Macrophage colony-stimulating factor enhances rituximab-dependent cellular cytotoxicity by monocytes

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Recent studies suggest that monocytes are the dominant effectors by which rituximab induces cell death in B-cell lymphoma. Because macrophage colony-stimulating factor (M-CSF) can enhance the cytotoxicity of monocytes, the authors examined whether this growth factor can enhance their ability to kill lymphoma cells *in vitro*. Monocytes derived from a healthy volunteer were cultured for 48 h in the presence or absence of M-CSF. Monocytes stimulated with M-CSF were significantly more cytotoxic to Daudi B-cell lymphomas than unstimulated monocytes. Flow cytometry revealed that M-CSF increased monocyte expression of Fcγ receptors III and I by 1.6- and 1.5-fold, whereas the expression of Fcγ receptor II remained unchanged. These results suggest that pretreatment with M-CSF can improve the therapeutic efficacy of rituximab against intractable CD20⁺ lymphoma. (*Cancer Sci* 2007; 98: 1368–1372)

Rituximab, a chimeric anti-CD20 IgG1 monoclonal antibody, has dramatically improved the treatment of both follicular and aggressive CD20⁺ B-cell non-Hodgkin lymphomas.[1–5] However, the advantage of rituximab is not whole. A substantial number of patients suffer from relapse after rituximab containing chemotherapy or refractoriness to it.[1–5] Approximately half of patients with relapsed or refractory non-Hodgkin lymphoma do not exhibit a durable clinical response to rituximab, despite continued expression of CD20 by lymphoma cells.[1,3,6]

Although the means by which rituximab inhibits the growth of lymphoma is not fully understood, accumulating evidence indicates that it is mostly mediated by antibody-dependent cellular cytotoxicity (ADCC) rather than induction of apoptosis and complement-dependent cytotoxicity.[7–12] In addition, studies in a mouse model of rituximab immunotherapy revealed that B cells depletion via ADCC is mostly mediated by monocytes rather than T or natural killer cells.[13]

Macrophage colony-stimulating factor (M-CSF), also known as colony-stimulating factor 1, promotes the differentiation of progenitor cells into mature monocytes and macrophages and prolongs macrophage and monocyte survival.[14–20] Previous studies have demonstrated that M-CSF activates monocytes, leading to greater ADCC against target cells, including human lymphoma and leukemic cell lines.[18,19] In the present study, the authors investigated whether M-CSF can enhance the ability of monocytes to kill CD20⁺ lymphoma cells via a rituximab-dependent mechanism.

Materials and Methods

Cell lines. Daudi Burkitt's lymphoma cells, Molt-4 acute lymphoid leukemia cells, and THP-1 acute monocytic leukemia cells were purchased from the Riken Cell Bank (Tsukuba City, Japan). Daudi cells express CD20 on their surface, whereas Molt-4 cells lack cell surface CD20. Cell lines were cultured in complete medium, which contained Roswell Park Memorial Institute (RPMI) 1640 (Gibco Laboratories, Grand Island, NY, USA), 10% heat-inactivated fetal bovine serum (Gibco Laboratories), 2 mmol/L l-glutamine (Gibco Laboratories), and penicillin-streptomycin (Gibco Laboratories). Cells in the late logarithmic phase of growth were passaged regularly every 4–5 days prior to cytotoxicity assays. The cell lines used for experiments were ≥90% viable according to Trypan blue exclusion.

Monoclonal antibodies and cytokines. The chimeric anti-CD20 monoclonal antibody rituximab was purchased from Roche Pharmaceuticals (Basel, Switzerland). Mouse fluorochrome-conjugated isotype control antibodies, phycoerythrin-conjugated anti-CD32 and anti-CD14, and fluorescein isothiocyanate-conjugated anti-CD64 and anti-CD16 antibodies were purchased from BD Biosciences (San Jose, CA, USA). Anti-CD16 blocking antibody was purchased from Abcam (Cambridge, UK), anti-CD32 blocking antibody from StemCell (Vancouver, British Columbia, Canada), and unconjugated anti-CD64 antibody from BD Biosciences (San Jose, CA, USA). Recombinant human M-CSF was purchased from Peprotech (London, UK).

Flow cytometry. Flow cytometric phenotyping of target and effector cells was carried out on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). The levels of Fcγ receptor (FcγR) I, II, and III on monocytes were quantified from the median fluorescence intensities obtained using monoclonal antibodies against CD64, CD32, and CD16, respectively. Two-parameter dot plots were generated using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), and acquired cytometric data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). The mean fluorescence intensity of each marker was compared between freshly isolated monocytes and monocytes that had been cultured for 48 h with or without M-CSF.

Isolation of monocytes. Peripheral blood mononuclear cells from eight healthy volunteers were isolated using the Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala, Sweden) and used for negative selection of blood monocytes by depleting T cells, B cells, natural killer cells, and granulocytes using a StemSep Monocyte Enrichment Kit (StemCell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's instructions. The purified cell fraction contained more than 90% monocytes as determined by flow cytometry and microscopic examination of the cell morphology.

Stimulation of monocytes with M-CSF. Isolated monocytes were cultured for 48 h in the presence or absence of 66 ng/mL M-CSF at 37°C in 5% CO_2. The concentration of M-CSF for optimal stimulation of the monocytes was determined as described previously.[19]

Cytotoxicity assay. Cytotoxicity was measured using flow cytometry with a LIVE/DEAD Viability/Cytotoxicity Assay Kit

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(Molecular Probes, Eugene, OR, USA) as described previously.[21] In this assay, living cells are stained with calcein-AM (green fluorescence), and dead cells are stained with ethidium homodimer-1 (red fluorescence). Target cells were cultured with rituximab (5 μg/mL) or human IgG1 (control) for 30 min at room temperature. Effector cells were adjusted to 1×10^6/mL. Target and effector cells were cocultured in sterile polystyrene round bottom tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in complete medium at various effector-to-target ratios for 4 h at 37°C in 5% CO_2. At the end of incubation, cells were stained with EH-1 (100 nM). Next, 10^4 cells per sample were examined using FACScan flow cytometry without gating. Acquired cytometric data were analyzed with FlowJo software (Tree Star Inc.), and cytotoxicity was calculated according to the manufacturer's instructions. Cytotoxicity assays were repeated at least three times for each sample.

Statistical analysis. The data were expressed as means ± SD. Paired *t*-tests were performed to determine the statistical significance of differences between two groups. Values of $P < 0.05$ were considered to indicate a statistically significant difference.

Results

M-CSF enhances monocyte-mediated rituximab-induced death of CD20-positive lymphoma cells. In initial experiments, monocytes were incubated for 48 h in the presence or absence of M-CSF and then the killing of rituximab-coated lymphoma cells was measured (Figs 1,2). At an effector-to-target ratio of 5:1, monocytes treated with M-CSF were significantly more cytotoxic to Daudi CD20-positive lymphoma cells than untreated monocytes. In contrast, neither M-CSF-treated nor -untreated cultured monocytes induced the lysis of rituximab-coated, CD20-negative Molt-4 cells (Fig. 3). Also, regardless of whether the monocytes were treated with M-CSF, they did not cause substantial lysis of Daudi cells coated with isotype-matched control IgG (Fig. 2). Furthermore, in the absence of effector cells, rituximab alone did not exhibit substantial cytotoxicity against Daudi (Fig. 2) or Molt-4 cells (Fig. 3), indicating that the lysis of Daudi cells was due to rituximab-dependent, monocyte-mediated ADCC.

Analysis of FcγR on monocytes. The effect of M-CSF on the expression of FcγRI (CD64), FcγRII (CD32), and FcγR (CD16)

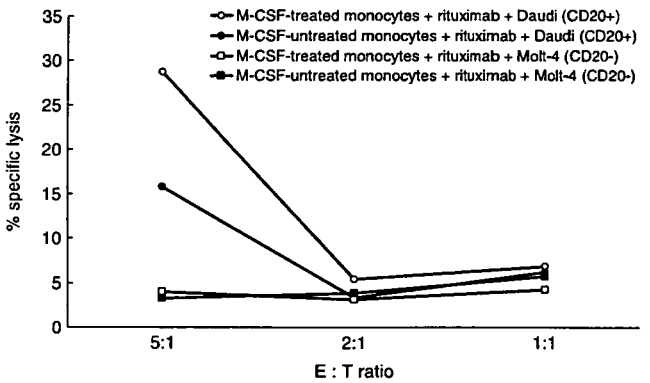

Fig. 1. Specific lysis of CD20-positive (Daudi) and CD20-negative (Molt-4) target cells at different effector-to-target (E : T) ratios in the presence of rituximab (5 μg/mL). Monocytes of healthy volunteers ($n = 8$) were isolated by negative selection and cultured in the presence or absence of macrophage colony-stimulating factor (M-CSF; 66 ng/mL) for 48 h. M-CSF-treated monocytes displayed greater cytotoxicity toward Daudi cells than M-CSF-untreated monocytes. Also, specific lysis of Daudi cells by M-CSF-treated monocytes in the presence of rituximab increased as the E : T ratio was increased. In contrast, the lysis of Molt-4 cells was not increased as the E : T ratio was increased. The results are representative of three independent experiments.

