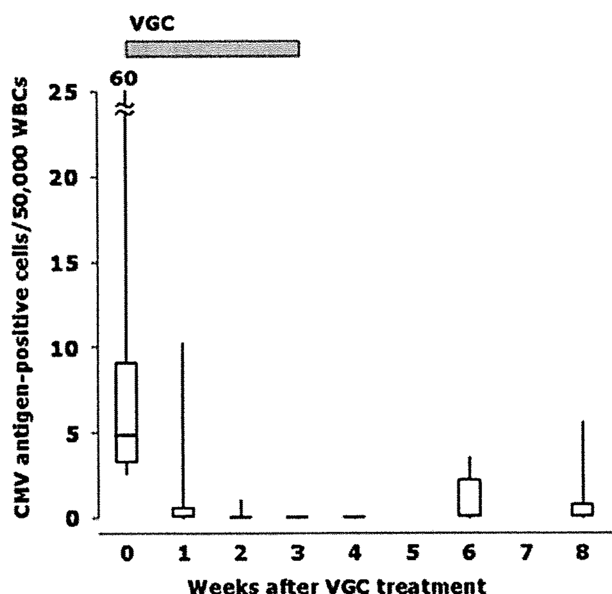

Fig. 1 Time course of the number of cytomegalovirus (CMV) antigen-positive cells after valganciclovir treatment. CMV antigenemia was reduced during treatment with valganciclovir. The *box plots* display the median, the 25th and 75th percentiles (*box*), and the smallest and largest values (*longitudinal line*). One patient discontinued valganciclovir on day 18 due to grade 4 neutropenia

in the subsequent 5 weeks after the completion of the VGC treatment.

CMV infection relapsed in four of the ten patients within 3–5 weeks after the completion of the preemptive VGC therapy. These four patients were successfully treated with IV-GCV.

3.3 Toxicity

Nine patients completed a 21-day course of VGC treatment, but one patient discontinued VGC due to grade 4 neutropenia. Due to impaired renal function (serum creatinine level, 1.68 mg/dL), this patient received a reduced VGC dose of 450 mg once per day for the first week. Renal function improved with the reduced dose, and the VGC dosage was increased to 450 mg twice per day in the second week of treatment. However, this patient developed grade 4 neutropenia (absolute neutrophil counts 0.17×10^9/L) after 17 days of treatment and then developed febrile neutropenia. The VGC was discontinued, and the patient immediately received granulocyte-colony stimulating factor (G-CSF) and antibiotic therapy. Neutrophil counts recovered to more than 1.0×10^9/L, and neutropenia resolved after five days. Recurrent CMV reactivation was not observed in this patient during the follow-up period. None of the patients developed thrombocytopenia (platelet count $<30 \times 10^9$/L)(Fig. 2).

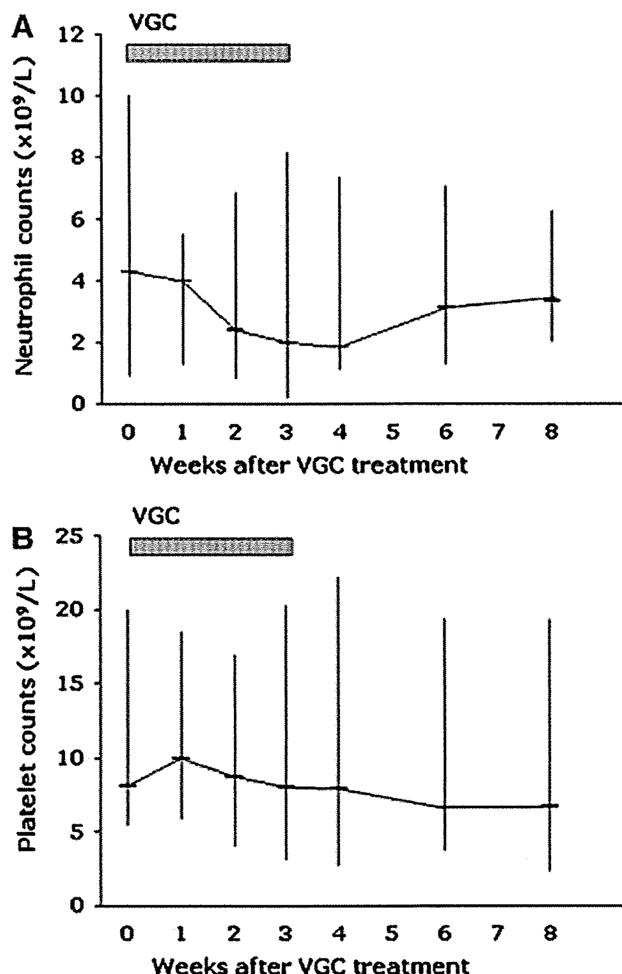

Fig. 2 Time course of neutrophils and platelets during valganciclovir treatment. Time course of neutrophil (**a**) and platelet numbers (**b**) during treatment with valganciclovir. The bar graph displays the median (*horizontal line*), and the smallest and largest values (*longitudinal line*). One patient discontinued valganciclovir on day 18 due to grade 4 neutropenia

