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ORIGINAL ARTICLE

Peripheral blood stem cell versus bone marrow transplantation from HLA-identical sibling donors in patients with leukemia: a propensity score-based comparison from the Japan Society for Hematopoietic Stem Cell Transplantation registry

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Abstract We retrospectively analyzed the results of 707 adult patients who underwent myeloablative peripheral blood stem cell transplantation (PBSCT) ($n = 365$) and myeloablative bone marrow transplantation (BMT) ($n = 342$) for leukemia from HLA-identical sibling donors between 2000 and 2005 using the propensity score method. The results were obtained from the Japan Society for Hematopoietic Cell Transplantation registry. Multivariate Cox analysis showed that PBSCT was associated with lower overall survival (OS) in standard-risk patients [adjusted hazard ratio (aHR) = 1.83; 95% confidence interval (CI) 1.04–3.23; $P = 0.036$], but not in high-risk patients (aHR = 1.11; 95% CI 0.76–1.61; $P = 0.599$). Hematopoietic recovery was significantly faster after

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PBSCT. The risk of acquiring grade III–IV acute graft-versus-host disease (GVHD) (aHR = 2.23; $P = 0.040$) and extensive chronic GVHD (aHR = 1.93; $P = 0.001$) were significantly higher after PBSCT. PBSCT was associated with higher non-relapse mortality in standard-risk patients (aHR = 2.30; 95% CI 1.08–4.88; $P = 0.030$), but not in high-risk patients (aHR = 1.29; 95% CI 0.65–2.54; $P = 0.468$). Relapse after transplantation did not differ between PBSCT and BMT either in standard-risk group or in high-risk group (aHR = 1.17; 95% CI 0.55–2.52; $P = 0.684$ and aHR = 0.81; 95% CI 0.52–1.28; $P = 0.370$, respectively). In this retrospective analysis, OS was significantly lower after PBSCT in standard-risk patients, but not in high-risk patients. PBSCT was associated with significant risks of grade III–IV acute GVHD and extensive chronic GVHD.

Keywords Bone marrow transplantation · Peripheral blood stem cell transplantation · Allogeneic · Graft-versus-host disease

1 Introduction

During the past decade, allogeneic peripheral blood stem cell transplantation (allo-PBSCT) has been increasingly used as an alternative to allogeneic bone marrow transplantation (allo-BMT) [1]. Furthermore, allo-PBSCT is associated with rapid hematopoietic recovery. Several prospective randomized controlled trials conducted in Western countries have shown an increased incidence of chronic graft-versus-host disease (GVHD) [2–11]. Nevertheless, there is still substantial controversy regarding survival, acute GVHD, non-relapse mortality (NRM), and relapse [12–14].

Ethnicity has been reported to affect the incidence and severity of GVHD [15]. Japanese patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) were found to have a lower incidence of acute GVHD than those from Western patients [16, 17]. Therefore, the outcome of allo-PBSCT compared with that of allo-BMT may differ according to the ethnic background.

Using the propensity score method, we retrospectively analyzed the clinical outcomes of 707 adult Japanese leukemia patients who received allogeneic HSCT with myeloablative conditioning from HLA-identical sibling donors. These data were obtained from the Japan Society for Hematopoietic Cell Transplantation (JSHCT) registry. A propensity scoring system was devised to estimate the effects of treatments by comparing outcomes of those subjects who were not randomly assigned to experimental or control groups in an observational study [18]. A randomized control trial is superior in eliminating the confounding factors of known and unknown covariates by random treatment assignment. The propensity score expresses the likelihood of being assigned to experimental or control treatments, and is calculated using logistic regression models, including variables measured prior to treatment as much as possible. Considering the propensity score in this analysis, we expected that a hypothetical evaluation of an experimental trial in an observational study would give results similar to those of an evaluation in a randomized controlled trial.

2 Patients and methods

2.1 Study population

Using a standardized reporting form, JSHCT collects data on individual transplant patients from each transplant center, and follow-up reports are submitted annually after transplantation. A total of 1,426 patients, who underwent allogeneic HSCT between 2000 and 2005 for acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic myelogenous leukemia (CML), have been reported to JSHCT. Patients were excluded from the study if their data were incomplete ($n = 205$), if they received a non-myeloablative or reduced-intensity conditioning regimen ($n = 223$), if they received grafts from other than HLA-identical siblings ($n = 217$), if they were less than 18 years of age ($n = 38$), if they had a previous history of HSCT ($n = 10$), and if they had non-allo-PBSCT or non-allo-BMT ($n = 16$). In Japan, most

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allo-HSCT patients have received granulocyte-colony stimulating factor (G-CSF) post-transplant [19]. The August 2006 data of the remaining 707 patients were analyzed. This study was approved by the Data Management Committee for the Nationwide Survey of JSHCT.

2.2 Definitions

Risk status at transplantation was categorized as either standard or high. Standard-risk diseases included acute leukemia in first complete remission (CR) and CML in first chronic phase (CP). Other disease status was categorized as high-risk disease [11]. The day of neutrophil engraftment was defined as the first of three consecutive days with an absolute neutrophil count (ANC) of more than 0.5×10^9/L. The day of platelet engraftment was defined as the first of seven consecutive days with a platelet count of more than 20×10^9/L without platelet transfusion. Acute GVHD was graded according to the standard criteria [20]. All patients who had no evidence of graft failure and survived beyond day 28 were considered to be evaluable for acute GVHD. GVHD persisting beyond day 100 and de novo GVHD occurring after day 100 were classified as chronic GVHD. The incidence of chronic GVHD was calculated in patients followed for more than 100 days, and the disease was classified as none, limited, or extensive [21]. Overall survival (OS) was defined as the duration of survival between transplantation and either death or the last follow-up.

Relapse was defined as disease progression with censored NRM. NRM included all causes of death other than relapse occurring at any time after transplantation. All deaths were considered in the estimation of OS.

2.2.1 Endpoints

The primary endpoint of comparison was OS. Secondary endpoints were hematopoietic recovery, acute GVHD (grade II–IV and III–IV), chronic GVHD (overall and extensive), NRM, and relapse.

2.2.2 Propensity score calculation

We calculated the propensity score using the pscore command in STATA version 10.1. (STATA, College Station, TX, USA) [22]. Factors included in the propensity score were as follows: age at HSCT in categories (<40, 40–49, and 50+) as an ordinal variable; sex (male/female) as an indicator variable; year of transplantation as a continuous variable; performance status at transplantation as an ordinal variable; risk status (CR1/CP1, CR2/CP2, or more advanced) as an indicator variable; a cumulative number of HSCT from related donors at an institution between 2000 and 2005 (1: 1–4, 62 institutions; 2: 5–11, 58 institutions; 3: 12 or more, 52 institutions) as an ordinal variable; and the percentage of allo-PBSCT out of total HSCT from HLA-identical siblings in tertile (1: <56%, 59 institutions; 2: 56–90%, 56 institutions; 3: 91% or more, 57 institutions) as an ordinal variable. We utilized as many variables as possible in the propensity score to evaluate the effects of known and unknown factors on the choice of treatment. After calculating the propensity score, the subjects were divided into four groups according to quartile. The numbers of subjects in quartiles 1–4 (allo-PBSCT/allo-BMT) were 23/154, 58/120, 126/50, and 158/18, respectively.

2.2.3 Statistical analysis

Patient characteristics and therapeutic outcomes were compared between allo-PBSCT and allo-BMT groups. OS was assessed using the Kaplan–Meier product limit method [23, 24]. Cumulative incidences of acute GVHD, chronic GVHD, NRM, and relapse were evaluated as 1 − (Kaplan–Meier estimate) instead of applying methods considering competing risks [25, 26] to maintain statistical consistency between logrank tests and methods of cumulative incidence estimation. Allo-PBSCT and allo-BMT groups were compared using the propensity score in quartiles [1–4], a stratified logrank test, and a stratified Cox proportional hazards model. Diagnosis (AML, ALL, and CML) and quartile of the propensity score were stratification factors. Confounders considered in the Cox proportional hazards model were as follows: year of diagnosis as a continuous variable; year of transplantation as a continuous variable; age at transplantation as a continuous variable; sex (male/female); sex matching (match/male to female/female to male/unknown); performance status (0, 1, 2, 3–4, and unknown); risk status (standard/high); GVHD prophylaxis [cyclosporin (CsA) + methotrexate (MTX), tacrolimus (TAC) + MTX, and others]; and conditioning regimen [total body irradiation (TBI)-containing regimen, busulfan and cyclophosphamide (BU/CY), and others]. All analyses were performed using STATA version 10.1, and P values less than 0.05 were considered statistically significant.

3 Results

3.1 Patient characteristics

The characteristics of patients are summarized in Table 1. The number of patients who underwent allo-PBSCT was 365, and that who underwent allo-BMT was 342. The median age at HSCT was 39 years (range 18–64 years) in the allo-PBSCT group and 39 years (range 18–59 years) in the allo-BMT group. The allo-PBSCT group included significantly more male patients from female donors than

Table 1 Characteristics of patients

	PBSCT *n* (%)	BMT *n* (%)	*P* value (Mann–Whitney test)
No. of patients	365	342	
Median patients age, years (range)	39, 18–64	39, 18–59	0.962
Patients sex (male/female)	210/155	189/153	0.543
Sex matching			
Matched	176 (48.2)	185 (54.1)	
Male to female	70 (19.2)	78 (22.8)	
Female to male	106 (29.0)	71 (20.8)	
Unknown	13 (3.6)	8 (2.3)	0.043
Risk group			
Standard-risk	149 (40.8)	202 (59.1)	
High-risk	216 (59.2)	140 (40.9)	<0.001
Diagnosis			
Standard-risk			
AML	58 (38.9)	76 (37.6)	
ALL	46 (30.9)	51 (25.2)	
CML	45 (30.2)	75 (37.2)	0.322
High-risk			
AML	128 (59.3)	75 (53.6)	
ALL	58 (26.9)	28 (20.0)	
CML	30 (13.8)	37 (26.4)	0.026
Performance status			
0	185 (50.7)	138 (40.4)	
1	73 (20.0)	55 (16.1)	
2	24 (6.6)	16 (4.7)	
3 or 4	12 (3.3)	2 (0.6)	
Unknown	71 (19.5)	131 (38.3)	<0.001
Conditioning regimen			
TBI-based	225 (61.6)	205 (59.9)	
Bu/CY	110 (30.1)	118 (34.5)	
Others	30 (8.3)	19 (5.6)	0.23
GVHD prophylaxis			
CsA + MTX	308 (84.4)	300 (87.7)	
TAC + MTX	12 (3.3)	14 (4.1)	
Others	45 (12.3)	28 (8.2)	0.176

