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ORIGINAL PAPER

Clinical outcomes of adult and childhood rhabdomyosarcoma treated with vincristine, *d*-actinomycin, and cyclophosphamide chemotherapy

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Abstract
Background Outcomes in adult patients with rhabdomyosarcoma are poor, with a 5-year survival rate of approximately 30 %. The current study aimed to compare the clinical outcomes of adult and childhood rhabdomyosarcoma patients with local and metastatic disease and to examine the impact and timing of local therapy on metastasis.
Methods Clinicopathological features and patient outcomes were reviewed retrospectively for rhabdomyosarcoma patients receiving chemotherapy between 1981 and 2010 at our institution. Adults were defined as those aged 21 years or older.
Results Of the 98 patients identified, 36 were adults (median age, 29; range, 21–72) and 62 were children (median age, 11; range, 0.6–20). Median progression-free survival of localized and metastatic disease for children and adults was as follows: localized disease, 166.9 versus 22.4 months ($p = 0.005$), and metastatic disease, 13.3 versus 13.3 months ($p = 0.949$), respectively. Multivariate regression analysis revealed that older age ($\geq$21 vs. $<$21) was a significant poor prognostic factor in localized disease. Conversely, age was not related to survival in metastatic disease. Receiving radiotherapy to the primary site was an independent factor indicating a better prognosis. An analysis of the optimal timing of local therapy was performed for 53 patients; however, its significance on survival could not be determined.
Conclusions Age was a negative prognostic factor in rhabdomyosarcoma patients with localized disease, but it did not affect the survival in metastatic disease. For metastatic disease, although local therapies may be effective for survival, the timing of such therapies should be determined individually.

Keywords Adult · Child · Rhabdomyosarcoma · Clinical outcome

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Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children, but it is far less common in adults (Ferrari et al. 2003). Improvements in multimodal treatment of RMS have improved survival in children from 25 to approximately 70 % over the past 40 years (Pappo et al. 1997; Raney et al. 2001; Breitfeld and Meyer 2005; Leaphart and Rodeberg 2007). In contrast, the prognosis for adult RMS remains poor, with a 5-year survival rate of approximately 30 % (Sultan et al. 2009; Ferrari et al. 2003;

La Quaglia et al. 1994; Little et al. 2002; Esnaola et al. 2001; Hawkins et al. 2001).

Metastatic RMS affects approximately 15 % of all children with RMS (Breneman et al. 2003). According to the risk categories identified by the Intergroup Rhabdomyosarcoma Study (IRS), low- and intermediate-risk patients have improved outcomes, with 96–97 % of low-risk patients achieving 5-year survival (Meza et al. 2006) and 79 % of intermediate-risk patients attaining 4-year survival (Arndt et al. 2009). However, survival for high-risk patients remains poor, with 34 % achieving 3-year survival (Oberlin et al. 2008) and 24 % achieving 5-year survival (Carli et al. 2004). Consequently, standard treatment for high-risk patients remains controversial (Klingebiel et al. 2008; Pappo et al. 2007; Lager et al. 2006).

The treatment strategy for RMS requires multidisciplinary therapy, including chemotherapy, surgery, and radiation therapy. Vincristine and *d*-actinomycin, with or without cyclophosphamide (VAC) regimens, are considered the standard option for RMS (Maurer et al. 1988, 1993; Crist et al. 1995, 2001; Meza et al. 2006; Arndt et al. 2009), with wide surgical resection of tumor and postoperative radiation therapy required for local control of localized RMS (La et al. 2011; Rodeberg et al. 2011; Schuck et al. 2004). The timing of radiation therapy is critical: for localized RMS, radiation is appropriate to start during weeks 9–12, and for parameningeal RMS with intracranial extension, local radiation treatment should begin during the first 1–2 weeks of chemotherapy (Michalski et al. 2004; Raney et al. 2002). However, the impact and optimal timing of local therapy for metastatic disease is unknown. Therefore, the purpose of the current study was to compare the clinical outcomes of local or metastatic adult and childhood RMS and to examine the impact and timing of local therapies on metastatic disease.

Patients and methods

Patients

All patients included in this analysis met the following criteria: histologically diagnosed with RMS, treated at the National Cancer Center Hospital in Tokyo between 1981 and 2010, and received VAC or VAC-like chemotherapy. Medical records were then retrospectively reviewed to obtain the following information: date of birth, gender, date of diagnosis, primary tumor site, histopathology, initial tumor size, presence of central nervous system (CNS) invasion, clinical stage, group category as defined by the IRS (Breitfeld and Meyer 2005), date of treatment initiation, chemotherapy regimen, best response, chemotherapy administration schedule, date of radiotherapy, date of surgery, date of progression, date of last follow-up, and survival status.

Treatment

The VAC regimen after the year of 2000 consisted of vincristine at a dose of 1.5 mg/m^2 (up to 2 mg/body), given intravenously on days 1, 8, and 15; cyclophosphamide at a dose of 2.2 g/m^2, given intravenously on day 1; and *d*-actinomycin at a dose of 1.5 mg/m^2 (up to 2.5 mg/body), given intravenously on day 1. The treatment course was repeated according to IRS-IV or Children's Oncology Group (COG) Study (D9803) protocols (Arndt et al. 2009). Before the year of 2000, the VAC-like regimen includes the following regimens: vincristine, *d*-actinomycin, and either ifosfamide, etoposide, or doxorubicin. The treatment course was administered according to IRS-II or III protocols.

Local therapy includes surgery, radiation therapy, or both. Surgical resection defined in this manuscript includes only total gross resection. Microscopic complete resection (Group I) was confirmed microscopically later on. Radiation therapy was delivered to each primary tumor site and regional lymph nodes where applicable. The total dose ranged from 30 to 56.3 Gy, with a median dose of 45 Gy.

Definitions of terms

We defined adults as patients aged 21 years or more, and considered the remaining patients to be children. The induction phase was defined as the first six cycles of the VAC/VAC-like regimen, and the maintenance phase was defined as the later cycles following the induction phase. Response to chemotherapy was compared with baseline status. A complete response (CR) was defined as the disappearance of tumors with no evidence of disease. A partial response (PR) was a 50 % or greater decrease in the sum of tumor diameters. Stable disease (SD) was a less than 50 % decrease in the sum of tumor diameters. Progressive disease (PD) was defined as a 25 % or greater increase in the sum of tumor diameters and/or the appearance of new lesions.

Surgery or radiation therapy in this study means resection or radiation therapy to the primary site during primary treatment, respectively. Resection or radiation therapy to a relapsed site or metastatic site is not included in this category; likewise, biopsy only is not included in this definition.

Statistical analyses

Progression-free survival (PFS) was defined as the time from the date of initial chemotherapy to the date when

local recurrences or distant metastases were recognized. Overall survival (OS) was defined as the time from the date of initial chemotherapy to the date of death due to any cause. Patients who survived were treated as censored observations on the last day of follow-up. PFS and OS were estimated using the Kaplan–Meier method, and survival curves were compared using the log-rank test. Multivariate Cox regression analysis was used to estimate the hazard ratios and 95 % confidence intervals (CI). A two-sided $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS (version 9.1.3; SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics

We identified 98 patients who met the eligibility criteria. The distributions of clinical and pathologic characteristics of patients are listed in Tables 1 and 2. There were 36 adults (age: median, 29; range 21–72) and 62 children (age: median, 11; range, 0.6–20). Seventy-three patients had localized disease (22 adults and 51 children) , while 25 patients had metastatic disease (14 adults and 11 children). The most common histology was embryonal in localized disease (56.2 %) and alveolar in metastatic disease (68.0 %). Botryoid was found in only one patient. Common primary sites in localized disease were parameningeal (38.4 %), head and neck (21.9 %), and extremity (15.1 %), while common primary sites in metastatic disease sites were parameningeal (32.0 %), extremity (28.0 %), and other (24.0 %) (Table 3). The most common metastatic

Table 1 Characteristics of patients with localized rhabdomyosarcoma ($n = 73$)

	Adults ($n = 22$)		Children ($n = 51$)	
	No.	%	No.	%
Gender				
Female	5	22.7	24	47.1
Male	17	77.3	27	52.9
Tumor size				
<5 cm	9	40.9	23	45.1
≥5 cm	13	59.1	28	51.9
Site				
Favorable	5	22.7	22	43.1
Unfavorable	17	77.3	29	56.9
Histology				
Alveolar	9	40.9	18	35.3
Embryonal	12	54.5	29	56.9
Other	1	4.5	4	7.8
Group				
1	3	13.6	9	17.6
2	3	13.6	5	9.8
3	16	72.7	37	72.5
Stage				
1	4	18.2	21	41.2
2	5	22.7	8	15.7
3	13	59.1	22	43.1
CNS invasion				
No	20	90.9	46	90.2
Yes	2	9.1	5	9.8

CNS central nervous system

Table 2 Characteristics of patients with metastatic rhabdomyosarcoma ($n = 25$)