Table 2 Adverse events other than hematological toxicities related to valganciclovir

Adverse events		No. of cases
Gastrointestinal		
Diarrhea	Grade 1	1/10
Hepatic		
AST/ALT	Grade 1	3/10

None of the patients experienced renal toxicity during the VGC treatment. Three patients developed grade 1 liver dysfunction, and one patient had grade 1 diarrhea (Table 2). However, none of these complications required discontinuation of the VGC.

4 Discussion

Effective preemptive therapy with IV-GCV reduced the incidence of early CMV disease to 5–10%; however, the risk of late CMV disease beyond day 100 after transplantation has increased over the past few years. Therefore, extended CMV monitoring beyond day 100 is currently recommended, especially in high-risk patients [2, 8]. There is a need for an effective oral anti-CMV drug that can be used for outpatient care. Oral VGC could be a useful alternative to IV-GCV in patients who require preemptive therapy for CMV infection. This study demonstrated the efficacy and safety of preemptive VGC therapy for CMV infection after allogeneic HSCT. There are four published studies that have shown the safety and the efficacy of VGC as preemptive therapy after allogeneic HSCT [20–23]. Although dosage and duration of the drug varied between studies, VGC therapy resulted in a rapid decrease of the viral load in all of the patients. In this study, we administered a dose of 900 mg twice daily for 3 weeks, and corroborated the efficacy and the tolerability of preemptive VGC therapy.

We demonstrated that VGC at a dose of 900 mg twice per day was effective and resulted in a rapid clearance of CMV antigen-positive cells in all patients. No CMV disease developed during the preemptive therapy or the subsequent 5 weeks after the completion of treatment. VGC was well tolerated as 90% of the patients completed the entire treatment course. However, four of the ten patients developed a recurrent CMV reactivation after the discontinuation of VGC treatment, and they were all successfully treated with IV-GCV. Because a guideline for preemptive VGC therapy has not been established for patients that have received allogeneic HSCT, further studies will be necessary to determine the optimal initial- and maintenance-dose of VGC.

We, and four other groups, have obtained good results with VGC starting-doses of 900 mg twice per day [20–23]. This dose was based on observations from previous pharmacokinetics studies in HIV-infected patients and liver transplant recipients. A VGC dose of 900 mg results in an area under the concentration-time curve for GCV similar to that of 5 mg/kg IV-GCV [26, 27], which is the recommended standard dose for preemptive CMV therapy [28, 29]. One of the concerns of using VGC after allogeneic HSCT is the absorption of oral VGC in patients suffering from severe gastrointestinal GVHD. Recently, Einsele et al. [30] conducted a randomized crossover clinical trial of IV-GCV and VGC in patients with or without intestinal GVHD. The results showed that patients without intestinal GVHD who took VGC were exposed to more GCV when compared to those administered IV-GCV. This was also true in patients with grade I and II intestinal GVHD. Thus, VGC may be as effective even in patients developing a mild form of intestinal GVHD as in patients without intestinal GVHD. However, a higher exposure of VGC may increase the toxicity of the drug, and the absorption of VGC was not evaluated in patients with severe intestinal GVHD. Recently, Candoni et al. [22] examined the efficacy of a lower dose of VGC. Preemptive therapy with 900 mg/day VGC was as effective for clearing CMV antigen-positive cells and preventing CMV disease as the standard dose of 1800 mg/day. These findings suggest that the initial dose of VGC could be reduced to 900 mg/day as preemptive therapy in low-risk patients.

The effective duration for preemptive VGC therapy is currently unclear. In the previous studies, patients received VGC for 2 weeks and then it was either discontinued or continued at a maintenance dose of variable duration dependant upon a negative CMV test result. Different from previous studies, we continued an initial dose of VGC for 3 weeks. The dosage and duration of VGC therapy likely affects the incidence of hematological toxicity such as neutropenia. In a study by Busca et al. [21], in which VGC was administered at a dose of 1,800 mg/day for 2 weeks, followed by 900 mg/day for an additional 2 weeks, 4 of the 15 patients failed to complete the 3-week scheduled therapy due to neutropenia and/or thrombocytopenia. In our study, only one of the ten patients failed to complete treatment. Thus, hematologic toxicity may be a significant problem after a 3 week treatment with VGC.

