Keywords Unrelated transplantation · Reduced-intensity conditioning · Hematologic malignancy

1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a possible curative approach for patients with various hematologic malignancies. Recently, the application of reduced-intensity conditioning (RIC) regimens, mostly incorporating fludarabine as a backbone agent, has been explored for patients whose age or concomitant medical conditions contraindicate the use of conventional myeloablative regimens [1–3]. Since only 30–40% of patients have an appropriate family donor available [4], the establishment of an unrelated donor transplantation program with RIC regimens is urgently needed.

Graft rejection, regimen-related toxicities and graft-versus-host disease (GVHD) have been the major problems in unrelated HSCT with RIC [5–13]. In unrelated transplantation, engraftment is influenced by the source of stem cells and superior results have been observed with peripheral blood stem cells (PBSC) compared to bone marrow [9, 14]. Nevertheless, PBSC has not yet been approved as a graft source for unrelated transplantation in Japan [15]. The level of regimen-related toxicities directly depends on the intensity of the regimen, and the incidence of GVHD increases with unrelated donors compared to related donors. Although attempts have been made to overcome these problems, a suitable procedure for unrelated bone marrow transplantation (BMT) with RIC regimens has not yet been established. To accumulate further expertise, we conducted a nationwide survey of Japanese patients with hematologic malignancy who had undergone BMT from an HLA-matched or -mismatched unrelated donor with RIC regimens. Although the present data were obtained from a limited population of patients, these findings may show a current status of unrelated BMT with RIC.

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2 Patients and methods

2.1 Data sources

This survey collected the data of 77 consecutive patients in 17 participating hospitals who received unrelated BMT with RIC for hematologic malignancies between 2000 and 2004. Data were derived from questionnaires distributed to each hospital. Additional questionnaires were sent to confirm the follow-up data, including the occurrence of GVHD. The minimum data required for inclusion of a patient in this study were age, sex, histological diagnosis, status at transplant, donor information, conditioning regimen, date of transplant, donor chimerism status, therapy-related complications, date of last follow-up, disease status at follow-up, date of disease progression (PD)/death and cause of death.

This study was approved by institutional review board of each individual center. All patients provided written informed consent according to the Declaration of Helsinki. Unrelated donors provided consent through the Japan Marrow Donor Program as part of its standard procedures. The indications, conditioning regimens, management of GVHD and supportive care for BMT were left to the discretion of each institution. Patients who had previously received allogeneic HSCT and those younger than 20 years were not included. Patients younger than 50 years who had organ dysfunction and/or have previously received high-dose chemotherapy with autologous HSCT were also included.

2.2 Definitions

RIC regimens were defined as previously reported [6, 9, 10], and conditioning regimens that included either beyond 4 Gy of total body irradiation (TBI), 8 mg/kg of busulfan or 140 mg/m^2 of melphalan were excluded from the study. Alleles at the HLA-A, -B, and -DRB1 loci were identified by middle-resolution DNA typing as described previously [16]. Risk status at transplantation was categorized as either standard risk or high risk. Standard-risk diseases included acute leukemia in first complete remission, chronic myeloid leukemia in first chronic phase, and refractory anemia of myelodysplastic syndrome (MDS). Other diseases were categorized as high-risk disease. Graft failure was analyzed in patients who survived more than 28 days posttransplant according to the criteria reported by Petersdorf et al. [17]. Briefly, the definition included failure of the absolute neutrophil count (ANC) to surpass 500/mm^3 before relapse, death or second transplantation, as well as a decrease in the ANC to less than 100/mm^3 on at least three consecutive determinations with a finding of severe hypoplastic marrow. The degree of donor chimerism among peripheral blood T cells was assessed several times

between day 28 and day 100 after HSCT using fluorescence in situ hybridization (FISH) to detect X and Y chromosomes for recipients of grafts from sex-mismatched donors, and polymerase chain reaction-based analyses of polymorphic microsatellite regions for recipients of sex-matched or sex-mismatched transplants. Mixed chimerism was defined as the detection of 5–90% of donor cells in the peripheral blood. Acute and chronic GVHD were graded according to the consensus criteria [18, 19]. Patients who survived 100 days were evaluable for the assessment of chronic GVHD. Overall survival (OS) was measured as the time from the day of transplantation until death from any cause, and progression-free survival (PFS) was the time from the day of transplantation until PD/relapse or death from any cause. Patients who died from transplantation-related causes were classified as non-relapse mortality (NRM) regardless of their disease status.

2.3 Statistical analysis

The primary endpoint of this study was OS and chimerism. The secondary endpoints were PFS, NRM, PD, and the incidence of acute and chronic GVHD. Descriptive statistical analysis was performed to assess patient baseline information. Patients were divided into two groups: age 60 or above and less than 60. OS and PFS were calculated using the Kaplan–Meier method. The cumulative incidence of acute GVHD was calculated using the method described by Gooley et al. [20] to eliminate the effect of competing risks. The competing event for acute GVHD was defined as death without grades II–IV acute GVHD. For each endpoint, a Cox proportional hazard model was used for univariate and multivariate analyses. The factors included in the analysis were HLA disparity (mismatch vs. identical), recipient age (age 60 or above vs. less than 60), use of TBI (yes vs. no), use of ATG (yes vs. no), diagnosis of AML (yes vs. no), risk status (high vs. standard) and acute GVHD (II–IV vs. 0–I). Acute GVHD in the model was treated as a time-varying covariate. We defined statistical significance as a P value less than 0.05. All statistical analyses were performed using STATA version 8 (College Station, TX).

3 Results

3.1 Patients and diagnoses

The patients' characteristics are listed in Table 1. The median age of the patients was 54 years (range, 25–68 years) as a whole. Twenty-one patients (27%) had acute myelogenous leukemia (AML), 2 (3%) had acute lymphoblastic leukemia, 5 (7%) had chronic myeloid leukemia, 20 (26%) had MDS or myeloproliferative disease (refractory anemia, $n = 8$; refractory anemia with excess blasts, $n = 9$; others, $n = 3$), 19 (25%) had non-Hodgkin lymphoma (follicular lymphoma, $n = 12$; diffuse large B-cell lymphoma, $n = 4$; mantle cell lymphoma, $n = 2$; peripheral T-cell lymphoma, unspecified, $n = 1$), 7 (9%) had adult T-cell leukemia/lymphoma, and 3 (4%) had multiple myeloma. Sixty-three patients (82%) had high-risk disease at the time of allogeneic BMT.

3.2 Conditioning regimens

Conditioning regimens are shown in Table 2. None received ex vivo T-cell depleted transplantation.

3.3 HSCT procedure and supportive care

Forty-seven patients (61%) were transplanted from a matched, 24 (31%) were from a 1 allele-mismatched, and 6 (8%) were from a 2 or 3 allele-mismatched unrelated donor. All patients received bone marrow as a source of stem cells. The prophylaxis of GVHD was either cyclosporine- or tacrolimus-based. Thirty-nine patients (51%) received cyclosporine with methotrexate, including five patients who received an ATG-containing preparative regimen. Nine patients (12%) received cyclosporine alone, including five patients who received ATG. Each patient received cyclosporine with mycophenolate mofetil and cyclosporine with prednisolone, respectively. Twenty-five patients (33%) received tacrolimus with methotrexate, including one patient who received ATG. Two patients (3%) received tacrolimus alone, including one who received ATG. Granulocyte colony-stimulating factor was administered intravenously from day +1 or +6 until neutrophil engraftment in all patients.