Standard-risk diseases: acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase; other disease status was categorized as high-risk diseases

PBSCT peripheral blood stem cell transplantation, *BMT* bone marrow transplantation, *AML* acute myelogenous leukemia, *ALL* acute lymphoblastic leukemia, *CML* chronic myelogenous leukemia, *TBI* total body irradiation, *Bu* busulfan, *CY* cyclophosphamide, *GVHD* graft-versus-host disease, *CsA* cyclosporin, *MTX* methotrexate, *TAC* tacrolimus

the allo-BMT group (Mann–Whitney test, $P = 0.043$). AML, ALL, and CML were diagnosed in 337, 183, and 187 patients, respectively. The allo-PBSCT group included significantly more high-risk patients than the allo-BMT group ($P < 0.001$). Among the high-risk patients, the allo-BMT group had significantly more CML patients than the allo-PBSCT group ($P = 0.026$). Conditioning regimen and GVHD prophylaxis were performed according to the protocol of each institution, and there were no differences between the two groups. The most frequently used conditioning regimens were BU/CY (busulfan 1 mg/kg × 4/day × 4 days with cyclophosphamide 60 mg/kg/day × 2 days) and CY/TBI (cyclophosphamide 60 mg/kg/day × 2 days with total body irradiation 10–12 Gy). CsA plus MTX was used most frequently for GVHD prophylaxis. Median follow-up period for the surviving patients at the time of analysis was 33 months (1.8–55 months) in the PBSCT group and 31 months (1–53 months) in the BMT group.

3.2 Primary endpoint

3.2.1 Overall survival

Three-year OS in standard-risk patients was 68% [95% confidence interval (CI) 59–75] after allo-PBSCT and 77%

(95% CI 70–82) after allo-BMT (by disease and quartile in the propensity-score stratified logrank test; $P = 0.023$). Three-year OS in high-risk patients after allo-PBSCT and allo-BMT was 38% (95% CI 31–45) and 54% (95% CI 44–62), respectively ($P = 0.587$) (Fig. 1). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for lower OS in the population with standard-risk [adjusted hazard ratio (aHR) = 1.83; 95% CI 1.04–3.23; $P = 0.036$], but not that with high-risk (aHR = 1.11; 95% CI 0.76–1.61; $P = 0.599$).

3.3 Secondary endpoints

3.3.1 Hematopoietic recovery

Engraftment occurred in all patients receiving allo-PBSCT and allo-BMT (allo-PBSCT, $n = 324$; allo-BMT, $n = 305$) surviving for more than 28 days. Allo-PBSCT patients showed significantly faster neutrophil and platelet recovery compared with allo-BMT patients. The median time of recovery to ANC $> 0.5 \times 10^9$/L was 14 days for the allo-PBSCT group and 16 days for the allo-BMT group, respectively (stratified logrank test, $P < 0.0001$). The median time of recovery to a platelet count $>20 \times 10^9$/L was 15 days for the allo-PBSCT group and 21 days for the allo-BMT group, respectively ($P < 0.0001$). In the multivariate Cox analysis, allo-PBSCT was a significant factor for faster neutrophil (aHR = 0.57; 95% CI 0.45–0.71; $P < 0.001$) and platelet (aHR = 0.56; 95% CI 0.44–0.71; $P < 0.001$) recovery compared with allo-BMT.

3.3.2 Acute GVHD

The cumulative incidence of grade II–IV acute GVHD was 31% (95% CI 27–35) in all patients, whereas that in allo-PBSCT and allo-BMT groups was 35% (95% CI 30–41) and 26% (95% CI 22–32) (stratified logrank test, $P = 0.221$), respectively. The aHR for grade II–IV acute GVHD after allo-PBSCT was 1.25 (95% CI 0.85–1.84; $P = 0.260$) by multivariate Cox analysis. The cumulative incidence of grade III–IV acute GVHD was 14% (95% CI 10–18) and 5.4% (95% CI 3.3–8.8) in the allo-PBSCT and allo-BMT groups, respectively ($P = 0.021$). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for the development of grade III–IV acute GVHD (aHR = 2.23; 95% CI 1.04–4.78; $P = 0.040$; Fig. 2).

3.3.3 Chronic GVHD

The risk of chronic GVHD in the first year after transplantation was significantly higher after allo-PBSCT than after allo-BMT (cumulative incidence at 1 year, 51%; 95% CI 44–58 after allo-PBSCT vs. 34%; 95% CI 28–41 after allo-BMT; $P = 0.0005$ with stratified logrank test). The extensive form of chronic GVHD was more prevalent in the allo-PBSCT group than in the allo-BMT group (26%; 95% CI 21–33 with allo-PBSCT and 15%; 95% CI 11–20 with allo-BMT; $P = 0.0017$). Multivariate Cox analysis showed that allo-PBSCT was a significant factor for the development of extensive chronic GVHD (aHR = 1.93; 95% CI 1.32–2.84; $P = 0.001$; Fig. 3).

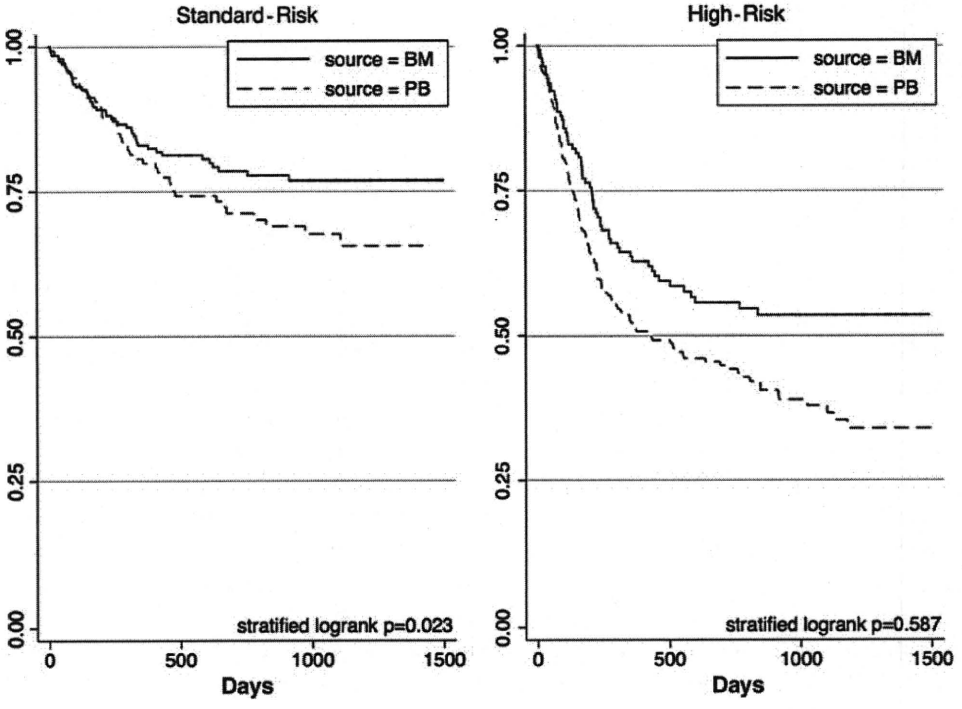

Fig. 1 Probabilities of overall survival after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

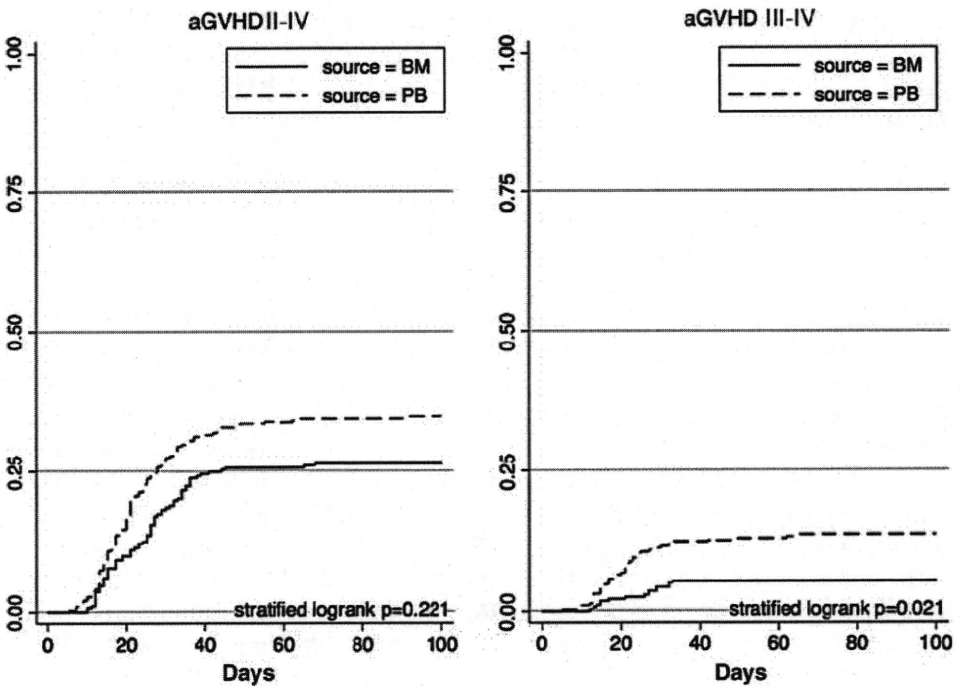

Fig. 2 Probabilities for grade II–IV and III–IV acute graft-versus-host disease (GVHD) after peripheral blood stem cell transplantation compared with bone marrow transplantation

