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bjh research paper

Outcome of unrelated umbilical cord blood transplantation in 88 patients with primary immunodeficiency in Japan

Tomohiro Morio,[1] Yoshiko Atsuta,[2] Daisuke Tomizawa,[1] Tokiko Nagamura-Inoue,[3] Koji Kato,[4] Tadashi Ariga,[5] Keisei Kawa,[6] Kazutoshi Koike,[7] Hisamichi Tauchi,[8] Michiko Kajiwara,[9] Toshiro Hara,[10] and Shunichi Kato[11] for the Japanese Cord Blood Bank Network

[1]Department of Paediatrics and Developmental Biology, Tokyo Medical and Dental University Graduate School of Medical and Dental Sciences, Tokyo, [2]Department of Haematopoietic Stem Cell Transplantation Data Management/Biostatistics, Nagoya University School of Medicine, Nagoya, [3]Department of Cell Processing and Transfusion, Research Hospital, Institute of Medical Science, University of Tokyo and Tokyo Cord Blood Bank, Tokyo, [4]Department of Paediatrics, Japanese Red Cross Nagoya First Hospital, Nagoya, [5]Department of Paediatrics, Hokkaido University Graduate School of Medicine, Sapporo, [6]Department of Haematology/Oncology, Osaka Medical Centre and Research Institute for Maternal and Child Health, Osaka, [7]Department of Paediatric Haematology, Ibaraki Children's Hospital, Ibaraki, [8]Department of Paediatrics, Ehime University School of Medicine, Ehime, [9]Department of Blood Transfusion, Tokyo Medical and Dental University Medical Hospital, Tokyo, [10]Department of Paediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, and [11]Department of Cell Transplantation and Regenerative Medicine, Tokai University School of Medicine, Isehara, Japan

Received 26 February 2011; accepted for publication 18 April 2011

Correspondence: Tomohiro Morio, MD, PhD, Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University Graduate School of Medical and Dental Sciences, 1-5-45 Yushima Bunkyo-ku, Tokyo 113-8519, Japan.
E-mail: tmorio.ped@tmd.ac.jp

Summary

We report the results of umbilical cord blood transplantation (UCBT) performed in 88 patients with primary immunodeficiency (PID) between 1998 and 2008 in Japan; severe combined immunodeficiency (SCID, $n = 40$), Wiskott–Aldrich syndrome (WAS, $n = 23$), chronic granulomatous disease ($n = 7$), severe congenital neutropaenia (SCN, $n = 5$) and other immunodeficiencies ($n = 13$). Five-year overall survival (5-year OS) for all patients was 69% [95% confidence interval (CI), 57–78%], and was 71% and 82% for SCID and WAS, respectively. The main cause of death before day 100 was infection (17/19), while that after day 100 was graft-versus-host disease (GVHD) (5/7). Using multivariate analyses, pre-transplant infection, no conditioning, ≥2 human leucocyte antigen (HLA) mismatches or diagnosis other than SCID, SCN or WAS were all associated with poor prognosis. Reduced-intensity conditioning was associated with decreased overall mortality compared with myeloablative therapy. The cumulative incidence of grade 2–4 acute GVHD at day 100 was 28% (95% CI, 19–38%), and that of chronic GVHD at day 180 was 13% (95% CI, 7–23%). We conclude that UCBT should be considered for PID patients without an HLA-matched sibling. The control of pre-transplant infection and selection of HLA-matched donors will lead to a better outcome.

Keywords: primary immunodeficiency, severe combined immunodeficiency, Wiskott-Aldrich syndrome, cord blood transplantation, reduced-intensity conditioning.

First published online 14 May 2011
doi:10.1111/j.1365-2141.2011.08735.x

Allogeneic haematopoietic stem cell transplantation (HSCT) has been successfully used as a curative therapy for most severe forms of primary immunodeficiency (PID) (Zeidler *et al*, 2000; Antoine *et al*, 2003; Sakata *et al*, 2004; Rao *et al*, 2005; Kobayashi *et al*, 2006; Mazzolari *et al*, 2007; Dvorak & Cowan, 2008; Griffith *et al*, 2008; Cuvelier *et al*, 2009). Stem cell transplantation from a human leucocyte antigen (HLA)-identical family donor provides better prognosis than bone marrow transplantation from an unrelated donor (Antoine *et al*, 2003). Survival with this type of transplantation from a matched unrelated donor has improved significantly over the years in patients with severe combined immunodeficiency (SCID), whereas no improvement in survival has been observed with this transplantation in non-SCID patients (Antoine *et al*, 2003). The optimal stem cell source for PID patients with no HLA-identical sibling remains to be determined (Dvorak & Cowan, 2008; Griffith *et al*, 2008; Cuvelier *et al*, 2009).

Umbilical cord blood grafts from unrelated donors have been successfully used, primarily in children and subsequently in adults (Kurtzberg *et al*, 1996; Wagner *et al*, 1996; Gluckman *et al*, 1997; Rubinstein *et al*, 1998; Rocha *et al*, 2000, 2004; Laughlin *et al*, 2004). Theoretically, unrelated cord blood transplantation (UCBT) has the following distinct advantages in PID patients: (i) the cord blood product is rapidly accessible in most cases; (ii) the incidence and severity of graft-versus-host disease (GVHD) is not excessive, even in mismatched transplantation and (iii) the risk of latent viral transmission is low. The disadvantages of UCBT include slower haematopoietic/immunological reconstitution and graft failure, which have been observed with UCBT for malignant disorders, and naivety of lymphocytes to pathogens (Brown *et al*, 2008; Griffith *et al*, 2008; Szabolcs *et al*, 2008). Rapid immune reconstitution is particularly important in PID patients with ongoing infection who undergo UCBT.

The limited data available show that UCBT can be a curative measure in patients with SCID, Wiskott–Aldrich syndrome (WAS), chronic granulomatous disease (CGD) and severe congenital neutropaenia (SCN) (Knutsen & Wall, 2000; Bhattacharya *et al*, 2003, 2005; Fagioli *et al*, 2003; Knutsen *et al*, 2003; Kobayashi *et al*, 2006). Most of the available data have come from a single centre, and thus, detailed information on the outcome and problems associated with UCBT in PID patients is still lacking. In this study, we report the results of UCBT performed in 88 PID patients between 1998 and 2008 in Japan.

Methods

Collection of data

All UCBTs carried out for PIDs through the Japan Cord Blood Bank Network (JCBBN) between August 1998 and January 2008 was enrolled in this study. Eighty-eight patients with PID underwent UCBT during this period. All data were provided by JCBBN, which collects recipients' clinical information at day 100 after transplantation. Recipients' data on survival, disease status and long-term complications are renewed annually by administering follow-up questionnaires. Latest data acquisition was performed in November 2009. The present study was approved by the institutional ethical and data management committees of JCBBN.

Patients

A summary of patients enrolled in this study is shown in Table I. Forty patients had SCID (45%) and 48 (55%) had non-SCID. Patients with familial haemophagocytic syndrome were not included in this study. The median age at the time of transplantation was 10 months (range, 0–248 months).