3.4 Engraftment and chimerism

Five patients died before the engraftment evaluation, with a median survival time of 15 days (range, 2–17 days). Seventy-one patients (92%) achieved initial neutrophil recovery, but three patients (two AMLs and one MDS) later experienced secondary graft failure; one each with AML and MDS after unrelated BMT from an HLA-1 allele-mismatched donor received a second transplantation when they failed to achieve subsequent complete donor-type chimerism, but both died of infectious complications. The other patient with AML after unrelated BMT from an HLA-6 allele-matched donor achieved initial complete chimerism, but later developed secondary graft failure upon the administration of ganciclovir for cytomegalovirus antigenemia. However, this patient achieved the spontaneous recovery of autologous marrow function and is currently surviving beyond 2,000 days.

Table 1 Patient characteristics

Variable	Younger than 60 years ($n = 60$)	60 years or older ($n = 17$)
Patient age (range, median)	25–59, 52	60–68, 63
Disease		
Acute myelogenous leukemia	16 (27%)	5 (29%)
Acute lymphoblastic leukemia	2 (3%)	0
Chronic myeloid leukemia	5 (8%)	0
Myelodysplastic syndrome or myeloproliferative disease	12 (20%)	8 (47%)
Malignant lymphoma	16 (27%)	3 (18%)
Adult T-cell leukemia/lymphoma	7 (12%)	0
Multiple myeloma	2 (3%)	1 (6%)
Risk status		
Standard	13 (22%)	1 (6%)
High	47 (78%)	16 (94%)
HLA disparity		
Matched	37 (62%)	10 (59%)
One-mismatched	19 (32%)	5 (29%)
Two or more mismatched	4 (7%)	2 (12%)
Donor–recipient sex match		
Male–male	20 (33%)	11 (65%)
Male–female	16 (27%)	2 (12%)
Female–male	9 (15%)	4 (24%)
Female–female	15 (25%)	0
GVHD prophylaxis		
Cyclosporine ± methotrexate	38 (63%)	10 (59%)
Tacrolimus ± methotrexate	21 (35%)	6 (35%)
Others	1 (2%)	1 (6%)
Median nucleated cell dose infused ($\times 10^8$/kg, range)	2.80 (0.39–5.52)[a]	2.92 (0.76–4.30)

HLA Human leukocyte antigen, *GVHD* graft-versus-host disease

[a] The data of two patients were excluded because infused nucleated cell dose was unknown

Chimerism was evaluated in 68 patients (88%), with short tandem repeats analysis ($n = 52$), variable number of tandem repeats analysis ($n = 5$) and FISH analysis in the case of sex mismatch ($n = 11$). Complete donor chimerism was confirmed in 58 (85%) within day 100. Mixed chimerism was confirmed in nine patients (13%), but two later reverted to recipient type. One patient failed to achieve donor-type chimerism due to disease relapse on day 20. The incidence of complete donor chimerism was similar in those younger and older than 60 years (85 and 86%), with a similar incidence of mixed chimerism (15 and 14%). No patients received donor lymphocyte infusion.

3.5 GVHD

Acute GVHD occurred in 41 of the 68 evaluable patients (60%), grades II–IV in 34 (50%) and grades III–IV in 14 patients (21%). Chronic GVHD occurred in 26 of the 42 evaluable patients (62%), with extensive type in 23 (55%). The incidence of grades II–IV acute GVHD was the same in patients younger and older than 60 years (50%). The incidence of grades III–IV acute GVHD (22 and 14%) and extensive chronic GVHD (56 and 50%) was similar. In unrelated BMT, from HLA-6 allele-matched ($n = 40$), HLA-1 allele-mismatched ($n = 23$), and HLA-2 or 3 allele-mismatched ($n = 5$) donors, grades II–IV acute GVHD occurred, respectively, in 18 (45%), 10 (43%) and 3 patients (60%), and chronic GVHD occurred in 15 (38%), 9 (39%) and 2 patients (40%). In univariate and multivariate analyses, an ATG-containing regimen was significantly associated with a decreased risk of the onset of grades II–IV acute GVHD (data not shown).

3.6 Survival

Thirty-three patients are currently alive with a median follow-up of 439 days (28–2,002 days), with an OS of 50% at 1 year and 46% at 2 years. The OS of patients younger than 60 years was 49% at 2 years (95% confidence interval [CI], 34–62%), and this could not be defined in older patients (95% CI, 15–45%). Patients younger than 60 years

Table 2 Conditioning regimens

Conditioning regimens	Younger than 60 years ($n = 60$)	60 years or older ($n = 17$)
TBI-containing		
Fludarabine 180 mg/m^2 (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, TBI 4 Gy	30 (50%)	6 (35%)
Fludarabine 125–180 mg/m^2, melphalan 80–140 mg/m^2, TBI 4 Gy	5 (8%)	3 (18%)
Fludarabine 180 mg/m^2 (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, TBI 2 Gy	2 (3%)	0 (0%)
Fludarabine 180 mg/m^2, TBI 4 Gy	0 (0%)	1 (6%)
ATG-containing		
Fludarabine 180 mg/m^2 (or cladribine 0.66 mg/kg), oral busulfan 8 mg/kg, ATG	5 (8%)	4 (24%)
Fludarabine 180 mg/m^2, cyclophosphamide 60 mg/kg, ATG	1 (2%)	0 (0%)
Fludarabine 180 mg/m^2, ATG	1 (2%)	0 (0%)
TBI and ATG-containing		
Fludarabine 180 mg/m^2, oral busulfan 8 mg/kg, TBI 4 Gy, ATG	1 (2%)	1 (6%)
Non-TBI and non-ATG		
Fludarabine 180 mg/m^2, oral busulfan 8 mg/kg	6 (10%)	2 (12%)
Fludarabine 125–180 mg/m^2, melphalan 140 mg/m^2	5 (8%)	0 (0%)
Fludarabine 180 mg/m^2, oral busulfan 8 mg/kg, cyclophosphamide 60 mg/kg	2 (3%)	0 (0%)
Fludarabine 180 mg/m^2, oral busulfan 8 mg/kg, thiotepa 10 mg/kg	1 (2%)	0 (0%)
Fludarabine 180 mg/m^2, cyclophosphamide 60 mg/kg	1 (2%)	0 (0%)

TBI Total body irradiation, *ATG* antithymocyte globulin (ATG-Fresenius 10 mg/kg or thymoglobulin 5 mg/kg)

tended to show better survival than older patients ($P = 0.124$). The HLA disparity (match vs. mismatch), TBI vs. non-TBI, ATG vs. non-ATG-containing regimen, and disease category (AML vs. MDS or myeloproliferative disease vs. lymphoid malignancies) was not significantly associated with OS (data not shown). Patients with standard risk tended to show better survival than those with high risk ($P = 0.129$). In univariate and multivariate analyses, no variables were significantly associated with OS (data not shown).

3.7 NRM and PD

Thirty-six patients (47%) died of therapy-related complications, with a cumulative incidence of NRM at 1 year of 43% (95% CI, 31–56%). Of the patients who died of therapy-related complications, 23 (30%) died within day 100 of transplantation and 13 (17%) died thereafter. The NRM at 1 year in patients younger and older than 60 years was 38% (95% CI, 25–53%) and 61% (95% CI, 36–85%), respectively, as shown in Fig. 1. The causes of NRM were infection (23%), regimen-related toxicity (14%) and GVHD (9%). GVHD-related mortality was found in 26%. Infection was the major cause of death in patients younger than 60 years. Regimen-related toxicity, mainly pulmonary complications, was the major cause of treatment failure for patients older than 60 years. In univariate and multivariate analyses, no variables were significantly associated with NRM (data not shown). Relapse or progression of primary disease after unrelated BMT with RIC regimens was observed in 13 patients (17%; 10 patients younger than 60 years and 3 older than 60 years). There were no relapsed patients after transplantation in standard risk group. The incidence of death due to relapse or progression of primary disease was 14%. In univariate and multivariate analyses, no variables were significantly associated with PD although patients with grades II–IV acute GVHD showed a relatively lower incidence of PD (data not shown).