	Adults ($n = 14$)		Children ($n = 11$)	
	No.	%	No.	%
Gender				
Female	6	42.9	6	54.5
Male	8	57.1	5	45.5
Tumor size				
<5 cm	2	14.3	4	36.4
≥5 cm	12	85.7	7	63.6
Site				
Favorable	1	7.1	1	9.1
Unfavorable	13	92.9	10	90.9
Histology				
Alveolar	11	78.6	6	54.5
Embryonal	2	14.3	5	45.5
Other	1	7.1	0	0
CNS invasion				
No	14	100	10	90.9
Yes	0	0	1	9.1

CNS central nervous system

Table 3 Primary site of rhabdomyosarcoma in localized and metastatic disease

	Localized disease No. (%)	Metastatic disease No. (%)
Extremity	11 (15.1)	7 (28.0)
Parameningeal site	28 (38.4)	8 (32.0)
Head and neck	16 (21.9)	1 (4.0)
Genitourinary primary site (non-bladder/prostate)	6 (8.2)	0 (0.0)
Orbit	5 (6.9)	1 (4.0)
Genitourinary primary site (bladder/prostate)	2 (2.7)	2 (8.0)
Unfavorable other	5 (6.9)	6 (24.0)

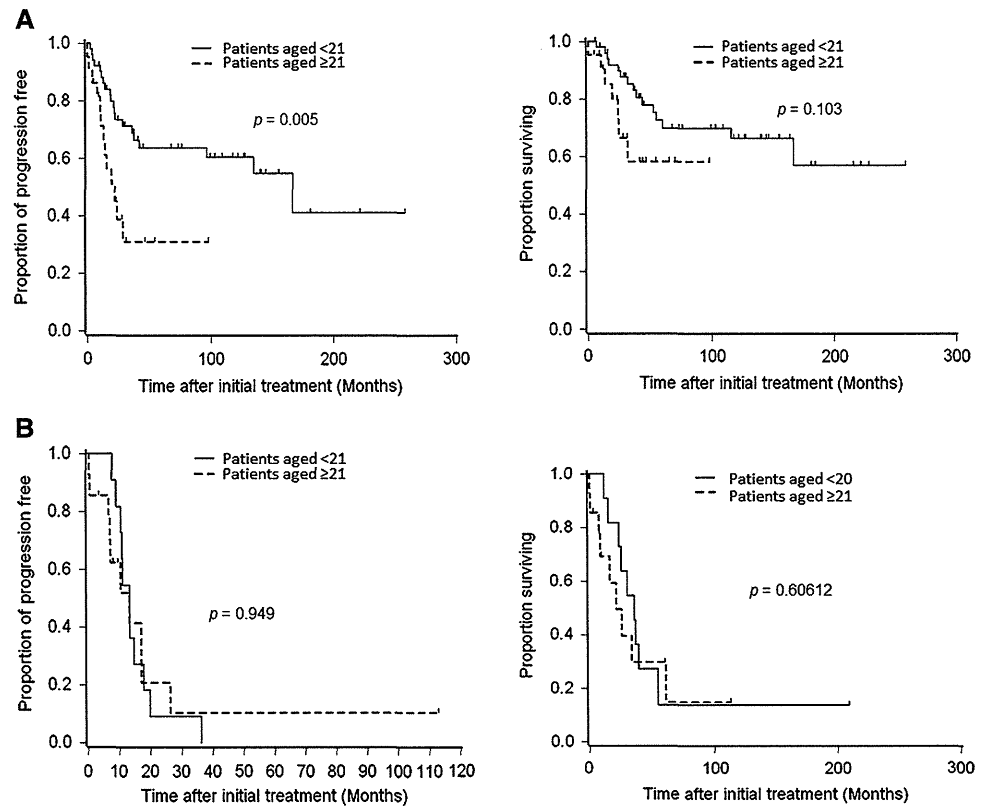

Fig. 1 **a** Kaplan–Meier curve of progression-free survival and overall survival in patients with localized disease of adults (*dashed line*) and children (*solid line*). **b** Kaplan–Meier curve of progression-free survival and overall survival in patients with metastatic disease of adults (*dashed line*) and children (*solid line*)

sites were bone ($n = 17$), bone marrow ($n = 7$), and lung ($n = 6$). Eleven patients had metastases to multiple sites.

Thirty-one patients (31.6 %) underwent surgical resection at RMS diagnosis. Ten patients were classified as IRSG Group I, 7 as Group II, and 14 as Groups III/IV. Surgery (primary tumor resection at diagnosis or second-look surgery) was performed in 43 patients (58.9 %) with localized disease and in 5 patients (20.0 %) with metastatic disease. We identified 59 patients (60.2 %) who had received VAC regimens and 39 patients (39.8 %) who had received VAC-like regimens. Radiation therapy was performed in 52 patients (71.2 %) with localized disease and in 18 patients (72.0 %) with metastatic disease during the course of treatment.

Patient outcomes in adults and children

The best responses to chemotherapy were as follows: among those with localized disease, 65 patients (89.0 %) achieved CR/PR, 5 patients (6.9 %) achieved SD/PD, and the data are not available for 3 patients (4.1 %); among patients with metastatic disease, 22 patients (88.0 %) achieved CR/PR and 3 patients (12.0 %) achieved SD/PD ($p = 1.000$). The overall median follow-up period was 37 months (range, 0–263 months); 37 months in metastatic disease (range, 0–213 months); and 43 months in localized disease (range, 0–263 months). At the time of analysis, 50 patients (51.0 %) experienced recurrence, and 41 of these patients (41.8 %) later died. Sites of first recurrence/progression were locoregional in 26 patients and distant metastases in 24 patients. Seven patients in whom the sites of first recurrence/progression were locoregional achieved CR and were still alive following second-line treatment.

Adult patients with localized disease had a significantly greater probability of poor outcome compared with children. The median PFS times for localized and metastatic disease for children and adults were as follows: localized disease, 166.9 versus 22.4 months ($p = 0.005$) (Fig. 1a), and metastatic disease, 13.3 versus 13.3 months ($p = 0.949$) (Fig. 1b), respectively. Median OS times were not statistically different in patients with metastatic disease for both adults and children.

Analyses of prognostic factors in localized and metastatic disease

To determine the independent predictors of survival, we used a multivariate Cox regression model. The results of multivariate analysis for PFS and OS in localized and metastatic disease are shown in Tables 4 and 5. According to Table 4, age (<21 vs. ≥21) was the only statistically significant negative predictor of PFS for patients with localized disease ($p = 0.018$). In contrast, for metastatic disease, age was not significantly different with respect to

Table 4 Multivariate analyses of PFS and OS in localized rhabdomyosarcoma ($n = 73$)

	No. of patients	PFS			OS		
		HR	95 % CI	p value	HR	95 % CI	p value
Age							
<21	51						
≥21	22	2.60	1.18–5.70	0.018	1.67	0.62–4.52	0.311
Stage							
1	25						
2	13	2.73	0.90–8.29	0.076	7.36	1.38–39.2	0.019
3	35	2.42	0.94–6.24	0.069	5.66	1.19–26.9	0.029
Radiotherapy							
No	21						
Yes	52	0.69	0.29–1.63	0.394	0.95	0.32–2.85	0.924
Surgery							
No	30						
Yes	43	0.60	0.29–1.24	0.167	0.63	0.26–1.54	0.312
Presence of CNS invasion							
No	66						
Yes	7	1.68	0.52–5.36	0.384	1.75	0.52–5.84	0.363

CNS central nervous system, *PFS* progression-free survival, *OS* overall survival, *HR* hazard ratio, *CI* confidence interval

Table 5 Multivariate analyses of PFS and OS in metastatic rhabdomyosarcoma ($n = 25$)

	No. of patients	PFS			OS		
		HR	95 % CI	p value	HR	95 % CI	p value
Age							
<21	11	1.00			1.00		
≥21	14	0.97	0.37–2.55	0.947	1.03	0.35–3.06	0.960
Radiotherapy							
No	7						
Yes	18	0.14	0.04–0.51	<0.001	0.24	0.07–0.82	0.023
Surgery							
No	20						
Yes	5	1.15	1.15–3.95	0.394	0.30	0.06–1.47	0.137
Presence of CNS invasion							
No	24						
Yes	1	1.85	0.20–16.8	0.585	0.99	0.12–8.45	0.995

CNS central nervous system, *PFS* progression-free survival, *OS* overall survival, *HR* hazard ratio, *CI* confidence interval

PFS and OS. Radiotherapy was the only significant factor in improved PFS or OS in metastatic disease.

Local therapy for metastatic disease

Of 25 patients with metastatic disease, 21 patients (84.0 %) received local therapy as part of their primary treatment that included radiotherapy ($n = 16$), surgery and radiotherapy ($n = 3$), and surgery ($n = 2$). Sixteen of these 21 patients (76.2 %) experienced relapse at the following sites: distant metastatic site ($n = 11$) and primary site progression ($n = 5$). Among four patients with metastatic disease who did not receive local therapy, three patients relapsed: two experienced local relapse and one relapsed at a distant metastatic site. In the 25 patients with metastatic disease, median PFS times in patients with or without local therapy (surgery and/or radiotherapy) were 13.4 versus 7.0 months ($p < 0.001$) (Fig. 2a), respectively, and median OS times were 36.1 versus 7.6 months ($p < 0.001$), respectively (Fig. 2b).