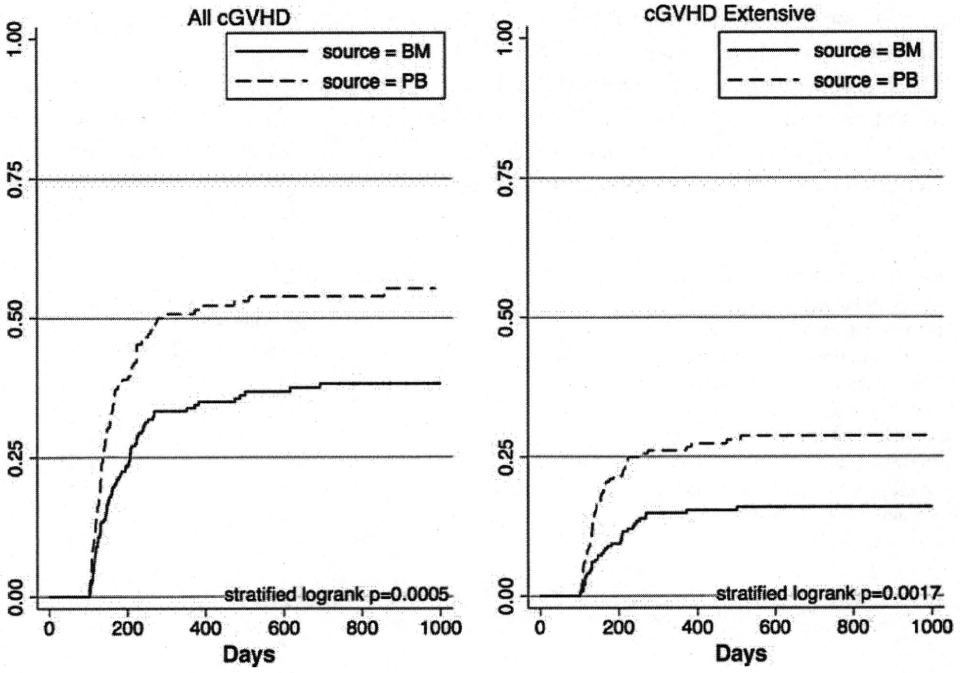

Fig. 3 Probabilities for chronic GVHD and extensive chronic GVHD after peripheral blood stem cell transplantation compared with bone marrow transplantation

3.3.4 Non-relapse mortality

The cumulative incidence of NRM for the standard-risk group at day 100 was 4.7% (95% CI 2.3–9.7) after allo-PBSCT and 6.0% (95% CI 3.4–10.2) after allo-BMT, and that at 1 year was 14.2% (95% CI 9.4–21.1) after allo-PBSCT and 11.2% (95% CI 8.0–17.2) after allo-BMT (stratified logrank test, $P = 0.047$). The cumulative incidence of NRM for the high-risk group at day 100 was 11.2% (95% CI 7.6–16.4) after allo-PBSCT and 8.9% (95% CI 5.1–15.1) after allo-BMT, and that at 1 year was 24.4% (95% CI 18.7–31.4) after allo-PBSCT and 14.7% (95% CI 9.6–22.2) after allo-BMT (stratified logrank test, $P = 0.221$) (Fig. 4).

Multivariate Cox analysis showed that allo-PBSCT was a significant factor for higher NRM in the standard-risk (aHR = 2.30; 95% CI 1.08–4.88; $P = 0.030$), but not in the high-risk (aHR = 1.29; 95% CI 0.65–2.54; $P = 0.468$).

3.3.5 Relapse

The cumulative incidence of relapse at 1 year for the standard-risk group was similar for allo-PBSCT (13.8%; 95% CI 8.9–21.0) and allo-BMT (9.7%; 95% CI 6.1–15.2) ($P = 0.518$ by stratified logrank test). Similarly, in the high-risk group the incidence was 32.4% (95% CI 25.6–40.3) for allo-PBSCT and 31.5% (95% CI 23.7–41.1) for allo-BMT ($P = 0.200$) (Fig. 5).

Multivariate Cox analysis showed no significant difference in the risk of relapse after allo-PBSCT and allo-BMT either in the standard-risk group or in the high-risk group (aHR = 1.17; 95% CI 0.55–2.52; $P = 0.684$ and aHR = 0.81; 95% CI 0.52–1.28; $P = 0.370$, respectively).

4 Discussion

In the present study, we analyzed results for 707 patients who underwent myeloablative HSCT for leukemia from HLA-identical sibling donors between 2000 and 2005. These data were obtained from the JSHCT registry. Health insurance coverage of allo-PBSCT was approved in Japan in 2000, and since then the number of allo-PBSCTs rapidly increased and exceeded the number of allo-BMTs between 2000 and 2003. Subsequently, the number of allo-PBSCTs decreased, and the numbers of allo-PBSCTs and allo-BMTs became equivalent in 2005. Thus, this analysis indicates the rather immature status of allo-PBSCT in Japan.

The Stem Cell Trialists' Collaborative Group [27] reported an individual patient data meta-analysis of nine randomized trials by comparing outcomes of allo-PBSCT versus allo-BMT from HLA-matched related donors for the treatment of hematologic malignancies. Allo-PBSCT was associated with a higher probability of 5-year OS in the subset analysis of patients with late disease due to decreased relapse. International Bone Marrow Transplant registry/European Group for Blood and Marrow Transplantation (IBMTR/EBMT) registry data of 398 adult allo-BMT and 208 allo-PBSCT patients with leukemia were analyzed using information on 6 or more years of follow-up [28]. OS in patients with early and advanced leukemia did not differ significantly between the two groups. The IBMTR report comparing outcomes after allo-PBSCT and allo-BMT for acute leukemia in children and adolescents showed that OS was lower after allo-PBSCT [29]. These controversial data indicate that the difference in stem cell source can affect OS depending on the underlying disease, disease status, and the patients' age.

In our study, OS was lower after allo-PBSCT than after allo-BMT in the standard-risk patients, but not in the high-risk patients. Considering the difference in stem cell source, factors affecting OS include hematopoietic and

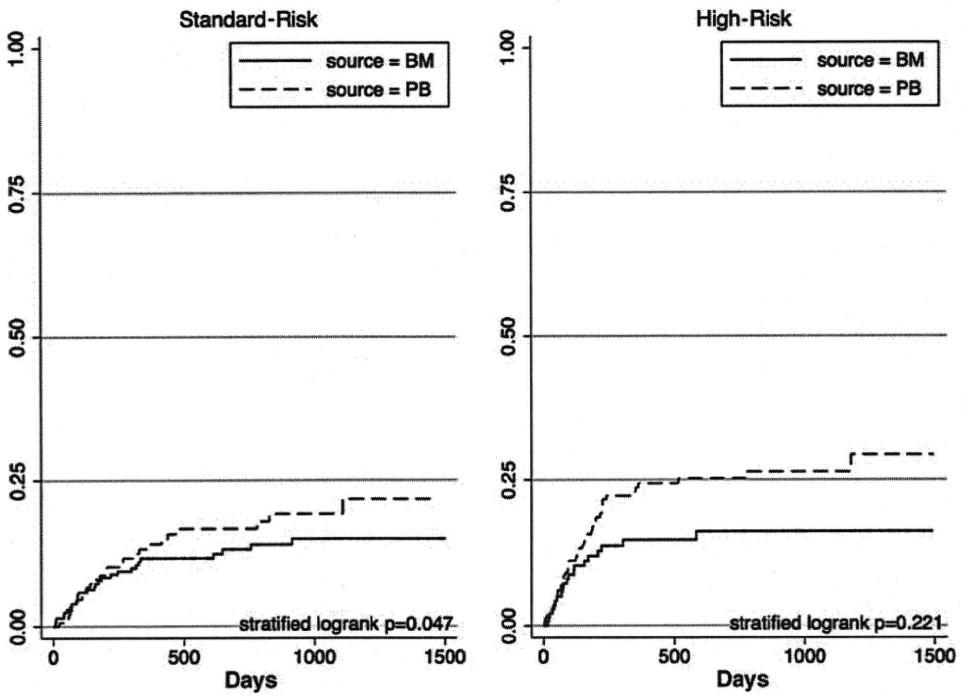

Fig. 4 Cumulative incidences of non-relapse mortality (NRM) after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

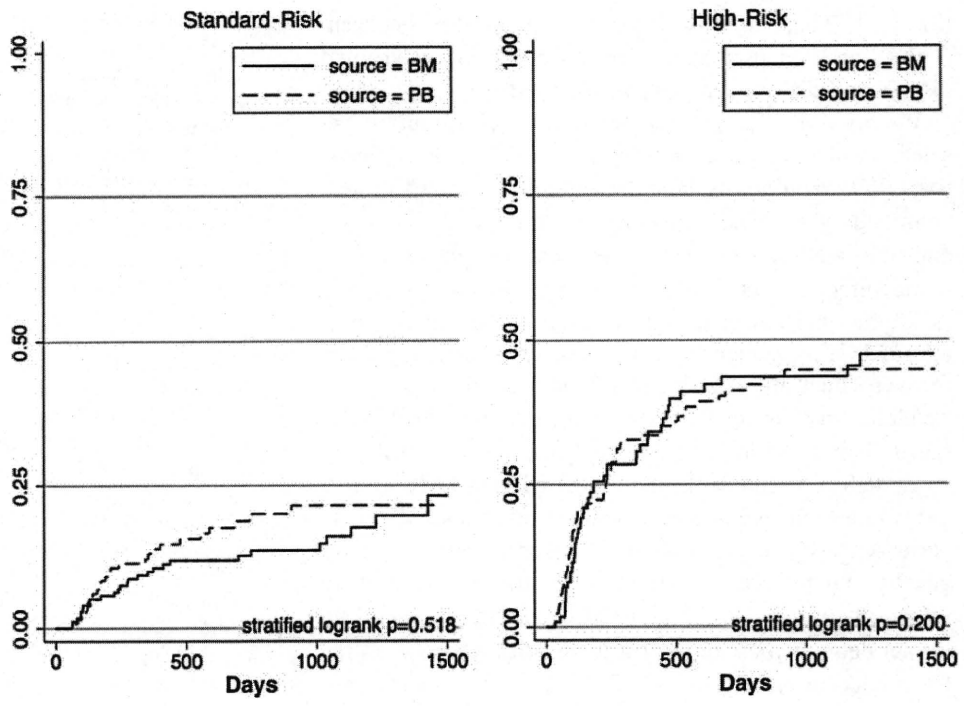

Fig. 5 Cumulative incidences of relapse after peripheral blood stem cell transplantation compared with bone marrow transplantation. Standard-risk diseases included acute leukemia in first complete remission and chronic myelogenous leukemia in first chronic phase. Other diseases were categorized as high-risk diseases

immune recovery, acute and chronic GVHD, and graft-versus-leukemia (GVL) effect or relapse [30].