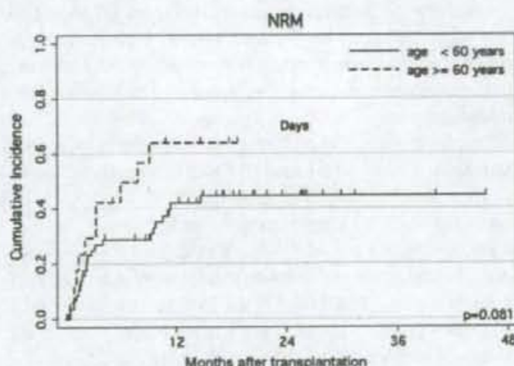

Fig. 1 Non-relapse mortality stratified according to patient age, younger or older than 60 years

4 Discussion

This report reviews the current experience of unrelated BMT with RIC regimens in Japan, with particular focus on the risk factors for engraftment, GVHD, NRM, survival and PD. Although the engraftment rate has been reported to be lower when RIC unrelated transplantation was performed with bone marrow compared to peripheral blood cells [9, 10], we observed that sustained engraftment was achieved in 99% of evaluable patients, with complete donor chimerism confirmed in 85%. The incidence of graft failure was not different from that in RIC transplantation from related donors in Japan; 3.7% in recipients with an HLA-matched donor and 5.7% in those with a 1-locus-mismatched donor [21]. Complete donor chimerism in our study was comparable with that reported from the National Marrow Donor Program (85 vs. 84%) [22]. In our study, two-thirds of patients successfully received 2–4 Gy TBI-containing regimens, which were aimed at the enhancement of engraftment, as suggested in a previous report with patients with aplastic anemia [23], while 2 of the 12 patients who received an ATG-containing regimen had late graft failure, similar to a previous report which noted an incidence of 19% [5]. It has been reported that the Japanese population is more homogenous than others in terms of the distribution of HLA. Thus, it would be possible that the impact of minor HLA disparities on engraftment may become prominent after RIC transplantation.

Despite the observed satisfactory engraftment rate, we confirmed a high NRM rate (47%) after unrelated BMT with variable RIC regimens, due mostly to GVHD-related complications, including infections under steroid therapy, as previously designated by Wong et al. [10]. On the other hand, the incidence of death due to relapse or progression of primary disease was low (14%). Hence, successful prophylaxis and treatment of GVHD is particularly important in this procedure, and studies with ATG [5, 24] or alemtuzumab [25–27] have reported encouraging results. Although the number of patients was still small, in our study an ATG-containing regimen resulted in a decreased incidence of acute and chronic GVHD, despite the use of a lower dose (ATG-Fresenius 10 mg/kg or Thymoglobulin 5 mg/kg) than reported elsewhere. This study showed that age older than 60 years tended to be associated with a higher risk of NRM after unrelated HSCT with RIC regimens, though this relation was not statistically significant in a multivariate analysis. This finding, however, is limited by the small sample size. Additional use of ATG may reduce the incidence of GVHD-related NRM even in older patients but ATG should be carefully incorporated since about 20% of patients who received an ATG-containing regimen developed late graft failure in our study.

This study suggested that the onset of grades II–IV acute GVHD was associated with a lower incidence of PD, although this was not statistically significant in a multivariate analysis, possibly due to the small sample size. However, GVHD in turn resulted in a higher incidence of NRM, and a desirable graft-versus-leukemia or lymphoma effect would be offset, particularly in older patients [10, 28]. Hence, our observation echoes the warning that the intentional induction of GVHD should be avoided.

Compared to the long-term follow-up data after unrelated HSCT with RIC from the NMDP reported by Giralt et al. [22], our NRM at 1 year was worse (43 vs. 30%), but OS was likely to be better (50% at 1 year and 46% at 2 years vs. 44% at 1 year, 28% at 3 years and 23% at 5 years). In their report, disease stage, performance status, stem cell source, HLA matching, and timing of transplant were the most important prognostic factors for survival after RIC unrelated donor transplantation. This study suggested that high risk and HLA-mismatched patients were associated with worse OS, although this was not statistically significant in the multivariate analysis. Interpretation of these results, however, should be careful because of relatively short period of follow-up and the small sample size in our study. Although high risk patients was 82%, rate of relapse were unexpectedly low in our study. This might be due to earlier mortality, which precludes estimate of relapse rate. Alternately, more patients (60%) received more intense conditioning composed of 8 mg/kg of busulfan or 80–140 mg/m^2 of melphalan and 4 Gy TBI in our study.

In conclusion, we confirmed that unrelated BMT with RIC regimens can be a curative therapeutic option in a subset of patients with advanced hematologic malignancy, but at the expense of a high risk of severe complications and NRM. The incorporation of low-dose TBI may be advantageous for enhancing engraftment, and a suitable prophylaxis for GVHD still remains a primary target of clinical research. Based on the observed data, a prospective trial is currently underway to determine the value of a lower dose of ATG (ATG-Fresenius 5 mg/kg) to be added to the combination of fludarabine and busulfan.

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Case Report

Successful Treatment of Primary Plasma Cell Leukaemia by Allogeneic Stem Cell Transplantation from Haploidentical Sibling

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Primary plasma cell leukaemia (PCL) is a rare, aggressive neoplasm of plasma cell dyscrasia. Conventional chemotherapy is usually ineffective, with an overall survival of only 8 months. Here, we describe a 42-year-old man with primary PCL, who was successfully treated with haploidentical (2-HLA loci mismatched) haematopoietic stem-cell transplantation (HSCT). To overcome the human leukocyte antigen (HLA) disparity, *in vivo* T-cell purging by the pre-transplant administration of antithymocyte globulin followed by a conventional prophylactic treatment against graft-versus-host disease (GVHD) resulted in an avoidance of severe GVHD as well as infectious complications. The patient has maintained complete remission for 13 months after haploidentical HSCT, indicating that a graft-versus-PCL effect might be preserved. Haploidentical HSCT can be a potentially curative treatment for patients with primary PCL who do not have an HLA-identical donor.

Key words: plasma cell leukaemia – haploidentical – stem cell transplantation – ATG

INTRODUCTION

Primary plasma cell leukaemia (PCL) is a very rare, aggressive variant of multiple myeloma (MM) accounting for 2–3% of all plasma cell dyscrasias (1). It is defined as malignant proliferation first diagnosed in the leukaemic phase without preceding MM. Patients with this entity usually have clinical presentations in advanced stages and multiple organ insufficiency due to the involvement of PCL at greater levels than MM. They then usually have a fulminant course and poor prognosis (1–4).

Treatment with standard alkylating agents and steroids is poorly effective (median survival 2 months) (5,6), although a combination of chemotherapy such as VAD chemotherapy, M-80 protocol or hyper-CVAD regimen might has provided slight better but not yet promising results (4,7). Recently, several preliminarily reports have demonstrated that high-dose chemotherapy followed by allogeneic haematopoietic stem-cell transplantation (HSCT) resulted in the sustained long-term survival of eligible patients (8), suggesting the possible effect of graft-versus-PCL as shown for MM (9,10). In this report, we describe the first case of a chemotherapy-resistant primary PCL, which was successfully treated with allogeneic HSCT from a haploidentical (2-HLA loci mismatched) sibling donor. *In vivo* T-cell depletion by antithymocyte globulin (ATG) contained in the conditioning regimen might avoid the development of severe graft-versus-host disease (GVHD), whereas a graft-versus-PCL effect still might be preserved (11) in this case. Considering the aggressive nature of this disease, haploidentical HSCT can be a treatment option based on graft-versus-PCL effects in addition to intensive conditioning regimens for patients with primary PCL who do not have an HLA-identical donor.