Procedures

Cryopreserved, unrelated cord blood cells were used as the source of haematopoietic stem cells. The type of conditioning used and median cell dose infused are shown in Table I.

In most cases, HLA matching was performed by both serological and DNA typing for HLA-A, HLA-B and HLA-DRB1. In this study, HLA mismatch was defined according to serological or low-resolution molecular typing for HLA-A and HLA-B and high-resolution molecular typing for HLA-DRB1. Of the UCB donors, 29 (33%) were HLA fully compatible. Of the mismatched donors, 40 (45%) were 1-antigen mismatched, 15 (17%) were 2-antigen mismatched and four (5%) were 3-antigen mismatched (Table I). In 48 patients in whom high-resolution genotypical typing was performed for HLA-A, HLA-B and HLA-DRB1, 11 were fully matched, 13 were 1-antigen mismatched, 16 were 2-antigen mismatched, five were 3-antigen mismatched and three were 4-antigen mismatched.

Immunosuppressive prophylaxis against GVHD after UCBT consisted of ciclosporin A (CyA)- and tacrolimus-based regimens in 48 and 35 patients, respectively. Five patients were not administered any immunosuppressive drug after UCBT.

Various techniques including karyotyping, HLA typing and fluorescence *in situ* hybridization for the XY chromosome and variable number of tandem repeats were used to confirm the engraftment of donor cells.

Definitions

Neutrophil recovery was defined by an absolute neutrophil count of at least $0{\cdot}5 \times 10^9$/l for three consecutive days. Platelet recovery was defined by a count of at least 20×10^9/l, unsupported by transfusion for 7 d. Reticulocyte recovery was defined by a count of at least 20‰.

Patients without conditioning or with only anti-thymocyte globulin (ATG) were categorized as receiving no conditioning. Patients administered busulfan (BU)/cyclophosphamide (CY) ± total body irradiation (TBI) or total lymphoid irradiation

Table I. Age at the time of transplantation, type of conditioning and HLA disparity.

	Patients (*N*)	Median age at transplantation (months) (range)	Median cell dose ($\times 10^7$/kg) (range)	Second or third transplantation (*N*)	Conditioning			HLA disparity			
					No (*N*)	RIC (*N*)	MAT (*N*)	0 (*N*)	1 (*N*)	2 (*N*)	3 (*N*)
Total	88	9 (0–248)	8·60 (1·89–31·1)	8	14	31	43	29	40	15	4
SCID	40	6·5 (0–27)	11·4 (4·55–31·1)	1	12	18	10	17	15	5	3
WAS	23	14 (4–84)	6·49 (2·89–13·6)	1	0	2	21	7	10	6	0
CGD	7	63 (31–248)	6·00 (1·89–12·3)	5	1	4	2	2	4	1	0
SCN	5	10 (4–124)	5·99 (4·16–9·19)	0	0	1	4	1	4	0	0
Others	13	37 (6–194)	8·11 (3·01–19·8)	1	1	6	6	2	7	3	1

RIC, reduced-intensity conditioning; MAT, myeloablative therapy. Definition of conditioning regimens are described in *Methods* section. 'Others' include four CD40L deficiency, two common variable immunodeficiency and one of each of the following disorders: Major histocompatibility complex (MHC) class II deficiency, DiGeorge syndrome, X-linked lymphoproliferative disorder, NEMO (NF-κ-B essential modulator) deficiency, IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome, Idiopathic CD4 lymphopenia and Blau syndrome.

(TLI), BU/CY + ATG ± TLI, BU/CY + fludarabine (Flu) or CY/etoposide/high-dose cytarabine were categorized as receiving myeloablative therapies (MATs). CY dose ranged from 120 to 240 mg/kg (median, 200 mg/kg) in patients receiving MAT.

TBI < 4 Gy was classified as 'low-dose TBI'. Patients administered Flu/melphalan (L-PAM) ± low-dose TBI or TLI, Flu/BU ± TLI or Flu/CY (50–60 mg/kg) ± low-dose TBI/TLI, Flu + low-dose TBI or Flu + ATG were categorized as receiving reduced-intensity conditioning (RIC). L-PAM dose was ≤140 mg/m^2 in patients receiving RIC.

GVHD was graded according to the standard criteria (Przepiorka *et al*, 1995).

Statistical analyses

The probability of survival was estimated by the product-limit method, and the log-rank test was used for group comparisons. Cumulative incidence curves were used in a competing-risks setting to calculate the probability of neutrophil, platelet and reticulocyte recovery and that of acute and chronic GVHD. Death before recovery was the competing event for haematological recovery, and death without GVHD was the competing event for GVHD. Gray's test was used for group comparisons of cumulative incidence (Gray, 1988; Gooley *et al*, 1999). The Cox regression model was used to analyse data for the identification of prognostic factors. Factors found to be significant ($P < 0·05$) or marginally significant ($P < 0·1$) in univariate analysis were included in multivariate analysis. The variables considered were patient age at the time of transplantation, diagnosis, duration from diagnosis to transplantation, second or third transplantation, HLA disparity, presence of infection at the time of transplantation, conditioning regimen and cell dose infused. Variables with >2 categories were included in the final model using dichotomized dummy variables when at least one of the categories showed significant effect on survival. Continuous variables were dichotomized for the prognostic factor analyses. Variables were dichotomized as follows; patient age greater or <12 months at transplantation, dichotomized at a median nucleic cell dose of $<8·2 \times 10^7$/kg vs. $\geq 8·2 \times 10^7$/kg and CD34 cell dose of $<2·1 \times 10^5$/kg and $\geq 2·1 \times 10^5$/kg, shorter than or equal to or longer than 180 d for time to transplant. All *P*-values were two-sided.

Results

Engraftment

Sixty-seven patients (76%) achieved stable engraftment. The cumulative incidence of neutrophil, platelet and reticulocyte recovery at day 100 after transplantation was 77% [95% confidence interval (CI), 66–85%], 56% (95% CI, 45–65%) and 64% (95% CI, 53–73%) respectively (Fig 1A, B; data not shown). The median time for neutrophil, platelet and reticulocyte recovery was 19 d (range, 9–104 d), 40 d (range, 10–122 d) and 27 d (range, 12–98 d), respectively. The cumulative incidences of neutrophil recovery were not statistically different among the disease groups (SCID, 74%; WAS, 91% and others, 68% at day 100 after transplantation) (Fig 1C), although incidence was low in CGD patients ($N = 7$, 43%).

The time required for neutrophil recovery was similar in all disease groups, while that required for platelet recovery varied to some extent among the different disease groups. Platelet engraftment was slightly delayed in WAS patients, but the time required for engraftment in these patients was not significantly different from that required in other patients (Fig 1D).

Forty-three, 31 and 14 patients received MAT, RIC and no conditioning, respectively. No difference was observed in the incidence of neutrophil recovery between the MAT and RIC groups (84% vs. 87% at day 100). Similarly, no difference was observed in platelet recovery between these two groups (data not shown).

The cell dose infused ranged from 1·89 to $31·1 \times 10^7$/kg, with a median of $8·60 \times 10^7$/kg. No correlation was observed between the cell dose infused and engraftment.