In our study, four of the ten patients treated with VGC developed recurrent CMV reactivation 3–5 weeks after the discontinuation of VGC. This was somewhat similar to the 10–53% recurrence rates in previous studies [20–23]. Thus, careful monitoring after the completion of VGC therapy is recommended. We continued an initial dose of VGC for 3 weeks. However, when considering hematological toxicity and frequent recurrence of CMV antigenemia, the duration of treatment and/or maintenance should be decided by monitoring CMV.

As previously reported [20–23], we found neutropenia to be the main toxic effect of VGC. One patient, who had impaired renal function before the preemptive therapy that required a dose reduction, discontinued the drug on day 17 due to grade 4 neutropenia. In high-risk patients, especially outpatient should be closely monitored, although any other toxicity profile different from IV-GCV was not observed in this study.

Our study demonstrated that the oral VGC preemptive therapy at a dose of 900 mg daily seemed to be as effective as conventional IV-GCV at a dose of 10 mg/kg daily to clear CMV antigen-positive cells. However, as shown in Fig. 1, CMV antigen-positive cells seem to decrease in numbers much faster after VGC treatment than those observed after standard dose of IV-GCV treatment.

Furthermore, hematological toxicities were considerable. Although pharmacokinetic data was not available in this study, these observations coincide with the previous pharmacokinetic study in HSCT recipients that showed the exposure of GCV after administration of 1800 mg daily VGC was significantly higher compared with 10 mg/kg IV-GCV even in patients without gastrointestinal GVHD [30]. Careful monitoring of neutrophil counts will be useful to improve the safety of VGC in HSCT recipients, especially with reduced renal function. Kanda et al. [14] showed the efficacy of response-oriented preemptive therapy using a low initial dose of IV-GCV that resulted in a successful reduction of the total dose of IV-GCV and decreased hematological toxicities. A lower dose of VGC could be also used as preemptive therapy by close CMV monitoring. Similar studies with a large number of patients will be required to define the optimal treatment schedule for preemptive VGC therapy.

Despite a limited number of patients, our results suggest that oral VGC is an effective alternative to IV-GCV for preemptive therapy to prevent CMV disease in allogeneic HSCT patients. Studies with a larger number of patients will be necessary to assess the efficacy and long-term effect of this preemptive therapy.

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Infectious complications in patients receiving autologous CD34-selected hematopoietic stem cell transplantation for severe autoimmune diseases

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Abstract: Long-term analysis of infectious complication after high-dose immunosuppressive therapy with CD34-selected autologous hematopoietic stem cell transplantation for patients with severe autoimmune diseases (AD) was performed. Theoretically, CD34 selection can reduce the risk of reinfusion of autoreactive lymphocytes. However, it is also associated with a significant reduction in T cells, natural killer cells, and monocytes, which in turn may compromise immune reconstitution, thereby increasing the risk of infection. Moreover, AD compromises host immunity and causes organ damage resulting in dysfunction of the cutaneous or mucosal barrier. In this study, the incidence rate of infections is reported in 14 patients who underwent high-dose (200 mg/kg) cyclophosphamide therapy followed by reinfusion of CD34-selected autologous peripheral blood stem cells. Bacterial complication occurred in 3 of 14 (21%) patients. Cytomegalovirus reactivation and adenovirus hemorrhagic cystitis were observed in 9 (64%) and 2 (14%) patients, respectively. As for late infectious complications, 7 patients (50%) developed dermatomal varicella zoster virus infection. No infection-related mortality was seen in this case series. Because the risk for infections approaches that seen in allogeneic transplant recipients, infection surveillance, diagnostic workup, and prophylactic strategies similar to those applicable to allogeneic recipients are warranted.

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Key words: Infection; stem cell transplantation; autologous; hematopoietic; CD34-selected; peripheral blood stem cells; autoimmune disease; cytomegalovirus; varicella zoster virus; adenovirus; bacteremia

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Pilot studies comprising high-dose immunosuppressive therapy followed by transplantation of autologous hematopoietic stem cells (HSC) were conducted to obtain safety and preliminary efficacy data in patients with severe autoimmune diseases (AD) (1–5).

Both unselected and CD34-selected peripheral blood stem cells (PBSC) have been used as sources of HSC (1–5). Theoretically, CD34 + cell selection of PBSC can reduce the possibility of reinfusion of autoreactive lymphocytes. However, the superiority of CD34-selected PBSC over unmanipulated PBSC has not been established. The safety and efficacy of CD34-selected autologous PBSC transplantation (PBSCT) for refractory AD have been investigated at our institute (6).