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CASE REPORT

A 42-year-old man was referred to our hospital because of leukocytosis and acute renal failure in May 2006. On admission, he manifested high fever and severe diarrhoea;

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however, spleen, liver and lymph nodes were not palpable. Endoscopic colonoscopy and histological findings of colon revealed a mild chronic inflammation. Haemoglobin concentration was 15.5 g/dl, the platelet count 103×10^9/l and the white blood cell (WBC) count 53.3×10^9/l with 55% abnormal cells. The serum level of total protein was 8.0 g/dl, and the serum level of creatinine and blood urea nitrogen increased up to 8.71 mg/dl and 84 mg/dl, respectively. The serum level of IgG, M, A, D and E was 2244 mg/dl, 1411 mg/dl, 550 mg/dl, 2.5 mg/dl and 1135 IU/ml, respectively. A bone marrow aspirate was hypercellular with 54% abnormal plasma cells, which were positive for IgM, CD138, CD19, CD30, CD35 and CD38, but negative for CD20, CD34 and CD56. Bone marrow smear showed abnormal plasma cells with classical eccentric nuclei, prominent paranuclear hof and abundant basophilic cytoplasm. Cytogenetic analysis of bone marrow showed normal in all metaphase cells. Radiological studies such as computed tomography and scintigraphy demonstrated that the patient had no extramedually masses and bone lesion. Serum and urinary protein electrophoresis revealed no monoclonal protein, indicating that these myeloma cells were categorized as non-secretary type. On the basis of these findings, a diagnosis of primary PCL was made. Acute renal failure was considered due to both dehydration and PCL. The patient was also found to be positive for hepatitis B virus (HBV) surface antigen and envelope antigen, and his serum HBV-DNA level was 3.8 log of the genome equivalent per millilitre. Therefore, he was also diagnosed as an HBV carrier.

On the day of admission, the patient received a VAD regimen consisting of vincristine 0.4 mg and doxorubicin 10 mg/m^2 on days 1–4, and dexamethasone 40 mg on days 1–4 and 13–16. Concurrently, to prevent the reactivation of HBV, treatment with lamivudine (100 mg daily) was started. Diarrhoea and high fever were gradually improved and resolved by day 10. On day 14, WBC count and serum creatinine level returned to normal levels. However, on day 17, the patient developed high fever again, and WBC count increased to 13.12×10^9/l with 24% of leukaemia cells. Therefore, the patient received chemotherapy according to the hyper-CVAD regimen, comprising cyclophosphamide 300 mg/m^2 every 12 h for days 1–3, doxorubicin 50 mg/m^2 on day 4, vincristine 2 mg on days 4 and 11 and dexamethasone 40 mg on days 1–4. Nine days after treatment with hyper-CVAD regimen, a WBC count was normalized to 4.7×10^9/l without leukaemia cells. On day 12, however, leukaemia cells appeared again in the peripheral blood, and the patient successively underwent intensive treatment with cytosine arabinoside 2.0 g/m^2 every 12 h on days 1–5 and mitoxantrone 10 mg/m^2 on days 1 and 2, and he achieved complete remission (CR) that was evaluated by flow cytometry. Then, he was administered interferon alpha (six million units) three times per week (12) as a post-remission therapy.

Since the patient presented with rapid clinical course due to the aggressiveness of the disease, we decided to perform allogeneic HSCT. However, the patient had no HLA-identical donor among his family or the Japan Marrow Donor Program, and there was no appropriate umbilical cord blood with sufficient CD34$^+$ cell doses for HSCT in the Japan Cord Blood Bank Network. In addition, his disease status did not allow us to search or wait for appropriate donors. Therefore, we selected his sister who had haplotype-identical, two loci-mismatch HLA phenotype (A and DR loci) as an alternative donor. The patient's HLA phenotype was A1101, A2402, B1501, B5401, DR0901 and DR1405, and that of the donor was A2602, A2402, B1501, B5401, DR1406 and DR1405. She was not a non-inherited maternal antigen (NIMA) mismatched donor according to microchimerism analysis. Written informed consent for this therapy was obtained.

On August 2006, the patient underwent allogeneic peripheral blood stem-cell transplantation (PBSCT) from his sister with HLA mismatched at two loci at CR state (Fig. 1). Unmanipulated PBSCs (CD34$^+$ cells, 3.68×10^6/kg) were infused after a conditioning regimen including total body irradiation (TBI, 12 Gy), cyclophosphamide (120 mg/kg) and rabbit ATG (Nippon Zoki Pharmaceutical Co. Ltd) 1.5 mg/kg on days −5 to −2. Acute GVHD prophylaxis comprised methotrexate (10 mg/m^2 on day 1, 7 mg/m^2 on days 3 and 6) and tacrolimus (0.03 mg/kg). Engraftment was documented on day 14 after allogeneic PBSCT, confirmed by complete donor chimerism using short tandem repeat-based polymerase chain reaction assay. On day 12, grade II acute GVHD developed, confined to the skin; this responded to methylprednisolone (mPSL, at a dose of 2 mg/kg daily), which was then gradually tapered to a maintenance dose of PSL 10 mg daily (Fig. 1).

On day 14, cytomegalovirus (CMV) antigenemia was detected (10 CMV-positive cells out of 57 900 cells), and pre-emptive treatment with ganciclovir (10 mg/kg daily) was started (Fig. 1). Flow cytometric analysis of peripheral blood on day 41 demonstrated that CD4$^+$ and CD8$^+$ cells comprised 20.2% (92/μl) and 65.1% (295/μl) mononuclear cells, indicating the severe cellular immunocompromised state of

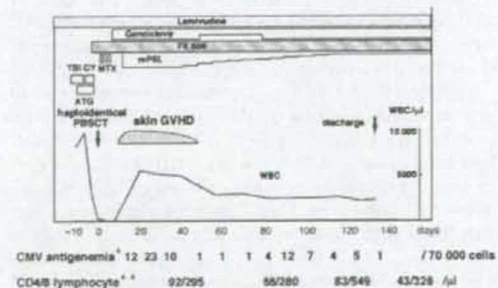

Figure 1. Clinical course of the patient. *Number of CMV-positive cells detected out of 70 000 leukocytes. **Serial change in numbers of CD4$^+$ and CD8$^+$ T cells after haploidentical HSCT. TBI, total body irradiation; ATG, antithymocyte globulin; GVHD, graft-versus-host disease; PBSCT, peripheral blood stem-cell transplantation; CMV, cytomegalovirus.

this patient. A delayed reconstitution of $CD4^+$ T cells was sustained after allogeneic PBSCT (68/μl on day 86, 83/μl on day 121 and 43/μl on day 202) (Fig. 1); however, on day 125 using tetrameric HLA-A2402 CMV peptide complex assay (13), CMV-specific T cells were found to be 0.23% out of all lymphocytes, suggesting a still weak but significant immune reconstitution against CMV. CMV antigenemia continued to be detected thereafter and the patient was treated with ganciclovir every day as an outpatient until day 205, when CMV antigenemia finally disappeared. An increase in HBV-DNA level was not documented throughout the treatment with lamivudine. The patient has maintained CR for 12 months after allogeneic PBSCT without GVHD, nor shown reactivation of CMV and HBV.

DISCUSSION

Prognosis of the primary PCL is extremely poor owing to the biologically aggressive nature of the disease despite intensive chemotherapy (1–4). The optimal treatment for the primary PCL is not yet well defined; however, recent studies have indicated the potential role of autologous or allogeneic HSCT to prolong overall survival (8,14). Taken together, high-dose chemotherapy and subsequent HSCT would be required to cure such patients with primary PCL who usually manifest a rapid and fatal course. Because PCL cells were not eradicated from the peripheral blood by the first two courses of chemotherapy, autologous PBSC might not have been proper for the stem cell source because of the possible contamination of PCL cells in PBSC products in this case. Thus, our patient, who had no HLA-identical donor, received haploidentical (2-HLA loci mismatched) HSCT 3 months after the initial diagnosis. In this setting, immunosuppressive therapy to prevent severe GVHD would be expected to be much more intensive than usual, and it became more likely to impair graft-versus-PCL effects and to develop severe opportunistic infections. We successfully performed haploidentical HSCT using an *in vivo* T-cell depletion by ATG-containing conditioning followed by a standard GVHD prophylaxis regimen, which resulted in the avoidance of severe GVHD and infectious complications.