Timing of local therapy

We further sought information about the optimal timing of local therapy in 53 patients who received local therapy (both radiotherapy and surgery) during their course of treatment (localized disease, $n = 38$; metastatic disease, $n = 15$). PFS and OS were not significantly different

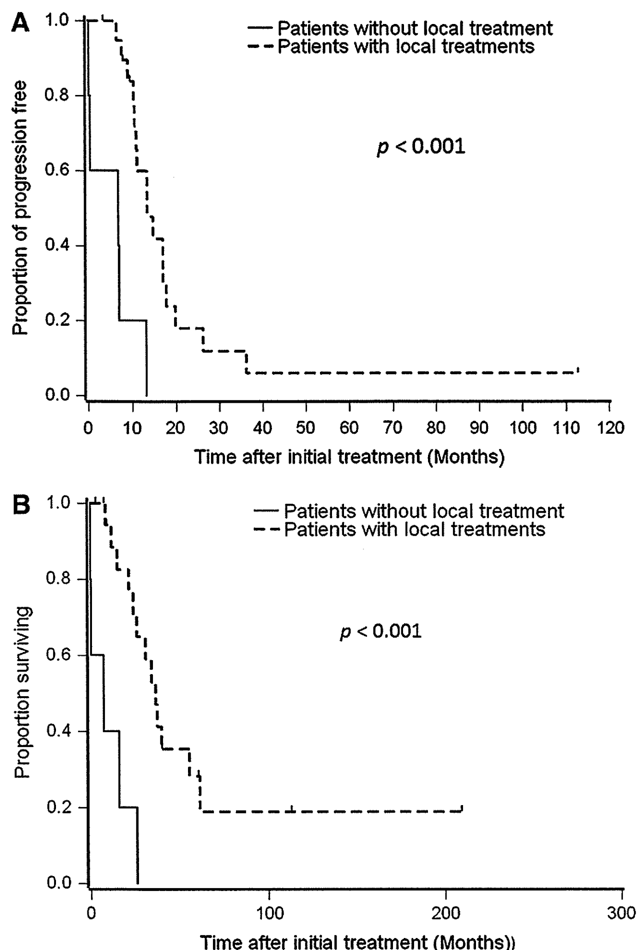

Fig. 2 Kaplan–Meier curve of progression-free survival (**a**) and overall survival (**b**) in patients with metastatic disease with local therapy (*dashed line*) and without local therapy (*solid line*)

between patients who received local therapy within or after 18 weeks of starting initial treatment (see Table 6 for our multivariance analysis of OS). In the 38 patients with localized disease, median PFS was 134.7 months in patients who received radiotherapy in the induction phase and 101.6 months in patients who received it during the maintenance phase ($p = 0.921$); median OS was similar for patients during both phases ($p = 0.277$). Median PFS and OS times were also similar in patients who received surgery either in the induction phase or in the maintenance phase ($p = 0.304$ for PFS and $p = 0.502$ for OS).

In the 15 patients with metastatic disease, the median PFS in patients who received radiotherapy was similar for both phases (induction, 18.4 months; maintenance, 13.3 months, $p = 0.177$); however, median OS was significantly longer for the patients receiving radiotherapy in the induction phase than for those receiving radiotherapy in the maintenance phase (60.7 and 25.7 months, respectively, $p = 0.048$). Median PFS and OS were similar in patients who received surgery in either the induction or maintenance phase ($p = 0.304$ for PFS and $p = 0.214$ for OS).

Discussion

In this study, we evaluated the clinical outcomes of adults and children with RMS who received VAC/VAC-like chemotherapy as their initial treatment. This study resulted in two main findings. First, we showed that age was an independent negative prognostic factor for PFS in RMS with localized disease, but it was not associated with survival in metastatic disease. Second, local therapy to the

Table 6 Multivariate analysis of OS to determine the significance of timing for local therapy in localized and metastatic rhabdomyosarcoma

	Localized disease ($n = 38$)			Metastatic disease ($n = 15$)		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
Age						
<21	1			1		
≥21	1.04	0.24–4.49	0.961	0.63	0.11–3.66	0.610
Stage						
1	1			–	–	–
2	30.74	0.68–1,390.3	0.078	–	–	–
3	12.98	0.50–339.5	0.124	–	–	–
Timing of radiotherapy						
≥18 weeks	1			1		
<18 weeks	0.89	0.23–3.36	0.857	0.30	0.04–2.31	0.246
Timing of surgery						
≥18 weeks	1			1		
<18 weeks	0.45	0.04–3.31	0.429	3.11	0.07–138.2	0.558
Presence of CNS invasion						
No	1			1		
Yes	1.45	0.38–5.56	0.587	2.81	0.18–45.0	0.465

CNS central nervous system, *OS* overall survival, *HR* hazard ratio, *CI* confidence interval

primary tumor site during the treatment course may be necessary for metastatic RMS, as the patients who received local therapies showed significantly longer survival than those who did not. Although our findings suggest that patients with metastatic RMS should be treated at an early stage with local radiotherapy to improve OS, this aspect of our results requires more research; thus, the timing of local therapy should be individually determined depending on patient conditions.

Several studies have reported that age is associated with poor survival in patients with RMS. Sultan et al. reported on the prognosis of pediatric (age $\leqq$ 19 years) and adult (age > 19 years) RMS patients, and their findings suggested that the 5-year survival rate was significantly poorer in adults compared to that in children (5-year OS, 27 and 61 %, respectively; $p < 0.0001$) (Sultan et al. 2009). Another study clarified that the outcomes of patients with intermediate-risk RMS varied depending on age (Meza et al. 2006). Oberlin et al. (2008) also reported on the prognosis of metastatic RMS, and their data suggested that the 3-year event-free survival rate was significantly poorer in RMS patients <1 year and >9 years of age compared to that in RMS patients aged 1–9 years ($p < 0.001$). In our study, age was a negative prognostic factor of PFS in RMS with localized disease, but outcomes for metastatic disease were not different between adults and children. Previous studies have mostly focused on age in children, but our study reported different prognoses for adults and children in both localized and metastatic disease. Therefore, our results would be expected to be different than those of Oberlin et al. (2008). The poor prognosis in adult metastatic RMS may depend on the tumor biology and drug delivery.

Histopathological classification of adult RMS is somewhat difficult to categorize conventional subtypes. Although our data include alveolar subtype most, pleomorphic and spindle subtypes in part may be included in the heterogeneous tumor and these subtypes are suggested poor prognosis (Mentzel, 2000 #3204). For the drug delivery, unpublished data in our institute suggest that the dose intensity of vincristine and cyclosporine is lower in adult when compared to children as hematological toxicities and neurotoxicity are severe. These data suggest that categorizing adult RMS and its treatment may be necessary to be developed independently to that of child RMS.

Radiation therapy and surgery are important for local tumor control and survival in the treatment of RMS. However, the optimal timing of radiation therapy is unclear. IRSG and COG protocols incorporate radiation therapy scheduled at weeks 9 or 12 after the induction of initial chemotherapy (Crist et al. 2001; Arndt et al. 2009). Minn et al. (2010) analyzed the risk of early treatment failure in intermediate-risk RMS, and the majority of patients with early progression experienced local failure. Earlier radiation therapy may improve outcomes by the prevention of early local progression, and the current COG study (ARST0531, http://www.clinicaltrials.gov) plans to perform radiation therapy at week 4 for intermediate-risk RMS. Although there has been no randomized trial to compare the timing of local therapy in RMS, early initiation of local treatment would seem to be preferable. In our study, local therapy was effective in improving survival even in metastatic disease. We could find the efficacy of radiotherapy in metastatic patients but not for surgery probably because of the shortage of patients number included. However, except for local radiotherapy in patients with metastatic disease, the timing of local therapy had no significantly different effect on outcomes in patients who received local therapy during the induction phase versus the maintenance phase. The threshold we used for dichotomization (within 18 weeks or later than 18 weeks after initial treatment onset) may have been a factor in our inability to detect a significant difference in outcomes. This result implies that the timing of local therapy for metastatic disease, whether radiotherapy or surgery, may be varied depending on the individual patient's characteristics, that is, the radiotherapeutic field or the operability of the patient's local site.

Several limitations to our study should be mentioned. Our analysis was limited by its retrospective design and small sample size. Patients receiving VAC-like chemotherapy had undergone chemotherapy during the period prior to 2000, and the dose intensity of chemotherapy varied by protocol. The dose of irradiation and radiation methods also varied. Further, the patients who received local therapy might have been in better general condition or had a smaller primary tumor, which could be included in one radiation field, compared with those who did not. To reduce these biases, we compared the baseline characteristics of each group and demonstrated their similarity. However, adult RMS is a rare cancer; thus, our results should contribute to further advances in this field of oncology.

In conclusion, we showed that age was a negative prognostic factor for PFS and OS in RMS patients with localized disease, but age was not associated with survival in metastatic disease. For metastatic disease, local therapy may have a beneficial effect on survival, but the optimal timing of local therapy is unclear and should be determined individually. Future clinical trials for metastatic RMS should focus on the timing of local therapy, and evaluation of treatment strategies limited to adult RMS patients is warranted.

Conflict of interest None.