In our analysis, allo-PBSCT was associated with more rapid hematopoietic recovery than allo-BMT as has been shown in most previous studies [4, 5, 11, 31]. Most randomized trials demonstrated that neutrophil recovery generally occurs 5–7 days earlier after allo-PBSCT compared with allo-BMT without G-CSF post-transplant [27, 32]. The EBMT study reported by Schmitz et al. [5] showed that neutrophil recovery was achieved 3 days earlier after allo-PBSCT than after allo-BMT with G-CSF post-transplant, and transplantation-related mortality did not differ between allo-PBSCT and allo-BMT groups. In Japan, most allo-HSCT patients receive G-CSF post-transplant, and in our study neutrophil recovery was observed 2 days earlier after allo-PBSCT than after allo-BMT. Accordingly, infectious complications may not decrease after allo-PBSCT compared to allo-BMT.

With regard to acute GVHD, the meta-analysis showed that allo-PBSCT was associated with a significant increase in the development of grade III–IV acute GVHD, but not grade II–IV acute GVHD [27]. In the present analysis, allo-PBSCT was also a significant factor in the incidence of grade III–IV acute GVHD. The increased incidence of grade III–IV acute GVHD in allo-PBSCT would have a negative effect on OS [33].

Extensive chronic GVHD was more frequent after allo-PBSCT than after allo-BMT in our study. This finding is in line with those of previous reports [5, 9, 11, 19, 31, 34].

In our analysis, NRM was higher after allo-PBSCT in the standard-risk patients, but not in the high-risk patients. The higher NRM after allo-PBSCT in the standard-risk group was likely due to increased grade III–IV acute GVHD and extensive chronic GVHD. Increased NRM after allo-PBSCT has been reported from children and adolescents suffering with acute leukemia [29]. A higher risk of mortality due to acute and chronic GVHD may counteract any benefit of more rapid hematopoietic recovery in the early transplant period.

In the allo-BMT setting, the development of both acute and chronic GVHD is associated with decreased relapse of leukemia, whereas the effect of GVHD on OS appears to be different depending on the study population [33, 35, 36]. The meta-analysis showed that allo-PBSCT was associated with a significant decrease in relapse in both early and late-stage disease patients [27]. On the contrary, increased extensive chronic GVHD in the allo-PBSCT group did not lead to a decrease in relapse in our analysis. We do not have a good explanation for this, but a similar observation was reported from the IBMTR/EBMT [28] registry data of adult patients with leukemia and the IBMTR [29] study in children and adolescents with acute leukemia. The advantage in term of the GVL effect with the cost of increased GVHD after allo-PBSCT relative to after allo-BMT remains controversial [27–29]. The allogeneic GVL effect varies from one disease to another, with the stage of the disease, and with donor histocompatibility. The GVL effect is believed to act while the leukemic burden is relatively

low [37]. Thus, to investigate the relationship between GVHD and relapse, subgroups differing in underlying disease and disease status would be needed.

We used the propensity score method to minimize selection bias. However, retrospective analysis has limitations. We could not exclude the possibility of unidentified confounding variables affecting the transplant outcomes and the inability to adjust the data for unknown or unmeasured factors. For example, we did not have data regarding pre-transplant infectious complications. Since allo-PBSCT is associated with more rapid hematopoietic recovery than allo-BMT, patients with serious infectious problems may have a tendency to undergo allo-PBSCT rather than allo-BMT. In this analysis, standard-risk diseases included acute leukemia in first CR and CML in first CP, while high-risk diseases included other diseases [11]. However, even in first CR acute leukemia patients, cytogenetic and molecular markers affect the prognosis with respect to survival in the allo-HSCT setting [38, 39]. We cannot deny the possibility that higher-risk patients in first CR tended to undergo allo-PBSCT. Thus, the results presented here should be interpreted with caution. It is also important to realize that our analysis was based on matched sibling myeloablative HSCT not on non-myeloablative HSCT. However, contrary to the result of the meta-analysis [27], multivariate Cox analysis showed that the allo-PBSCT group was associated with a lower OS in the populations with standard-risk. Prospective randomized trials are necessary to elucidate the advantages and disadvantages of allo-PBSCT in comparison with allo-BMT from HLA-identical sibling donors for the treatment of adult Japanese patients with leukemia.

Conflict of interest statement The authors declare no financial conflict of interest.

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ORIGINAL ARTICLE

Qualitative and quantitative differences in the intensity of Fas-mediated intracellular signals determine life and death in T cells

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Abstract Fas stimulation has been reported to promote the activation and proliferation of T lymphocytes, but the intracellular signalling pathways that mediate non-apoptotic responses to Fas are poorly defined. To distinguish between the activation signalling and the death-inducing pathway downstream of Fas, we generated a novel T cell line expressing a chimeric hCD8-FasC protein and found that stimulation with the anti-CD8 antibodies induced tyrosine phosphorylation of TCR-proximal proteins, activation of Raf-1/ERK, p38 and JNK, and increased expression of CD69, Fas, and Fas ligand. Stimulation of hCD8-FasC-induced activation of an atypical NF-κB pathway, partial cleavage of caspases, and increased expression of TRAF1, $FLIP_L$ and $FLIP_S$, thereby protecting T cells from FasL-mediated apoptosis. The proliferative response transmitted through hCD8-FasC chimeric receptors was converted into death signals when cells were stimulated, resulting in increased expression of IL-2 and Nur77 and increased caspase cleavage. Surprisingly, both the enhanced expression of $FLIP_L$ and $FLIP_S$ and the complete inhibition of $FLIP_S$ expression were functionally associated with cell death induction. These findings imply that Fas is able to trigger intracellular signalling events driving both apoptosis and activation of T cells but that cell fate is determined by quantitative and qualitative differences in intracellular signalling following Fas stimulation.

Keywords Fas · Apoptosis · Activation signals · T cells

1 Introduction

Fas (CD95/APO-1), which is a member of the tumour necrosis factor receptor (TNF-R) family, plays a fundamental role in tissue homeostasis, development, and

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regulation of the immune system [1–3]. The engagement of Fas results in the formation of the death-inducing signalling complex (DISC), in which FADD binds to the cytoplasmic domain of Fas via its death domain (DD) and recruits the death effector domain (DED)-containing caspase-8 to the DISC [1, 3–5]. Pro-caspase-8 then undergoes auto-proteolysis so that it can leave the DISC and gain access to other substrates that must be proteolysed for the cell to die [6, 7]. The c-FLIP protein, which is structurally similar to caspase-8, contains DEDs but has no enzymatic activity and has been shown to have opposing functions in Fas-mediated apoptosis depending upon the level of its expression [8, 9]. In certain cells, such as hepatocytes and pancreatic β cells, Fas-mediated apoptosis requires amplification through caspase-8-mediated proteolytic activation of Bid, which is dispensable in lymphoid cells [10, 11]. Death receptor-mediated activation-induced cell death [AICD, recently proposed to be called restimulation-induced cell death (RICD)], occurs as a result of a strong activation signal and plays an important role in the deletion of autoreactive T cells in the thymus, peripheral autoreactive T cells with specificity for autoantigens, and activated T cells at the termination of an immune response [1, 4, 12]. In contrast, other studies have shown that a decline in the levels of cytokines that promote T cell survival initiates AICD by triggering the activation of the pro-apoptotic BH3-only protein Bim [10, 13]. More recently, the killing of activated T cells during the chronic immune response has been shown to require both Fas-induced apoptotic signalling and Bim [2, 4, 14].

Despite these findings, the other activities of Fas ligand (FasL)-Fas signalling, such as the induction of cellular proliferation and differentiation, remain poorly understood. It has been reported that Fas promotes the proliferation of human T lymphocytes and the maturation of dendritic cells in culture [15]. Additionally, stimulation of Fas with agonistic anti-Fas monoclonal antibodies (mAbs) has been demonstrated to accelerate liver regeneration in mice after partial hepatectomy, and this process was delayed in $Fas^{lpr/lpr}$ mutant mice [16]. In tissue culture, enzymatic inhibitors of caspase-8 or specific FADD inhibitors were found to impair T cell activation and proliferation in response to mitogenic or antigenic stimulation [17, 18]. The identities of the intracellular non-apoptotic signalling pathways triggered by Fas stimulation and the mechanisms by which the signalling molecules involved in this process are activated remain unknown.

To investigate the molecular nature of non-apoptotic signalling events induced by Fas, we generated stable Jurkat T cell transfectants expressing an hCD8-FasC fusion protein on the cell surface to enable the differentiation of Fas-mediated activation signals from cell death-inducing signals. We found that activation of the hCD8-FasC chimeric receptor by the anti-CD8 mAbs UCHT4 and OKT8 not only induced early and late activation signals leading to cell proliferation but also activated an atypical NF-κB pathway that inhibited FasL-induced cell death. Moreover, an increase in intracellular Ca^{2+} or PKC activation converted activation signals through the hCD8-FasC chimeric receptor into death signals by enhancing IL-2 and Nur77 expression. In contrast to previous results demonstrating a concentration-dependent anti-apoptotic function of c-FLIP, our data suggest that two independent signalling pathways in which the expression of both $FLIP_L$ and $FLIP_S$ is increased by phorbol 12-myristate 13-acetate (PMA) or $FLIP_S$ expression is inhibited by ionomycin (IM) may play an important role in the conversion of Fas-mediated activation to cell death. These results provide the first molecular evidence that qualitative and quantitative differences in signalling through the Fas receptor can modulate the fate of T cells, driving them toward either cell death or activation, and our study may have clinical benefits to the Fas-associated dysfunction such as self reactivity, immune dysfunction, malignant transformation [19].

