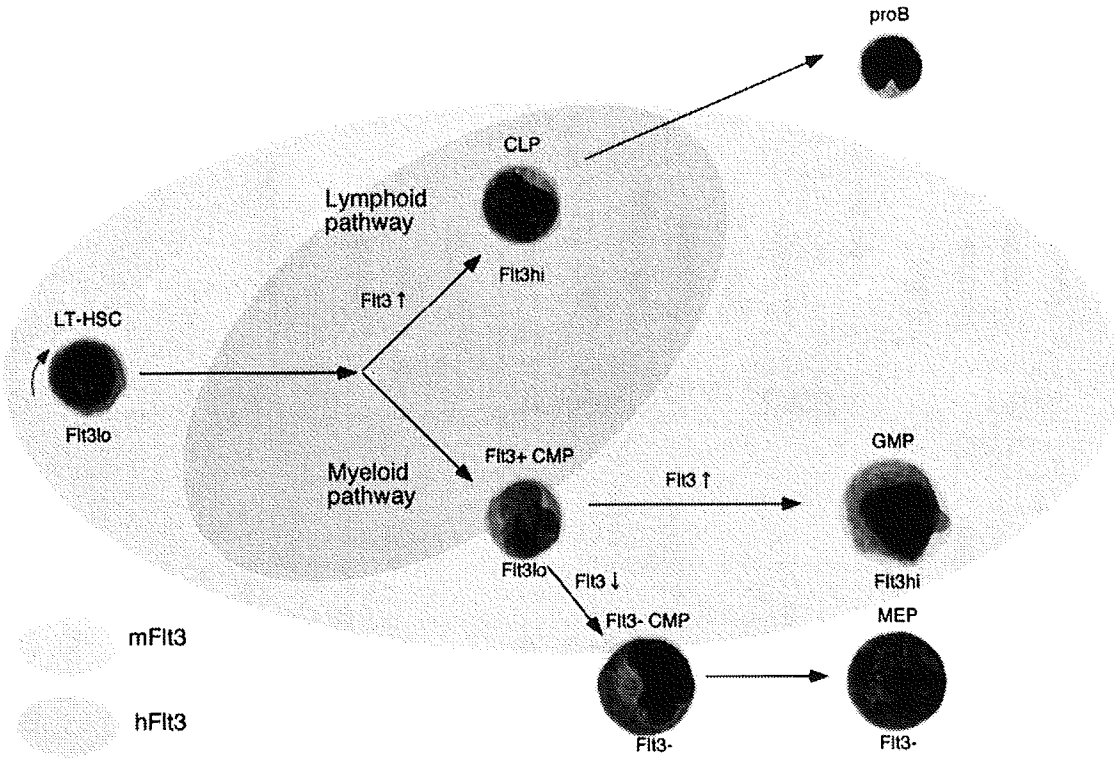

FIGURE 7. Proposed differential expression of human and mouse Flt3 in steady-state hematopoiesis. Cellular morphology of directly sorted each progenitors (May-Giemsa ×1000) is shown here. In human, the most primitive LT-HSC expressed hFlt3 at a low level and its expression is up-regulated at the early GM and the lymphoid progenitor stages, while it is down-regulated in MEPs. In contrast, the mouse LT-HSC lacks mFlt3 expression, and mFlt3 is expressed in cells primed to the lymphoid pathway, including CLPs and a fraction of CMPs.

5*B*). These data strongly suggest that Flt3 signaling does not instruct hematopoietic lineage commitment in hFlt3-expressing myeloid progenitors, but it does promote their survival.

SCF, the ligand for c-Kit, has also been shown to play a critical role in the maintenance of survival in early hematopoiesis. Both c-Kit and Flt3 belong to the class III receptor tyrosine kinase (RTK) family, sharing their major signaling cascade (44). Human HSCs, CMPs, and GMPs expressed both c-Kit and Flt3 at the single cell level (Fig. 1). Thus, we tested the anti-apoptotic effect of SCF in this system. As shown in Fig. 5*C*, in all HSC, CMP, and GMP populations, SCF also displayed anti-apoptotic effects whose impact on cell survival is similar to that of FL. Furthermore, in HSCs and CMPs, the combination of FL and SCF further increased percentages of live cells as compared with those in the presence of either FL or SCF alone, suggesting that SCF and FL signals collaborate to maintain cell survival of HSCs and CMPs.