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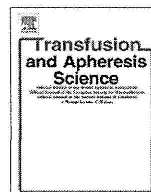

Maintenance of surface antigens and the absence of an apoptotic marker are observed during storage of granulocyte concentrates collected by bag separation method

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ABSTRACT

Granulocytes were collected by the bag separation method and stored in whole blood for up to 72 h. We evaluated the expressions of various surface antigens: CD62L, CD11b, CD18, CD64, CD16b, and CD95. Apoptosis was assessed both by flow cytometry and by light microscopy. Expression levels of all the surface antigens were shown to be maintained during storage for up to 72 h. Approximately 80% of granulocytes were annexin V negative until 72 h after collection. The storage of granulocyte concentrates collected by the bag separation method may maintain granulocyte surface antigens and lack an apoptotic marker.
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1. Introduction

Granulocyte transfusion therapy (GTX) had been used for intractable infections in patients with severe neutropenia. However, this therapeutic option had nearly ceased by at the end of the 1980s because consistent therapeutic effects had not been achieved primarily owing to the inability to collect a sufficient number of functionally intact granulocytes. Recently, mobilization using granulocyte-colony-stimulating factor (G-CSF) has facilitated the collection of a large number of granulocytes from healthy donors [1], and GTX has been reconsidered as a useful option for treating intractable infections in patients with severe neutropenia [2]. There is a general agreement that collected granulocytes should be transfused as soon as possible after collection. If granulocytes can be stored with their functions maintained for a longer period, GTX will be a more useful strategy. We previously showed that the phagocytosis level, oxidative killing level, and the viability of granulocytes collected by the bag separation method were well maintained during storage for up to 72 h after collection [3]. Cell viability was maintained above 96%, phagocytosis level was above 70%, and oxidative killing level was above 95% for up to 72 h after collection. In this study, we evaluated the expression of various surface antigens, which play important roles in phagocytosis and cell adhesion, during storage for up to 72 h. We also evaluated the apoptosis of granulocytes during storage until 72 h after collection.

2. Materials and methods

2.1. Subjects

The subjects were five healthy adult volunteers who gave their consent to participate in this study after being fully informed about the adverse effects of G-CSF and the bag separation method. The written informed consent were obtained. The study protocol was approved by the Institutional Review Board of Fukushima Medical University.

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2.2. Dose and administration of G-CSF

Donors received recombinant human G-CSF (Nartograstim, Kyowa Hakko, Tokyo, Japan) subcutaneously at a dose of 200 μg 12 h before granulocyte collection. In addition, we collected granulocytes without the administration of G-CSF to obtain baseline (control) values.

2.3. Granulocyte collection by bag separation method

We collected granulocytes by the bag separation method as previously reported [3,4]. In brief, 200 mL of whole blood was collected into a blood transfusion bag using the gravity-flow principle. After centrifugation, the buffy-coat layer and the upper one-third of the erythrocyte layer, both of which are rich in granulocytes, were collected into a polyvinyl chloride bag containing the CPD solution. As a result, approximately 25–40 mL of granulocyte concentrate was obtained from the 200 mL of whole blood sample. The remaining erythrocyte and plasma components were returned to the donor. The granulocyte concentrates were irradiated at 15 Gy using Liniac (NELAC-1012A, NEC, Tokyo, Japan), which simulates clinical conditions. In Japan, blood components from branches of the Japanese Red Cross Blood Center are irradiated at 15 Gy (or more), thus granulocyte concentrates are irradiated before administration to the patient at a dose between 15 and 50 Gy [5].

2.4. Storage

All the collected granulocyte concentrates were stored in whole blood at room temperature without agitation for up to 72 h. We then analyzed the stored granulocyte samples as described below.

2.5. Immunophenotyping of granulocytes

The expressions of various surface antigens on granulocytes: CD62L (L-selectin), CD11b, CD18, CD64 (Fcγ Receptor I), CD16b (Fcγ Receptor III), and CD95 (Fas; a death receptor), were assayed by direct immunofluorescence flow cytometry (FACS), using saturating concentrations of commercially available monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) at time points of 24, 48, and 72 h after collection. The following mouse monoclonal antibodies were obtained from Beckman Coulter (Fullerton, CA, USA): CD62L-FITC-conjugated IgG1 antibody, CD11b-FITC-conjugated IgG1 antibody, CD64-FITC-conjugated IgG1 antibody, and CD16b-FITC-conjugated IgM antibody. The following mouse monoclonal antibodies were obtained from Invitrogen (Carlsbad, California, USA): CD18-FITC-conjugated IgG1 antibody and CD95-FITC-conjugated IgG1 antibody. In brief, each antibody was added to granulocyte concentrates and mixed gently. After 30 min of incubation at 4 °C, erythrocyte lysis was carried out twice. Precipitated granulocytes were washed in PBS and centrifuged at 750*g* for 5 min, and the resulting cell pellets were resuspended in PBS. These samples were analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, Tokyo, Japan). Forward and side scatter gating was performed to retain granulocytes and to exclude monocytes and lymphocytes. Results were presented as mean fluorescence intensity (MFI), which is defined as the mean amount of cell surface antigens per cell.

2.6. Measurement of apoptosis

Apoptosis of granulocytes was evaluated by flow cytometry using an annexin V-FITC apoptosis assay kit (Beckman Coulter, Fullerton, CA, USA) at time points of 24, 48, and 72 h after collection.

In brief, an erythrocyte-lysing reagent (NH_4Cl) was added to granulocyte concentrates and the concentrates were incubated for 15 min at room temperature in the dark. After centrifugation at 500*g* for 5 min at 4 °C, the precipitated granulocytes were used as samples. The samples were washed with PBS once, then they were centrifuged at 500*g* for 5 min at 4 °C. After discarding the supernatant, the cell pellets were resuspended in an ice-cold 1× binding buffer. Then, annexin V-FITC solution and PE-labeled CD13 were added to a granulocyte suspension, and the suspension was mixed gently. The granulocyte suspension in tubes was kept on ice for 15 min in the dark, after which ice-cold 1× binding buffer was added and mixed gently. The samples were analyzed using a flow cytometer (FACS Calibur, Becton Dickinson, Tokyo, Japan). The cells double-positive for annexin V and CD13 were counted as apoptotic granulocytes, and apoptosis was expressed as the percentage of double-positive cells with respect to the total number of CD13-positive cells. Apoptosis of granulocytes was also assessed by light microscopy. The samples of granulocyte concentrates were spotted on glass slides and stained with May–Grünwald–Giemsa stain. Cells showing the morphologic features of apoptosis (nuclear condensation and fragmentation) were counted at time points of 0, 24, 48, and 72 h of storage after granulocyte collection.

2.7. Statistics analyses

All values are expressed as mean ± standard deviation. The collected data were evaluated by two-factor repeated measure analysis of variance (two-factor ANOVA). If we found no significant interaction between storage time and mobilization (with or without G-CSF), we used multiple *t*-tests with the Tukey test for multiple comparisons. If we found interaction between storage time and mobilization, we revaluated the data by one-factor analysis of variance (one-factor ANOVA), and then used multiple *t*-tests with the Tukey test for multiple comparisons. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Granulocyte concentrates

Two-hundred milliliters of whole blood was processed by the bag separation method and approximately 25–40 mL of granulocyte concentrate was obtained. The recovery rate of granulocytes of the G-CSF mobilized group and that of the control group were 54.5 ± 11.4% and 53.2 ± 17.1%, respectively. The granulocyte count per

200 mL of whole blood samples of the G-CSF mobilized group and that of the control group were $4.8 \pm 1.1 \times 10^9$ and $8.2 \pm 2.7 \times 10^8$, respectively. The granulocyte count per obtained granulocyte concentrates of the G-CSF mobilized group and that of the control group were $2.6 \pm 0.6 \times 10^9$ and $4.2 \pm 1.6 \times 10^8$, respectively.

3.2. Immunophenotyping of granulocytes

CD11b, CD18, CD64, CD16b, and CD95 expressions did not show a significant interaction between storage time and mobilization. Only the expressions of CD62L showed interaction between storage time and mobilization. The CD11b expression level in the G-CSF mobilized group was greater than that in the control group at 48 and 72 h after collection. The CD11b expression level increased in a time-dependent manner for up to 72 h after collection in both groups. The CD16b expression level in the G-CSF mobilized group was less than that in the control group. The CD62L expression level in the G-CSF mobilized group was less than that in the control group at 24 h. The CD62L expression level decreased in a time-dependent manner for up to 72 h after collection in the control group, whereas it remained unchanged during storage for up to 72 h in the G-CSF mobilized group. The CD64 expression level in the G-CSF mobilized group was greater than that in the control group (Fig. 1).

3.3. Apoptosis

The percentage of CD13-positive granulocytes of the G-CSF mobilized group and that of the control group at 24 h were 86.0 ± 9.4% and 52.8 ± 11.0%, respectively. No significant interaction between storage time and mobilization was observed for the annexin V-positive cells. The number of both annexin V- and CD13- positive cells increased in a time-dependent manner for up to 72 h after collection in both groups.