2 Materials and methods

2.1 Construction of CD8 chimeric receptors

We constructed two chimeric receptors in which portions of Fas and CD8 are fused (Supplementary Fig. 1A). In the hCD8-FasTC chimera, the extracellular domain of Fas was replaced with the extracellular domain of CD8. The other chimera, hCD8-FasC, contains the extracellular and transmembrane domains of CD8 and the cytoplasmic domain of Fas. For construction of hCD8-FasC, cDNA fragments of the extracellular and transmembrane domains of human CD8 and the cytoplasmic domain of human Fas were amplified by PCR and cloned into the pcDNA3 eukaryotic expression vector at the *Xba*I, *Bgl*II and *Bam*HI restriction sites. For construction of the hCD8-FasTC construct, the PCR-amplified human CD8 extracellular domain and human Fas transmembrane and cytoplasmic domains were cloned into pcDNA3. These DNA constructs were confirmed by sequencing.

2.2 Antibodies and reagents

OKT3 and OKT8 hybridomas were purchased from the American Type Culture Collection (ATCC; Manassas, VA), and secreted antibodies were purified as previously described [20]. The following antibodies were used: anti-p-Tyr (clone 4G10), anti-p-Tyr-agarose conjugate, anti-ICE, anti-Fyn, and anti-Fas (clone ZB4) from Upstate Biotechnology (Lake Placid, NY); anti-caspase-3 and anti-c-Raf-1 from BD Transduction Laboratories (San Jose, CA); anti-Fas (clone

ZB4) from Medical and Biological Laboratories (Nagoya, Japan); anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-p38, anti-phospho-p38, anti-IκB-α, anti-phospho-IκB-α, anti-ERK-1/-2, anti-phospho-ERK-1/-2, and anti-phospho-Akt from New England Biolabs (Beverly, MA); anti-human CD8 UCHT-4 from Sigma (St. Louis, MO); anti-phospho-p38, anti-phospho-JNK, anti-Bcl-2, anti-PARP, anti-Vav, anti-Daxx, anti-ZAP70, and anti-PLC-γ from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-human Fas ligand, anti-human Fas (clone G254-274), and anti-CD69 from BD PharMingen (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA), ionomycin, bisindolylmaleimide I (BIM-I), Z-YVAD-CMK peptides, Z-DEVD-FMK peptides, Z-VAD-fmk peptides, and PD98059 were obtained from Calbiochem Inc. (San Diego, CA). Sodium salicylate was obtained from Aldrich Chemical (Milwaukee, WI). RiboQuant multi-probe RNase protection assay system was purchased from BD PharMingen (San Diego, CA). Other antibodies and chemicals used were obtained from Sigma Chemical (St. Louis, MO).

2.3 Transfection

For stable transfection, Jurkat cells (1×10^7) were washed with PBS supplemented with 0.1 M $MgCl_2$ and incubated with 25–50 μg plasmid on ice for 10 min and then electroporated using an ECM 600 electroporator (BTX Inc. Holliston, MA) at 1.2 kV and 25 μF. After pulsing, the cells were incubated in T75 culture flasks for 48 h at 37°C and plated in 96-well plates with medium containing 2.2 mg/ml geneticin (Gibco BRL, Rockville, MD). 293T cells were transiently transfected with 1 μg plasmid using LipofectAMINE$_{TM}$ Reagent (Gibco BRL, Rockville, MD) according to the manufacturer's instructions.

2.4 RT-PCR analysis and RPA assay

Total cellular RNA was extracted from the activated cells as indicated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), and cDNA was obtained using murine Moloney leukaemia virus reverse transcriptase (MMTV-RT, Gibco BRL) as previously described [21]. The cDNA was amplified using the Takara PCR amplification kit (Takara Biotechnology, Shiga, Japan), and PCRs were carried out in a PerkinElmer thermal cycler. Sequences of primer pairs specific for IL-2, β-actin, and nur77 have been previously described. These primers were purchased from Bioneer (Daejeon, Korea), and their sequences were determined in previous experiments [21]. RPA was performed using the RiboQuant multi-probe RNase protection assay system (PharMingen, San Diego, CA) following the manufacturer's instructions.

2.5 Apoptosis analyses

To analyse DNA fragmentation, cells (3×10^6) were lysed in 2× lysis buffer (200 mM HEPES, pH 7.5, 2% Triton X-100, 400 mM NaCl, 20 mM EDTA) and incubated with RNase at 37°C for 1 h. DNA was extracted with phenol, precipitated with 5 M ammonium acetate and absolute ethanol, and analysed by electrophoresis in 2% agarose.

2.6 Immunoprecipitation and western blot analysis

After incubation of 3×10^7 hCD8-FasC transfectants with the cross-linked OKT8 and UCHT4 antibodies (1 μg/1×10^6 cells) at 37°C, cells were lysed in lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 mM Na_3VO_4, 200 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 1% NP-40). Tyrosine-phosphorylated proteins were immunoprecipitated using anti-p-Tyr antibody coupled to agarose beads and washed in 1:5-diluted lysis buffer. Immunoprecipitates or total cell lysates were fractionated by SDS-PAGE and transferred to Immunobilon-P (Millipore, Bedford, MA) membranes. Membranes were incubated with primary antibodies, washed, incubated with horseradish peroxidase-conjugated secondary antibodies, and developed with ECL (Amersham, Uppsala, Sweden).

3 Results

3.1 hCD8-FasC chimeric receptor-mediated stimulation transduces activation signals but not cell death signals in T cells

To investigate the molecular nature of Fas-mediated non-apoptotic signals, two fusion constructs that express hCD8-Fas chimeric receptors were generated. One of these constructs expresses a fusion of the extracellular domain of human CD8 and the transmembrane (TM) and cytosolic domains of Fas (hCD8-FasTC), and the other expresses a fusion of the CD8 extracellular and transmembrane domains and the Fas cytosolic domain (hCD8-FasC) (Supplementary Fig. 1A). After transfection of 293T cells with hCD8-FasTC or hCD8-FasC, surface expression of each chimeric receptor was confirmed by FACS analysis (data not shown). The cells were incubated with either two immobilised anti-CD8 antibodies (OKT8 and UCHT4) or an anti-Fas antibody for 12 h or 24 h, and the levels of apoptosis were assessed by propidium iodide (PI) assays. OKT8 and UCHT4 stimulation resulted in cell death in the hCD8-FasTC transfectants but not in the hCD8-FasC transfectants (Fig. 1a). We next generated stable Jurkat transfectants expressing the hCD8-FasC chimeric receptor

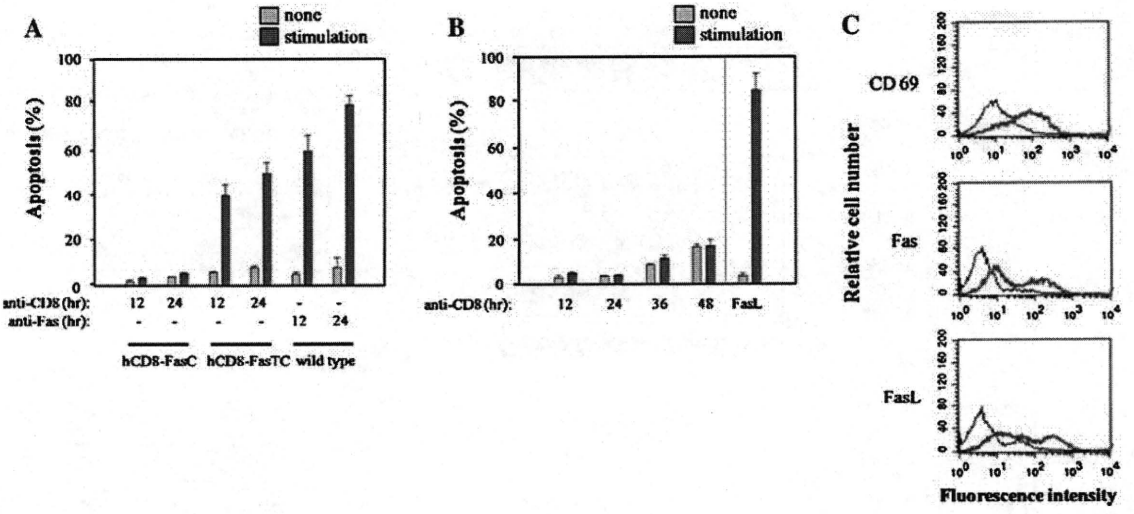

Fig. 1 Cell death was not induced by stimulation of hCD8-FasC chimeric receptor. **a** 293T cells transiently transfected with the hCD8-FasC DNA construct were stimulated with immobilised OKT8 and UCHT4 antibodies (1 μg/10^6 cells) for 12 or 24 h, and cell death was analysed. As a control, cells were stimulated with immobilised anti-Fas antibodies. **b** Stable Jurkat T cell transfectants expressing the hCD8-FasC chimeric receptor were stimulated for the indicated periods with immobilised OKT8 and UCHT4 or with NIH3T3 cells stably expressing FasL, and cell death was analysed by PI exclusion. **c** hCD8-FasC Jurkat transfectants were stimulated for 8 h with immobilised OKT8 and UCHT4 antibodies (*red lines*). Surface expression levels of CD69, Fas and FasL were analysed by flow cytometry

and confirmed the surface expression of this receptor by FACS analysis (Supplementary Fig. 1B). The hCD8-FasC Jurkat transfectants were consistently killed by FasL stably expressed on the surface of NIH3T3 cells but not by UCHT4 and OKT8 (Fig. 1b). To examine whether expression of T cell activation markers was induced in hCD8-FasC Jurkat transfectants upon stimulation with OKT8 and UCHT4, the expression levels of CD69, Fas, and FasL in anti-CD8-stimulated hCD8-FasC Jurkat cells were analysed by FACS (Fig. 1c). Activation through the hCD8-FasC chimeric receptor significantly increased the cell surface expression of CD69, Fas, and FasL. The apoptotic signal appears to be dependent on the multimerization of the chimeric receptor. Thus, apoptosis was not triggered can be the evidence which the trimeric complex was not formed in hCD8-fasC system. Or, it may not enough for inducing apoptosis for the intensity of the signal through hCD8-FasC is weaker than the wildtype trimeric complex. These results suggest that signalling through the cytoplasmic domain of Fas transduces activation signals upon stimulation, and the transmembrane domain of Fas is important for formation of the death-inducing structural conformation.