Haploidentical HSCT is a treatment option for most patients who do not have an HLA-identical donor. To overcome the histcompatibility disparity barrier, several efforts have been made to prevent severe GVHD (15). Classically, *ex vivo* T-cell depletion methods were commonly used; however, T cell-depleted HSCT was complicated by a very high risk of graft failure and recurrent malignancy. Recently, Aversa *et al.* (16,17) developed the transplantation of 'megadoses' of CD34-selected PBSCs from haploidentical donors after ATG-containing myeloablative conditioning but no post-transplant GVHD prophylaxis: engraftment was obtained in 94 out of 101 patients (93%), and greater than grade II acute GVHD occurred in only 8 out of 100 patients. They deliberated that a megadose of highly purified stem cells might be crucial for promoting engraftment across the histocompatibility barrier as well as avoidance of severe GVHD. In contrast, other approaches using intensification of GVHD prophylaxis or potent pre-transplant immune suppression with ATG (18,19) or alemtuzumab (20) have described similarly favourable engraftment rates and protection from GVHD, if unmanipulated grafts were transplanted. All these methods are still experimental and there is no 'gold standard' in GVHD prophylaxis. Practically, *ex vivo* manipulation methods sometimes require commercially unavailable antibody and special devices, and are expensive. Thus, considering availability in our case, an ATG-containing myeloablative regimen was chosen to deplete T cells *in vivo* and overcome two-loci mismatched disparity.

Haploidentical HSCT has another disadvantage of delayed and incomplete immune reconstitution, resulting in the high incidence of severe infectious complications, especially viral infections (15). In this case, $CD4^+$ and $CD8^+$ T cells were strongly suppressed throughout the patient's clinical course after HSCT; CMV reactivation was first documented even on day 14 and CMV antigenemia continued to be detectable for 6 months despite treatment with ganciclovir. On the other hand, as an advantage of haploidentical HSCT, the more potent graft-versus-tumour effect would be expected through the histcompatibility disparity (15). In fact, the curative potential of allografts relies on an immune attack of donor cells against MM (9,10), although TBI also could confer durable cytoreductive responses. Moreover, Mohty et al. have disclosed that the potent immunosuppression with ATG as a conditioning regimen did not impair the graft-versus-myeloma effect (11). In this context, haploidentical HSCT can be a treatment option for patients with primary PCL who have poor prognoses and no HLA-identical donor.

In conclusion, we describe the first case with primary PCL to be successfully treated, to our knowledge, with haploidentical (2-HLA loci mismatched) HSCT after potent pre-transplant immune suppression with an ATG-containing myeloablative regimen. Given the aggressiveness of this disease, allogeneic HSCT is a potentially curative therapy for patients with primary PCL based on graft-versus-PCL effects in addition to intensive conditioning regimens.

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Conflict of interest statement
None declared.

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ORIGINAL ARTICLE

Allo-SCT using reduced-intensity conditioning against advanced pancreatic cancer: a Japanese survey

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Pancreatic cancer is a frequent cause of cancer-related mortality and has an extremely poor prognosis. To evaluate the efficacy of allogeneic hematopoietic SCT with reduced-intensity conditioning (RICT) against pancreatic cancer, we analyzed the clinical data of 22 patients. After a fludarabine-based conditioning regimen followed by the infusion of PBSCs, all but two achieved engraftment. Complete, partial and minor response was observed in 1, 2 and 2 patients, respectively, with an overall response rate of 23%. Median survival was only 139 days and the major cause of death was tumor progression. Poor performance status before RICT and a lower number of infused CD34-positive cells were associated with shorter survival after RICT. Patients who developed chronic GVHD tended to survive longer than those who did not. These findings support the investigation of a novel treatment strategy to enhance the immunological effect against pancreatic cancer.
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Keywords: reduced-intensity conditioning; SCT; mini-transplantation; pancreatic cancer; graft-versus-tumor effect

Introduction

Allogeneic hematopoietic SCT is an established treatment for a variety of hematological disorders. However, its application has been limited to young patients because of various complications including regimen-related toxicities, GVHD, infection and so on. Therefore, SCT with reduced-intensity conditioning (RICT) has been investigated for use in older or clinically infirm patients. The antitumor effect of this therapeutic approach depends not only on the antineoplastic agents and/or irradiation in the conditioning regimen, but also on the immunological graft-versus-tumor effect after RICT.[1] Although RICT has not been clearly shown to have a clinical advantage over conventional chemotherapy, some studies have suggested that RICT may be beneficial in elderly patients with hematological malignancies.[2]

Since the late 1990s, several studies of RICT against advanced solid tumors have been performed to harness the graft-versus-tumor effect.[3] A clinical tumor response after RICT was observed in several solid tumors, especially in renal cell cancer and breast cancer.[4–6] Pancreatic cancer is the fifth most common cause of cancer-related mortality in Japan and the United States, and carries an extremely poor prognosis. The median duration of survival in advanced pancreatic cancer is less than 6 months, even when patients are treated with gemcitabine.[7] The combination of gemcitabine with the other chemotherapeutic agents failed to significantly improve survival.[8–10] Furthermore, although the combination of gemcitabine and erlotinib, a molecular targeting agent against epidermal growth factor receptor, significantly prolonged survival, the difference in median survival was only 2 weeks.[11] Because of this poor prognosis by chemotherapy, treatment strategies to enhance immunological effects against pancreatic cancer have been investigated. One of these is a vaccination targeting tumor-specific antigens such as CA19-9 and CEA.[12] Another strategy is RICT to harness a strong allogeneic immunological antitumor effect. The first successful application of RICT against pancreatic cancer was reported in 2001.[13] Several other reports have suggested the existence of an immunological graft-versus-tumor effect against pancreatic cancer, but the number of patients in each report was too small to draw any meaningful conclusion.[14,15] Therefore, we collected the clinical results of RICT against pancreatic cancer from transplantation centers in Japan, in which a prospective clinical trial of RICT against pancreatic cancer had been performed.

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Patients and methods

We surveyed transplantation centers in Japan and identified three centers (Komagome Hospital, Kyushu University and National Cancer Center Hospital) that were performing a prospective clinical trial against various advanced solid tumors including pancreatic cancer. The University of Tokyo Hospital was performing a trial that exclusively included patients with advanced pancreatic cancer. Two of these trials have already been published.[14,15] We collected the clinical results of all patients with pancreatic cancer who participated in these studies from the published papers or using a questionnaire.

The reduced-intensity conditioning regimens were exclusively fludarabine-based, but varied among centers. The most intensive regimen was the combination of fludarabine (30 mg/m^2/day for 6 days), BU (4 mg/kg/day for 2 days) and gemcitabine (1000 mg/m^2/day for 3 days) at the University of Tokyo Hospital, whereas the combination of fludarabine (30 mg/m^2/day for 3 days) and TBI at 2 Gy (Kyushu University) was the least intensive. CY (60 mg/kg/day for 2 days) was combined with fludarabine (25 mg/m^2/day for 5 days) in the Komagome Hospital. Prophylaxis against GVHD was performed with CYA either alone or in combination with MTX or mycophenolate mofetil. PBSCs were mobilized with G-CSF, cryopreserved using standard techniques without *ex vivo* manipulation, thawed and infused on day 0. Host/donor T-cell chimerism was analyzed by sex-chromosome FISH or the short tandem repeat method after transplantation.[16]

The tumor response to treatment was evaluated as described previously.[15] Briefly, CR (complete response) was defined as disappearance of all clinical evidence of tumor for a minimum of 4 weeks by computed tomography scan. MR (minor response) and PR (partial response) were defined as decreases of 25–50% and greater than 50%, respectively, in the sum of the products of the maximum diameter and its perpendicular diameter of all measurable lesions for a minimum of 4 weeks.[7]

Engraftment was defined as a neutrophil count more than 500/mm^3 for 3 consecutive days after RICT. Engraftment failure was diagnosed as when engraftment was not achieved at any time after transplantation. The probability of survival was calculated using the Kaplan–Meier method. The incidence of chronic GVHD was evaluated in 13 patients who survived longer than 100 days after RICT. Univariate comparisons for dichotomous and time-to-event variables between groups were performed with the Fisher exact test and the log-rank test, respectively, and multivariate analyses were performed using logistic regression analysis and proportional hazards modeling, respectively. Factors associated with at least borderline significance ($P<0.10$) in the univariate analysis were subjected to a multivariate analysis using backward stepwise selection of covariates. All *P*-values were two sided and values of 0.05 or less were considered statistically significant.