As shown in Fig. 2A, the percentage of double-positive granulocytes in the G-CSF mobilized group and those in the control group were 13.3 ± 7.3% and 6.1 ± 3.1% at 24 h, 18.7 ± 7.2% and 15.2 ± 6.1% at 48 h, and 23.0 ± 2.0% and 19.8 ± 2.5% at 72 h, respectively. The values of both annexin V and CD13 positive cells in the G-CSF mobilization group was significantly greater than those in the control group at all observation time points. In light microscopy, the percentage of apoptotic cells increased 72 h after collection in both groups. As shown in Fig. 2B, the percentage of apoptotic cells in the G-CSF mobilized group and those of the control group were 0.24 ± 0.22% and 0.2 ± 0.27% at 0 h, 0.6 ± 0.65% and 1.0 ± 0.61% at 24 h, 1.9 ± 1.1% and 8.7 ± 10% at 48 h, and 9.4 ± 4.2% and 27.1 ± 21% at 72 h, respectively. The percentage of apoptotic cells in the G-CSF mobilized group was less than that in the control group at all observation time points.

4. Discussion

In this study, we evaluated the changes in surface antigen expression levels on granulocytes and the apoptosis of granulocytes, which were collected by the bag separation method and stored in whole blood.

Concerning adhesion molecules on granulocytes, CD11b/CD18 (Mac-1) expression level has been shown not to change during storage for up to 48 h [6] or to increase during storage for up to 24 h [7]. On the other hand, CD62L (L-selectin) expression level has been shown to decrease during storage for up to 48 h [8,9]. In this study, the CD62L expression level in the G-CSF mobilized group did not change during storage, which suggests that the adhesion ability of granulocytes mobilized by G-CSF is maintained for up to 72 h. The CD64 (Fcγ Receptor I) and CD16b (Fcγ Receptor III) play an important role in phagocytosis, and their expressions on granulocytes that were collected from G-CSF-mobilized donors have been shown not to change during storage for up to 48 h [6]. In this study, the expressions of CD64 and CD16b were also unchanged during storage for up to 72 h in both groups. These findings support our previous finding that the phagocytosis level of granulocytes is maintained in vitro for up to 72 h after collection [3]. In general, granulocytes are very abundant, short-lived polymorphonuclear leukocytes, which survive in the circulation for 24–36 h before undergoing apoptosis [10]. Annexin V enables the detection of cells undergoing apoptosis based on the changes in the expression levels of negatively charged lipids by flow cytometric assay. In the early phase of apoptosis, cell membrane integrity is maintained, but the symmetry of cell membrane phospholipids is lost, and phosphatidylserine (PS), which is usually located in the inner leaflet of the cell membrane, is exposed at the surface of the cells. Annexin V thus enables the detection of early apoptotic cells via high affinity binding to PS in the presence of Ca2+. In this study, the number of granulocytes stained by annexin V increased for up to 72 h after collection, indicating that the number of apoptotic cells increases in a time-dependent manner during storage. The number of cells with morphologic features of apoptosis has been reported to highly correlate with the percentage of annexin V-positive cells [11,12]. However, in this study, the number of cells with morphologic features of apoptosis was less than the percentage of annexin V-positive cells at any time points of 24, 48, and 72 h after collection. The difference between our findings and previous results may be due to the following: light microscopy reveals the features of cells in the final phase of apoptosis, not those in the early phase, which can be detected by annexin V staining. The number of annexin V-positive granulocytes in the G-CSF mobilized group was significantly greater than that in the control group. Storage of high-cell density environment might affect acceleration of apoptosis. Inversely, the number of granulocytes with morphologic features of apoptosis in the G-CSF mobilized group was significantly less than that in the control group. It has been reported that G-CSF delays neutrophil apoptosis in vitro [13–15], G-CSF might affect the apoptotic process of granulocytes, such as morphologic changes in the late phase.

In this study, granulocytes were collected by the bag separation method and stored in whole blood. Approximately 80% of granulocytes are annexin V negative until 72 h after collection. Hodge et al. have reported that the levels of apoptosis in isolated peripheral blood mononuclear cultures

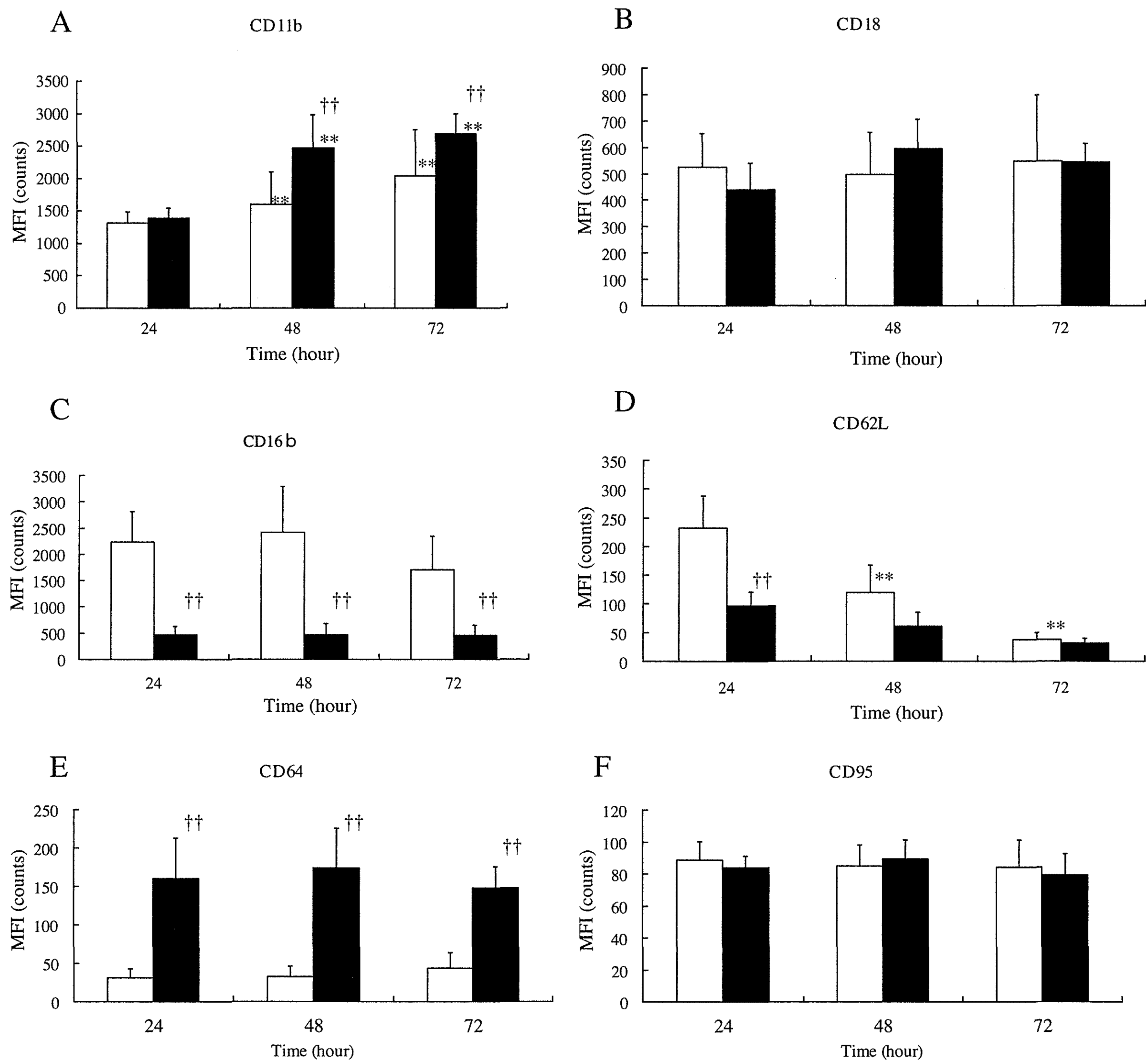

Fig. 1. The expressions of various surface antigens on granulocytes ($n = 5$). Results are presented as mean fluorescence intensity (MFI) (mean ± standard deviation) from 24 to 72 h of storage after granulocyte collection. Open bars indicate the non-G-CSF-mobilized (control) group, and black bars indicate the G-CSF-mobilized group. *Significant difference in comparison with the values at 24 h (*$P < 0.05$, **$P < 0.01$). †Significant difference between the control and the G-CSF mobilization groups (†$P < 0.05$, ††$P < 0.01$).

were significantly greater than those in whole-blood cultures at several cell concentrations [16]. In addition, Hannah et al. have reported that neutrophil survival was enhanced when cells were cultured in a medium containing serum protein [17]. Furthermore, Schwanke et al. have reported that phagocytosis level and oxidative burst were maintained for up to 72 h when granulocytes were stored in autologous plasma [18,19]. Considering the above findings, storage of granulocyte concentrates in whole blood containing autologous plasma may have the benefits of extending granulocyte survival. We collected granulocytes by the bag separation method in whole blood rather than by apheresis to make donation easier for granulocyte donor. Because of this, we couldn't make a comparison between storing in whole blood and storing in various suspension media including autologous plasma alone.

In the present study, the expression levels of surface antigens, which play important roles in phagocytosis and cell adhesion, were shown to be maintained during storage for up to 72 h after collection. Approximately 80% of granulocytes did not bind annexin V for at least 72 h after collection. We previously showed that the phagocytosis level, oxidative killing level, and the viability of granulocytes collected by the bag separation method were well maintained during storage for up to 72 h after collection [3]. Other functional assays, such as those which measure cell migration should be performed in future studies.