3.2 hCD8-FasC-mediated signalling activates multiple intracellular proteins related to T cell activation

To examine whether the tyrosine phosphorylation of various intracellular signalling proteins, a hallmark of T cell activation, can be induced by hCD8-FasC stimulation, hCD8-FasC transfectants were activated using UCHT4 and OKT8 antibodies. Various proteins with molecular weights of approximately 230, 210, 155, 80, 67, 64, 45, 40, 28, and 25 kDa were rapidly tyrosine phosphorylated (Supplementary Fig. 2). The resultant phosphorylated proteins were immunoprecipitated with an anti-pTyr antibody and analysed by western blotting with corresponding mAbs. Upon stimulation of hCD8-FasC, TCR-proximal proteins (Fyn, ZAP-70, Vav and PLC-γ) and MAP kinases (Erk1/2, JNK and p38) became tyrosine-phosphorylated (Fig. 2a). Surprisingly, Daxx, which is essential for Fas-mediated apoptosis, was not tyrosine-phosphorylated after hCD8-FasC-mediated activation. We next examined the kinetics of MAP kinase activation. hCD8-FasC-mediated stimulation induced prolonged phosphorylation of Raf-1, Erk1/2 and JNK, whereas phosphorylation of p38 was induced rapidly and then gradually decreased (Fig. 2b). Because PKC activation is involved in the activation of c-Raf, MEK 1/2 and Erk MAP kinase [20], we examined whether the activation of c-Raf, Erk, JNK and p38 MAP kinases through hCD8-FasC was affected by the PKC-specific inhibitor BIM-I (Fig. 2c). hCD8-FasC transfectants were pre-treated with BIM-I and then stimulated with UCHT4 and OKT8 for different periods. hCD8-FasC stimulation-induced phosphorylation of Raf-1, Erk1/2, JNK and p38 MAP kinases was dramatically suppressed by BIM-I, suggesting that activation of Erk, JNK, p38 MAP kinases following stimulation of hCD8-FasC occurs in part through a PKC-mediated signalling pathway. Because previous reports have indicated that Fas-induced JNK and p38

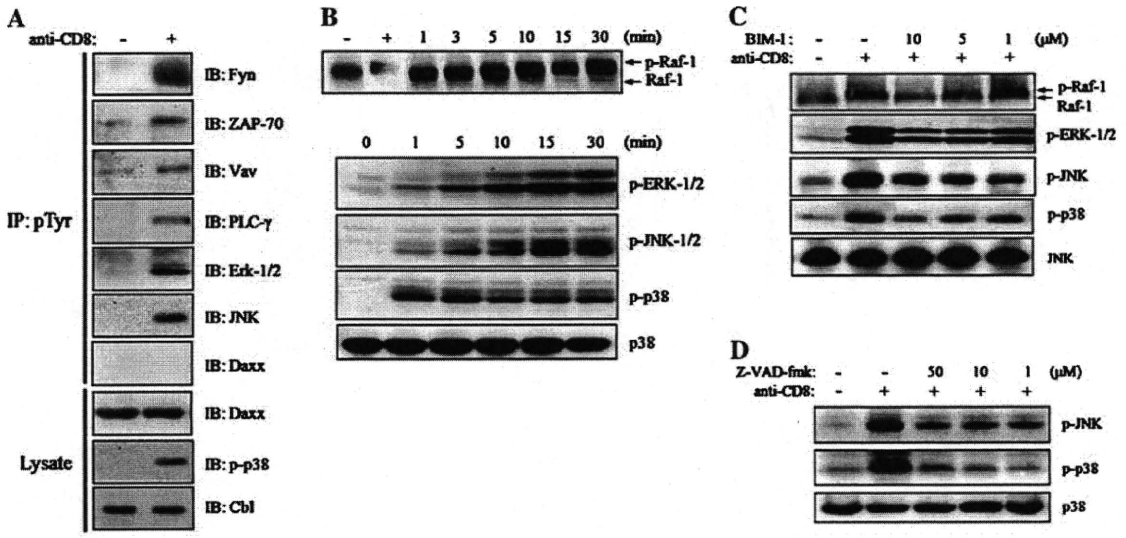

Fig. 2 hCD8-FasC-mediated signalling activates multiple intracellular proteins involved in T cell activation. **a** hCD8-FasC Jurkat transfectants were stimulated for 10 min with or without cross-linked OKT8 and UCHT4 antibodies. Cell lysates were immunoprecipitated with anti-pTyr Ab-conjugated agarose beads and immunoblotted with the indicated antibodies. **b** hCD8-FasC Jurkat transfectants were stimulated for the indicated periods with cross-linked OKT8 and UCHT4 antibodies, and cell lysates were immunoblotted with antibodies specific to phospho-Erk1/2, phospho-JNK or phospho-p38. hCD8-FasC Jurkat transfectants were treated for 1 h with or without BIM-I (1, 5, or 10 μM) (**c**) or Z-VAD-fmk (1, 10, or 50 μM) (**d**) and stimulated with OKT8 and UCHT4 antibodies for 10 min. Cell lysates were immunoblotted with antibodies specific to phospho-Erk1/2, phospho-JNK or phospho-p38

activities depend upon the activation of caspases in T lymphocytes [8, 21], we also tested whether hCD8-FasC-mediated phosphorylation of JNK and p38 MAP kinases could be blocked by caspase inhibitors (Fig. 2d). Pre-treatment of hCD8-FasC transfectants with a pan-caspase inhibitor (Z-VAD-fmk) significantly suppressed phosphorylation of JNK and p38 MAP kinases in a dose-dependent manner, suggesting that JNK and p38 MAP kinase activation through hCD8-FasC is partially dependent on the activity of caspase-8, caspase-1 and caspase-3. These results imply that intracellular signals initiated by the cytoplasmic domain of Fas activate many mediators of T cell activation, such as Fyn, ZAP-70, Vav, PLC-γ and MAP kinases, but that these signals do not activate the pro-apoptotic protein Daxx, which is essential for Fas-mediated apoptosis. Additionally, we conclude that caspase and PKC activation are important for the activation of ERK and JNK/p38 MAP kinases downstream of hCD8-FasC.

3.3 NF-κB activation downstream of CD8-Fas chimeric receptor inhibits Fas-mediated apoptosis

Previous reports have implicated the NF-κB pathway in Fas-mediated cell survival; however, these findings remain controversial [15]. To determine whether hCD8-FasC-mediated activation signalling induces the activation of NF-κB, hCD8-FasC transfectants were stimulated with UCHT4 and OKT8, lysed, and immunoblotted with anti-phospho-IκB-α antibody. Activation of the T cells through hCD8-FasC immediately induced phosphorylation of IκB-α (Fig. 3a), and this hCD8-FasC-mediated NF-κB activation was dependent on caspase activity, but not on the canonical PKC-mediated NF-κB activation pathway (Fig. 3b). We next performed an RPA assay to identify anti-apoptotic proteins induced by hCD8-FasC-mediated activation (Fig. 3c). Stimulation of hCD8-FasC induced a significant increase in expression of TRAF1, but not TRAF2, and this increase in TRAF1 expression was completely inhibited by the NF-κB inhibitor sodium salicylate (Na-Sal).

To test whether NF-κB activation induced by hCD8-FasC is critical for preventing the activation of apoptotic signals in hCD8-FasC transfectants, the cells were pre-treated with Na-Sal and stimulated with immobilised UCHT4 and OKT8. Inhibition of NF-κB activation significantly increased hCD8-FasC-mediated cell death (Fig. 3d, upper panel), and cell death was accompanied by increased cleavage of caspase-3 (Fig. 3d, lower panel). We next examined whether hCD8-FasC-mediated NF-κB activation protects hCD8-FasC transfectants from anti-Fas-mediated apoptosis. The cells were pre-stimulated with immobilised UCHT4 and OKT8, incubated with NIH3T3 cells stably expressing FasL, and then analysed for cell death. Interestingly, cells stimulated through hCD8-FasC were resistant to FasL-induced apoptosis (Fig. 3e). These results suggest that hCD8-FasC-mediated activation of atypical NF-κB pathways induces the expression of TRAF1 and potent inhibitors of Fas-mediated apoptosis