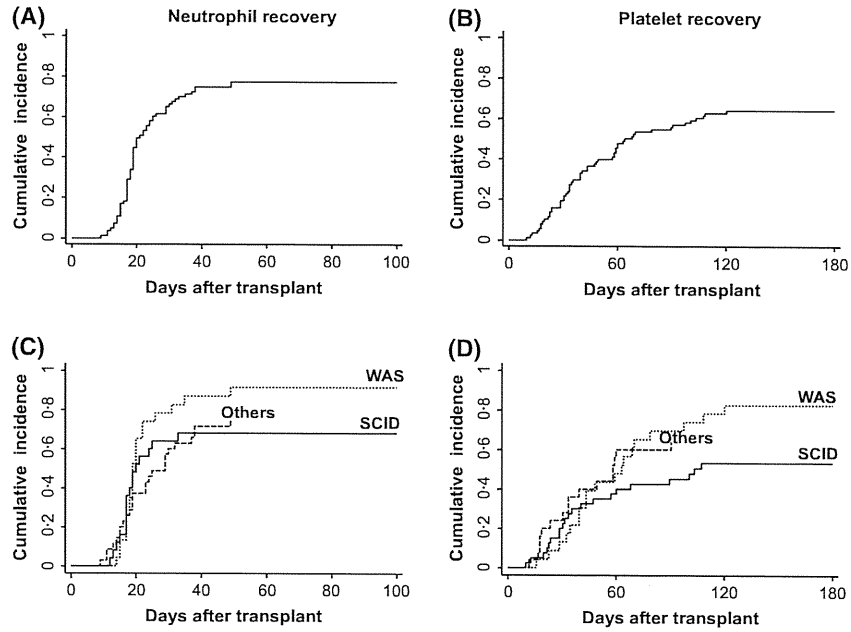

Fig 1. Cumulative incidence of neutrophil and platelet recovery after UCBT. (A) The cumulative incidence of neutrophil recovery 77% (95% CI, 66–85%). (B) The cumulative incidence of platelet recovery 56% (95% CI, 45–65%). The cumulative incidence of neutrophil (C) and platelet (D) recovery according to disease category is shown.

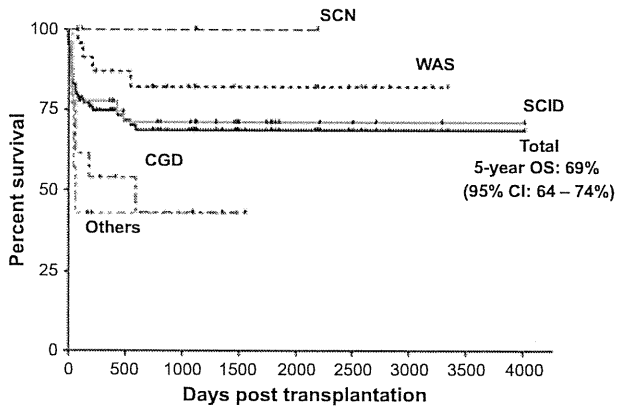

Fig 2. Kaplan–Meier estimates of overall survival after umbilical cord transplantation.

Five of 21 patients with engraftment failure received a second transplantation. Two WAS patients achieved successful engraftment in the second transplantation, while one SCID and two CGD patients did not survive the second transplantation. Only two of the remaining 16 patients who rejected the UCB graft remained alive at the latest data analysis.

Survival and causes of death

Of the 88 PID patients who underwent UCBT, 62 remained alive at the latest follow-up. Five-year OS for all patients was 69% (95% CI, 57–78%) (Fig 2), while that for SCID and WAS patients was 71% and 82%, respectively. All five SCN patients remained alive, although one patient had rejected the graft on day 79 after UCBT. Three of seven CGD patients survived UCBT; this low survival rate may be due to the fact that UCBT was selected in five patients after the first or second failed bone marrow transplantation (BMT). Seven of 14 patients categorized as 'other diseases' remained alive at the latest follow-up.

Table II summarizes the survival and causes of death after UCBT. Of the 26 patients who died, 19 had died within day 100 (17 from infection) and seven (SCID, six and congenital CD4 lymphopenia, one) had died within day 28 after UCBT.

Causes of early death (≤28 d) were cytomegalovirus (CMV) disease (three patients), *Pneumocystis* pneumonia (one patient), interstitial pneumonia (one patient), bacterial infection (one patient) and veno-occlusive disease (VOD) (one patient). All those who died of CMV disease had CMV pneumonia before transplantation.

The cause of death between days 28 and 100 in the remaining 12 patients was bacterial infection (seven had concomitant fungal infection, one also had VOD and one had CMV disease), CMV disease (two patients), fungal infection (one patient), multiple organ failure (one patient) and VOD (one patient). Four of seven CGD patients died of bacterial or fungal infection without engraftment. Although detailed data on bacterial/fungal infections at the time of transplantation were not collected, all the CGD patients were administered both antimicrobial and antifungal agents at the time of transplantation.

The causes of death after day 100 were GVHD (five patients), Epstein–Barr virus (EBV)-associated post-transplant lymphoproliferative disorder (EBV-PTLD, one patient) and

Table II. Survival and causes of death.

	Cases (*N*)	Alive (*N*)	Death (day) <28 (*N*)	<100 (*N*)	≥100 (*N*)	Infection at CBT (*N*)	Cause of death (<day 100) Bac/Fung infection (*N*)	Viral infection (*N*)	Others (*N*)	Cause of death (≥day 100) GVHD (*N*)	Others (*N*)
Total	88	62	7	19	7	18	10	7	VOD 3 MOF1	5	PTLD 1 AI 1
SCID	40	29	6	9	2	11	2	6	1 (VOD)	1	1 (AI)
WAS	23	19	0	1	3	1	1	0	0	3	0
CGD	7	3	0	4	0	5	4	0	1 (VOD)	0	0
SCN	5	5	0	0	0	0	0	0	0	0	0
Others	13	6	1	5	2	1	3	1	1 (VOD) 1 (MOF)	1	1 (PTLD)

Bac/Fung infection, bacterial and/or fungal infection. VOD, veno-occlusive disease; MOF, multiple organ failure; AI, adrenal insufficiency; PTLD, post-transplant lymphoproliferative disorder. Cause of death total does not equal the number of deceased patients because one patient died of VOD and bacterial infection.

adrenal insufficiency (one patient). None of the other patients died of infection after day 100.

GVHD

All but five patients in the present study received either CyA- or tacrolimus-based immunosuppressant prophylaxis for GVHD. The cumulative incidence of grade 2–4 acute GVHD at day 100 was 28% (95% CI, 19–38%), and that of grade 3–4 GVHD was 8% (95% CI, 4–15%) (Fig 3A, D).

The incidence of grade 2–4 GVHD was higher in patients who underwent 2- or 3-antigen-mismatched UCBT compared with those who underwent HLA-matched or HLA-1-antigen–mismatched UCBT, but it was not statistically significant ($P = 0{\cdot}071$) (Fig 3B). On the other hand, no difference was observed in the incidence of grade 3–4 GVHD between <2-antigen-mismatched and >2-antigen-mismatched transplants (Fig 3E), although grade 3–4 GVHD was not observed by high-resolution DNA typing in patients who underwent genotypically HLA-matched transplantation.