We previously showed that the levels of cytokines (IL-1β, IL-6, IL-8, and TNF-α) increased with time for up to 72 h in stored granulocyte concentrates [3]. Bashir et al. also have reported that the levels of IL-1β and IL-8 increased during storage of the pooled granulocyte

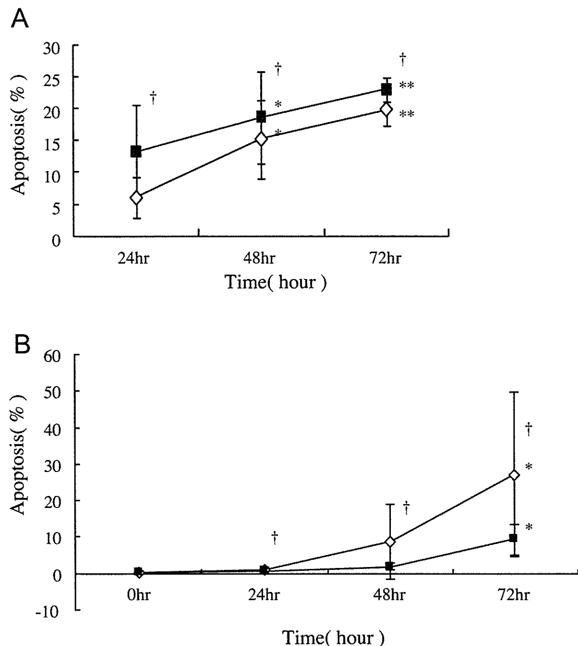

Fig. 2. (A) Comparison of granulocyte apoptosis from 24 to 72 h of storage after granulocyte collection ($n = 5$). Cells were stained with FITC-labeled annexin V and PE-labeled CD13, and were analyzed by flow cytometry. Double-positive cells were counted as apoptotic granulocytes, and the apoptosis was expressed as the percentage of double-positive cells with respect to the total number of CD13-positive cells. The results are presented as mean ± standard deviation. Open diamonds indicate the non-G-CSF-mobilized group, and black squares indicate the G-CSF-mobilized group. *Significant difference in comparison with the values at 24 h ($^*P < 0.05$, $^{**}P < 0.01$). †Significant difference between the control and the G-CSF-mobilized groups ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$). (B) Comparison of granulocyte apoptosis from 0 to 72 h of storage after granulocyte collection, as determined by light microscopy. ($n = 5$). The samples of granulocyte concentrates were stained with May-Grünwald–Giemsa stain, and the number of granulocytes with the morphologic features of apoptosis were counted. Results are presented as mean ± standard deviation. Open diamonds indicate the non-G-CSF-mobilized (control) group, and black squares indicate the G-CSF-mobilized group. There was no statistically significant difference between the two groups. *Significant difference in comparison with the values at 0 h ($^*P < 0.05$, $^{**}P < 0.01$). † Significant difference between the control and the G-CSF-mobilized groups ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$).

component for 40 h [20]. Increased levels of these cytokines have the potential to cause non-immune transfusion-related adverse reactions after GTX.

One limitation of this study is that our samples are small. Further larger studies will be required to better document improvements in the quality of granulocyte concentrates for clinical applications. Clinical studies will be required to demonstrate safety in GTX therapy.

Acknowledgments

A.K. and K.M. were responsible for the conception and design of the study. H.O. and M.H. supervised the study and reviewed the paper. M.A. and K.M. performed the laboratory work for this study. M.A., K.M., K.N., M.I., H.S., and S.K. participated in the data analysis and interpretation. M.A. wrote the paper.

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EBV-associated T/NK–cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases

Hiroshi Kimura, Yoshinori Ito, Shinji Kawabe, Kensei Gotoh, Yoshiyuki Takahashi, Seiji Kojima, Tomoki Naoe, Shinichi Esaki, Atsushi Kikuta, Akihisa Sawada, Keisei Kawa, Koichi Ohshima and Shigeo Nakamura

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EBV-associated T/NK–cell lymphoproliferative diseases in nonimmunocompromised hosts: prospective analysis of 108 cases

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EBV-associated T/NK–cell lymphoproliferative disease (T/NK-LPD) is defined as a systemic illness characterized by clonal proliferation of EBV-infected T or NK cells. We prospectively enrolled 108 nonimmunocompromised patients with this disease (50 men and 58 women; median onset age, 8 years; age range, 1-50 years) evidenced by expansion of EBV$^+$ T/NK cells in the peripheral blood; these were of the T-cell type in 64 cases and of the NK-cell type in 44, and were clinically categorized into 4 groups: 80 cases of chronic active EBV disease, 15 of EBV-associated hemophagocytic lymphohistiocytosis, 9 of severe mosquito bite allergy, and 4 of hydroa vacciniforme. These clinical profiles were closely linked with the EBV$^+$ cell immunophenotypes. In a median follow-up period of 46 months, 47 patients (44%) died of severe organ complications. During the follow-up, 13 patients developed overt lymphoma or leukemia characterized by extranodal NK/T-cell lymphoma and aggressive NK-cell leukemia. Fifty-nine received hematopoietic stem cell transplantation, 66% of whom survived. Age at onset of disease (≥ 8 years) and liver dysfunction were risk factors for mortality, whereas patients who received transplantation had a better prognosis. These data depict clinical characteristics of systemic EBV$^+$ T/NK-LPD and provide insight into the diagnostic and therapeutic approaches for distinct disease. (*Blood*. 2012;119(3): 673-686)

Introduction

EBV-associated lymphoproliferative diseases (LPDs) have a vast spectrum from reactive to neoplastic processes in the transformation and proliferation of lymphocytes spanning B, T, and NK cells,[1-3] and are clinically complicated by the interaction between the biologic properties of EBV$^+$ lymphocytes and the host immune status. Our understanding of these diseases is now evolving and has led to the recognition of a variety of EBV$^+$ diseases, including Burkitt lymphoma,[3] age-related EBV$^+$ B-cell LPD,[4] extranodal NK/T-cell lymphoma of nasal type (ENKL),[5] aggressive NK-cell leukemia (ANKL),[6] classic Hodgkin lymphoma,[3] and immunodeficiency-associated lymphoproliferative disorders.[1] EBV-associated T- and NK-cell LPD (T/NK-LPD) was first incorporated into the 4th World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues, in which systemic EBV$^+$ T-cell LPD of childhood and hydroa vacciniforme–like lymphoma are proposed as distinct entities.[7-8] Historically, based on their broad clinical manifestations, these diseases have been described under various nosological terms from indolent (eg, severe mosquito bite allergy[9] and hydroa vacciniforme[10]) to aggressive or fulminant forms (eg, EBV-associated hemophagocytic lymphohistiocytosis [HLH],[11] chronic active EBV disease [CAEBV] of the T/NK–cell type,[12] fulminant EBV$^+$ T-cell LPD of childhood,[13] and fatal infectious mononucleosis[3]).

CAEBV originally referred to chronic or recurrent infectious mononucleosis-like symptoms.[14-16] A severe form of CAEBV was found to be prevalent in east Asian countries and was characterized by clonal expansion of the EBV-infected T or NK cells,[12,17-18] whereas in Western countries CAEBV is mostly associated with EBV-infected B cells.[19-20] The term EBV-associated HLH was coined to describe hemophagocytosis involving BM or other organs and resulting in pancytopenia in the peripheral blood. This disease is also frequently seen in east Asian countries,[11] and involves a clonal expansion of EBV$^+$ T or NK cells, which produce inflammatory cytokines that induce the activation of macrophages and hemophagocytosis.[21-23] Apart from these systemic diseases, accumulating evidence indicates that 2 cutaneous diseases, hydroa vacciniforme and severe mosquito bite allergy, are closely associated with EBV$^+$ T or NK cells. Hydroa vacciniforme is characterized by recurrent vesiculopapules usually occurring on sun-exposed areas and seen in children and adolescents.[10] In some of these patients, systemic symptoms including fever, wasting, lymphadenopathy, and hepatosplenomegaly have been recorded.[24-26] Severe mosquito bite allergy was determined to be associated with EBV$^+$ NK cells, but rarely with EBV$^+$ T cells, and to progress into overt lymphoma or leukemia in the long-standing clinical course.[9,27] These EBV$^+$ cutaneous diseases had the same geographic distribution as the other EBV$^+$ T/NK–cell lymphomas and LPDs among

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Table 1. Definitions of EBV+ T/NK-LPDs in this study