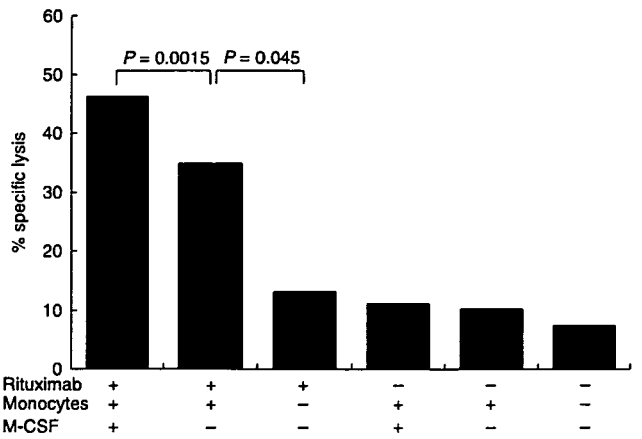

Fig. 2. Effect of macrophage colony-stimulating factor (M-CSF) on the lysis of CD20-positive cells (Daudi) by monocytes at an effector-to-target (E : T) ratio of 5:1 in the presence of rituximab (5 μg/mL). Monocytes were derived from healthy volunteers ($n = 8$) and cultured in the presence or absence of M-CSF (66 ng/mL) for 48 h. Specific lysis was significantly higher in M-CSF-treated monocytes than in monocytes that were not treated with M-CSF (mean ± SD, 46.2% ± 28.6% *vs* 34.7% ± 31.9%, respectively [$n = 8$]; $P = 0.0015$). Specific lysis by rituximab (5 μg/mL) in the absence of effector cells was 13.1% ± 8.2%. Specific lysis by monocytes in the absence of rituximab was 12.6% ± 8.1% for M-CSF-treated monocytes and 11.3% ± 7.0% for M-CSF-untreated monocytes. In the absence of rituximab, monocytes did not cause substantial cells lysis irrespective of M-CSF stimulation compared to Daudi cells' autolysis.

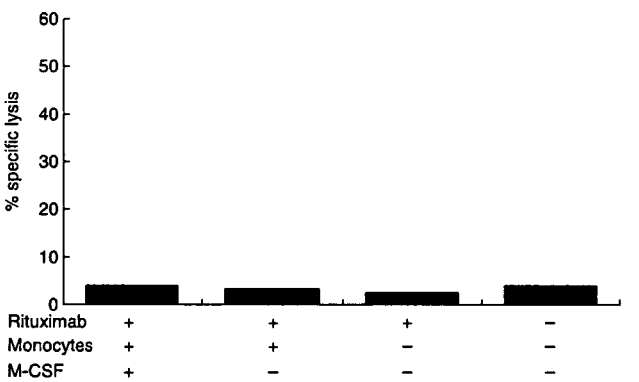

Fig. 3. Macrophage colony-stimulating factor (M-CSF)-stimulated monocytes do not cause rituximab-mediated cell death of CD20-negative cells (Molt-4). Monocytes from eight healthy volunteers were used for cytotoxicity assay. Specific lysis of target cells was 3.94% by M-CSF-stimulated monocytes in the presence of rituximab (5 μg/mL), 3.29% by M-CSF-untreated monocytes in the presence of rituximab, and 2.56% by rituximab in the absence of monocytes. The results are representative of two independent experiments.

on $CD14^+$ monocytes was examined next using flow cytometry. As shown in Fig. 4, M-CSF caused a statistically significant increase in the level of FcγRI (1.6-fold; $P = 0.00031$) and FcγRIII (1.5-fold; $P = 0.039$) on monocytes. The expression of FcγRII, however, was not affected by stimulation with M-CSF ($P = 0.25$).

Blocking of cytotoxicity by anti-FcγR antibodies. Acute monocytic leukemia cell line THP-1 cells can induce ADCC using FcγR on their cell surface, which is a good model with which to test the cytotoxic mechanisms of monocytes.[22]

Stimulation of THP-1 cells with M-CSF increased their cytotoxicity against Daudi cells coated with rituximab by 20%, and amplified their expression of FcγR I and III but not FcγR II

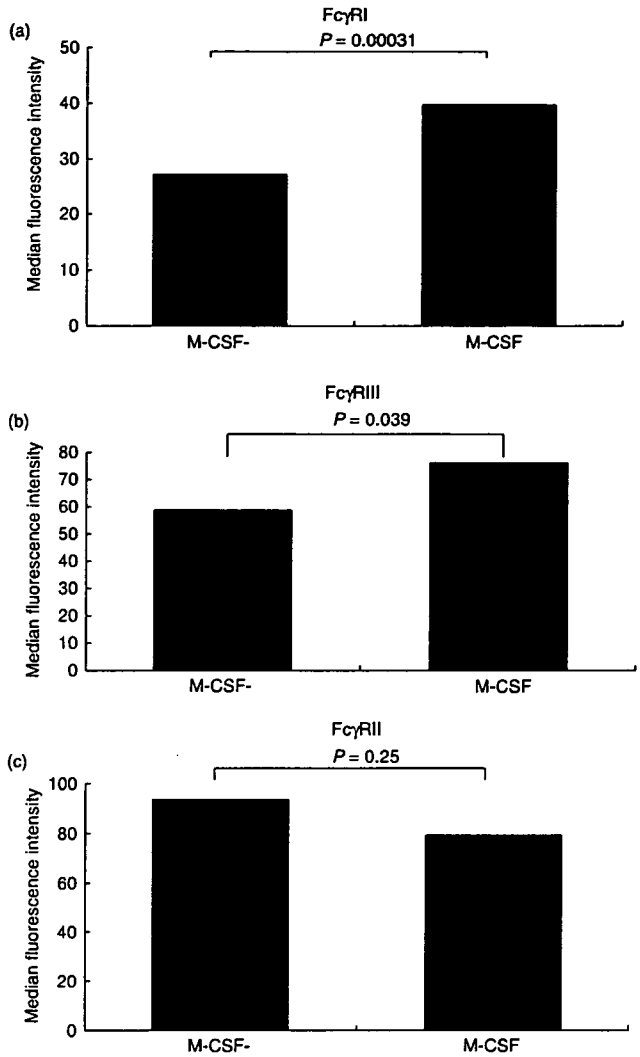

Fig. 4. Effect of M-CSF on the expression of Fcγ receptor (FcγR) by monocytes. The levels of FcγR on the surface of monocytes from eight healthy volunteers were measured using flow cytometry after a 48 h treatment in the presence or absence of macrophage colony-stimulating factor (M-CSF). The median fluorescence intensities of FcγR on monocytes from the same volunteers were compared using a paired *t*-test. (a) The expression of FcγRI on monocytes was significantly enhanced by treatment with M-CSF (60% increase; $P = 0.00031$). (b) The expression of FcγRIII on monocytes was significantly enhanced by treatment with M-CSF (50% increase; $P = 0.039$). (c) The expression of FcγRII was not significantly changed by treatment with M-CSF.

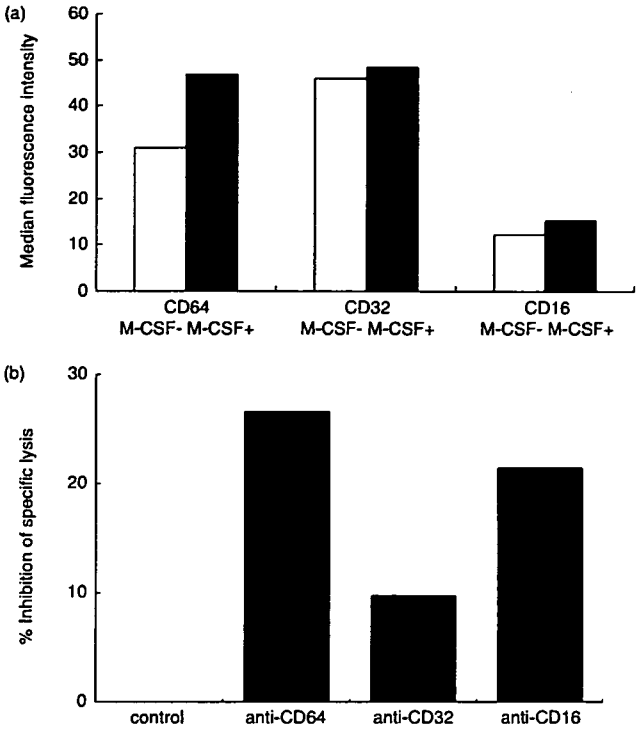

Fig. 5. (a) The expression of CD64 and CD16 on THP-1 were significantly enhanced by treatment with macrophage colony-stimulating factor (M-CSF). (b) Blocking of cytotoxicity mediated by THP-1 against Daudi cells. A total 2.5×10^5 THP-1 cells treated with M-CSF were incubated with 5×10^4 Daudi cells in the presence of 5 μg/mL rituximab in medium containing isotype control IgG (control) or indicated purified monoclonal blocking antibodies (40 μg/mL): anti-CD16, CD32, CD64. The percentage inhibition was calculated using the mean percentage of specific lysis determined from triplicate cultures after 4 h of incubation.

(Fig. 5a), as seen in freshly isolated monocytes. Blocking of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) inhibited the specific lysis of rituximab-coated Daudi cells by M-CSF treated THP-1 (Fig. 5b), suggesting that the FcγR expression on effector cells may be pivotal for rituximab-mediated ADCC by monocytes.

Discussion

In the present study, it was demonstrated that M-CSF enhanced monocyte-induced, rituximab-mediated ADCC of $CD20^+$ lymphoma cells. Although the mechanism by which M-CSF enhances monocyte cytotoxicity remains unknown, the present results suggest that this may partly be due to an increase in monocytes expression of FcγRI and FcγRIII. This hypothesis is supported by a previous study showing that rituximab-mediated depletion of B cells depends on monocytes expression of FcγRI and FcγRIII.[13] The importance of FcγR is also supported by the finding that follicular lymphoma patients with the FcγRIIIa 158 V allotype exhibit a higher affinity for human IgG1 and show better clinical responses to rituximab than those with the FcγRIIIa 158F allotype, who exhibit a lower affinity for human IgG1.[11,23] Monocyte enhancement of rituximab-mediated ADCC by M-CSF may also be due to prolonged activation of signal transduction pathways that promote cell survival, including the mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways,[15,16,19] although additional studies are needed to examine this possibility in detail.