Flt3 signaling up-regulates Mcl-1, but not Bcl-2 or Bcl-x_L, expression in human hematopoietic stem and progenitor cells

The question: is the mechanism of cell survival enhancement by signaling of RTKs, such as Flt3 and c-Kit? We have shown that in murine hematopoiesis, Mcl-1, a Bcl-2 homologue, is indispensable for hematopoietic stem and progenitor cell survival, and that c-Kit signaling is one of the most critical inducers for Mcl-1 expression in mHSCs (45). We therefore hypothesized that Flt3, as well as c-Kit, signaling may up-regulate Mcl-1 to maintain cell survival in human hematopoiesis as well.

Fig. 6*A* shows the distribution of the transcripts of Bcl-2 family molecules including Mcl-1, Bcl-2, and Bcl-x_L in human stem and progenitor cells. Mcl-1 is expressed at the highest level in HSCs. CMPs and CLPs expressed similar levels of Mcl-1, and MEPs expressed Mcl-1 at the lowest level. This expression pattern of Mcl-1 transcript in human hematopoiesis is consistent with that in murine hematopoiesis (45). In contrast, Bcl-2 was highly expressed in GMPs and CLPs, whereas Bcl-x_L was expressed in MEPs at the highest level.

Purified stem and progenitor populations were incubated with FL and/or SCF in serum-free media. Both FL and SCF dramatically up-regulated the expression of Mcl-1 in a dose-dependent manner, and it reached its peak 30 min after initiation of culture at a concentration of 5 ng/ml (data not shown). Fig. 6*B* shows the relative expression level of Mcl-1, Bcl-2, and Bcl-x_L in the presence of 20 ng/ml FL and/or SCF. We found that both FL and SCF significantly up-regulated the expression of Mcl-1, but not of Bcl-2 or Bcl-x_L, in HSCs, CMPs, and GMPs. These data collectively suggest that one of the important functions of these class III RTKs is to specifically activate Mcl-1 expression. Interestingly, in HSCs, FL and SCF displayed an additive effect on the up-regulation of Mcl-1. Therefore, Flt3 and c-Kit signaling collaborate to protect $Flt3^+$ HSCs and early myeloid progenitors from apoptotic cell death, presumably through activating anti-apoptotic Mcl-1 transcription. In CLPs, however, FL activated not only Mcl-1 but also Bcl-2 transcription.

Discussion

In this study, by using a multicolor FACS and a highly efficient xenograft system, we provide evidence that the distribution of Flt3 RTK is quite different in human and mouse hematopoiesis. First, although mouse LT-HSCs do not express mFlt3, the HSC-enriched $hCD34^+hCD38^-hLin^-$ population, that can reconstitute human hematopoiesis for a long-term in our xenogenic mouse model, uniformly expresses hFlt3 in both BM and CB. It is still unclear whether SCID-repopulating cells directly correspond to hLT-HSCs. However, because the $hCD34^+hCD38^+hLin^-$ cells never

reconstituted in xenogenic hosts for a long-term in our and others' experiments (42), it is highly likely that $hCD34^+hCD38^-hLin^-$ population is highly enriched for hLT-HSCs. Therefore, it is suggested that the negative expression of hFlt3 does not mark LT-HSCs in human, while mFlt3 does in mouse (16, 17). Second, in contrast to mouse hematopoiesis, where mFlt3 expression is restricted within progenitor populations of lymphoid potential including CLPs and a minority of CMPs that can differentiate into B cells (20), hFlt3 is expressed in human CMPs and GMPs, as well as in CLPs. The Flt3 expression is suppressed after cells are committed into the MegE lineage in both human and mouse. The distribution of Flt3 in mouse and human hematopoiesis is schematized in Fig. 7. The significant difference of Flt3 distribution in human and mouse hematopoiesis suggests that the critical role of Flt3 signaling in hematopoietic development could also be different between these species.