PBSCT-related complications include regimen-related toxicities and various infections. For the treatment of AD, PBSCT is a more toxic treatment modality than the conventional immunosuppressive therapies. Organ damage due to AD puts the patients at risk of regimen-related toxicities. Thus, careful selection of refractory AD patients for PBSCT is essential to minimize transplant-related mortality.

Infections are major contributors to morbidity and mortality in PBSCT. In hematological malignancies, CD34-selected autologous PBSCT has been reported to increase incidences of opportunistic infections compared with non-CD34-selected autologous PBSCT (7–11). Most AD patients had undergone immunosuppressive therapy, including cyclosporine and corticosteroids, before transplantation. AD

itself compromises host immunity to various infections. Furthermore, AD causes organ damage such as skin ulcers, esophageal dysmotility, and interstitial pneumonia (IP) resulting in dysfunction of the cutaneous or mucosal barrier. Thus, the understanding of infectious complications is important in increasing the safety of CD34-selected autologous PBSCT for AD.

Here, we retrospectively analyze infectious complications during the course of CD34-selected autologous PBSCT for severe AD.

Materials and methods

Protocol

The protocol of this phase I/II clinical trial (6) was approved by the ethics committee of Kyushu University Hospital. Written informed consent was obtained from all patients.

Patients and eligibility

Patients between 16 and 65 years of age were eligible at the time of pre-transplant evaluation. Patient eligibility depended on the diagnosis of AD, as previously described (6).

PBSC mobilization and CD34 + cell selection

PBSC were mobilized during hematological recovery after administration of cyclophosphamide (CY) (2 g/m^2/day) for 2 days, followed by a recombinant human granulocyte-colony stimulating factor (G-CSF, filgrastim; Kirin Brewery, Tokyo, Japan) at a dosage of 2 μg/kg/day. After collecting PBSC to obtain 2×10^6 CD34 + cells/kg or more by apheresis, CD34 + cells were positively selected using immunomagnetic beads with an anti-CD34 monoclonal antibody (CliniMACS, Miltenyi Biotec, Cologne, Germany).

Autologous PBSCT and supportive care

Patients were kept in HEPA-filtered rooms until engraftment. For pre-transplant conditioning, high-dose CY (50 mg/kg/day) was administered for 4 days, from days −5 to −2. Frozen-thawed CD34-selected PBSC were infused on day 0. All immunosuppressive and disease-modifying agents were discontinued upon HSC procurement, except systemic corticosteroids, which were tapered to a relatively low dose (5–15 mg of prednisolone/day) over 2–6 months after PBSCT. Acyclovir (intravenous 250 mg/day, from days 1 to 18), ciprofloxacin (by mouth [PO] 600 mg/day, from days −7 to 14), fluconazole (PO 200 mg/day, from days −7 to 30), and trimethoprim-sulfamethoxazole (TMP-SMX) (each 1920 mg/day; from days −14 to −2, and twice a week from days 30 to 180, respectively) were prophylactically administered, as previously described (6). Neutropenic fever was treated with intravenous administration of broad-spectrum cephalosporins according to the guidelines for the use of antimicrobial agents in neutropenic patients (12). After engraftment, weekly monitoring of cytomegalovirus (CMV) pp65 antigenemia was conducted until day 100 after transplant (13). If CMV antigenemia was detected, preemptive therapy was initiated with ganciclovir.

Diagnosis and definition of infections

The day of onset of infection was defined as the day the diagnostic test was performed.

Bacterial infections were categorized as bacteremias and site-specific infections (14). Varicella zoster virus (VZV) infections were defined as typical cutaneous vesicular lesions. CMV infection and disease were defined as previously described (13, 15). In brief, CMV infection was defined as isolation of the CMV virus or detection of the viral proteins or nucleic acids in body fluid or tissue specimen. CMV disease is defined by the presence of organ-specific signs and/or symptoms with the detection of CMV in test specimens (e.g., bronchoalveolar lavage in the lungs or biopsy samples in other organs). CMV infection with unexplained fever for at least 2 days within a 4-day period and the presence of neutropenia or thrombocytopenia is considered CMV syndrome. Hemorrhagic cystitis (HC) due to adenoviruses (AdV) was diagnosed when AdV were detected by either viral culture or polymerase chain reaction in macroscopic hematuria with clinical signs of cystitis. To exclude regimen-related HC, patients with *de novo* hematuria at least 10 days after HSC transplantation (HSCT) and no tendency toward generalized bleeding or bacteriuria were considered to have AdV HC (16). Fungal infection was defined by proven or probable invasive fungal infection (17) and clinical or radiological manifestation along with positive microbiological tests.