Colony-stimulating factor 1^{op} mice, which are M-CSF-deficient and lack bone marrow macrophages and blood monocytes,[24] exhibit slow clearance of circulating B cells after treatment with CD20 monoclonal antibody and do not clear all of the mature spleen B cells.[13] In contrast, in mice lacking functional T cells and in perforin-deficient mice, which have defective natural killer cell function, more than 95% of blood and spleen B cells are eliminated after treatment with CD20 monoclonal antibody. Furthermore, classical or alternative pathway C activation does not contribute to B-cell depletion in the M-CSF-deficient mouse model. Based on these findings, it has been concluded that monocytes, as well as the innate monocyte network, are the major effectors mediating depletion of $CD20^+$ B cells *in vivo*. This may also be supported by the authors' findings that monocyte

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activation by M-CSF significantly improves the killing of CD20+ malignant cells via rituximab-mediated ADCC. Although the Daudi Burkitt's lymphoma cell line that was used as *in vitro* model of B-cell lymphoma is not a major target of rituximab therapy in the clinical setting, several reports[25,26] have shown Burkitt's lymphoma cell lines are acceptable as targets to evaluate rituximab-mediated ADCC.

Several studies have examined how cytokines that augment effector cell numbers and function and/or induce antigen expression on target cells affect ADCC in the context of rituximab therapy.[25,27–30] Interferon-α,[31] interleukin (IL)-2,[28] and IL-12[32] have been effectively combined with rituximab in small-group trials; however, the substantial toxicity of such proinflammatory cytokines might limit their overall utility in clinical settings.

Granulocyte colony-stimulating factor (G-CSF), which has a relatively low toxicity profile, and granulocyte-monocyte colony-stimulating factor (GM-CSF) have been reported to enhance the *in vitro* efficacy of rituximab by enhancing the ADCC of neutrophils.[25] In addition, in a severe combined immunodeficiency mouse lymphoma model, the concurrent administration of G-CSF augments the biological activity of rituximab, probably by increasing neutrophil counts.[33] Such a boost in the efficacy of rituximab does not occur with GM-CSF, although it also increased the neutrophil count. In preliminary clinical trials for relapsed or refractory B-cell non-Hodgkin lymphoma, the administration of G-CSF[30] and GM-CSF[34] appeared to enhance the effect of rituximab. Similar clinical trials using M-CSF in place of G-CSF and GM-CSF are warranted because of the lower toxicity of M-CSF.[35] Because administration of M-CSF causes a 10-fold increase in the numbers of blood monocytes and an increase in the numbers of macrophages in the liver, spleen, and peritoneal cavity,[20] M-CSF priming could be a reasonable approach for improving the therapeutic efficacy of rituximab against intractable CD20+ lymphoma, particularly in the late stages of the disease.

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ELSEVIER

Safety and Efficacy of Foscarnet for Preemptive Therapy Against Cytomegalovirus Reactivation After Unrelated Cord Blood Transplantation

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ABSTRACT

In association with the increased use of unrelated cord blood transplantation (UCBT) in adults, numerous patients have developed cytomegalovirus (CMV) reactivation concomitant with cytopenia. Although foscarnet appears to offer similar efficacy and higher safety as a preemptive therapy against CMV infection than ganciclovir, little is known about the usefulness of foscarnet in such patients. Foscarnet was administered as preemptive therapy against CMV antigenemia in 10 UCBT recipients who were unable to receive ganciclovir due to cytopenia or poor response to ganciclovir. Fatal CMV disease developed in one patient, whereas CMV antigenemia resolved without progression to CMV disease in the remaining nine patients. Foscarnet was well tolerated without serious hematotoxicity and was not discontinued due to adverse events in any patient. Foscarnet represents a safe and effective agent for preemptive therapy against CMV infection and may offer a feasible alternative to ganciclovir in UCBT recipients.

COMPARED TO ALLOGENEIC bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT), the advantages of unrelated cord blood transplantation (UCBT) include ease and safety of cell collection, low risk of transmitting viral infections, prompt availability of stem cells, and reduced incidence and severity of graft-versus-host disease (GVHD). Conversely, UCBT is disadvantageous in that slow marrow recovery and immunological immaturity after transplantation lead to an increased risk of infectious complications, and these account for most transplant-related deaths in adult patients receiving UCBT. Patients undergoing UCBT not only develop cytomegalovirus (CMV) reactivation more frequently but also earlier posttransplant compared to patients undergoing BMT or PBSCT.[1,2] For patients who develop CMV reactivation in the early post-UCBT period, preemptive treatment with ganciclovir might promote neutropenia that could place the patient at risk of fatal infectious complications. In contrast to ganciclovir, the antiviral drug foscarnet (trisodium phosphonoformate) appears to lack significant hematotoxicity in allograft recipients.[3–5] Dose-limiting toxicities of foscarnet are nephrotoxicity and neurotoxicity. Foscarnet might thus represent a feasible alternative for preemptive therapy against CMV reactivation in patients after UCBT. To evaluate the safety and efficacy of foscarnet for preemptive therapy against CMV infection after UCBT, the present study used this drug as an alternative to ganciclovir for patients who were unable to receive ganciclovir due to cytopenia or poor response to ganciclovir.

MATERIALS AND METHODS

Patients

Patients with CMV-positive antigenemia detected at least once were considered eligible for the study if absolute neutrophil count (ANC) was $<1.0 \times 10^9$/L or platelet count was $<20 \times 10^9$/L or response of ganciclovir against CMV antigenemia was tardy as assessed by a physician. Exclusion criteria were serum creatinine clearance <40 mL/min or therapy with foscarnet before study inclusion. Among 28 consecutive adult patients receiving UCBT at Kanazawa University Hospital between 2001 and 2005, 10 patients entered this study. Written informed consent was obtained from all patients. Foscarnet was administered as initial preemptive therapy in 5 of the 10 patients due to cytopenia. Pharmacotherapy was switched from ganciclovir to foscarnet due to ganciclovir-induced

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cytopenia in three patients and tardy response to ganciclovir in two patients.

CMV Antigenemia Monitoring

Monitoring CMV-pp65 antigen levels assessed CMV antigenemia. CMV antigenemia assays were performed as previously described.[6] Degree of CMV antigenemia was expressed as the number of CMV antigen-positive cells per 5×10^4 leukocytes, and CMV antigenemia was defined as ≥1 antigen-positive cell.

Preemptive Therapy With Foscarnet for Prevention of CMV Disease

Foscarnet was intravenously administered over 2 hours at 60 mg/kg every 12 hours for 14 days as induction treatment. Treatment was stopped if two consecutive CMV antigenemia assays yielded negative results. When CMV antigenemia remained detectable in peripheral blood assay after induction treatment, patients then received intravenous foscarnet at a maintenance dosage of 90 mg/kg/d over 3 hours while antigenemia persisted. Dosage of foscarnet was adjusted according to decreased renal function based on predetermined guidelines. Renal impairment was considered present for an increase in serum creatinine ≥100% or a decrease in creatinine clearance ≥50% from baseline values.

Statistical Analysis

Parameters in the two groups were compared by Fisher exact test.

RESULTS

Efficacy of Foscarnet

The 10 patients had a total of 28 episodes of CMV antigenemia one to five episodes a patient). Median duration from transplantation to initiation of foscarnet treatment was 43 days (range, 14 to 52 days; Table 1). Median duration of exposure to foscarnet in one episode was 10 days (range, 7 to 20 days). In 9 of the 10 patients CMV antigenemia ended, which was defined as attainment of no CMV antigenemia for ≥3 months without antiviral treatment, at a median of 60 days (range, 7 to 104 days) after starting foscarnet treatment. The remaining one patient received foscarnet starting on day 14 due to ganciclovir-associated neutropenia, but it resulted in no response and the patient died of interstitial pneumonia (IP) on day 83.

Safety of Foscarnet

Although one patient developed impaired renal function during treatment with foscarnet, only dose adjustment was required. Although hypocalcemia, hypomagnesemia, hypokalemia, and hypophosphatemia occurred in seven, five, seven, and one patients, respectively, electrolyte disturbances were improved by supplementation through intravenous infusions, and no clinical symptoms or signs attributable to changes in serum electrolyte levels were identified. No seizures or parasthesias occurred. Nausea and vomiting occurred in two patients and were resolved using antiemetic treatments. Median ANC on onset of foscarnet was 1.3×10^9 (range, 0.4×10^9/L to 4.3×10^9/L), and median maximal drop in ANC was 0.2×10^9/L (range, -1.6×10^9/L to 12.9×10^9/L). No patient developed foscarnet-induced severe neutropenia ($<0.5 \times 10^9$/L), although one patient whose ANC was 0.4×10^9/L at the start of foscarnet therapy received transient treatment with granulocyte colony-stimulating factor in association with foscarnet. No adverse events required discontinuation of foscarnet treatment.

Comparison Between UCBT Patients and Unrelated BMT Patients

When the UCBT patients were compared to seven unrelated BMT patients who received foscarnet as preemptive therapy against CMV infection, there was no statistical difference between the two groups in parameters on the efficacy and toxicity of foscarnet (Table 1).

DISCUSSION

CMV infection is still a major concern following allogeneic hematopoietic transplantation because CMV pneumonia is fatal in 70% of patients, even when treated with a combination of antiviral therapies and CMV hyperimmune immunoglobulin.[7] The determinants for development of CMV infection and disease are seropositivity for CMV prior to transplant, GVHD, HLA-mismatched donor, unrelated bone marrow or cord blood donor, treatment with steroids, and modifications of the graft such as in vitro and in vivo T-cell depletion. In contrast to patients treated with high-

Table 1. Efficacy and Toxicity of Foscarnet

	UCBT (n = 10)	Unrelated BMT (n = 7)
Median time from SCT to treatment start with foscarnet, d (range)	43 (14–52)	34 (25–70)
Median time of foscarnet treatment, d (range)	10 (7–20)	8 (6–60)
Patients with clearance of CMV antigenemia (%)	90	100
Patients with CMV disease (type)	1 (IP)	1 (enteritis, before the initiation of foscarnet)
Transplant-related deaths		
Infections	2	1
CMV-induced IP	1	0
Median ANC on onset of foscarnet (range), $\times 10^9$/L	3 (0.4–15.4)	3.1 (1.7–10.5)
Median platelet counts on onset of foscarnet (range), $\times 10^9$/L	26 (13–113)	75 (40–223)
Median maximal drop in ANC from the start of foscarnet during foscarnet treatment (range), $\times 10^9$/L	0.2 (−1.6–12.9)	0 (−2.4–6.9)
Patients with discontinuation of foscarnet	0	0

dose chemotherapy and autologous stem cell transplantation, patients after allogeneic stem cell transplantation are at a much higher risk of development of CMV infection because of the delayed recovery of T- and B-cell functions. Thus the rate by which immune function recovers after hematopoietic reconstitution significantly influences the incidence of CMV infection and disease after stem cell transplantation.