Results

Clinical data of 22 patients with a median age of 57 years (range: 36–68 years) were collected (Table 1). There were 15 male and seven female patients. Fifteen patients had metastatic disease, whereas 7 had locally advanced diseases. All but one patient had received chemotherapy with gemcitabine either alone or in combination with other antineoplastic agents before RICT. In all, 10 had received local irradiation in addition to chemotherapy. Eastern Cooperative Oncology Group performance status (ECOG-PS) was equal to or greater than 2 in 10 patients. The conditioning regimen was fludarabine-BU-based in 10, fludarabine-CY in 7 and fludarabine-TBI in 5. The donors were HLA-matched relatives except in one patient who received graft from an HLA-mismatched family donor. The number of CD34-positive cells infused was greater than 4.0×10^6 cells/recipient body weight (kg) in 10 patients. CYA was used for GVHD prophylaxis: alone in 8, combined with MTX in 10 and combined with mycophenolate mofetil in 4.

Engraftment was observed in all but two patients with a median duration from RICT of 12 days (range: 6–42 days). Complete donor-type T-cell chimerism was confirmed in 18 patients, whereas mixed chimerism persisted in 4 patients. A total of 12 patients developed grade II–IV acute GVHD. Limited and extensive chronic GVHD was observed in three and five patients, respectively, among the 13 patients who survived longer than 100 days after RICT.

The best response after RICT was CR in one, PR in two, MR in two and stable disease in eight. The overall response

Table 1 Characteristics of the patients

Age (years)	
Median	57
Range	36–68
Sex	
Male	15
Female	7
Disease	
Locally advanced	7
Metastatic	15
ECOG-PS	
0–1	12
2–4	10
Regimen	
Flu + BU + Gem	7
Flu + CY	6
Flu + TBI	6
Flu + BU	3
Donor	
HLA-matched sibling	21
Mismatched family donor	1
CD34+ cells in graft	
$\leqslant 4.0 \times 10^6$/kg	12
$> 4.0 \times 10^6$/kg	10
GVHD prophylaxis	
CsA alone	8
CsA + MTX	10
CsA + MMF	4

Abbreviations: ECOG-PS = Eastern Cooperative Oncology Group performance status; Flu = fludarabine; Gem = gemcitabine; MMF = mycophenolate mofetil.

rate (CR + PR + MR) was 23%. A univariate analysis to identify possible relationships between clinical parameters and overall response failed to show any statistically significant factors. The conditioning regimen did not significantly affect the response rate, although the statistical power was not enough due to the small number of patients in each group. Response was observed in two of the seven patients who received the most intensive regimen including fludarabine, BU and gemcitabine, while it was seen in one of the six patients who received the least intensive regimen with fludarabine and low-dose TBI. None of the patients with mixed chimerism showed a response, but this difference was not statistically significant. DLI (donor lymphocyte infusion) was performed in four patients who had progressive disease after RICT, and the number of infused CD3-positive cells was between 2.7×10^7 and 1.8×10^8 cells/kg. One patient showed tumor shrinkage after DLI, but the response was transient.

Figure 1a shows overall survival after RICT. Median survival was only 139 days and the major cause of death was tumor progression. Other causes of death included infection in one and chronic GVHD in two. In a univariate analysis, ECOG-PS below 2 and infused CD34-positive cell dose greater than 4.0×10^6 cells/kg were associated with significantly longer survival after RICT (Table 2; Figures 1b and c). A multivariate analysis revealed that these two factors were almost independently significant (Table 2). With regard to post transplantation factors, while the development of grade II–IV acute GVHD did not significantly affect survival ($P = 0.76$), the eight patients who developed chronic GVHD tended to survive longer than those who survived longer than 100 days after RICT but did not develop chronic GVHD ($P = 0.092$; Figure 2). This analysis was unlikely to be biased by the fact that patients who survived longer had more chance to develop chronic GVHD, as most of the patients developed chronic GVHD as a progressive type from acute GVHD.

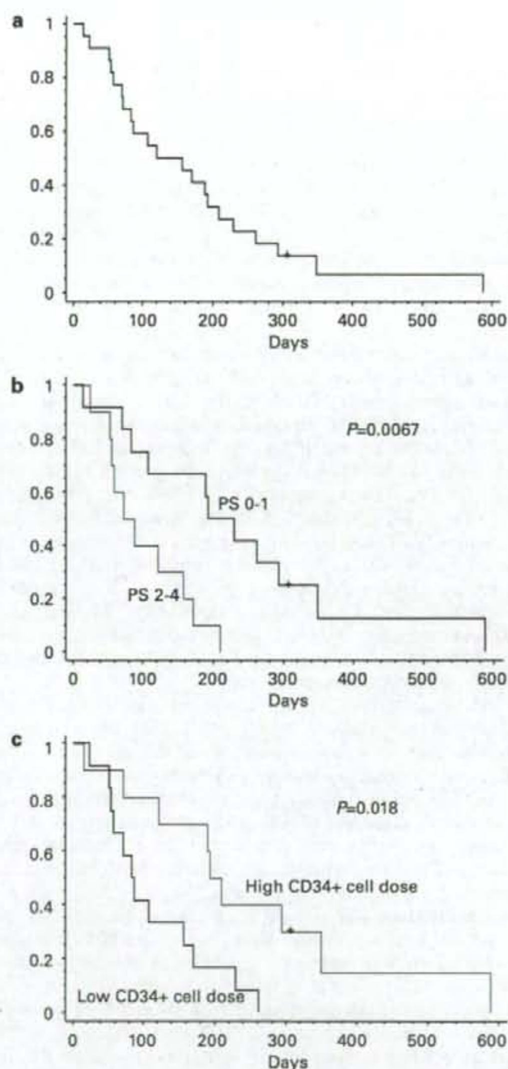

Figure 1 Patient survival, overall (**a**) and grouped according to risk factors (**b** and **c**).

Discussion

To summarize these findings, 23% of the 22 patients in this series showed a response to RICT. However, the duration of the response was generally short and most of the patients eventually died with progressive disease. The median survival after RICT was only 139 days and only one survived longer than 1 year after transplantation. Good ECOG-PS and higher number of CD34-positive cells in the graft were independently associated with longer survival.

The relationship between the number of infused CD34-positive cells and transplant outcome has been studied in PBSC transplantation for hematological malignancies.[17] The infusion of a higher number of CD34-positive cells has been associated with faster recovery of neutrophils and plts, but chronic GVHD was more frequently observed in patients who received a very high dose of CD34-positive cells (that is, $>8.0 \times 10^6$ cells/kg). In this study, two patients failed to achieve engraftment, and both had received less than 4.0×10^6 cells/kg of CD34-positive cells. However, a statistically significant survival advantage was confirmed even after these two patients were excluded from the analysis. If we consider that the major cause of death in this study was progressive disease, the infusion of a higher number of CD34-positive cells might have protected patients from disease progression by a graft-versus-host reaction, although we failed to show a significant difference between the number of infused CD34-positive cells and tumor response or the incidence of chronic GVHD, probably due to the small number of patients. Patients who developed chronic GVHD showed better survival than those who did not, with a borderline significance, suggesting that they had some immunological protection against the progression of pancreatic cancer.