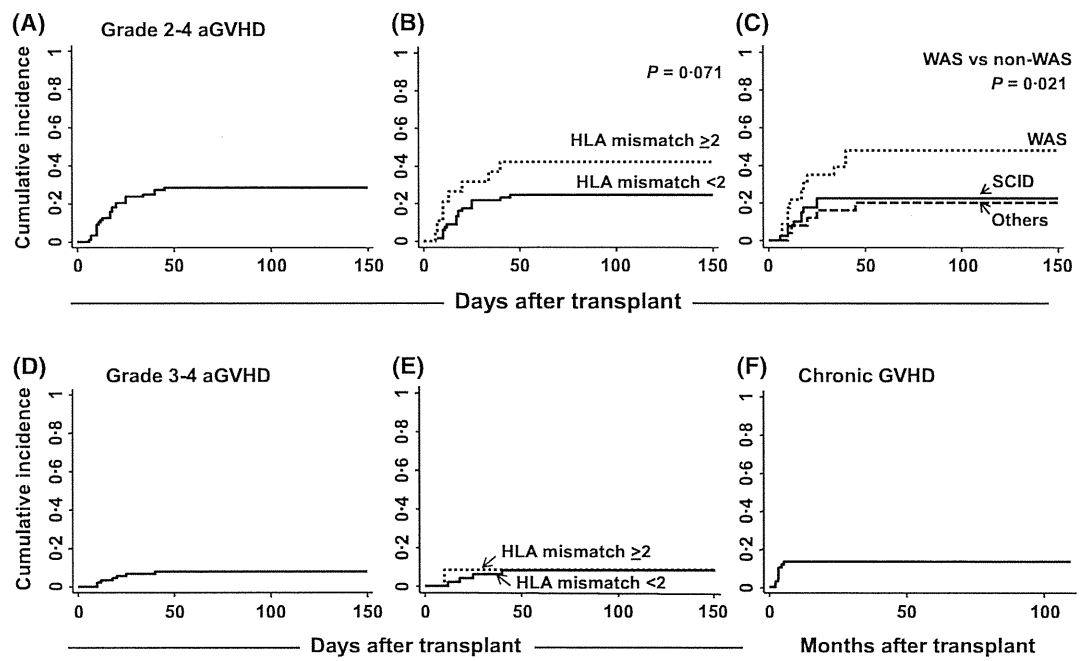

Fig 3. Cumulative probability of acute and chronic GVHD after UCBT. The cumulative incidence of grade 2–4 acute GVHD (aGVHD) at day 100 was 28% (95% CI, 19–38%) (A). The incidence was higher in transplantation mismatched for ≤2 antigens (B) and in that for WAS patients (C). The cumulative incidence of grade 3–4 acute GVHD at day 100 was 8% (95% CI, 4–15%) (D) and the incidence was not different between patients undergoing transplantation for ≥2-antigen mismatched transplant and those undergoing <2-antigen mismatched transplant (E). The cumulative incidence of chronic GVHD at day 180 was 13% (95% CI, 7–23%) (F).

When focusing on differences among the disease groups (Fig 3C), a significantly higher incidence of grade 2–4 GVHD was observed in WAS patients than in non-WAS patients, $P = 0{\cdot}021$. In addition, three of five WAS patients who developed grade 3–4 GVHD died of either GVHD (two patients) or VOD (one patient).

Chronic GVHD was observed in nine patients, and its cumulative incidence at day 180 was 13% (95% CI, 7–23%) (Fig 3F).

Infections

Twenty-eight patients (SCID, 11; WAS, eight; CGD, three and other diseases, six) developed bacterial infection after UCBT. Sixteen of the 28 patients remained alive at the time of data collection.

Fungal infection mainly caused by *Aspergillus* species was observed in eight patients (CGD, three; SCID, two; WAS, two and X-linked hyperIgM syndrome, one). Three of the eight patients died of bacterial infection, bacterial/fungal infection or GVHD.

Twenty patients (SCID, eight; WAS, four; CGD, two; SCN, two and others, four) developed CMV disease after UCBT. CMV was detected before conditioning in all eight SCID patients of which four patients died of CMV disease after transplantation. Twelve of the 20 patients remained alive at the time of analysis.

Other notable virus-related complications were respiratory syncytial virus bronchiolitis accompanied by chronic GVHD in one SCID patient and EBV-PTLD in one patient with Blau syndrome; both infections led to a fatal outcome. One WAS patient had severe haemorrhagic colitis caused by Coxsackie virus B infection, which was treated successfully by infusion of expanded CD4 T cells prepared from the infusion residual of donor cord blood (Tomizawa *et al*, 2005). Another WAS patient had persistent norovirus infection. Interstitial pneumonia not due to CMV or *Pneumocystis* was noted in three patients of which one patient had parainfluenza/rhinovirus infection, while the causative agent for infection in the remaining two patients was not identifiable.

Risk factors for overall mortality

Lastly, we analysed the factors contributing to overall survival. Using univariate analyses, the following were found to be significant contributory factors to a poor prognosis: HLA mismatch of ≥2 antigens, time to transplant >180 d, second or third transplantation, ongoing infection at the time of transplantation, no conditioning for UCBT and diagnosis other than SCID, SCN or WAS (Table III). The dose of transfused nucleated cells or CD34-positive cells did not affect the 5-year OS.

Using multivariate regression analyses, the following were found to be significant contributory factors to patient death: infection at the time of transplantation, no conditioning, HLA mismatch of >2 antigens and diagnosis other than SCID, SCN or WAS (Table IV). RIC was determined to be the favourable factor for patient survival ($P = 0{\cdot}01$) (Fig 4 and Table IV).

Table III. Univariate analyses of factors that contributed to 5-year OS.

Factors	Hazard ratio	95% CI	*P*-value
Age: ≥12 months	1·73	(0·78–3·83)	0·175
Diagnosis			
WAS and SCN	1·00		
SCID	2·34	(0·75–7·36)	0·145
Other diseases	5·39	(1·70–17·0)	0·004*
Nucleic cell dose: ≥$8{\cdot}2 \times 10^7$/kg	1·51	(0·69–3·29)	0·299
CD34 cell dose: ≥$2{\cdot}1 \times 10^5$/kg	0·86	(0·36–2·08)	0·744
HLA disparity			
6/6 matched	1·00		
5/6 matched	1·68	(0·58–4·83)	0·337
4/6 matched	3·78	(1·23–11·60)	0·020*
3/6 matched	3·24	(0·63–16·74)	0·160
4/6 or 3/6 matched	2·64	(1·20–5·83)	0·016*
Time to transplant: ≥180 d	1·89	(0·85–4·17)	0·117
Infection at transplant	6·24	(2·61–14·9)	<0·0001*
Second or third transplantation	3·37	(1·26–9·02)	0·016*
Conditioning			
MAT	1·00		
RIC	0·41	(0·13–1·23)	0·111
No conditioning	2·89	(1·21–6·93)	0·017*

*Significant contributory factors to the poor prognosis.