The current study has shown that foscarnet can be effective as a preemptive therapy against CMV reactivation after UCBT. Only 1 of 10 patients developed CMV disease and CMV antigenemia resolved in the remaining nine patients during the study period, appearing comparable to treatment results with ganciclovir and foscarnet for recipients of allogeneic BMT and PBSCT.[3,5,8–10]

Despite effectiveness in preventing CMV disease after BMT and PBSCT, use of ganciclovir is associated with marked toxicity to the bone marrow, and severe neutropenia is reported in up to 35% of patients who receive ganciclovir treatment after allogeneic BMT or PBSCT.[11–14] This long duration of neutropenia places patients receiving ganciclovir at significant risk of bacterial and fungal infection,[14] and 8% also develop CMV disease.[13] Furthermore, neutropenia in ganciclovir recipients has been shown to represent an independent risk factor for mortality.[11]

Foscarnet was well tolerated and was not associated with any serious hematotoxicity, even in patients with cytopenia at the start of treatment. In addition, foscarnet did not interfere with hematopoietic engraftment despite being administered in the early posttransplant period after UCBT. These results suggest that foscarnet not only can be used from the very early phases of engraftment but also might be started before transplantation for prophylaxis against CMV reactivation. Of note is the fact that foscarnet was not discontinued due to adverse events in any patient. All adverse events, including nephrotoxicity, were resolved with supportive medication or a reduction in foscarnet dose.

In conclusion, the present results may suggest that foscarnet offers an effective and safe alternative to ganciclovir for CMV prophylaxis in recipients of allogeneic UCBT who are unable to receive treatment with ganciclovir due to cytopenia or poor response to ganciclovir. Randomized, comparative studies between ganciclovir and foscarnet are warranted for better evaluation of the preemptive and treatment roles of these agents in patients after UCBT.

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Tacrolimus as Prophylaxis for Acute Graft-Versus-Host Disease in Reduced Intensity Cord Blood Transplantation for Adult Patients With Advanced Hematologic Diseases

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Background. Myeloablative cord blood transplantation (CBT) for adult patients offers a 90% chance of engraftment with a 50% rate of transplant-related mortality, mostly attributable to infection. We have demonstrated the feasibility of reduced-intensity CBT (RI-CBT) for adult patients, in which cyclosporine was used for acute graft-versus-host disease (GVHD) prophylaxis. Transplantation-related mortality (TRM) was 27% within 100 days. Therefore our objective was to evaluate the feasibility of RI-CBT with tacrolimus as GVHD prophylaxis for adult patients with hematologic malignancies.

Methods. Thirty-four patients with a median age of 56.5 years (range; 22–68) with hematologic diseases underwent RI-CBT at Toranomon Hospital between November 2003 and September 2004. Preparative regimen comprised fludarabine 25 mg/m2 on days −7 to −3, melphalan 80 mg/m2 on day −2, and 4 Gy total body irradiation on day −1. GVHD prophylaxis was continuous intravenous infusion of tacrolimus 0.03 mg/kg, starting on day −1.

Results. Thirty-one patients achieved neutrophil engraftment at a median of day 20. Median infused total cell dose was 2.4×10E7/kg (range; 1.6–4.8). Thirty-two patients achieved complete donor chimerism at day 60. Grade II–IV acute GVHD occurred in 45% of patients, with a median onset of day 26. Primary disease recurred in five patients, and TRM within 100 days was 12%. Estimated 1-year overall survival was 70%.

Conclusion. This study demonstrated the possible improvement in transplant-related mortality by tacrolimus as GVHD prophylaxis in adult RI-CBT recipients.

Keywords: Tacrolimus, Acute graft-versus-host disease, Reduced intensity cord blood transplantation.

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Cord blood transplantation (CBT) represents an attractive alternative for patients with hematologic diseases who lack matched related or unrelated donors. The value of CBT using myeloablative preparative regimens has been confirmed for pediatric and adult patients (*1–5*). Myeloablative CBT for adult patients offers a 90% chance of engraftment with a 50% rate of transplant-related mortality, mostly attributable to infection (*1–4, 6*).

We and other groups have demonstrated the feasibility of reduced-intensity CBT (RI-CBT) for adult patients, in which cyclosporine was used for acute graft-versus-host disease (GVHD) prophylaxis (*7, 8*). Transplantation-related mortality (TRM) was 27% within 100 days (*7*). Posttransplant immune disorders including pre-engraftment immune reactions (PIR) and acute GVHD were problematic in RI-CBT for adult patients (*7, 9*). These reactions and/or additional immunosuppressive therapy might have increased the risk of infection and/or organ dysfunction, leading to a high TRM (*10, 11*).

We employed tacrolimus for acute GVHD prophylaxis in place of cyclosporine from November 2003 in RI-CBT. It might reduce the incidence and severity of PIR and acute GVHD. We will summarize the results of RI-CBT using tacrolimus to investigate its safety and efficacy for acute GVHD prophylaxis after RI-CBT.

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MATERIALS AND METHODS

Study Patients

The study population consisted of 34 adult patients with hematologic disorders, who underwent a protocol of RI-CBT with GVHD prophylaxis using tacrolimus alone at Toranomon Hospital between November 2003 and September 2004. All of the patients were incurable with conventional treatments and were considered inappropriate for conventional allogeneic stem-cell transplantation (allo-SCT) due to the lack of an human leukocyte antigen (HLA)-identical sibling or a suitable unrelated donor, age >50 years old, and/or organ dysfunction. Patients with no suitable HLA-matched related donor were eligible for this protocol if a matched unrelated bone marrow donor was unavailable as a first treatment option. If there was insufficient time for an unrelated bone marrow donor search due to rapidly progressive disease or if the preliminary search indicated a low possibility of obtaining a matched unrelated bone marrow donor, we attempted to locate cord blood grafts. Patients who received RI-CBT as second allo-SCT were excluded from this study. The clinical protocol was approved by the Institutional Review Board of Toranomon Hospital, and written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

HLA Typing and Donor Matching

HLA-A and HLA-B antigens were determined by serologic typing. HLA-DRB1 alleles were identified by high-resolution molecular typing using polymerase chain reaction method (PCR) with sequence-specific primers (SSP). All cord blood grafts were evaluated by HLA-A, HLA-B, and HLA-DRB1 typing and nucleated cell counts. Preferred cord blood units were those matched to 4 or 6 of 6 HLA loci and contained at least a cell count of 2×10E7 nucleated cells/kg of recipient body weight before freezing. All cord blood units came from cord blood bank in the Japan Cord Blood Bank Network (*12*).

Preparative Regimen and GVHD Prophylaxis

Preparative regimen comprised fludarabine 25 mg/m^2 on days −7 to −3, melphalan 80 mg/m^2 on day −2, and 4 Gy total body irradiation (TBI) in two fractions on day −1 (*7*). GVHD prophylaxis was continuous intravenous infusion of tacrolimus 0.03 mg/kg, starting on day −1. Once oral intake could be tolerated, patients were administered oral tacrolimus at a dose ratio of 1:2–3, in two divided doses based on the latest intravenous dose. In the absence of GVHD, it was tapered from day 100 until day 150. Tacrolimus was reduced when serum creatinine levels were elevated above 1.5 times baseline or other serious adverse effects occurred. If grade II–IV acute GVHD developed, methylprednisolone at a dose of 1 to 2 mg/kg was added to tacrolimus.

Supportive Cares

All of the patients were managed in reverse isolation in laminar airflow-equipped rooms and received trimethoprim/sulfamethoxazole for *Pneumocystis carinii* prophylaxis. Fluoroquinolone and fluconazole were administered for prophylaxis of bacterial and fungal infections, respectively. Prophylaxis of herpes virus infection with acyclovir was also given. Neutropenic fever was managed according to the guidelines (*13*). Cytomegalovirus (CMV) pp65 antigenemia was monitored once a week. If positive results were identified, preemptive therapy with foscarnet was initiated. Hemoglobin and platelet counts were maintained at >7 g/dL and 10×10E9/L, respectively, with in-line filtered and irradiated blood transfusions. All the patients received granulocyte colony stimulating factor (G-CSF) at a dose of 5 μg/kg intravenously, starting on day 1 until durable neutrophil recovery was achieved.

Assessment of Engraftment, GVHD, Regimen-Related Toxicity (RRT) and Survival

Engraftment was defined as the first of two consecutive days in which white blood cell counts >1.0×10E9/L or the absolute neutrophil counts (ANC) >0.5×10E9/L. The date of platelet recovery was defined as the first of seven consecutive days during which the nontransfused platelet count was at least 20×10E9/L.

Chimerism was assessed using fluorescent in situ hybridization in sex-mismatched donor-recipient pairs. In sex-matched pairs, PCR for variable number of tandem repeats was used with donor cells detected at a sensitivity of 10% (*14*). Whole blood and CD3-positive cell chimerism was assessed at the time of granulocyte engraftment. When engraftment was delayed, chimerism was assessed at least once during life.