Disease	Eligibility criteria	Exclusion criteria	Lineages/clonality	References
Clinical category				
CAEBV of T/NK–cell type	(1) Illness ≥ 3 mo in duration (EBV-related illness or symptoms including fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme-like eruptions, and hypersensitivity to mosquito bites)*	(1) No evidence of previous immunological abnormalities or other recent infection that might explain the observed condition	T/NK cell	12
	(2) Increased amounts of EBV detected by Southern blot hybridization or EBER+ cells in affected tissues or peripheral blood; ≥ $10^{2.5}$ copies/μg of EBV DNA in PBMCs	(2) Congenital immunodeficiency including X-linked lymphoproliferative disorders	Polyclonal, oligoclonal, monoclonal	16,29
HLH	(1) Clinical criteria (fever and splenomegaly)	(1) Hemophagocytic syndrome in accelerated phase of CAEBV of T/NK cell type	T/NK cell	11
	(2) Laboratory criteria (cytopenia affecting 2 of 3 lineages in the peripheral blood, hypertriglyceridemia, and/or hypofibrinogenemia) (3) Histological criteria (hemophagocytosis in the BM, spleen, or lymph nodes)	(2) Congenital immunodeficiency including familial HLH	Polyclonal, oligoclonal, monoclonal	
Severe mosquito bite allergy*	Hypersensitivity to mosquito bites characterized by high fever after bites, ulcers, necrosis, and scarring*	Any systemic symptoms in addition to the cutaneous lesions were categorized to CAEBV of T/NK cell type	T/NK cell, polyclonal, oligoclonal, monoclonal	9,39
Hydroa vacciniforme*	Recurrent vesiculopapules with central umbilication and crust formation mimicking herpetic vesicles usually occurring on sun-exposed areas	Any systemic symptoms in addition to cutaneous lesions categorized as CAEBV of T/NK cell type	T/NK cell, polyclonal, oligoclonal, monoclonal	10,39
Pathological classification				
Systemic EBV+ T-cell LPD	(1) Illness or symptoms including fever, persistent hepatitis, lymphadenopathy, hepatosplenomegaly, hemophagocytosis, and interstitial pneumonia (2) Can occur shortly after primary EBV infection or in the setting of CAEBV (3) Monoclonal expansion of EBV-infected T cells with an activated cytotoxic phenotype in tissues or peripheral blood	Other overt leukemia and lymphoma such as extranodal NK/T-cell lymphoma, aggressive NK-cell leukemia, and peripheral T-cell lymphoma	T-cell, monoclonal	7
Hydroa vacciniforme-like lymphoma	(1) Recurrent vesiculopapules with central umbilication and crust formation usually occurring on sun-exposed areas with or without systemic symptoms including fever, wasting, lymphadenopathy, and hepatosplenomegaly (2) Monoclonality of EBV-infected cells	Other overt leukemia and lymphoma such as extranodal NK/T-cell lymphoma, aggressive NK-cell leukemia, and peripheral T-cell lymphoma	T/NK cell, monoclonal	7

*"Severe mosquito bite allergy" and "hydroa vacciniforme" were used as clinical categories, whereas "hypersensitivity to mosquito bites" and "hydroa vacciniforme-like eruptions" were used to designate symptoms.

east Asians and Native Americans in Central and South America and Mexico,[8] and were encountered as a part of the initial and accompanying symptoms of the systemic EBV+ T/NK-LPDs.[28-30] However, the mutual relationship and clinicopathologic distinctiveness of these EBV+ T/NK-LPDs are unfounded, posing diagnostic and therapeutic problems for pathologists and hematologists, respectively. These patients appear to exist in the gray zone between systemic EBV+ T-cell LPD of childhood and hydroa vacciniforme–like lymphoma according to the 4th WHO classification. The former encompasses CAEBV of T-cell type, EBV+ HLH, and EBV+ T-cell lymphomas with prodromal phase, whereas the latter may include all cases with EBV+ hydra vacciniforme despite the presence or absence of the systemic disease in the patient's history.

The aim of the present study was to clarify the clinicopathologic characteristics of these EBV+ T/NK-LPDs and the biologic properties of the proliferating cells by analyzing a large number of patients. We previously performed a nationwide survey for CAEBV of T/NK–cell type and determined its prognostic factors.[29] Similarly, a nationwide study for HLH was recently performed in Japan.[31] However, these studies were retrospective and lacked the precise diagnosis of the current level because of their study design. In 1998, we established an EBV-DNA quantification system using real-time PCR,[32-33] which allowed for the determination of the phenotype of EBV-infected cells in the peripheral blood with the combination of fractionation to the lymphocyte subset.[12,34-35] More recently, we developed the simultaneous staining method for surface antigens and nuclear EBV-encoded small RNA (EBER) to more precisely determine EBV-infected cell phenotypes.[36] Using these techniques, we enrolled and prospectively followed patients with definitive cases of EBV+ T/NK-LPDs in 1998. In this study, 108 nonimmunocompromised patients with EBV+ T/NK-LPDs were analyzed for clinical and virological characteristics to obtain an understanding of their pathogenesis and for refining their

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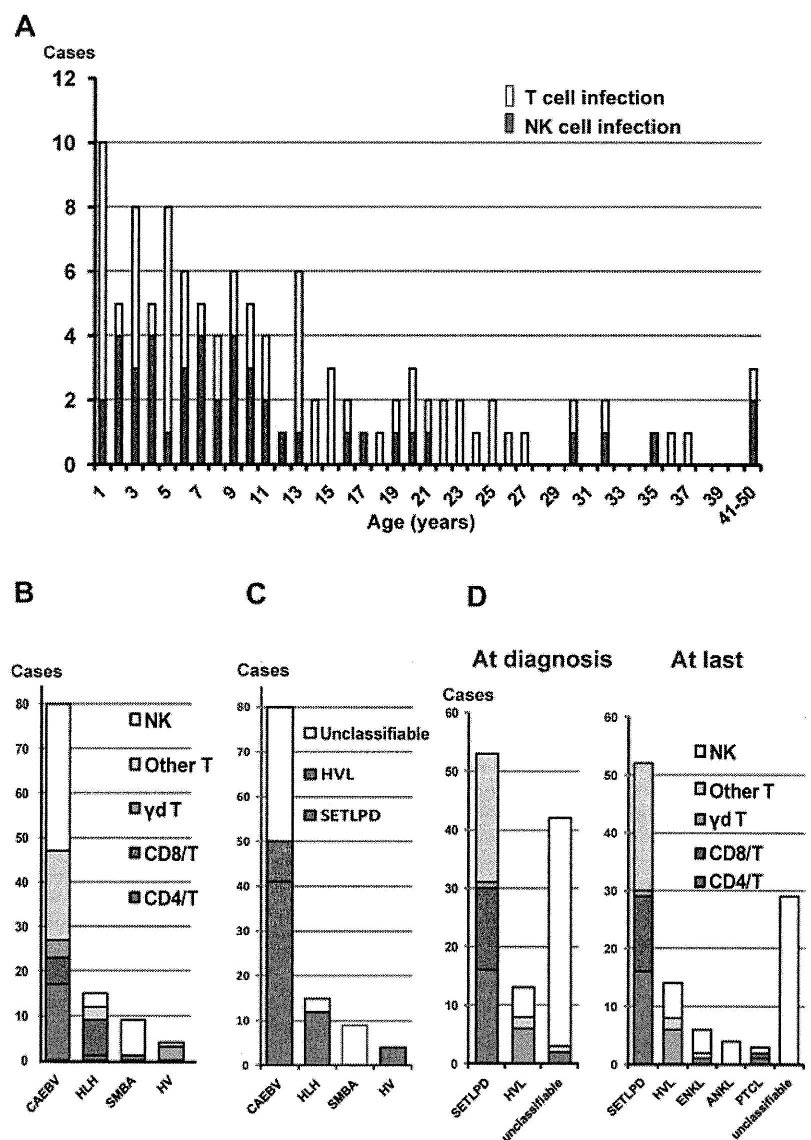

Figure 1. EBV-infected cell phenotypes of EBV+ T/NK lymphoproliferative diseases. (A) Age distribution of patients with T-cell and NK-cell types. (B) EBV-infected cells among categories of clinical groups. Infected T cells were further divided into CD4+ T cells, CD8+ T cells, γδ T cells, and "other T cells." The 25 cases of "other T cells" were defined as either phenotypically different T-cell subsets (2 patients were CD4−CD8−, 1 patient was CD4+CD8+, and 1 patient had 2 lineages consisting of CD4+CD8− and CD4−CD8+ cells) or ill-defined T cells (n = 21). In the majority of the ill-defined T-cell patients, Abs against CD4 or CD8 could not be used to define their CD4/CD8 phenotype because the number of recovered PBMCs was not sufficient. SMBA indicates severe mosquito bite allergy; and HV, hydroa vacciniforme. (C) The 4th WHO pathologic classification of each clinical group at the time of diagnosis. SETLPD indicates systemic EBV+ T-cell lymphoproliferative disease of childhood; and HVL, hydroa vacciniforme–like lymphoma. (D) EBV-infected cells among categories of the pathologic classification at diagnosis and at the last follow-up or death. Patients in CR were classified according to the data and status before remission.

classification. Furthermore, prognostic factors and the efficacy of therapeutic interventions including hematopoietic stem cell transplantation (HSCT) were analyzed.