Discussion

This paper reports the outcome of UCBT for 88 PID patients, the largest cohort of PIDs to receive UCBT to date. The overall survival rate for PID patients undergoing UCBT was comparable to that previously reported for 46 Japanese PID patients undergoing BMT from either HLA-identical siblings or unrelated donors (Sakata *et al*, 2004), and also to that reported by the European Society of Immunodeficiency and other stem cell transplantation centres for PID patients receiving BMT from HLA-matched related donors, HLA-mismatched related donors or unrelated donors (Antoine *et al*, 2003; Rao *et al*, 2005; Dvorak & Cowan, 2008). The time for haematopoietic recovery was comparable to or better than the median recovery time observed in a large cohort of UCBT in children with haematopoietic disorders (Thomson *et al*, 2000; Michel *et al*, 2003) and in adults with leukaemia (Laughlin *et al*, 2004; Atsuta *et al*, 2009). The incidence of grade 2–4 GVHD (28%) in UCBT was lower compared with that reported in unrelated donor BMT in PID patients in Japan (47%) (Sakata *et al*, 2004), with that reported in BMT in 90 SCID patients (34%) (Neven *et al*, 2009) and with that observed in the studies of UCBT for childhood haematological malignancies (Thomson *et al*, 2000; Michel *et al*, 2003; Sawczyn *et al*, 2005). The incidence of chronic GVHD (13%) after UCBT was slightly

Table IV. Multivariate analyses of factors that contributed to 5-year OS.

Factors	HR	95% CI	*P*-value
Diagnosis			
WAS and SCN	1·00		
SCID	1·71	(0·39–7·38)	0·475
Other diseases	7·50	(2·06–27·19)	0·002*
HLA disparity			
6/6 matched	1·00		
5/6 matched	1·53	(0·50–4·66)	0·454
4/6 matched	5·64	(1·66–19·14)	0·006*
3/6 matched	1·04	(0·68–23·96)	0·124
4/6 or 3/6 matched	3·87	(1·63–9·19)	0·002*
Infection at transplant	4·61	(1·74–12·16)	0·002*
Conditioning			
MAT	1·00		
RIC	0·20	(0·06–0·69)	0·011†
No conditioning	4·87	(1·79–13·3)	0·002*

*Significant contributory factors to an unfavourable prognosis.
†Significant contributory factors to a favourable prognosis.

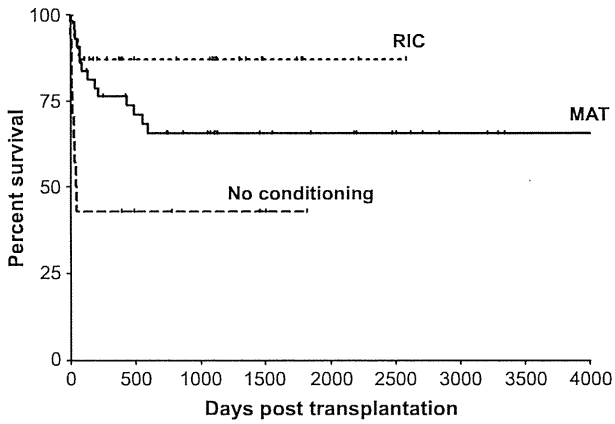

Fig 4. Kaplan–Meier estimates of overall survival after umbilical cord transplantation. Comparison of overall survival between reduced intensity conditioning (RIC), myeloablative therapy (MAT), and no conditioning is shown. For 5-year OS, MAT *versus* RIC, $P = 0{\cdot}111$, MAT *versus* no conditioning, $P = 0{\cdot}017$ in univariate analysis.

lower than that after URBMT in PID patients in Japan (20%) (Sakata *et al*, 2004), and was lower compared to that in UCBT studies for childhood leukaemia (Michel *et al*, 2003; Sawczyn *et al*, 2005). Thus, UCBT in PID patients in the present study was associated with a good survival rate, good engraftment rate, rapid haematological recovery and a lower incidence of acute and chronic GVHD.

Given that the 5-year OS for SCID patients (71%) was better than that for SCID patients receiving bone marrow from HLA-mismatched related donors in both Japan (5-year OS, 36%, Imai, Morio, Kamachi, Kumaki, Ariga, Nonoyama, Miyawaki, and Hara, unpublished observations) and Europe (5-year OS, 52%, Antoine *et al*, 2003), UCBT would be particularly beneficial for patients requiring rapid access to donor units yet lacking a matched related donor.

The present study found that several key risk factors were associated with overall mortality. First, infection was the major cause of mortality during the first 100 d after UCBT in PID patients, and the frequency was much higher than that observed in other disorders following UCBT (Rocha & Gluckman, 2006; Kurtzberg *et al*, 2008, Szabolcs *et al*, 2008). As predicted and reported in previous studies (Antoine *et al*, 2003; Cuvelier *et al*, 2009), infection at the time of transplantation was associated with poor survival ($P < 0{\cdot}0001$), suggesting that the control of pre-existing infection at the time of UCBT is critically important.

Eight of 11 SCID patients who had active infection, mainly CMV pneumonia, died before day 50, while 26 of 28 patients without infection at the time of UCBT remained alive at the time of data collection. UCBT without conditioning was selected for 12 patients, of which seven had CMV infection and one had *Pneumocystis* pneumonia at the time of transplantation. Six out of the seven patients died of CMV infection; and one patient with *Pneumocystis* pneumonia did not survive UCBT.

UCBT in WAS patients achieved a good 5-year OS, as reported in a previous study of 15 cases (Kobayashi *et al*, 2006). One of the key factors would have been the time from diagnosis to transplantation. In our WAS patients, UCBT was performed at a median age of 14 months (range, 4–84 months), when most patients were thrombocytopenic, but did not yet have uncontrolled infection or autoimmunity.

Four CGD patients died of bacterial or fungal infection without engraftment. Although these patients were not categorized as those with active infection at the time of transplantation, they required intravenous administration of antimicrobial and antifungal agents before and after transplantation.

Second, HLA disparity was a risk factor associated with overall mortality. Lower survival was observed in UCB recipients transplanted with a ≥2 antigen-mismatched graft compared with those transplanted with a <2 antigen-mismatched graft [Hazard Ratio (HR) = 3·87, $P = 0{\cdot}002$]. Although no difference was observed in 5-year OS between recipients of HLA-matched and those of HLA 1-antigen mismatched UCBT in the present study, we would need data from a larger number of patients with information on more extensive and sensitive HLA typing to discuss the impact of fully matched HLA on transplant outcome.

Finally, non-SCID/SCN/WAS patients showed a significantly lower survival rate (HR = 5·40, $P < 0{\cdot}0001$ by multivariate analyses). Although a previous large-scale study showed that results of HSCT according to disease did not show obvious disease-specific findings (Antoine *et al*, 2003), it is not yet known if UCBT is suitable for all types of PIDs. This may indicate donor source other than UCB is preferable for certain types of PID. Although the success of UCBT noted for X-linked hyperIgM syndrome, bare lymphocyte syndrome and

X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency (Tono *et al*, 2007) is encouraging, optimization of transplantation procedures and determination of suitable timing for UCBT may be necessary for this group of patients. Alternatively, this may simply indicate an expansion of transplantation to less favourable clinical conditions or to less favourable transplantation conditions. Studies on a larger cohort are necessary for drawing any conclusion on whether diagnosis is significant overall.