Primary graft failure was defined as peripheral cytopenia and marrow hypoplasia occurring later than day 60, without detection of donor markers by cytogenetic and/or molecular techniques (*15*). Late graft failure was defined among the patients who attained neutrophil engraftment as a decline of ANC to less than 0.5×10E9/L for at least seven consecutive days with evidence of severe hypocellularity of bone marrow confirmed by histopathological examination. PIR was diagnosed as reported previously (*9*). When febrile patients (body temperature ≥38 °C) with no evidence of infection or adverse effects of medication exhibited skin eruption, diarrhea, jaundice (serum total bilirubin >2.0 mg/dL) or body weight gain >10% of baseline, these changes were defined as immune reactions. Immune reactions developing ≥6 days before engraftment were defined as PIR. Acute and chronic GVHD were diagnosed and graded according to standard criteria (*16, 17*). GVHD was clinically diagnosed in combination with skin or gut biopsies after engraftment or attainment of 100% donor chimerism. All the patients who had evidence of donor cell engraftment were considered to be evaluable for acute GVHD. Response to corticosteroid was evaluated according to the report by Martin et al. (*18*). Chronic GVHD was evaluated in patients who survived without relapse or disease progression for at least 100 days after transplantation. Patients with hematologic malignancies in complete remission (CR) at the time of transplant, in chronic phase of chronic myelogenous leukemia (CML), with refractory anemia (RA), or refractory anemia with ringed sideroblasts (RARS) of myelodysplastic syndrome (MDS) and with nonmalignant disease were defined as being at standard risk. The other patients were defined as being at high risk. Chemotherapy resistance was defined as relapse after initial cytotoxic chemotherapy or failure to achieve remission.

Overall survival (OS) was applied to all the patients and

measured from the date of transplantation (day 0) to the date of death from any cause. Event-free survival (EFS) was evaluated in patients who survived in remission for at least 30 days after transplantation and measured from the date of transplantation to the date of relapse or death. Patients who have never achieved CR after transplant were considered to have had a recurrence on day 0. Surviving patients were censored at the time of the last follow-up. RRT was defined as any nonhematologic organ dysfunction from day 0 to day 28 and was graded according to the Bearman's criteria (*19*). Toxicities of foscarnet were evaluated using the Common Terminology Criteria for Adverse Events version 3.0 (*20*). TRM was defined as death without the primary disease progression.

Endpoints and Statistical Analysis

Data were analyzed as of December 2005. The primary end point of this study was to evaluate the feasibility of GVHD prophylaxis using tacrolimus in the setting of RI-CBT. The secondary end points were to assess the incidences of RRT, acute and chronic GVHD, infections, EFS and OS. Cumulative incidences were estimated for engraftment, achievement of donor chimerism, acute GVHD and cytomegalovirus reactivation to take account of a competing event (e.g., death) (*21*). Gray's test was used to compare different cumulative incidence curves (*21*). The probabilities of EFS and OS were estimated from the time of transplantation, according to the Kaplan-Meier product limit method. Log-rank test was used to compare these two outcomes. The following patient or transplant characteristics (baseline factors) were analyzed using a Cox regression model for their prognostic value on EFS and OS: patient age, sex, performance status, disease risk, previous history of autologous stem-cell transplantation (yes/no), disparity of HLA-A, -B, -DR antigen (one/two antigen mismatched), number of infused nuclear cells and number of CD34-positive cell dose. Stat View 5.0, Statistical Analysis System (SAS; SAS Institute Inc., Cary, NC) and S Plus 2000 (Mathsoft, Seattle, WA) were used for all statistical analyses.

RESULTS

Characteristics of the Study Patients

Patient characteristics and cord blood grafts are summarized in Table 1. Median age was 56.5 years (range, 22–68), and median weight was 57 kg (range, 40–75 kg). Twenty-two patients were refractory to cytotoxic chemotherapies. Another nine patients had chemosensitive diseases including acute myeloid leukemia (AML) in second CR (n=4), CML in second chronic phase (n=2), adult T-cell leukemia in partial remission (n=2), and malignant lymphoma in second CR (n=1). Two patients who had AML with prior MDS (n=1) had not received pretransplantation chemotherapy. The remaining patient had transfusion-dependent severe aplastic anemia.

TABLE 1. Patient characteristics

Variables	N or median (range)
Total patients	34
Sex (male/female)	
Male	11
Female	23
Age, median years (range)	56.5 (22–68)
Weight, mean kg (range)	57 (40–75)
Underlying diseases (n)	
Acute myeloid leukemia	13[a,b]
Myelodysplastic syndrome	3[c,d]
Acute lymphoblastic leukemia	3[e,f]
Adult T-cell leukemia	6[g]
Chronic myeloid leukemia	3[h]
Malignant lymphoma	5[i]
Severe aplastic anemia	1
Risk of underlying diseases	
High	26
Low	8
Previous history of autologous stem-cell transplantation	
Yes	2
No	32
HLA mismatches	
One	40
Two	30
Number of infused nuclear cells/kg, median (range)	2.4 (1.6–4.8)

Patients with hematologic malignancies in complete remission at the time of transplant, in chronic phase of chronic myelogenous leukemia, with refractory anemia or refractory anemia with ringed sideroblasts of myelodysplastic syndrome, and with nonmalignant diseases were defined as being at standard risk. The other patients were defined as being at high risk.

[a] Three patients were in the second complete remission. The primary diseases were refractory to chemotherapy in 8 patients. The remaining 2 patients had not received prior chemotherapy.

[b] Data of Chromosomal abnormalities were available in 10 patients. Those revealed normal karyotype (n=8) and complex karyotype (n=2).

[c] The primary diseases were refractory to chemotherapy in 2 patients. The remaining 1 patient had not received prior chemotherapy.

[d] Data of chromosomal abnormality was available in 1 patient. It revealed 47XY, +21 [1], 46XY [29].

[e] The primary diseases were refractory to chemotherapy in all the 3 patients.

[f] Chromosomal abnormalities reveals t (9; 22) (q34; q11) and 45 X, -Y [4], 69, XXY [1], 46, XY [14] (n=1).

[g] The primary diseases were refractory to chemotherapy in 5 patients. The remaining 1 patient received retransplantation due to graft failure of reduced-intensity cord blood transplantation.

[h] The disease status of those patients were the second chronic phase (n=1), accelerated phase (n=1), and blast crisis (n=1).

[i] One patient was in complete remission. The primary diseases were refractory to chemotherapy in the remaining 4 patients.

Engraftment

Thirty-one patients achieved neutrophil engraftment at a median of day 20 (range, 12–33). Platelet engraftment was achieved in 27 patients, at a median of day 38 (range, 24–216). All of the three patients without primary engraftment died at a median of day 26 (range, 20–34) due to sepsis (n=2) and intracranial hemorrhage (n=1). Neither primary nor late graft failure was diagnosed in any of the 34 patients.

TABLE 2. Prognostic factors of neutrophil engraftment and 100% donor chimerism

	N	Percent (95% CI)	*P* value
Neutrophil engraftment			
Variable			
Total cell dose			0.010
≥3×10E7/kg	7	100 (68–100)	
<3×10E7/kg	27	89 (69–100)	
HLA disparities			0.0097
HLA 5/6 match	4	100 (64–100)	
HAL 4/6 match	30	90 (71–100)	
100% donor chimerism			
Total cell dose			0.25
≥3×10E7/kg	7	100 (68–100)	
<3×10E7/kg	27	93 (72–100)	
HLA disparities			0.96
HLA 5/6 match	4	100 (64–100)	
HAL 4/6 match	30	93 (75–100)	

TABLE 3. Regimen-related toxicity according to Bearman's criteria

Grade	0	I	II	III	IV
Central nervous system	32	0	1	0	1
Lung	33	0	0	1	0
Kidney	28	4[a]	2[a]	0	0
Liver	22	10[b]	2[b]	0	0
Heart	34	0	0	0	0
Gut	10	19	5	0	0

[a] Median serum creatinine level: 1.2 mg/dL (range, 0.9–1.8).
[b] Median serum aspartate aminotransferase and alanine aminotransferase levels of those patients were 29 IV/L (range 18–274) and 24 IV/L (range 12–593), respectively.

Chimerism Analysis

Chimerism data were obtained from all the 34 patients. Thirty-two patients (94%) achieved complete donor chimerism at day 60. Median time to complete donor chimerism was 22 days (range, 13–38). One patient who died of TRM within 28 days of RI-CBT had complete donor chimerism before neutrophil engraftment. All the surviving patients were monitored for chimerism every 3 months, showing complete donor chimerism during the follow-up even after the discontinuation of immunosuppressants.

No significant association was identified between complete donor chimerism and either infused cell dose or HLA disparity (Table 2).

Cause of Death

Nine patients died during follow-up. Four patients died due to TRM at a median day of 30 (range, 20–46). The remaining five patients died due to the primary disease progression at a median of day 171 (range, 103–203).

Regimen-Related Toxicity and Transplantation-Related Mortality

RRT was shown in Table 3 Grade III–IV RRT developed in two patients. One patient developed fatal intracranial hemorrhage on day 26. TRM within 100 days of RI-CBT was 12% (95% confidence interval [CI], 1–23%). Primary causes of death were sepsis (n=2), encephalitis (n=1), and intracranial hemorrhage (n=1). Three patients developed creatinine level abnormality associated with tacrolimus: grade 1 in one, grade 2 in one, and grade 3 in one patient. Those patients required dose modifications of tacrolimus for toxicities. No patients developed tacrolimus-associated hypertension, diabetes, neurotoxicity, or microangiopathy.

Pre-Engraftment Immune Reactions

Fifteen of the 34 patients (44%; 95% CI, 27–61%) developed PIR. PIR was treated supportively without corticosteroid in all the patients.

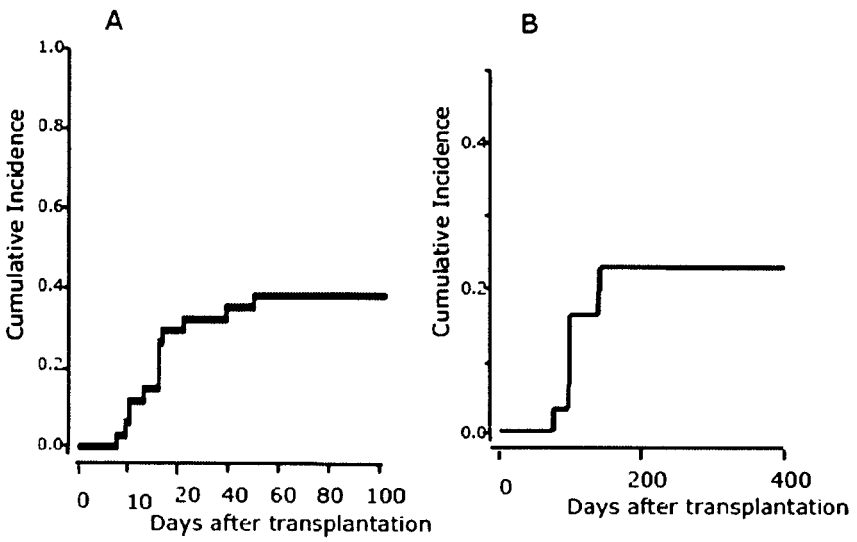

FIGURE 1. (A) Cumulative incidence of grade II–IV acute GVHD. Fourteen of 31 patients (45%; 95% CI, 28%–63%) who achieved primary engraftment developed grade II–IV acute GVHD. (B) Cumulative incidence of chronic GVHD. Seven of the evaluable 26 patients (27%) developed chronic GVHD.