Table 2 Univariate and multivariate analyses for overall survival

Factor	Median survival (days)	P-value
A. Univariate		
Age (years)		
<55	115	0.63
⩾55	180	
Sex		
Male	87	0.69
Female	170	
ECOG-PS		
0–1	211	0.0067
2–4	80	
Stage		
Locally advanced	192	0.21
Metastatic	121	
Serum CEA		
Negative	122	0.70
Positive	192	
Serum CA19-9		
Negative	157	0.84
Positive	132	
Regimen		
Flu + BU based	191	0.25
Flu + CY	156	
Flu + TBI	71	
CD34+ cell dose		
$\leqslant 4.0\times10^6$/kg	85	0.018
$>4.0\times10^6$/kg	201	
GVHD prophylaxis		
CsA alone	132	0.55
CsA + MTX	191	
CsA + MMF	96	
B. Multivariate	*Relative risk (95% CI)*	P-value
ECOG-PS		
0–1	1.00	0.032
2–4	3.39 (1.11–10.3)	
CD34+ cell dose		
$\leqslant 4.0\times10^6$/kg	1.00	0.068
$>4.0\times10^6$/kg	0.37 (0.13–1.07)	

Abbreviations: CI = confidence interval; ECOG-PS = Eastern Cooperative Oncology Group performance status; Flu = fludarabine; MMF = mycophenolate mofetil.

This study was limited by the heterogeneity of transplantation procedures among centers. However, considering the difficulty of performing a large-scale prospective study on RICT against pancreatic cancer, this small survey may currently represent the best evidence of the efficacy of this novel treatment strategy against advanced pancreatic cancer and may suggest a future direction for improving the treatment outcome. We showed that pancreatic cancer can be a possible target for allogeneic immunotherapy. However, the immunological effect was not strong or durable enough to prevent tumor progression. A possible strategy for enhancing a graft-versus-tumor effect against pancreatic cancer without enhancing GVHD is a combination with specific immunotherapy using antigens including CA19-9, CA242, CEA, Her-2, mutated K-ras and MUC-1.[12] Among these, CEA is attractive, since it is expressed in 85–90% of pancreatic cancer, and a specific immunotherapy against CEA could also be applied to other gastrointestinal cancers. An increase in the serum anti-CEA antibody level associated with a tumor response was observed in the University of Tokyo Study.[15] In addition, Kim *et al.*[18] showed that a peptide CEA652, TYACFVSNL, binds to HLA-A24 and induces CEA-specific cytotoxic T cells. Therefore, vaccination with such a peptide may be promising as a post transplantation immunotherapy against pancreatic cancer. Another approach is to add molecular targeting agents such as erlotinib after RICT. This may induce tumor cell death, leading to the enhanced presentation of tumor antigens to donor T cells. In addition, RICT can be combined with surgical resection, since the prognosis of pancreatic cancer is very poor even after complete resection.[19,20] Maximum graft-versus-tumor effect can be expected when the tumor load is at its lowest level.

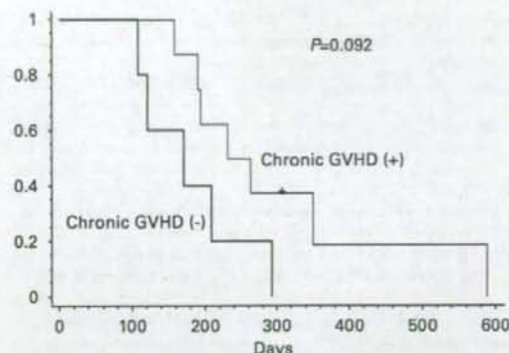

Figure 2 Overall survival of patients who survived at least 100 days after transplantation grouped according to the presence or absence of chronic GVHD.

In conclusion, a tumor response was observed in approximately one-fourth of the patients who underwent RICT against advanced pancreatic cancer. Although the response was not durable, our findings, such as the relationship between longer survival and the infusion of a higher number of CD34-positive cells or the development of chronic GVHD, should support a future study to enhance the specific immunological effect against pancreatic cancer.

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

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

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

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

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

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

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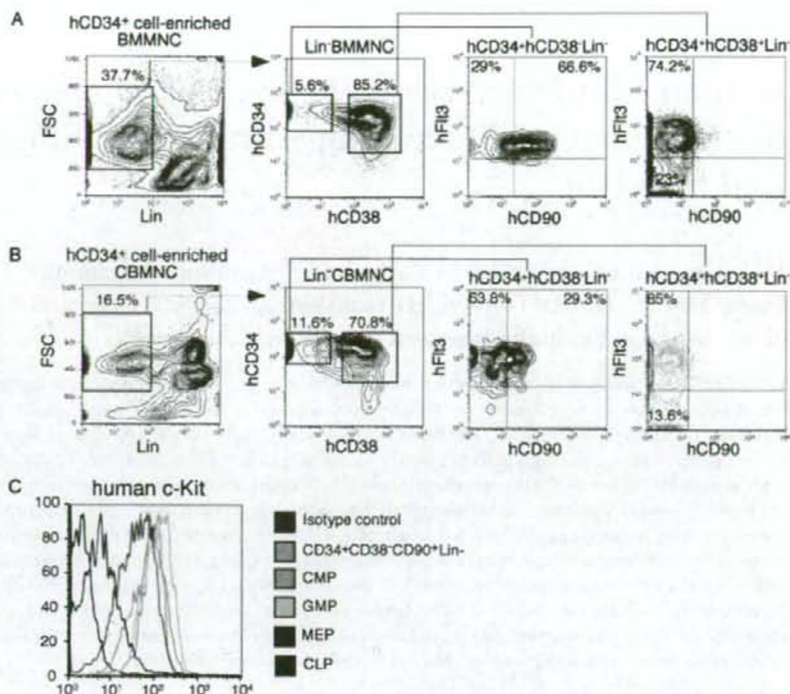

FIGURE 1. A flow cytometric analyses of human early hematopoietic populations in the BM and the CB. In $hCD34^+hCD38^-$ immature BM (*A*) and CB (*B*) cells, hFlt3 was expressed at a low level in both hCD90 (Thy1) positive and negative fractions. In contrast, the $hCD34^+hCD38^+$ BM and CB progenitor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