Results

Patients

Fourteen patients (4 males, 10 females) with a median age of 54 years (range 21–63 years) were examined (Table 1). Patients No. 1–11 were diagnosed as diffuse systemic sclerosis (SSc). Patient No. 1 was suffering from systemic lupus er-

Clinical characteristics of the autoimmune patients receiving CD34-selected transplant

Patient number	Disease	Age (years)	Sex	Complication	Previous therapies	Follow up (months)[1]
1	SSc/SLE	54	F	IP, digital ulcer	DEX, IVCY	72
2	SSc	55	M	IP, digital ulcer	PSL, IVCY	65
3	SSc	58	M	IP	PSL, IVCY	61
4	SSc	54	F	IP	PSL, IVCY	58
5	SSc	53	F	IP	PSL	56
6	SSc	49	F	IP	m-PSL, CsA, IVCY	52
7	SSc	33	F	IP	–	21
8	SSc	63	F	IP	PSL	36
9	SSc	61	F	IP	PSL, CsA	31
10	SSc	44	F	IP	PSL, IVCY	27
11	SSc	52	M	IP, digital ulcer	PSL, CsA, IVCY	23
12	DM	54	F	IP	PSL pulse, CsA, IVCY	70
13	DM	44	F	IP, skin ulcer	PSL pulse, CsA	12
14	WG	21	M	Exophthalmos	PSL pulse, IVCY	55

[1]After transplantation.
SSc, systemic sclerosis; SLE, systemic lupus erythematosus; IP, interstitial pneumonia; DEX, dexamethasone; IVCY, intravenous cyclophosphamide; PSL, prednisolone; m-PSL, methyl prednisolone; CsA, cyclosporine; DM, dermatomyositis; WG, Wegener's granulomatosis.

Table 1

ythematosus (SLE) for 22 years and SSc for 2 years. Although SLE was inactive, she had progressive IP and severe digital ulcers due to SSc. Patients No. 2–11 (SSc) and 12 and 13 (dermatomyositis) developed IP, which did not respond to immunosuppressive agents. Patients No. 3–6, 8, 9, and 11 showed severe skin sclerosis. Patient No. 3 had been in complete remission from non-Hodgkin's lymphoma for 1 year and was considered eligible. Patient No. 14 (Wegener's granulomatosis) presented with severe exophthalmos due to granuloma formation (18 mm in diameter) in the upper lateral region of the left orbit affecting the superior rectus muscle. He needed monthly steroid pulse therapy to prevent further growth of the granuloma. The Eastern Cooperative Oncology Group performance status (18) was <3 in all patients. CY and cyclosporine were administered to 9 patients and 5 patients, respectively. All patients, except Patient No. 7, were treated with corticosteroids. The median follow-up duration was 53.5 months after transplant (range 8–72 months).

Results are reported as of March 2008.

Infections

Bacterial

Nine of 14 patients developed febrile neutropenia at a median of 6 (0–9) days after PBSCT (Table 2). Among these, Patients No. 6 and 11 revealed *Streptococcus mitis* bacteremia on days 8 and 9 after PBSCT, respectively. Both were empirically treated with broad-spectrum cephalosporins. Vancomycin was added when the blood culture was reported positive. Patient No. 12 developed high-grade fever without signs of local infection on day 119 post PBSCT, and a blood culture turned out to be positive for *Listeria monocytogenes*. Empirical therapy was initiated with broad-spectrum cephalosporin but switched to penicillin/β-lactamase inhibitor after detection of the microbe. All patients responded to the therapy, and no fatal complications occurred.

Patient No. 14 developed *Mycobacterium gordonae* pneumonia 1343 days after PBSCT. However, he was on anti-tumor necrosis factor (TNF) antibody therapy because of the relapse at that time; thus the case is omitted from this study.