Graft-Versus-Host Disease

Fourteen of 31 patients (45%; 95% CI, 28–63%) who achieved primary engraftment developed grade II–IV acute GVHD: grade II (n=6) and grade III (n=8) (Fig. 1-A). Its median onset was day 26 (range, 12–90). Ten patients with grade II–IV acute GVHD received corticosteroids. The initial response to corticosteroid was CR in seven, partial response (PR) in two, and mixed response in one patient. No patients required second line immunosuppressive therapy for acute GVHD. Seven of the evaluable 26 patients (27%) developed chronic GVHD including extensive-type (n=2) and limited-type (n=5) (Fig. 1B). Neither acute nor chronic GVHD was fatal in any patients.

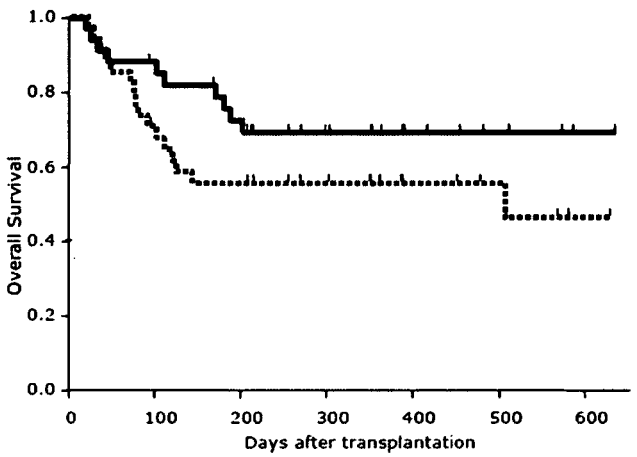

FIGURE 2. Probability of overall survival (OS) and event-free survival (EFS). As of December 2005, the median follow-up after RI-CBT for surviving patients was 12.1 months (range 3.1–21.1). The Kaplan-Meier probability of OS and EFS at 1 year were 70% and 55% (95% confidence interval: 38%–72%), respectively.

Infection

Twelve patients developed bacteremia (n=12). It was fatal in two patents. One patient developed disseminated tuberculosis, which was successfully treated by antitubercular drug (*11*). Reactivation of CMV was documented in 15 patients at a median of day 33 (range, day 17–87). Six patients developed CMV enterocolitis. It was successfully treated by ganciclovir or foscarnet. One developed viral hemorrhagic cystitis, which was successfully treated by vidarabine. Two patients developed encephalitis caused by human herpes virus 6. It was fatal in one patient despite foscarnet use.

Event-Free and Overall Survival

As of October 2005, the median follow-up after RI-CBT for surviving patients was 12.1 months (range 3.1–21.1). Five patients died due to disease progression. The Kaplan-Meier probability of EFS and OS at 1 year were 55% (95% CI, 38%–72%) and 70% (95% CI: 54%–85%), respectively (Fig. 2).

Prognostic Factors

Univariate analysis failed to identify any significant risk factors of OS, and multivariate analysis was therefore not conducted (Table 4).

Univariate analysis showed borderline significances between EFS and either risk of underlying diseases or HLA disparity; however, multivariate analysis failed to identify any prognostic factors of EFS (Table 3).

TABLE 4. Prognostic factors of overall and event-free survival

Univariate factors	Hazard ratio	95% CI	*P* value
Overall survival			
Age (years)	1.01	0.96–1.068	0.63
Sex (female vs. male)	1.99	0.42–9.39	0.38
Performance status (1 vs. 0)	4.15	0.88–19.56	0.072
Risk of underlying diseases (high vs. low)	3.20	0.41–25.31	0.27
Previous history of autologous stem-cell transplantation (yes vs. no)	1.68	0.36–7.93	0.51
Disparity of HLA-A, -B, -DR antigen (two- vs. one-antigen mismatched)	0.56	0.12–2.64	0.46
Number of infused nuclear cells	0.58	0.17–1.97	0.38
Number of infused CD34 positive cells	0.54	0.14–2.14	0.38
Event-free survival			
Age (years)	1.02	0.98–1.06	0.38
Sex (female vs. male)	0.57	0.21–1.55	0.27
Performance status (1 vs. 0)	2.35	0.81–6.79	0.11
Risk of underlying diseases (high vs. low)	2.76	0.63–12.18	0.18
Previous history of autologous stem-cell transplantation (yes vs. no)	1.00	0.23–4.44	1.00
Disparity of HLA-A, -B, -DR antigen (two- vs. one-antigen mismatched)	0.36	0.10–1.31	0.12
Number of infused nuclear cells	1.21	0.58–2.55	0.61
Number of infused CD34 positive cells	1.10	0.56–2.15	0.79
Multivariate factors		0.74–6.37	0.16
Performance status (1 vs. 0)	2.18	0.12–1.55	0.19
Disparity of HLA-A, -B, -DR antigen (one- vs. two-antigen mismatched)	0.43		

TABLE 5. Clinical characteristics of patients treated with cyclosporine and tacrolimus

Variables	Cyclosporine[a]	Tacrolimus	*P* value
N	30	34	
Age, median years (range)	58.5 (20–70)	56.5 (22–68)	0.21
HLA matching (n)			
5/6	6	4	0.37
4/6	24	30	
Pre-engraftment immune reactions (%)	66.7	44.1	0.07
Grade II–IV acute GVHD (%)	37.5	45.2	0.57
Transplant-related mortality before day 100 (%)	26.7	11.8	0.13
Overall survival at 1 year (%)	26.4	69.5	0.02

[a] Patient characteristics were described in reference 7.

Clinical Impact of Cyclosporine and Tacrolimus as GVHD Prophylaxis

We summarized the clinical characteristics of the patients with between cyclosporine and tacrolimus in Table 5. The characteristics of patients with cyclosporine were previously described (*7*).

DISCUSSION

The present study suggests that GVHD prophylaxis using tacrolimus is feasible in adult RI-CBT recipients. Intensification of GVHD prophylaxis can suppress postCBT immune reactions including PIR and GVHD. PIR is an immune reaction before engraftment, which is frequently associated with fever, diarrhea, rash, and weight gain (*9*). We previously reported that 78% of the RI-CBT recipients given cyclosporine developed PIR (*9*), while 15 of the 34 patients (44%; 95% CI, 27–61%) developed it in the present study. PIR was less frequent in the present study than in RI-CBT with cyclosporine (*7*). Interestingly, it was treated supportively without corticosteroid in all the patients, while 66% of the patients with PIR were given corticosteroid than in RI-CBT with cyclosporine (*7*). Severity of PIR was milder in patients given tacrolimus than those given cyclosporine.

GVHD is the most significant concern in allo-SCT. The frequencies of grade II–IV acute GVHD in adult myeloablative CBT using mainly cyclosporine were 25–72% (*22–28*). We previously reported that 66% of adult RI-CBT recipients developed grade II–IV acute GVHD, when cyclosporine was used for GVHD prophylaxis (*9*). In the present study, 14 of 31 patients (45%; 95% CI, 28%–63%) developed grade II–IV acute GVHD. The incidence of acute GVHD was lower in RI-CBT using tacrolimus than that using cyclosporine. Tacrolimus has been shown stronger in its immunosuppressant effects than cyclosporine in randomized controlled studies (*29–31*) and in vitro studies (*32*). It is reasonable to assume that immunosuppression using tacrolimus might suppress acute GVHD in the present study. Alternatively, suppression of PIR with tacrolimus might have contributed to the prevention of acute GVHD following RI-CBT, since PIR can trigger GVHD (*9*).

The present study suggests the possibility that intensification of GVHD prophylaxis decreases TRM after RI-CBT, improving the prognosis. GVHD prophylaxis in adult CBT is mostly cyclosporine, and the TRM ranges 27–52% (*3, 4, 7, 8*). In contrast, TRM in the present study with tacrolimus was 12% (95% CI, 1–23%), which was much lower than those in previous reports (*3, 4, 7, 8*). The major causes of TRM after CBT are infections and GVHD (*3, 4, 7, 8, 10*). As none died of GVHD, intensification of immunosuppression may have reduced TRM. Additional immunosuppression such as steroids for PIR and GVHD may increase the risk for infections. Intense GVHD prophylaxis by tacrolimus probably reduced steroid use, and hence the risk of severe infections.

It remains unknown whether intensification of GVHD prophylaxis using tacrolimus might hamper graft-versus-malignancy (GVM) effects, since they are closely associated with GVHD (*33*). In the present study, the cumulative probability of relapse at 1 year was 37%, and 55% of the RI-CBT recipients survived without disease progression at 1 year after transplantation. Considering the patients' backgrounds in this study, these findings suggest that RI-CBT using tacrolimus carries a considerable GVM effects. Since the impact of GVHD on a GVM effect varies according to disease status and patients' conditions, management of GVHD should be tailored. Further studies are warranted to establish a proper GVHD prophylaxis following RI-CBT.

Adverse effects of tacrolimus were tolerable in RI-CBT recipients in the present study. Its major adverse effects include renal insufficiency, hyperglycemia, and hypertension. Renal insufficiency by tacrolimus has been reported higher in incidence than by cyclosporine (*34*). Despite the advanced age in most of our patients, renal insufficiency by tacrolimus was reversible. While another concern about tacrolimus may be graft failure with intense immunosuppression (*35*), incidence of engraftment in the present study was comparable to those in the previous reports (*3, 4, 7, 8*). This study has demonstrated that tacrolimus is feasible in RI-CBT for patients with advanced age.