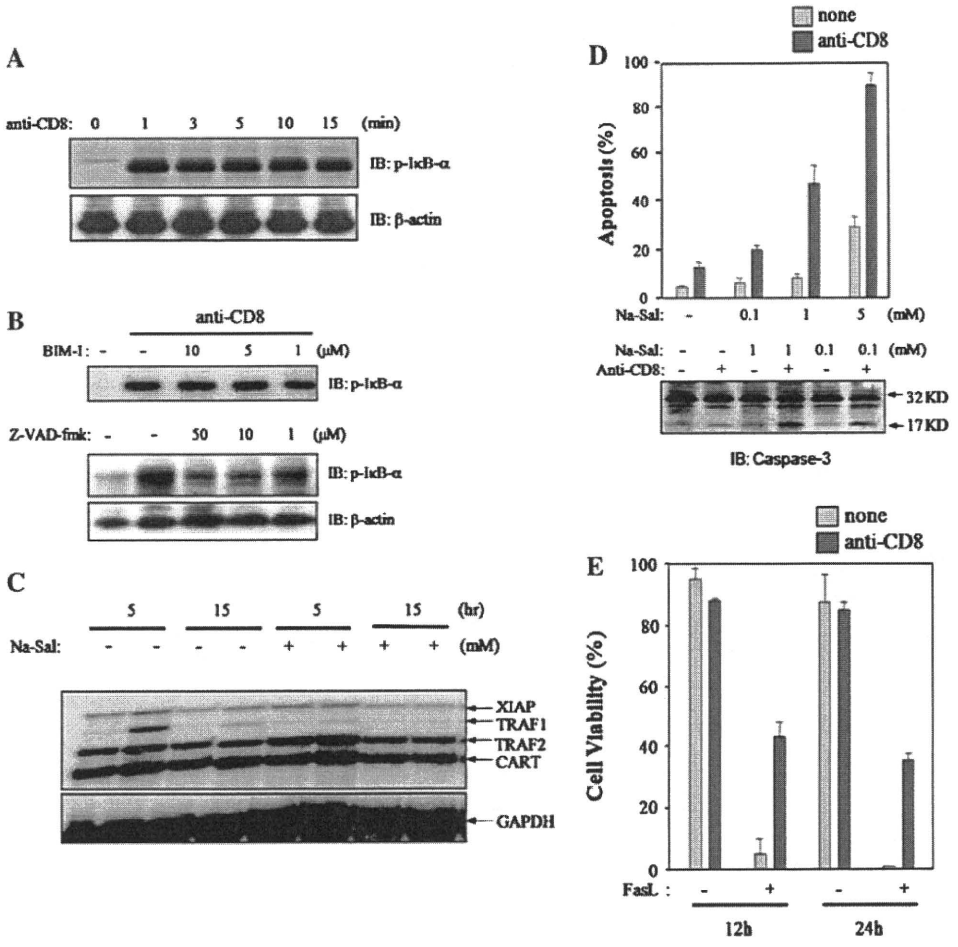

Fig. 3 hCD8-FasC-mediated NF-κB activation inhibits Fas-mediated apoptosis. **a** hCD8-FasC Jurkat transfectants were stimulated with cross-linked OKT8 and UCHT4 antibodies for the indicated periods, and cell lysates were immunoblotted with anti-phospho-IκB-α antibodies. **b** hCD8-FasC Jurkat transfectants were treated for 1 h with or without various concentrations of BIM-I (1, 5, or 10 μM) or Z-VAD-fmk (1, 10, or 50 μM) and stimulated with cross-linked OKT8 and UCHT4 antibodies for 10 min. NF-κB activation was detected by anti-phospho-IκB-α antibodies. **c** hCD8-FasC Jurkat transfectants were stimulated for 5 or 15 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 5 mM sodium salicylate. Induction of mRNAs encoding anti-apoptotic proteins was analysed by RPA. **d** hCD8-FasC Jurkat transfectants were stimulated for 12 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of sodium salicylate. Cell death was analysed by PI staining. The cleavage of caspase-3 was analysed by immunoblotting with anti-caspase-3 antibodies. **e** After hCD8-FasC Jurkat transfectants were stimulated for 12 h or 24 h with or without immobilised OKT8 and UCHT4 antibodies, cells were incubated with NIH3T3-FasL for 90 min. Cell death was analysed by PI staining

and plays a critical role in protecting cells from apoptosis induction.

3.4 Elevated PKC activation or intracellular Ca^{2+} convert hCD8-FasC-mediated activation signals into death signals

We next investigated whether hCD8-FasC-mediated T cell activation in combination with strong T cell signals such as elevated PKC activation or Ca^{2+} influx results in cell death. Treatment with PMA or IM induced a significant increase in cell death in hCD8-FasC transfectants stimulated through hCD8-FasC, as measured by DNA fragmentation (Fig. 4a). Induction of apoptosis upon stimulation through hCD8-FasC in the presence of PMA or IM was significantly blocked by cyclosporine A (CsA) or BIM-I, respectively, suggesting that the low levels of both PKC activation and Ca^{2+} influx induced by hCD8-FasC stimulation are required for hCD8-FasC-mediated apoptosis. Similar results were obtained in 293T cells transiently expressing hCD8-FasC (Fig. 4b). To confirm the effect of PKC activation or Ca^{2+} influx on Fas-induced cell death, we stimulated primary mouse T cells with two immobilised anti-Fas antibodies, which are known to induce apoptosis slowly, in the absence or presence of PMA or IM. Treatment with PMA or IM significantly enhanced Fas-mediated cell death in primary T cells (Fig. 4c). It has been demonstrated that caspase activation is required for induction of Fas-mediated apoptosis in Jurkat T

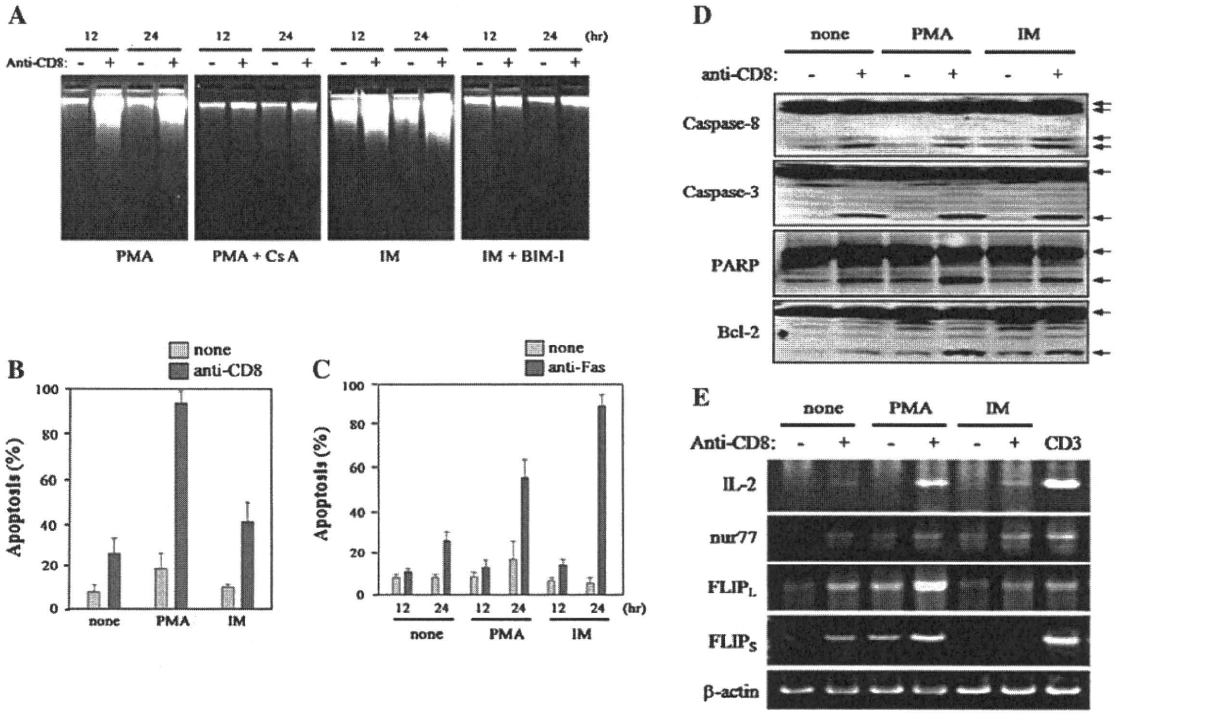

Fig. 4 PMA or ionomycin convert hCD8-FasC-mediated activation signals to death signals. **a** hCD8-FasC Jurkat transfectants were stimulated for 12 h or 24 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA, 1 ng/ml PMA and 10 μg/ml cyclosporine A, 50 nM IM, or 50 nM IM and 1 μM BIM-I. Cell death was analysed by DNA fragmentation assay. **b** 293T cells were transiently transfected with hCD8-FasC DNA and stimulated with cross-linked OKT8 and UCHT4 antibodies in the absence or presence of PMA or IM. Cell death was analysed by PI exclusion. **c** Peripheral T cells were stimulated for 12 or 24 h with two immobilised agonistic anti-Fas antibodies, ZB4 and G254-274, in the absence or presence of 5 ng/ml PMA or 100 nM IM. Cell death was analysed by PI exclusion. **d** hCD8-FasC Jurkat transfectants were stimulated for 1 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA or 50 nM IM, and cell lysates were immunoblotted with the indicated antibodies. **e** hCD8-FasC Jurkat transfectants were stimulated for 5 h with immobilised OKT8 and UCHT4 antibodies in the absence or presence of 1 ng/ml PMA or 50 nM IM. RNA was extracted from the cells, and RT-PCR analysis was performed using primers specific for IL-2, Nur77, $FLIP_L$ and $FLIP_S$. As a positive control, cells were stimulated with immobilised OKT3 antibodies in the presence of 1 ng/ml PMA or 25 ng PMA and 200 nM IM

cells [22]. While caspase-3 cleavage and activation was substantially enhanced by hCD8-FasC stimulation and even further increased by the addition of PMA or IM, proteolytic cleavage of caspase substrates such as PARP and Bcl-2 was significantly induced by hCD8-FasC stimulation in combination with PMA, but not in combination with IM. In contrast, efficient cleavage of caspase-8 was detected following hCD8-FasC-mediated activation, and additional stimulation with PMA or IM did not enhance caspase-8 cleavage (Fig. 4d).

Stimulation of the TCR complex has been reported to induce sensitivity to cell death by increasing the expression of Fas, FasL, IL-2, and Nur77 in T cell hybridomas [23]. To determine whether hCD8-FasC-mediated apoptosis induction in the presence of PMA or IM results in increased IL-2 and Nur77 expression, hCD8-FasC transfectants were stimulated with immobilised UCHT4 and OKT8 in combination with PMA or IM, and expression of IL-2, Nur77, $FLIP_L$ and $FLIP_S$ was analysed (Fig. 4e). Although hCD8-FasC stimulation resulted in only a slight induction of Nur77 expression, both PMA and IM significantly increased Nur77 mRNA expression levels. The levels of Nur77 mRNAs induced by hCD8-FasC stimulation in combination with PMA or IM were comparable to those observed upon TCR stimulation. Stimulation of hCD8-FasC induced a substantial increase in IL-2, $FLIP_L$ and $FLIP_S$ expression that was further enhanced only by PMA; IM treatment resulted in a significant abrogation of hCD8-FasC-mediated $FLIP_S$ induction. In contrast, hCD8-FasC stimulation in the presence of either PMA or IM effectively induced cell death. The level of IL-2 induction by hCD8-FasC was not increased by IM, suggesting that the signalling pathway leading to cell death downstream of TCR stimulation may be similar to that downstream of hCD8-FasC and PKC activation but different from that downstream of hCD8-FasC and increased Ca^{2+} influx. Taken together, these results demonstrate that enhanced PKC activation or an increase in intracellular Ca^{2+} influx

can convert T cell activation signals transmitted through the cytoplasmic domain of Fas into death signals. Changes in PKC activation or intracellular Ca^{2+} influx generate distinct intracellular signalling contexts that affect cell fate after Fas stimulation by altering expression of $FLIP_L$ and $FLIP_S$, activation of caspases and subsequent cleavage of their substrates.