Recent studies suggest improved survival after BMT for PID with the RIC regimen; however, to date, comparison of CBT using RIC *versus* MAT has not been made. In our study, 87% of patients on the RIC regimen and 66% on the MAT regimen remained alive at the latest follow-up. Multivariate analyses revealed that the RIC regimen is associated with a higher 5-year OS than the MAT regimen (HR = 0·20, $P = 0·011$). Although it is premature to conclude that RIC provides an equal or superior outcome to MAT for all PID patients, non-myeloablative treatment may be beneficial at least for certain types of PID. RIC was selected preferentially in SCID and CGD patients, with good survival rates: 17 of 18 SCID patients and three of four CGD patients remain alive. As a result of this, we are in the process of initiating a clinical trial of UCBT with RIC in SCID patients. On the other hand, only two of 23 WAS patients received RIC. Our previous data showed that a conditioning regimen other than BU/CY or BU/CY/ATG was the only independent factor associated with failure in HSCT for WAS patients (Kobayashi *et al*, 2006). However, whether this holds true for UCBT in younger WAS patients should be determined.

Notably, although the outcome of UCBT for WAS in this cohort was excellent compared with that from previously reported HSCT results using different donor sources (Kobayashi *et al*, 2006; Friedrich *et al*, 2009), UCBT in WAS patients was associated with a high rate of grade 2–4 acute GVHD (11 of 23 patients) and a post-transplant infectious episode (13 of 23 patients). Eight patients experienced bacteraemia/sepsis and six suffered a viral infection (CMV pneumonia, four; Coxsackie virus enterocolitis, one and persistent norovirus infection, one). The high rate of serious infections and GVHD in WAS patients after transplantation warrants further study in search of preventive measures that might include RIC for severe, transplantation-related toxicities.

Long-term follow-up of the clinical and immunological status is necessary when considering the lifespan of PID patients. Recent studies on the long-term outcome after HSCT for SCID revealed the presence of relatively late complications, such as chronic GVHD, autoimmune events, severe or recurrent infections, chronic human papilloma virus infection, nutritional problems and late rejection in 50% of patients (Mazzolari *et al*, 2007; Neven *et al*, 2009). Similarly, long-term follow-up of HSCT in WAS patients revealed that 20% of patients developed chronic GVHD-independent autoimmunity (Ozsahin *et al*, 2008). One possible measure that might be taken to avoid the chronic problems associated with CBT would be to select a HLA-matched UCB unit, as HLA disparity was a risk factor for both overall survival and the development of GVHD in our study. The advantage of RIC over MAT in preventing late complications needs careful assessment, together with data on mortality, engraftment and early post-transplant complications.

Finally, the issue of SCID patients who died before or without receiving SCT, most likely due to uncontrolled infection, still remains unresolved. This suggests that the early diagnosis of SCID and prevention of opportunistic infection within a protected environment and the administration of appropriate prophylactic drugs is critically important for the improvement of survival in SCID patients in general. To that end, neonatal screening with the employment of T cell receptor excision circles should be beneficial for an improved outcome in SCID patients (McGhee *et al*, 2005; Morinishi *et al*, 2009).

We report the results of UCBT for 88 PID patients in Japan. Despite the limitations of a retrospective, non-randomized study, our study suggests that unrelated umbilical cord blood can be considered as a promising stem cell source for children with congenital immunodeficiency when a HLA-matched related donor is not available.

Acknowledgements

This work was in part supported by Health and Labour Sciences Research Grants for Intractable Diseases (H22-03 and H22-113 to T.H. and T.M., respectively), and for Tissue Engineering (H17-014), Allergic Disease and Immunology (H20-015), and Cancer (H19-1) to S.K., and by Grants-in-Aid for Scientific Research by MEXT (20591245) to T.M.

Y. A., D. T., T. N.-I., K. Kato, and S. K. analysed the data; K. Kato, T. N.-I., T. A., T. N.-I., K. Kawa, K. Koike, T. H., and M. K. contributed to the acquisition and interpretation of data; Y. A. and S.K. edited the manuscript; T. M. designed research and wrote the manuscript; all authors reviewed and approved the manuscript.

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Leukemia (2011) 1-3

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Letter to the Editor

Expansion of NK cells from cord blood with antileukemic activity using GMP-compliant substances without feeder cells

Leukemia advance online publication, 6 December 2011; doi:10.1038/leu.2011.345

Neonatal cord blood (CB) cells have been demonstrated to contain a high percentage of natural killer (NK) cells, but the NK cells are immature, with a low level of cytolytic activity. However, expression levels of perforin and granzyme have been reported to be high in CB NK cells, and it has been suggested that CB NK cells are phenotypically and functionally mature. It has been suggested that CB is a source of stem cells that is as safe and effective as bone marrow or mobilized peripheral blood.[1,2] Also, there are a number of progenitor cell populations in CB that can be differentiated to NK cells. Therefore, CB is a useful source to expand NK cells for adoptive immunotherapy, particularly against malignant cells that express a low level of human leukocyte antigen class I molecules. Resting CB NK cells rapidly respond to cytokine stimulation by increasing cytolytic activity. Adoptive transfer of allogeneic NK cells is a potential immunotherapy to induce a graft-versus-leukemia (GVL) effect, without causing a graft-versus-host disease (GVHD).[3] In this study, we tried to expand NK cells from CB with antileukemic activity using good manufacturing practice (GMP)-compliant substances without feeder cells. We used tacrolimus (FK506) and low molecular weight heparin (dalteparin sodium) for expansion of NK cells. Kim *et al.*[4] showed impaired interleukin (IL)-2 signaling and a reduction in activating receptors in NK cells by tacrolimus. However, we have reported that tacrolimus enhances the cytolytic activity of inhibitory NK cell receptor (CD94/NKG2A)-expressing CD8T cells.[5] Also, Wang *et al.*[6] showed that a related compound (cyclosporine A) has essentially no effect on cytolytic activity of NK cells. There is conflicting data on the effect of calcineurin inhibitors on NK cell. Anyways, tacrolimus can inhibit T-cell proliferation. Heparin was reported to bind to several types of cytokines and to activate them, and also to have an important role in the expansion of hematopoietic progenitor cells.[7] Also, heparin sulfate and its related compounds were recognized by natural cytotoxicity receptors such as NKp30, NKp44 and NKp46, and soluble heparin enhanced the secretion of interferon-γ by NK cells.[8] Spanholtz *et al.*[9] reported efficient expansion of NK cells from CB $CD34^+$ cells, using low molecular weight heparin-based media containing various cytokines. Therefore, we tried to use tacrolimus to inhibit T-cell proliferation and dalteparin to support NK-cell proliferation during NK-cell expansion using IL-2, IL-15 and OKT3 *in vitro*.