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

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

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

Materials and Methods

BM and CB samples

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

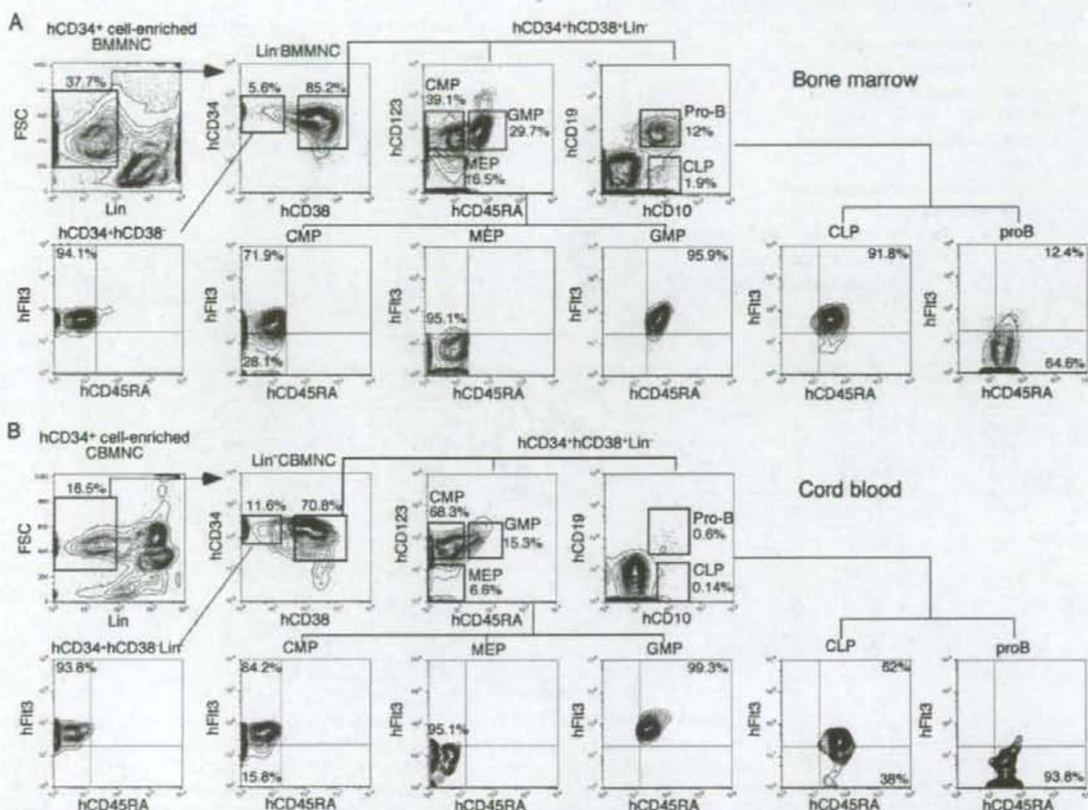

FIGURE 2. The expression patterns of hFlt3 are similar in early human BM (*A*) and CB (*B*) hematopoiesis. In the myeloid pathway in both the BM and CB, hFlt3 was up-regulated into the GM pathway, but was down-regulated in the MegE pathway; GMPs expressed hFlt3 at a high level, whereas MEPs did not express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

Cell preparation, flow cytometric analysis, and cell sorting

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

In vitro assays to determine the differentiation potential of progenitors

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

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

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

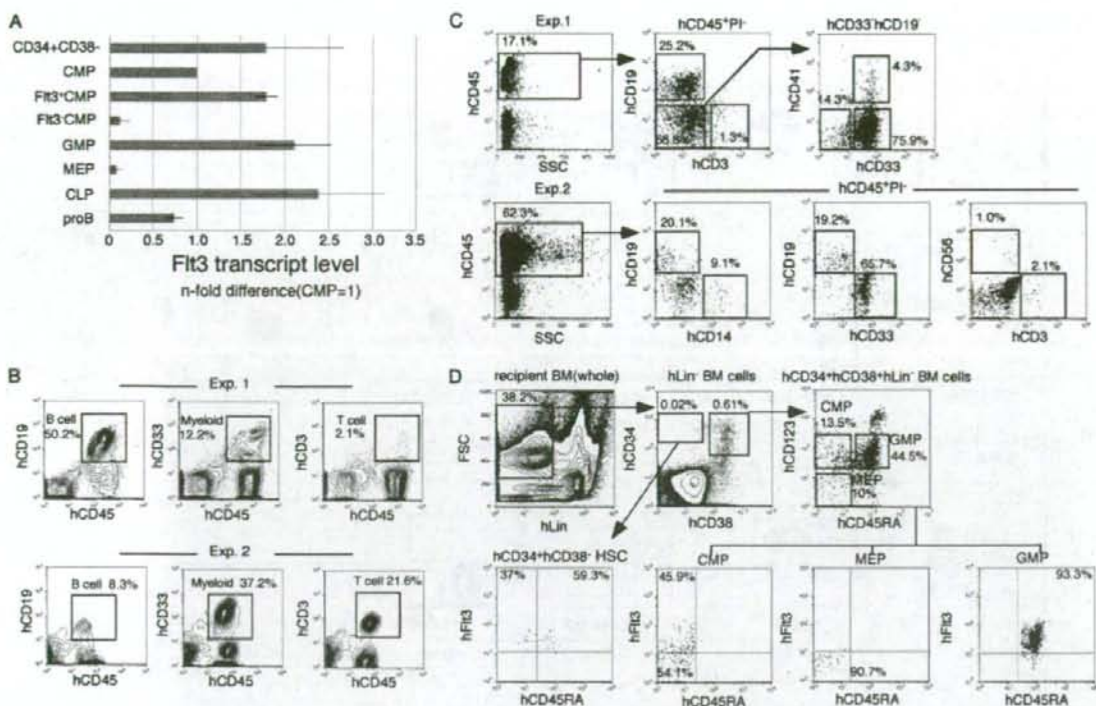

FIGURE 3. Long-term reconstitution potential of hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$Lin$^-$ cells in NOD/SCID/IL2rγ^{null} newborn mice. *A*, Analyses of the relative expression levels of hFlt3 transcript by real-time PCR. Each bar shows the *n*-fold difference of the level of hFlt3 mRNA in comparison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

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

In vivo assays to determine the differentiation potential and reconstitution capacity

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

Quantitative real-time PCR

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

Results

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

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

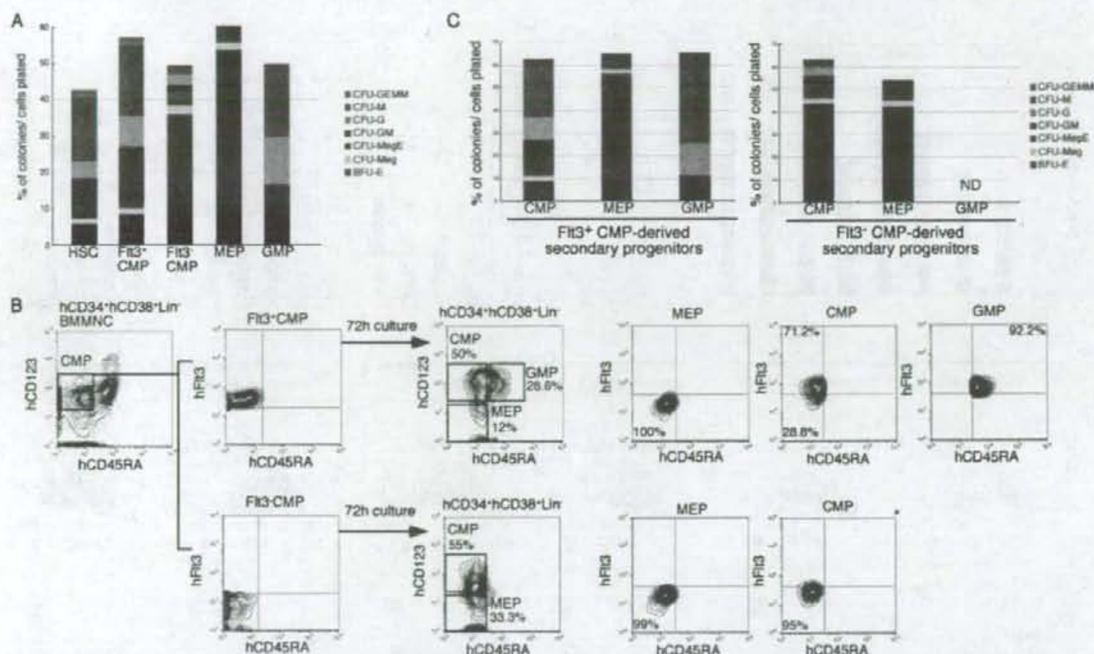

FIGURE 4. The lineage potential and the relationship of myeloid progenitor populations. *A*, Clonogenic colony formation of purified populations on methylcellulose in the presence of cytokine mixture. The hCD34$^+$hCD38$^-$ HSCs and hFlt3$^+$CMPs gave rise to various myeloid colonies including CFU-GEMM,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

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

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

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

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

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