Viral

CMV. Nine of 14 patients developed CMV antigenemia at a median of 28 days (range 10–60 days) after PBSCT. All patients who developed CMV infection were seropositive for CMV antibody before PBSCT, and this was considered as reactivation. Patients were preemptively treated with ganciclovir (5 mg/kg twice a day) and none developed CMV disease. High levels of antigenemia were detected in Patients No. 1 and 6. With their clinical symptoms, these patients were considered to have CMV syndrome. Foscarnet

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 hyper-reactivity 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

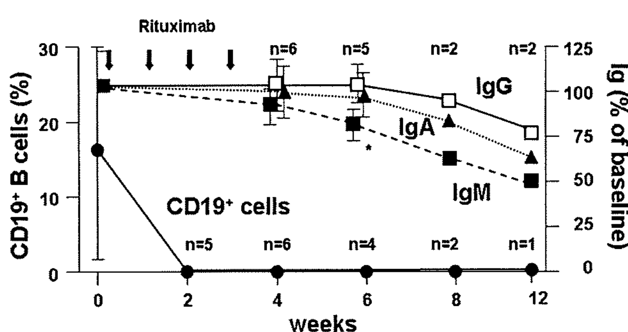

Fig. 1 Laboratory parameters over time after rituximab therapy. IgG, IgA and IgM levels are shown as percentage of baseline levels.*$P < 0.01$ compared with IgG or IgA

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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FLT3-ITD up-regulates MCL-1 to promote survival of stem cells in acute myeloid leukemia via FLT3-ITD–specific STAT5 activation

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Myeloid cell leukemia-1 (MCL-1) is an essential survival factor for hematopoiesis. In humans, hematopoietic stem cells (HSCs) express MCL-1 at the highest level in response to FMS-like tyrosine kinase-3 (FLT3) signaling. We here show that this FLT3-dependent stem cell maintenance system also plays a critical role in survival of leukemic stem cells (LSCs) in acute myeloid leukemia (AML). The $CD34^+CD38^-$ LSC fraction expresses high levels of FLT3 as well as MCL-1, even compared with normal HSCs. Treatment with FLT3 ligand induced further MCL-1 up-regulation in LSCs in all AML cases tested. Interestingly, the group of samples expressing the highest levels of MCL-1 constituted AML with FLT3–internal tandem duplications (ITD). In FLT3-ITD AML cell lines, cells expressed a high level of MCL-1, and an inhibition of MCL-1 induced their apoptotic cell death. A tyrosine kinase inhibitor suppressed MCL-1 expression, and induced apoptosis that was reversed by the enforced MCL-1 expression. Finally, transduction of FLT3-ITD into HSCs strongly activated MCL-1 expression through its signal transducer and activator of transcription 5 (STAT5)–docking domains. This effect was completely abrogated when STAT5 activation was blocked. Thus, the acquisition of FLT3-ITD ensures LSC survival by up-regulating MCL-1 via constitutive STAT5 activation that is independent of wild-type FLT3 signaling. (Blood. 2009;114:5034-5043)

Introduction

Apoptosis is an evolutionarily conserved cell death pathway essential for homeostasis, development, and removal of damaged cells. Abnormality in genes affecting apoptosis has been found in the majority of cancers, suggesting that resistance to apoptosis, often achieved by overexpression of antiapoptotic proteins, is one of the critical factors for cancer development.[1,2] B-cell leukemia 2 (BCL-2) family proteins regulate survival of many types of normal and malignant cells.[3,4] In fact, in collaboration with other oncogenes, the enforced expression of BCL-2 can significantly enhance the development of acute myeloid leukemia (AML) in several murine models,[5-7] suggesting that deregulation of cell survival plays a critical role in development of leukemias.

Recent studies have shown that myeloid cell leukemia-1 (MCL-1)[8] is the most critical BCL-2 family molecule for hematopoietic stem cell (HSC) survival. We have reported that MCL-1 is expressed in HSCs at the highest level in murine hematopoiesis, and is essential for maintenance of cell survival in HSCs, based on the fact that conditional disruption of *Mcl-1* in HSCs induced a complete ablation of hematopoiesis.[9] Human HSCs also express MCL-1 at the highest level.[10] In human lymphoid malignancies such as chronic lymphocytic leukemia and multiple myeloma, the high level of MCL-1 transcripts in malignant cells is a useful prognostic marker.[11-14] Suppression of MCL-1 but not of BCL-2 or BCL-xL triggered rapid apoptosis in chronic lymphocytic leukemia and myeloma cells,[13,15,16] suggesting that MCL-1 could be a therapeutic target in these lymphoid malignancies.