While the present study suggested the possible improvement in TRM by intensification of GVHD prophylaxis, there are some problems to be discussed. First, it is a small-sized, retrospective study; unrecognized biases might have affected the results. Large-scale prospective evaluations are required. Second, the follow up was rather short. Little information is available concerning chronic GVHD and GVM effects. Longer follow-up observations are necessary to investigate them. Third, we did not investigate the postCBT immune reconstitutions in the recipients with tacrolimus. Evaluation of immune parameters such as

CD4 and CD8 T cell might be worth investigating. The last optimal strategy should be established in the management of acute GVHD following RI-CBT. An appropriate protocol of tacrolimus use in RI-CBT for patients with advanced age remains unclear and requires further study.

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Invasive Fungal Infection Following Reduced-Intensity Cord Blood Transplantation for Adult Patients with Hematologic Diseases

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ABSTRACT

Invasive fungal infection (IFI) is a significant complication after allogeneic hematopoietic stem cell transplantation (HSCT); however, we have little information on its clinical features after reduced intensity cord blood transplantation (RICBT) for adults. We reviewed medical records of 128 patients who underwent RICBT at Toranomon Hospital between March 2002 and November 2005. Most of the patients received purine-analogbased preparative regimens. Graft-versus-host disease (GVHD) prophylaxis was a continuous infusion of either tacrolimus 0.03 mg/kg or cyclosporine 3 mg/kg. IFI was diagnosed according to the established EORTC/NIH-MSG criteria. IFI was diagnosed in 14 patients. Thirteen of the 14 had probable invasive pulmonary aspergillosis and the other had fungemia resulting from *Trichosporon* spp. Median onset of IFI was day 20 (range: 1-82), and no patients developed IFI after day 100. Three-year cumulative incidence of IA was 10.2%. Four of the 13 patients with invasive aspergillosis (IA) developed grade II-IV acute GVHD, and their IA was diagnosed before the onset of acute GVHD. The mortality rate of IFI was 86%. Multivariate analysis revealed that the use of prednisolone >0.2 mg/kg (relative risk 7.97, 95% confidence interval 2.24-28.4, P = .0014) was a significant risk factor for IA. This study suggests that IFI is an important cause of deaths after RICBT, and effective strategies are warranted to prevent IFI.

KEY WORDS

Invasive aspergillosis • Graft-versus-host disease • Corticosteroid

INTRODUCTION

Cord blood transplantation (CBT) is an attractive alternative for patients with hematologic diseases who lack a matched related or unrelated donor. The usefulness of CBT using myeloablative preparative regimens has been confirmed for pediatric patients [1,2]. Myeloablative CBT for adult patients achieves engraftment in 90% of the patients, but carries 50% risk of transplant-related mortality (TRM), mostly resulting from infection [3,4]. We and other groups have reported the feasibility of CBT using reduced-intensity regimens (RICBT) for adult patients with advanced hematologic diseases [5,6].

Because of delayed immune recovery and graft-versus-host disease (GVHD), infection is the leading cause of TRM after CBT using myeloablative preparative regimens [2-4,7]. However, studies on immune recovery following RICBT gave us hope that RICBT recipients may less frequently experience GVHD and infectious complications. Invasive fungal infection (IFI) has been 1 of the most feared infectious complications in conventional allogeneic marrow or peripheral blood stem cell (PBSC) transplantation [8,9], whereas we have little information on IFI following RICBT. We investigated its incidence and clinical features in patients who underwent RICBT for advanced hematologic diseases.

PATIENTS AND METHODS

Data Collection

We reviewed medical records of 128 recipients who underwent first reduced-intensity allogeneic hematopoietic stem cell transplantation (HSCT) using cord blood (CB) between March 2002 and November 2005 at Toranomon Hospital. Their characteristics are shown in Table 1. Of the 128 patients, 101 had high-risk diseases including acute myelogenous leukemia (AML) in relapse or the second and higher complete remission (CR; n = 42), acute lymphoid leukemia (ALL) except those in the first CR (n = 12), chronic myelogenous leukemia (CML) in blastic phase (n = 4), myelodysplasia except refractory anemia (n = 10), refractory lymphoma (n = 30), idiopathic myelofibrosis (n = 1), plasma cell leukemia in relapse (n = 1), and chronic myelomonocytic leukemia (n = 1).

Table 1. *Patients' Characteristics and Transplantation Procedures*

Variables	Number
Patients Characteristics	
Age, median (range)	56 (17-71)
Sex, male/female	80/48
Primary diseases	
AML/MDS	63
Malignant lymphoma	33
Acute lymphoblastic leukemia	17
Severe aplastic anemia	6
Chronic myelogenous leukemia	6
Chronic myelomonocytic leukemia	1
Plasmacytic leukemia	1
Idiopathic myelofibrosis	1
Risk of underlying diseases,*1 high/low	101/27
Prior autologous stem cell transplant, yes/no	9/119
Transplantation procedures	
Conditioning regimen	
Flu + Mel + TBI 2 Gy or 4 Gy/8 Gy	112/2
Flu + BU + TBI 4 Gy/8 Gy	8/1
Others	5
GVHD prophylaxis, cyclosporine/tacrolimus	64/64
Number of infused nucleated cell, median (range) $\times 10^7$/kg	2.7 (1.6-4.8)
HLA disparity (antigen), 2/1/0	108/17/3
Transplantation outcomes	
Neutrophil engraftment	99/128
Complete donor chimerism*2	90/99
Grade II-IV acute GVHD*2	45/99
Chronic GVHD*3*4	11/40
CMV antigenemia*3	48/93
CMV disease	10
Relapse*2	24/98

*1 We divided the risk of transplantation into two groups. The low-risk group was as follows: acute myelogenous or lymphoid leukemia in first and second remission, chronic myelogenous leukemia in chronic phase, and myelodysplastic syndrome refractory anemia. The other patients were defined as having high-risk diseases.

*2 Percentage was calculated based on 99 patients who achieved primary engraftment.

*3 Percentage was calculated based on the number of patients who achieved engraftment and evaluated.

*4 No patients received systemic corticosteroids for the treatment of chronic GVHD.

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndromes; ULN, upper limit of normal; GVHD, graft-versus-host disease; CMV, cytomegalovirus.

Transplantation Procedures and Supportive Cares

Transplantation procedures were shown in Table 1, and we previously reported details of the procedures [6]. GVHD prophylaxis was either tacrolimus 0.03 mg/kg or cyclosporine 3 mg/kg continuous infusion starting on day −1. Trough blood levels of these drugs were monitored 2-3 times a week and the dosage were modified to maintain the target level of 10-15 ng/mL for tacrolimus and 200-400 ng/mL for cyclosporine [10-12]. Immunosuppressants were tapered off from day 100 until day 150. If grade II-IV acute GVHD (aGVHD) developed, 1-2 mg/kg/day of methylprednisolone was added to cyclosporine or tacrolimus, and tapered from the beginning of clinical response.

The diagnosis and management of preengraftment immune reactions were reported previously [13].

Management of Infections

Patients were managed in reverse isolation laminar airflow-equipped rooms. All patients received tosufloxacin 450 mg/day from the start of conditioning until neutrophil engraftment. Fluconazole 200 mg/day or micafungin 150 mg/day, and acyclovir 600 mg/day were given from the start of conditioning until the discontinuation of GVHD prophylaxis, which were restarted when patients developed GVHD and were treated with steroids and immunosuppressants. They received prophylaxis with trimethoprim-sulfamethoxazole against *Pneumocystis jirovecii* infection from the start of conditioning until the discontinuation of immunosuppressants or disappearance of chronic GVHD (cGVHD). When patients develop neutropenic fever, tosufloxacin was changed to broad-spectrum antibiotics [14]. Intravenous administration of amphotericin B at a dose of 0.5 mg/kg/day was added when the fever persisted for more than 5 to 7 days. If the diagnosis of aspergillus infection was confirmed, the dosage of amphotericin B was increased to 1.0 mg/kg/day. We used blood tests, enzyme-linked immunosorbent assay for galactomannan antigen, (1-3)-beta-D glucan assay, and chest computed tomography for the early diagnosis of invasive aspergillosis (IA), as previously reported [15]. Because most patients had been heavily treated and received multiple transfusions prior to transplantation, anti-CMV antibodies were not examined before transplantation. All patients were monitored for cytomegalovirus pp65 antigenemia once a week. When CMV antigenemia exceeded 10/50,000, patients preemptively received foscarnet 30 mg/kg intravenously twice daily.

Diagnostic Criteria for IFI

Invasive fungal infection was diagnosed according to the established EORTC/NIH-MSG criteria [16]. Briefly, we diagnosed patients as having proved IFI when any 1 of the following examinations was positive: histopathologic or cytopathologic examinations for hyphae or yeasts in needle aspiration or biopsy specimens, fungal cultures obtained from normally sterile sites by sterile procedures, and *Cryptococcus* antigen in cerebrospinal fluid. Probable IFI was diagnosed when a patient satisfied at least 1 host factor, microbiologic criteria, and clinical criteria. Possible IFI was not included in this study. The day of diagnosis of IFI was defined as the day when the first diagnostic test was performed.

Endpoints and Statistical Analysis

The cumulative incidence of IA was evaluated using Gray's method, considering death without IA as a competing risk [17].

Potential confounding factors considered in the analysis of risk factors of IA were age, sex, disease status, previous stem cell transplantation, conditioning regimens, HLA mismatch, stem-cell dose (all nucleated cells, and CD 34-positive cells), GVHD prophylaxis, grade II-IV aGVHD, and use of prednisolone. Proportional hazard modeling was used to evaluate the influence of these factors on the incidences of IA treating the development of aGVHD and the use of prednisolone as time-dependent covariates. Factors associated with at least borderline significance ($P < .10$) in the univariate analyses were subjected to a multivariate analysis using backward stepwise proportional-hazard modeling. P-values of $<.05$ were considered statistically significant. Survival was estimated by the Kaplan-Meier method. Median follow-up of surviving patients was 628 days (range: 26-1347 days).