Umbilical CB cells (Hokkaido Cord Blood Bank, Sapporo, Japan; 1×10^6 per ml) were cultured with IL-15 (10 ng/ml; PeproTech Inc., Rocky Hill, NJ, USA), IL-2 (5 ng/ml; R&D Systems, Minneapolis, MN, USA) and anti-CD3 monoclonal antibody (mAb) (OKT3, 10-1000 ng/ml, Janssen Pharmaceutical Company, Tokyo, Japan), with or without tacrolimus (0.02-0.1 ng/ml, Fujisawa, Osaka, Japan) and dalteparin sodium (Fragmin, 5-10 U/ml, Pfizer Japan, Tokyo, Japan) in culture medium stem cell growth medium (SCGM) (CeeGenix, Freiburg, Germany), which was produced under GMP, with 5% human AB serum in 24-well plates or T25 flasks without feeder cells. Cell cultures were split approximately one-second to one-fourth after 3-4 days of culture, and fresh medium, cytokines and reagents were added. After 3 weeks culture of umbilical CB cells (1×10^6 per ml) with IL-15, IL-2 and anti-CD3 mAb without feeder cells, $CD56^+CD3^-$ NK cells had increased by more than 1000-fold with about 50% purity. Furthermore, addition of dalteparin sodium and tacrolimus efficiently augmented NK cell expansion (1700-fold expansion with 72.8% purity). Also, NK cell proportion was the highest (72.8%) after expansion with both dalteparin sodium and tacrolimus compared with expansion with cytokines only, dalteparin sodium only and tacrolimus only (Table 1, means ± s.d.s, $n = 5$). The proportion of $CD56^+CD3^-$ NK cells increased after more than 7 days of culture, and the proportion of CD56-expressing cells increased up to 90% after 3 weeks of culture (Figure 1a, bars indicate means ± s.d.s, $n = 5$). Finally, we could obtain about 40×10^6 NK cells from 1×10^6 unmanipulated CB cells under GMP-conditioned medium with 5% human AB serum without feeder cells. Furthermore, this method has also enabled to expand NK cells from adult peripheral blood mononuclear cells (PBMCs; preliminary data not shown).

These expanded NK cells expressed stimulatory NK cell receptor NKG2D and intracellular cytotoxic molecule granzyme (Figure 1b, bars indicate means ± s.d.s, $n = 5$). Also, the expanded $CD16^+CD56^+$ NK cells expressed high levels of inhibitory NK receptors, but significantly higher levels of stimulatory NK cell receptors including NKG2C, NKG2D, NKp30, NKp46 and especially, NKp44, than the levels of these receptors on $CD16^+CD56^+$ NK cells in resting CB before culture were noted (Figure 1c, $P < 0.01$, bars indicate means ± s.d.s, $n = 10$).

The cytolytic activities of expanded NK cells were tested against ^{51}Cr-labeled K562 human leukemic cell lines, patients' leukemic cells and allogeneic phytohemagglutin (PHA) blasts (5×10^3), using standard 4-h ^{51}Cr release assays. The expanded NK cells had

Table 1. Expansion of NK cells from CB samples

	Pre	*IL2+15*	*+Tacrolimus*	*+Fragmin*	*+T+F*
%	2.8 ± 0.8	48.3 ± 6.5	50.4 ± 3.7	65.0 ± 10.8[b]	72.8 ± 9.6[a,c,d]
Absolute number	0.028 ± 0.008	36.1 ± 10.4	34.7 ± 16.2	49.9 ± 17.7[e]	43.5 ± 14.3
Fold expansion	1	1422 ± 316	1360 ± 581	1989 ± 678[e]	1706 ± 389

Abbreviations: CB, cord blood; IL, interleukin; NK, natural killer. Values in the upper column indicate the percentage of $CD56^+CD3^-$ cells after 3-weeks expansion of CB cells with indicated factors (%). Significant differences were found in the values compared with the values for culture with only IL-2 and IL-15. Significant differences were also found in the values compared with the values for culture with 0.02 ng/ml of tacrolimus and 5 IU/ml of fragmin. Values in the middle and lower columns indicate the calculated absolute $CD56^+CD3^-$ cell number and fold expansion of NK cells after 3-weeks expansion of 1×10^6 CB cells. Significant difference was found only in the value for culture with IL-2, IL-15 and 0.02 ng/ml of tacrolimus, compared with the values for culture with IL-2, IL-15 and 5 IU/ml of fragmin (means ± s.d.s, $n = 5$). [a]$P < 0.01$, [b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.05$ and [e]$P < 0.05$.