We further found that the important function of hFlt3 should include the maintenance of cell survival via the up-regulation of anti-apoptotic Mcl-1 in early hematopoiesis. Previous studies have demonstrated that FL can support in vitro survival of human long-term culture-initiating cells (24, 46, 47). MCL-1 is a non-redundant anti-apoptotic protein, at least in mouse hematopoiesis, because the removal of Mcl-1 from hematopoietic cells in a conditional knockout system caused fatal hematopoietic failure, and because in vitro disruption of *Mcl-1* in mouse HSCs, CMPs, or CLPs rapidly induced their apoptotic cell death (45). The expression level of Mcl-1 was the highest at the HSC stage and gradually declined as HSCs differentiate into myeloid and lymphoid progenitors in mouse hematopoiesis (45). The pattern of Mcl-1 distribution is well preserved in human hematopoiesis (Fig. 6*A*), suggesting that Mcl-1 might also be essential for hHSC survival. In mouse HSCs, Mcl-1 is up-regulated by signals from cytokines including SCF, IL-6, and IL-11, and SCF exerts the most potent effect on the up-regulation of Mcl-1(45). In contrast to mouse LT-HSCs that express c-Kit but not Flt3, functional hLT-HSCs coexpress c-Kit and Flt3 (Fig. 1), and importantly, FL as well as SCF are potent inducers for Mcl-1 transcription (Fig. 6). The fact that FL and SCF activated only Mcl-1, but not Bcl-2 or Bcl-x_L, in turn suggests that Mcl-1 might be the most critical survival factor controlled by exogenous cytokine signals at the HSC stage. Although it remains unclear whether hFlt3 and/or c-Kit signaling is absolutely required for hHSC survival, our data suggest that, to maintain the Mcl-1 level in hHSCs, the Flt3/FL system could work as an alternative to the SCF/c-Kit system. This is of interest because the SCF/c-Kit system is non-redundant in mouse hematopoiesis (48), where mouse LT-HSCs express only c-Kit, but not Flt3.

The anti-apoptotic effect of hFlt3 signaling was also seen in hFlt3-expressing myeloid progenitor populations. The incubation of CMPs and GMPs with FL significantly prevented their apoptotic cell death in vitro, and FL, as well as SCF, rapidly activated the Mcl-1 transcription in these progenitors. Interestingly, in CLPs, FL activated not only Mcl-1 but also Bcl-2. In lymphopoiesis, Bcl-2 (49, 50), as well as Mcl-1 (51), is critical. FL may collaborate with IL-7 to maintain lymphoid cell survival by up-regulating both Bcl-2 and Mcl-1. Collectively, in humans, Flt3 signaling might support cell survival in early hematopoietic stages with only the exception of the MegE lineage developmental pathway.

Our data also provides an important insight into pathogenesis of AML with *FLT3* mutations. A total of 15–35% of AML patients have either internal tandem duplications (ITDs) in the juxtamembrane domain or mutations in the activating loop of *FLT3* (28, 29), resulting in ligand-independent constitutive signal activation. The *FLT3* mutations are rarely found in acute lymphoblastic leukemia (28, 29). The etiologic link of *FLT3* mutations with AML does not fit the lymphoid-only expression pattern of Flt3 in mouse hematopoiesis. In mouse models, however, the ectopic expression of *FLT3*-ITDs in the bone marrow promotes development of myeloproliferative disorders, but these mutations themselves do not cause leukemia (52). We have found that AML cells with *FLT3*-ITD mutations possess extremely high levels of Mcl-1, and transduction of *FLT3*-ITD into normal HSCs induces rapid up-regulation of Mcl-1 of up to >10-fold higher levels (G. Yoshimoto and K. Akashi, manuscript in preparation). Because the expression of *FLT3* mutations should occur in concert with that of normal Flt3, our data suggest that once *FLT3* mutations are acquired in human hematopoiesis, abnormal survival-promoting signals of Mcl-1 should be expressed in LT-HSCs, and is progressively up-regulated in GMPs. It has been shown that both LT-HSCs and GMPs are the critical cellular target for leukemic transformation. The reinforced survival of CMPs/GMPs by blocking two independent apoptotic pathways (53), or the enforced expression of bcr-abl together with survival-promoting Bcl-2 at the GMP stage (54), results in AML development in mouse models. In human bcr-abl-positive chronic myelogeneous leukemia, GMPs could be the target for blastic transformation by acquisition of β-catenin signaling (55). GMPs can also be converted into leukemic stem cells simply by transducing leukemia fusion genes, such as MLL-ENL (56) or MOZ-TIF2 (57). Thus, these data collectively suggest that the acquisition of *FLT3* mutations in human hematopoiesis might induce the reinforced survival of cells at the HSC and myeloid progenitor stages, where *FLT3* mutations might collaborate with other genetic abnormalities to achieve full AML transformation.

In conclusion, our data show that the distribution of Flt3 is quite different in mouse and human hematopoeisis. hFlt3 targets LT-HSCs and myeloid progenitors except for MEPs. Flt3 signaling might support cell survival in early hematopoiesis including the HSC and the myeloid progenitor stages through up-regulation of Mcl-1. This is a striking example that the expression pattern of key molecules could be significantly different between human and mouse. Accordingly, special considerations are required in using mouse models to understand the role of Flt3 and *FLT3* mutations in human hematopoiesis.

Disclosures

The authors have no financial conflict of interest.

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