RESULTS

Clinical Outcomes after RICBT

Ninety-nine (77%) patients achieved primary engraftment at a median of day 20 (range: 9-53 days). Of the remaining 29 patients who failed to achieve primary engraftment, 4 patients received second RICBT, and the other 25 patients died before engraftment. Their causes of death included bacteremia (n = 22), invasive pulmonary aspergillosis (IPA) (n = 1), and progression of primary disease (n = 2). Of the 99 engrafted patients, 45 and 22 patients developed grade II and grade III-IV aGVHD, respectively. The median onset of grade II-IV aGVHD was day 28 (range, 11-92). Eleven of 40 patients (28%) who survived longer than 100 days without disease progression developed cGVHD. Estimated 3-year overall survival was 33% (95% confidence interval (95% CI), 24%-42%). Causes of deaths comprised nonrelapse mortality (n = 31) and disease progression (n = 23). Infection was the leading cause of nonrelapse mortality (n = 20). Autopsy was performed in 5 patients (3.9%) in this series of patients.

Incidence and Clinical Features of IFI

Invasive fungal infection was diagnosed in 14 patients. Their clinical features are shown in Table 2. Thirteen of the 14 patients had probable IA, and the other had fungemia from *Trichosporon* spp. Three-year cumulative incidence of probable IA was 10.2% (Figure 1). Median onset of IFI was day 20 (range: 1-82), and no patients developed IFI after day 100. IFI was diagnosed after day 30 in 1 patient. Prophylactic uses of antifungal agents included fluconazole (n = 12) and micafungin (n = 1) among the 13 patients with IA. Of the 63 patients who survived 100 days or longer, none developed IFI after day 100. Four of the 13 patients with IFI developed grade II-IV aGVHD, and their diagnosis of IFI was before the onset of aGVHD (Table 2).

Seven patients were given prednisolone >0.2 mg/kg/day for the treatment of preengraftment immune reactions, of whom 5 developed grade II-IV aGVHD. Twelve of the 14 patients with IFI died, and the mortality rate was 86%. IFI was the primary cause of deaths in 4 patients.

Risk Factors of IPA

Table 3 shows the results of univariate and multivariate analyses. Reactivation of cytomegalovirus (CMV) is a well-known risk factor of IA [18,19]. However, it was not included in the analysis of this study, because the onset of IFI was earlier than the onset of CMV antigenemia. Use of prednisolone >0.2 mg/kg (relative risk [RR], 7.97; 95% CI, 2.24-28.4; $P = .0014$) was a significant risk factor in multivariate analysis.

DISCUSSION

The present study demonstrated that IFI early after RICBT is a significant complication. Among IFI, the incidence of IA was high, which was consistent with the studies on reduced intensity stem cell transplantation (RIST) using other stem cell sources [9,19]. Our results contrasted with the previous reports that the incidence of infection because of non-*Candidia albicans* species was high in myeloablative allogeneic stem cell transplantation [20]. The observations may be associated with the milder gastrointestinal mucosal toxicity by conditioning regimens in RIST than in myeloablative transplantation [21] and the less fre-

Table 2. *Clinical Characteristics of Patients with Invasive Aspergillosis*

UPN	Age	Sex	Primary Disease	Disease Status at Transplant	No. of Prior Regimens	Neutrophil Engraftment Day	Grade II-IV Acute GVHD Onset Day	PSL Started	Invasive Fungal Infection (IFI)*1	Onset (Day)	Other Infectious Complication	Overall Survival (Day)	Outcomes of IFI	Causes of Death
286	57	M	AML	PIF	3				Probable IA	4		4	dead	IFI
365	69	M	AML	RLI	3				Probable IA	3		14	dead	IFI
411	56	F	ML	PD	1			11	Probable IA	19	Bacteremia	24	dead	Bacteremia complicated with IA
196	61	M	AML	PIF	2	11	22		*T. cutaneum* fungemia	1		28	dead	IFI
202	62	M	AML	RLI	4	21	21	8	Probable IA	21	Bacteremia	28	dead	Bacteremia complicated with IA
344	55	F	ML	PD	1	19			Probable IA	4	Bacteremia	30	dead	IFI
262	59	M	AML	PIF	1			10	Probable IA	20		31	dead	PD
151	52	F	MDS	RAEB	3	20		7	Probable IA	12		33	improved	GI bleeding
114	52	F	ML	PD	4	13		0	Probable IA	23		39	dead	PD
153	70	M	AML	1st CR	2	14	30		Probable IA	29	Bacteremia	46	dead	IP
197	33	M	MDS	RA	0				Probable IA	28	Bacteremia	47	dead	Bacteremia complicated with IA
160	66	M	ML	PD	2	14	29	9	Probable IA	25		75	dead	MOF
120	70	F	SAA		0	13			Probable IA	82		1308+	improved	
127	20	M	SAA		3	31	55	12	Probable IA	3		1347+	improved	

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndromes; CR, complete remission; GI, gastrointestinal; IA, invasive aspergillosis; IFI, invasive fungal infection; SAA, severe aplastic anemia; RA, refractory anemia; PD, progressive disease; PIF, primary induction failure; RARB, refractory anemia with excess of blasts; MOF, multiple organ failure.

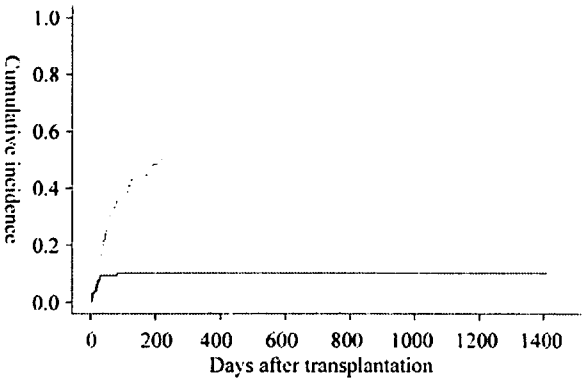

Figure 1. Cumulative incidence of invasive aspergillosis (IA) considering death without IA as a competing risk. Black line indicates incidence of death with IA, and gray line indicates incidence of death without IA.

quent and milder GVHD following CBT. Because gastrointestinal mucosal toxicity is milder in RICBT than in myeloablative transplantation, the incidence of infection from non-*Candida albicans* species as part of the gastrointestinal normal flora might be low, and hence aspergillus infection might become the majority of IFI.

The median onset of IFI was day 20 (range: 1-82) in the present study; the majority developed IFI early after RICBT. Majority of the patients who developed IFI died of causes other than fungal infection (Table 2), as reported previously by Saavedra et al. [7]. Our results were consistent with a previous report on CBT [18], and contrasted to reports on RIST using marrow or peripheral blood [19,22,23], in which IA develops late after transplantation. The low incidence of IA after day 100 would be related to the low incidence of cGVHD. The short duration from RICBT to IFI development suggests aggravation of latent infection, which would have existed before transplantation. These findings were consistent with a recent report by Martino et al. [23]. Given the possibility, several issues need to be addressed in the management of IFI following RICBT. First, selection of RICBT candidates would have to include accurate evaluation for the risk of fungal infection [24] and high-risk patients might need to be excluded from the indication of RICBT. Pretransplant CT scan of the chest and sinus would be useful in the screening of IA following RICBT, and bronchoalveolar lavage should be performed in patients with abnormal findings. Second, the importance needs to be stressed in prophylactic antifungal agents with antiaspergillus activity and attempts for early diagnosis of aspergillosis such as methods using molecular techniques [25], antigen tests [26], and imaging tests [15,22]. Third, the way of steroid use after RICBT requires further investigations. The present study showed that the administration of steroids 0.2 mg/kg and more was a strong risk factor of IA (RR, 7.97; 95% CI, 2.24-28.4; $p = .0014$). Our observation that the use of small-dose steroids was a risk factor of IFI after RICBT supports the previous results of severe immunosuppression early after RICBT [27]. In RICBT using our regimens, immunologic reactions such as a preengraftment immune reaction frequently develops in addition to GVHD, requiring steroid administration early after RICBT [13]. Because steroids suppress phagocyte activities and cellular immunity [28], the risk of fungal infection early after RICBT may be increased.

The incidence of late IFI was not high in the present study. Of 102 patients who survived longer

Table 3. *Univariate and Multivariate Analyses for the Incidence of IA*

	Incidence of IPA (95% CI)	P Value
Univariate analysis		
Pretransplantation factors		
Age		.30
<55	7%	
≥55	13%	
Sex		.94
Female	11%	
Male	10%	
Disease risk		.63
Standard	16%	
High	9%	
Previous ASCT		.55
No	11%	
Yes	0%	
Regimen		.72
FM-based	11%	
FB-based	10%	
HLA mismatch		.076
0 or 1 antigen	0%	
2 antigens	12%	
Cell dose		.42
ANC $<2.5 \times 10^7$/kg	8%	
ANC $\geq 2.5 \times 10^7$/kg	12%	
Cell dose		.03
CD34 $<0.8 \times 10^6$/kg	5%	
CD34 $\geq 0.8 \times 10^6$/kg	16%	
GVHD prophylaxis		.42
Cyclosporine	13%	
Tacrolimus	8%	
Fungal prophylaxis		.99
Fluconazole	10%	
Micafungin	10%	
Posttransplantation factors (time-dependent covariates)		
Acute GVHD		.96
Grade 0-I	1.00	
Grade II-IV	1.06 (0.12-9.40)	
Prednisolone		.001
<0.2 mg/kg/day	1.00	
≥0.2 mg/kg/day	7.97 (2.24-28.4)	
Multivariate analysis		
Prednisolone		.0014
<0.2 mg/kg/day	1.00	
≥0.2 mg/kg/day	7.97 (2.24-28.4)	

GVHD indicates graft-versus-host disease; ASCT, autologous stem cell transplantation.