In early hematopoiesis, MCL-1 expression is regulated at least by cytokines. Human HSCs and early myelolymphoid progenitors express type III receptor tyrosine kinase family proteins including FMS-like tyrosine kinase-3 (FLT3) and steel factor receptor (c-KIT),[10] and ligands for either receptor stimulate HSCs to express MCL-1, but not BCL-2 or BCL-xL.[10] FLT3 ligation induces receptor dimerization, triggers 2 major signal transduction pathways including phosphatidylinositol 3-kinase (PI3K)/Akt, and RAS/mitogen-activated protein kinase (MAPK) pathways, and provides growth-stimulating and antiapoptotic signals.[17]

In AML, FLT3 is expressed in AML blasts at a high level in most cases.[18] Of note, FLT3 mutation is one of the most frequent genetic alterations in AML[19] and marks a fraction of patients with poor prognosis.[20-22] FLT3 internal tandem duplication mutations (FLT3-ITD) induce constitutive activation of FLT3 signaling, and retroviral transduction of FLT3-ITD in murine bone marrow cells results in progression of myeloproliferative disorders,[23-29] but these mutations themselves do not cause AML.[24] In contrast to the wild-type FLT3 (FLT3-WT), FLT3-ITD is a potent activator of signal transducer and activator of transcription 5 (STAT5). Of note, the STAT5 Src homology 2 docking phosphorylation sites in the juxtamembrane domain of FLT3-ITD, tyrosines 589 and 591,

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which do not exist within the FLT3-WT, are necessary to recapitulate myeloproliferation in a FLT3-ITD knock-in mouse model.[30]

In this paper, we demonstrate that the leukemic stem cell (LSC) fraction of AML cells expresses a high level of MCL-1 even compared with normal HSCs, suggesting that AML LSCs use MCL-1 as a critical survival factor. AML LSCs expressed FLT3 and FLT3-ITD on their surface. MCL-1 expression in LSCs was further up-regulated by treatment with exogenous FLT3 ligands. Interestingly, LSCs with FLT3-ITD express extremely high levels of MCL-1, a process mediated by STAT5 signaling pathways that are not used in the FLT3-WT. Thus, both normal FLT3 and FLT3-ITD signaling maintain MCL-1 expression in LSCs presumably to prolong their survival, but FLT3-ITD exerts more potent effects on MCL-1 induction using a signaling pathway independent of FLT3-WT.

Methods

Patients

Bone marrow (BM) samples were analyzed from 30 adult patients with primary AML diagnosed according to French-American-British (FAB) criteria in Kyushu University Hospital. BM mononuclear cells were prepared by gradient centrifugation and cryopreserved until use for this study. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects.

Analysis of FLT3-ITD mutations

For detection of FLT3-ITD, polymerase chain reaction (PCR) amplification of genomic DNA was carried out using primers 11F and 12R located on FLT3 exons 14 and 15 as previously described.[31] PCR products were separated by electrophoresis using 3% Nusieve GTG agarose gel (Cambrex Bio Science Rockland) and cut out from the gel, purified with a QIAquick Gel Extraction kit (QIAGEN), and cloned into the PCR II-TOPO vector (Invitrogen) according to the manufacturer's recommendations. Sequencing was performed using the ABI PRISM BigDye Terminator V 1.1 Cycle Sequencing Kit and 21M13 and T7 primer on an ABI 310 Prism sequencer (Applied Biosystems).

Antibodies, cell staining, and sorting

$CD34^+$ cells were enriched from BM cells using the Indirect CD34 MicroBead Kit (Miltenyi Biotec) as described previously.[32] For analyses and sorting, cell samples were stained with cyanin 5–phycoerythrin (PE)– or phycoerythrin-Cy5–conjugated lineage mixture, including cyanin 5–PE– or phycoerythrin-Cy5–conjugated CD3 (HIT3a), CD4 (RPA-T4), CD8 (RPA-T8), CD10 (HI10a), CD20 (2H7), CD11b (ICFR44), CD14 (RMO13), GPA (GA-R2), fluorescein isothiocyanate–conjugated anti-CD90 (Thy-1) (5E10) or anti-CD45RA (HI100), PE-conjugated anti-CD117 (c-Kit) (YB5.B8) or anti-FLT3 (CD135) (4G8), anti-CD123 (interleukin-3Rα [IL-3Rα]) (6H6), allophycocyanin–conjugated anti-CD34 (8G12), and biotin-conjugated anti-CD38 (HIT2) antibodies. Streptavidin-conjugated allophycocyanin was used for visualization of the biotinylated antibodies (BD Pharmingen). Nonviable cells were excluded by propidium iodide (PI) staining. HSCs ($CD34^+CD38^-CD90^+Lin^-$ cells) and myeloid progenitors, including common myeloid progenitors ($CD34^+CD38^+CD123^+$ $CD45RA^{lo}Lin^-$), granulocyte-macrophage progenitors (GMPs: $CD34^+$ $CD38^+CD123^+$ $CD45RA^{lo}Lin^-$), and megakaryocyte-erythroid progenitors ($CD34^+CD38^+CD123^-CD45RA^-Lin^-$) were isolated from normal and AML BM cells by fluorescence-activated cell sorter (FACS, FACSAria; BD Biosciences).[32,33] The sorted cells were subjected to an additional round of sorting using the same gate to eliminate contaminating cells and doublets. HSCs and GMPs in the murine bone marrow were purified according to the method described previously.[34]