4 Discussion

Death receptors have been suggested to carry out several non-apoptotic functions, such as the induction of cellular activation, proliferation, differentiation, or migration, but the nature of the intracellular signalling pathways involved in these non-apoptotic functions is poorly understood. In addition to identifying the complexes formed upon death receptor stimulation, determining the exact stoichiometry of the signalling molecules involved may shed light on the molecular mechanisms driving life versus death decisions in T cells. It has been shown that Fas makes trimeric complex upon activation by ligands or antibodies. In the previous reports the proliferation of human T lymphocytes and the maturation of dendritic cells were also promoted by ligand- or agonistic mAb-mediated Fas stimulation. This process was delayed in $Fas^{lpr/lpr}$ mutant mice and deletion of Fas in T cells causes lymphopenia [15, 16]. These results suggest that stimulation-induced formation of trimeric Fas receptor can induce cell death as well as cell survival, and quantitatively or qualitatively differential signals through Fas receptor may determine the cell fate to death or survival. In this study, we generated a cell line system to differentiate the Fas-mediated nonapoptotic signalling pathway from the death-inducing pathway. Stimulation of an hCD8-FasC chimeric receptor, which contains the cytoplasmic domain of Fas, may induce T cell proliferative signals by activating key TCR-proximal proteins, such as Fyn, Zap-70, Vav, PLC-γ, Raf-1/ERK and p38/JNK MAP kinases, and increasing the expression of CD69, Fas, and Fas ligand. In contrast to the Fas-mediated death signalling pathway, hCD8-FasC-mediated signalling did not activate Daxx/Ask-1, which is an essential factor in cell death. We also found that hCD8-FasC-mediated activation of Raf, ERK and JNK/p38 MAP kinases is dependent on PKC and caspase activation. Importantly, the T cell activation signals downstream of hCD8-FasC-induced NF-κB activation in a caspase-dependent but not PKC-dependent manner, suggesting that these signals are mediated by an atypical NF-κB activation pathway. The low levels of caspase-8 and caspase-3 activation and subsequent NF-κB activation induced the expression of low levels of anti-apoptotic proteins such as TRAF1, Nur77, $FLIP_L$ and $FLIP_S$, which are important for maintaining hCD8-FasC-mediated signals as activation signals, but not for cell death signal.

In contrast, others have reported that the transmembrane and cytoplasmic domains of Fas in other chimeric receptors, such as CD40-Fas, TNFR1-Fas, CD44-Fas, mainly induce cell death upon activation by their cognate antibodies [24]. In accordance with these results, another chimeric receptor generated in our study, hCD8-FasTC, containing both the transmembrane and the cytoplasmic domain of Fas, was found to induce cell death similarly to the above death-inducing chimeric receptors. Together, these results suggest that the discrete mechanisms of Fas receptor stimulation by its cognate ligand and the presence of the transmembrane domain of Fas receptor are important in forming the unique conformation required to transmit the signal for cell death.

Surprisingly, activation signals downstream of hCD8-FasC or Fas receptor were converted into death signals in the presence of elevated PKC activation or Ca^{2+} influx; these death signals were reflected in increased expression of Nur77 and cleavage of caspase-3 and its substrates. The potential of PMA or IM to convert hCD8-FasC-mediated activation into a cell death signal was completely inhibited by CsA or a PKC inhibitor, respectively, demonstrating that balanced and low levels of both PKC activation and Ca^{2+} influx are required for hCD8-FasC-induced T cell activation, and the enhancement of either of these two signals can alter the intracellular activation status of T cells, resulting in cell death.

The essential roles of caspase-8, NF-κB activation and FLIP expression in the modulation of Fas-mediated cell death have been demonstrated in several studies and are widely accepted, but the function of these proteins in the activation of non-apoptotic signalling pathways is still controversial [3, 4]. Consistent with previous findings, our study demonstrated that low levels of cleavage and activation of caspase-8, caspase-3 and caspase-3 substrates were induced by hCD8-FasC-mediated activation signals, and treatment with PMA but not IM enhanced this caspase cleavage. Low levels of IL-2, Nur77, $FLIP_L$ and $FLIP_S$ expression were induced by stimulation of hCD8-FasC. PMA treatment substantially enhanced the expression of these proteins, leading to cell death, but IM treatment did not affect IL-2 expression and significantly inhibited induction of $FLIP_L$ and $FLIP_S$ expression. These results suggest that at least two independent signalling pathways, a PKC-mediated pathway and a Ca^{2+} influx-dependent pathway, may be involved in the conversion of Fas-mediated activation signals to death signals, and $FLIP_L$ and $FLIP_S$ may play different roles in driving cells toward apoptosis or survival. Recently, two N-terminal cleavage products of cFLIP, p43-FLIP and p22-FLIP, have been reported to play an important role in NF-κB activation. We are currently analysing the function of these two proteins in the modulation of Fas-mediated activation and apoptosis in our hCD8-FasC transfectants.

Several studies demonstrated that T cell proliferation induced by suboptimal anti-CD3 stimulation is enhanced when Fas is triggered [15]. Also, deletion of CD95 in T cells causes lymphopenia in mice, suggesting that CD95 expression by T cell is required for the survival, proliferation and activation. FADD, caspase-8, and c-FILP are known to link Fas to nonapoptotic pathways. The non apoptotic outcomes may result in response to particular circumstances as inhibition at the receptor level, inappropriate concentrations of caspase-8/caspase-10 or of downstream proapoptotic proteins such as Bax, upregulation of protective molecules, or activation of protective pathways. These studies could tell that the differences of specific microenvironments might decide the fate of the Fas signal rather than the indispensability of transmembrane domain or formation of trimeric structure of Fas.

Taken together, our findings suggest that Fas-mediated signals may be capable of inducing both cell death and activation, that the stimulation of Fas receptor in distinct intercellular contexts results in the formation of a discrete signalling conformation, and that the ensuing quantitative and qualitative differences in the intracellular signalosome are critical in determining the outcome of Fas-mediated signalling. Additionally, the differences of specific microenvironment around T cells expressing Fas, the expression level, localisation, extent of activation and modification of initiator and executor caspases and their substrates and adaptor proteins may be also essential in determining the fate of T cells after interaction of Fas and FasL rather than formation of trimeric complex of Fas or indispensability of its transmembrane domain. From clinical point of view, our results may lead to better understanding of the pathogenesis of immunological disease such as autoimmune lymphoproliferative syndrome (ALPS) to the development of measures to manipulate Fas-mediated signal in the patients [25].

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Patient Report

Prominent eosinophilia but less eosinophil activation in a patient with Omenn syndrome

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Key words cytokines, eosinophils, immunodeficiency, Omenn syndrome.

Abbreviations: ECP, eosinophil cationic protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; HES, hypereosinophilic syndrome; IFN, interferon; IL, interleukin; OS, Omenn syndrome; PBMC, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; RAG, recombination activating genes; SCID, severe combined immunodeficiency; Th, T helper; TNF, tumor necrosis factor.

Omenn syndrome (OS) is a form of severe combined immunodeficiency (SCID), characterized by the occurrence of diffuse erythrodermia, hepatosplenomegaly, generalized lymphadenopathy, eosinophilia and highly elevated serum immunoglobulin (Ig) E level,[1] together with activated, autoreactive T lymphocyte infiltration of various organs. Most of these findings are observed soon after birth or during early infancy. Unless treated by allogeneic hematopoetic stem cell transplantation (SCT), the prognosis of OS is fatal.

Missense mutation in the recombination activating genes (*RAG)1* or *RAG2* and partial recombinase activity may result in OS.[1] These genes are also involved in the creation of various T cell repertoires. Total defect of enzymatic activity function causes SCID without producing mature lymphoid cells. Partial defect with leaky recombinase activity may lead to variable clinical features reflecting the actual degree of the enzyme defect. Indeed, our case showed an oligoclonal expansion of T lymphocytes with multiple second-site mutations leading to a typical OS with *RAG1*-deficient SCID.[2] In this report, we analyzed eosinophilia, eosinophil activity, and production of several cytokines in this syndrome.

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Case Report

We report the case of a 3-month-old boy presenting with generalized exudative erythrodermia, hepatosplenomegaly, draining otitis externa, and alopecia, with a history of *Pseudomonas aeruginosa* bacteremia requiring systemic antibiotic treatment. He was the second child of consanguineous parents with an older healthy daughter. Laboratory evaluation showed mild anemia, leukocytosis (104.0×10^9/L) with marked lymphocytosis (53.0×10^9/L) and eosinophilia (21.8×10^9/L), hypoalbuminemia (2.4 g/dL), and low serum Ig level (IgG 148 mg/dL; IgA < 1 mg/dL; IgM 2 mg/dL; IgE < 2 U/mL). Some eosinophils in both peripheral blood and bone marrow showed nuclear hypersegmentation such as three- or four-lobed nuclei (data not shown). Skin biopsy specimens showed lymphocytic invasion in the dermis, consistent with the graft-versus-host disease (GvHD) (data not shown). Immunophenotypic analysis showed the following percentages of lymphocyte subpopulations: cluster of differentiation (CD)3^+ 91.8%; $CD4^+$ 44.4%; $CD8^+$ 49.9%; $CD16^+$ 3.5%; $CD20^+$ < 0.1%; T cell receptor (TCR)$\gamma\delta^+$ 0.3%; human leukocyte antigen (HLA)-$DR^+/CD3^+$ 95.5%; $CD45RO^+/CD4^+$ 99.8%; and $CD45RO^+/CD8^+$ 97.0%. Analysis of T cell receptor Vβ repertoire in the periphery showed extremely restricted heterogeneity. There was no evidence of materno-fetal transplantation (MFT) from the results of HLA typing and fluorescent *in situ* hybridization analysis. Finally, mutation in *RAG1* was detected by DNA sequencing analysis.[2] Thus, a definitive diagnosis of OS was made, and immunosuppressive therapy with prednisolone (1.5 mg/kg/day) was started with the addition of cyclosporin