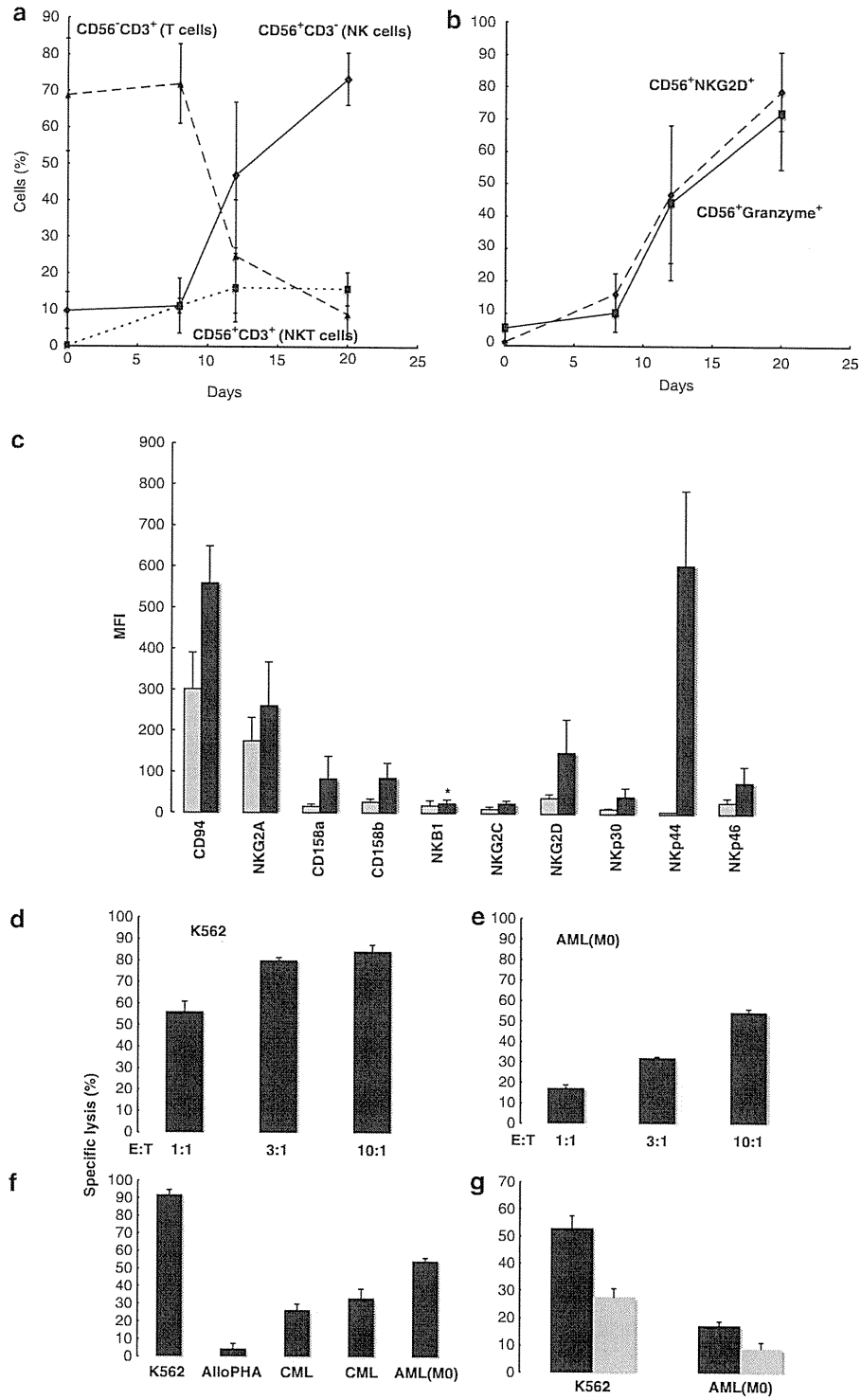

Figure 1. Time course profile of expanded cells during culture without feeder cells. $CD56^+CD3^-$ (NK cells), $CD56^-CD3^+$ (T cells) and $CD56^+CD3^+$ cells (NKT cells; **a**) and $CD56^+NKG2D^+$ cells and $CD56^+$granzyme$^+$ cells (**b**); bars indicate means ± s.d.s, $n = 5$. Mean fluorescence intensity (MFI) of NK receptors on $CD16^+CD56^+$ NK cells in CB before expansion (gray bars) and after expansion (black bars). There were significant differences between before and after expansion except for NKB1 *($P < 0.01$, $n = 10$; **c**). Cytolytic activities of expanded NK cells against K562 cells (**d**) and AML (M0) patient's leukemic cells (**e**). Data presented are means ± s.d.s (effector/target ratios of 1:1, 3:1 and 10:1). Cytolytic activities against K562 cells, allogeneic third-party PHA blasts and patients's leukemic cells from chronic myeloid leukemia and AML (M0; E:T ratio of 10:1; **f**). Inhibitory effect of anti-NKG2D monoclonal antibody (20 μg/ml, gray bars) against cytolytic activities of expanded NK cells (E:T ratio is 1:1; **g**).

a very high level of cytolytic activity against the K562 leukemic cell line, with specific lysis of more than 50% under the condition of an effector:target ratio (E:T ratio) of 1:1 and more than 80% under the condition of an E:T ratio of 3:1 (Figure 1d). Also, the expanded NK cells could attack patients' primary acute myeloid leukemia (AML; M0) and chronic myeloid leukemia (CP) leukemic cells, but could not attack allogeneic third-party PHA blasts (Figures 1e and f). Anti-NKG2D mAb (1D11, 20 μg/ml, Serotec, Oxford, UK)

suppressed the cytolytic activity against K562 cells and also patients' primary leukemic cells (Figure 1g). Therefore, the cytolytic activity of these expanded NK cells depended at least partially on NKG2D-activating receptor.

Allogeneic NK cells have been reported to have a strong GVL effect after haploidentical hematopoietic stem cell transplantation (HSCT) in patients with advanced AML, without causing GVHD.[3] Adoptive transfer of allogeneic NK cells may be a promising immunotherapy. However, expansion of NK cells seems to be difficult compared with expansion of T cells. About 20-fold expansion of NK cells was achieved by culture of PBMCs with cytokines for a short time and co-culture with K562 cells that had been transfected with, and expressed membrane-bound IL-15 and 4-1BBL. These expanded NK cells using artificial feeder cells had cytolytic activity against human AML cells and also pediatric solid tumors such as Ewing sarcoma and rhabdomyosarcoma. On the other hand, there have been several reports of NK cell expansion from PBMCs without using feeder cells. Alici *et al.*[10] reported the possibility of expanding NK cells without feeder cells from PBMCs of multiple myeloma patients with significant cytolytic activity against primary autologous multiple myeloma cells. It is more beneficial for clinical use if it is not necessary to use feeder cells for efficient expansion of NK cells *in vitro*. Also, Ayello *et al.*[11] reported a 20-fold expansion of NK cells from CB cells with depletion of adherent monocytes by *ex vivo* culture with IL-2, IL-7 and IL-12 for 7 days. Therefore, NK cells can be expanded from not only PBMCs, but also CB.

Clinical-scale NK cell purification has so far been performed by donor leukapheresis followed by CD3 depletion with or without CD56 enrichment. There have been several clinical reports on adoptive transfer of allogeneic NK cells for hematological malignancies. Passweg *et al.*[12] first reported the feasibility of allogeneic NK cell purification and infusion in five myeloid malignant patients after haploidentical HSCT. Miller *et al.*[13] reported that haploidentical NK cell infusions after cyclophosphamide and fludarabine treatment resulted in expansion of donor NK cells and induction of complete hematological remission in 5 of 19 AML patients with poor prognosis. A recent pilot study showed good results in pediatric patients who received haploidentical NK cells to consolidate chemotherapy for AML patients. Also, Nguyen *et al.*[14] reported a persistent and massive expansion of infused alloreactive NK cells in an AML patient who had relapsed after haploidentical HSCT. Yoon *et al.*[15] reported patients who underwent human leukocyte antigen-mismatched HSCT and subsequently received donor NK cells that were generated from CD34+ cells from donor leukapheresis products by *ex vivo* culture for more than 6 weeks (9.3×10^6 per kg, CD122/CD56$^+$ 64%, CD3$^+$ 1%). There were no signs of acute toxicity in 14 adult patients infused with these cells 6–7 weeks after transplantation, with one patient developing acute GVHD and five patients developing chronic GVHD. Therefore, clinical-grade allogeneic NK cell infusion is safe and feasible. However, many issues remain to be resolved, including selection of donor, NK cell selection and expansion procedure, type of conditioning regimen, survival and expansion of NK cells in the recipient after infusion, their localization and finally, the clinical effect of NK cell infusion.

In this study, we could obtain about 40×10^6 NK cells from 1×10^6 unmanipulated CB cells using tacrolimus and dalteparin sodium without feeder cells (more than 1000-fold expansion with more than 70% purity). Thus, alloreactive Killer cell immunoglobulin-like receptor (KIR)-mismatched expanded NK cells from CB can be used for adoptive NK cell immunotherapy to induce a strong GVL/tumor effect without severe GVHD for patients who do not have KIR-mismatched related donors.

CONFLICT OF INTEREST

A patent application for the composition for expanding NK cells and the use of them has been filed with Junji Tanaka as a sole inventor.

ACKNOWLEDGEMENTS

We thank Ms M Yamane, Ms M Mayanagi and Ms Y Ishimaru for their technical assistance.

J Tanaka, J Sugita, S Shiratori, A Shigematu, S Asanuma, K Fujimoto, M Nishio, T Kondo and M Imamura
Department of Hematology and Oncology, Hokkaido University Graduate School of Medicine, Sapporo, Japan
E-mail: jutanaka@med.hokudai.ac.jp

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