Cell culture and apoptosis measurement

To test the effect of FLT3 stimulation on the leukemic cells, the sorted cells were prepared in the fetal calf serum (FCS)–free condition media with human (h) FLT3 ligand (FL, 20 ng/mL) or human stem cell factor (SCF, 20 ng/mL). After culture of 30 minutes, cells were subject to analysis of gene expression. Leukemic cell lines, including FLT3-ITD–positive cell lines, MV4-11, were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) in the absence or presence of PKC-412 (LC Laboratories), Ly294002 (EMD Biosciences), AG490 (EMD Biosciences), or U0126 (Cell Signaling Technology). Cultured cell lines were picked up for analyses of changes in transcript and protein expression and apoptotic status. Apoptosis of cell lines treated with PKC-412 was evaluated using annexin V (BD Pharmingen) and PI staining. The living cells were defined as annexin V^-/PI^- among the live-gated cells.

Quantitative real-time PCR assay

Total RNA was extracted from 2000 purified cells using Isogen reagent (Nippon Gene) according to the manufacturer's protocols. RNA was reverse transcribed to cDNA using a TaKaRa RNA PCR Kit (Takara). The mRNA levels were quantified in triplicate using a real-time PCR (7500 Real-Time PCR system; Applied Biosystems). β_2-Microglobulin mRNA was separately amplified in the same plate to be used for internal control. The primers and probes were designed by Primer Express software (Applied Biosystems) as follows: BCL-2 (the forward primer, 5-ctgggatgcctttgtggaa-3; the reverse primer, 5-cagccaggagaaatcaaacaga-3; and the probe, 5-FAM-tgtacggccccagcatgcgg-TAMRA-3), BCL-xL (the forward primer, 5-aatgaccacctagagccttgga-3; the reverse primer, 5-tgctgcattgttcccatagagt-3; and the probe, 5-FAM-cggcggctgggatacttttg-TAMRA-3), MCL-1 (the forward primer, 5-caaaacgggactggctagttaaa-3; the reverse primer, 5-ccttctaggtcctctacatggaaga-3; and the probe, 5-FAM-aaagaggctgggatgggtttgtggag-TAMRA-3), and β_2-microglobulin (the forward primer, 5-tgactttgtcacagcccaagata-3; the reverse primer, 5-aatgcggcatcttcaaacct-3; and the probe, 5-FAM-ttaagtgggatcgagacatgtaagcagcatc-TAMRA-3). Collected data were analyzed with Sequence Detector software (Applied Biosystems). All the samples and PCR analyses were carried out in duplicate, and negative controls were included in each run. Data were expressed as fold changes in gene expression relative to those from normal BM or normal HSCs.

Intracellular flow cytometry

Cells were fixed and permeabilized, and labeled with the rabbit polyclonal antibody MCL-1 (S-19; Santa Cruz Biotechnology), followed by the second staining with fluorescein isothiocyanate– or PE-conjugated antirabbit antibodies for visualization.

Western blot analysis

Cells (10^6) were lysed as described.[35] Lysates were then denatured in an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer, resolved by a 10% SDS–polyacrylamide gel electrophoresis gel, and electrotransferred to nitrocellulose membranes in non–SDS-containing transfer buffer (25mM tris(hydroxymethyl)aminomethane, 0.2M glycine, 20% methanol, pH 8.5). Western blotting was performed with rabbit anti–human MCL-1 sera (1:5000; Sigma-Aldrich), anti–human phospho-Akt (Cell Signaling Technology), anti–human phospho-mitogen-activated protein kinase kinase 1/2 (MEK1/2; Cell Signaling Technology), or mouse anti–β-actin monoclonal antibody (1:5000; Sigma-Aldrich), followed by 1:15 000 dilution of antirabbit or antimouse horseradish peroxidase–conjugated immunoglobulin G (Jackson ImmunoResearch Laboratories). Blots were developed with enhanced chemiluminescence plus kit (Amersham Biosciences). The chemiluminescence intensity was monitored by laser3000 (FujiFilm) instrument. We quantitated band intensity of the proteins using ImageGauge software (FujiFilm) and normalized their expression in reference to β-actin levels. Using these normalized data, relative expression (RE) is subsequently calculated as fold changes in protein expression compared with the controls.