Methods

Eligibility criteria

Informed consent was obtained from all participants or their guardians in accordance with the Declaration of Helsinki. This study was approved by the institutional review board of Nagoya University Graduate School of Medicine. From 1998 to 2010, patients whose samples were sent to Nagoya University Graduate School of Medicine for determination of the EBV-infected cell phenotype and who fulfilled the following criteria were prospectively enrolled in this study: (1) EBV-associated T/NK-LPD suspected or diagnosed based on clinical and/or histopathological findings; (2) high EBV load detected in PBMCs by quantitative PCR ($\geq 10^{2.5}$ copies/μg of EBV-DNA)[12,32]; and (3) EBV infection in T or NK cells in the peripheral blood confirmed by either immunobead sorting followed by quantitative PCR[34-35] or FISH.[36] Exclusion criteria were: (1) pathologically defined ENKL,[5] ANKL,[37] or peripheral T-cell lymphoma (PTCL)[38]; (2) congenital immunodeficiency; (3) HIV positivity; and (4) other immunodeficiencies requiring immunosuppressive therapies or underlying diseases with potential immunosuppression. Patients were recruited through an announcement by the Japanese Association for Research on Epstein-Barr Virus and Related Diseases and on the homepage of our institute's website. Approximately 240 hematology units and 400 departments of pediatrics were included in the association.

On entry into the study, peripheral blood was collected and sent to Nagoya University Graduate School of Medicine to examine EBV-DNA quantification and EBV-infected cell determination along with detailed clinical data. Clonality analyses were also performed at this time if possible. Primary EBV infection was determined based on serological findings, detection of antiviral capsid Ag-IgM, and seroconversion of either antiviral capsid Ag-IgG or anti-EBV nuclear Ag. A total of 108 patients from 40 hospitals were enrolled in the study (25 from Nagoya University Hospital, 13 from Osaka Medical Center and Research Institute for Maternal and Child Health, 9 from Fukushima Medical University, and 61 from other hospitals). Each patient enrolled in the study was treated according to physician decision at each hospital. The physicians completed questionnaires regarding the administered treatment and outcome every 3 years (2001, 2004, and 2007); the final questionnaire was sent and collected in December 2010. Compared with data provided by previous national surveys for CAEBV and HLH,[29,31] we estimated that approximately 15%-20% of systemic EBV+ T/NK-LPD cases during the study period were recruited by this registry.

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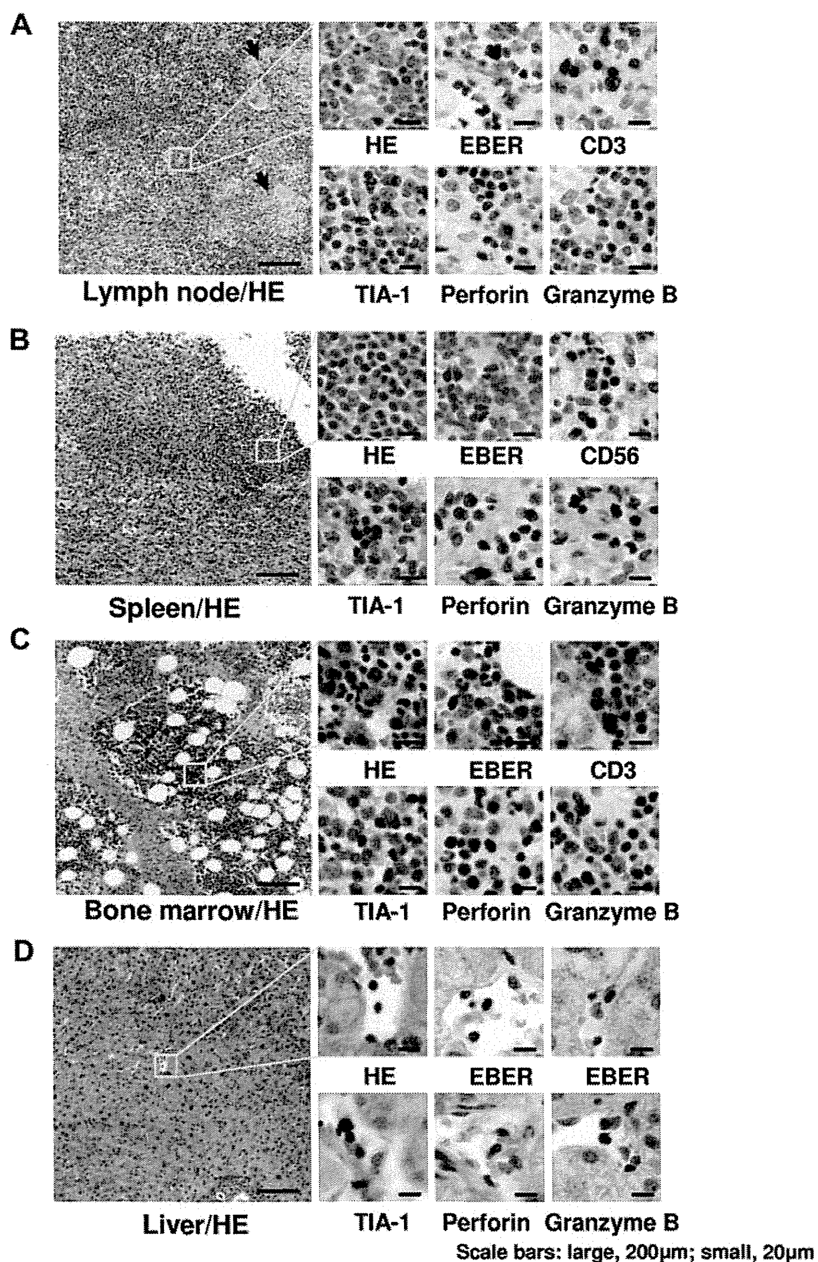

Figure 2. Histopathological findings of representative patients. (A) Cervical lymph node from a 6-year-old boy with chronic active EBV disease with T-cell infection (patient 3). Follicles and paracortical hyperplasia including a mild increase in transformed lymphocytes were seen. Focal epithelioid reactions were detected (arrows). Medium-sized transformed lymphocytes in the paracortex were positive for EBER. TIA-1 and perforin were positive, but granzyme B was negative. (B) Spleen from a 13-year-old boy with chronic active EBV disease with NK-cell infection (patient 6). White pulp was atrophic and red pulp showed congestion. Small lymphocytes infiltrating in the red pulp were positive for EBER. TIA-1 and perforin were positive, but granzyme B was negative. (C) BM from a 25-year-old female with chronic active EBV disease with T-cell infection (patient 17). In the mild hyperplastic BM, small lymphocytes were positive for EBER. TIA-1, perforin, and granzyme B were positive. (D) Liver from a 42-year-old female with chronic active EBV disease with NK-cell infection (patient 60). Small lymphocytes infiltrating in vessels and sinusoid were positive for EBER. TIA-1, perforin, and granzyme B were positive. HE indicates H&E staining. Images of sections were obtained by a microscopy (BX50, Olympus Corp) with CCD camera (D5-5M-L1, Nikon Corp). Each micrograph was represented at either a 100× or 400× magnification using 10× or 40× objective lens (UPlanFL, Olympus Corp), respectively.

Patient criteria

Patients were clinically divided into 4 groups according to the clinical categorization at the 2008 National Institutes of Health meeting: (1) CAEBV of T/NK–cell type, (2) EBV-associated HLH, (3) hydroa vacciniforme, and (4) severe mosquito bite allergy.[39] The clinical diagnosis was made at entry into the study. Definitions of each clinical category are listed in Table 1. CAEBV was defined according to previously proposed criteria.[16,29] HLH was defined based on the criteria proposed by an international treatment study group.[11] Severe mosquito bite allergy and hydroa vacciniforme were applied for cases with only skin symptoms and lacking systemic symptoms. In this study, "severe mosquito bite allergy" and "hydroa vacciniforme" were used as clinical categories, whereas "hypersensitivity to mosquito bites" and "hydroa vacciniforme–like eruptions" were used as terms for symptoms; "hydroa vacciniforme–like lymphoma" was used as a term for pathologic classification.

Patients were also classified according to the 4th WHO classification for tumors of hematopoietic and lymphoid tissues.[7] The definitions of pathologic classification are listed in Table 1. The classification was made both at the diagnosis and at the last follow-up or death. Patients diagnosed with ENKL, ANKL, or PTCL were excluded from the study, but some developed these diseases during the follow-up period. Of 108 patients, 54 were biopsied (liver, n = 15; skin, n = 15; lymph nodes, n = 10; intestine, n = 3; spleen, n = 2; muscle, n = 2; others, n = 7), and 6 were autopsied. For differential diagnosis, BM examination was performed in most patients (79%), even though there were no hematologic abnormalities of the peripheral blood. When abnormal findings were detected in BM or peripheral blood, EBER/immunohistochemical staining was performed. Histopathology was reviewed by the Central Pathology Review Board (Shigeo Nakamura, Nagoya University and Koichi Ohshima, Kurume University).

Disease status was defined as follows: stable disease, partial remission (PR), and complete remission (CR). Patients with PR had no symptoms but had significant EBV loads in PBMCs (EBV-DNA $\geq 10^{2.5}$ copies/μg of DNA).[12,32] CR patients had no symptoms and continuously low or no EBV loads in PBMCs (EBV-DNA $< 10^{2.5}$ copies/μg DNA). Disease activity was assessed before HSCT and was classified as either active or inactive as described previously.[40] Active disease was defined by the existence of symptoms and signs such as fever, persistent hepatitis, lymphadenopathy,

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Table 2. Comparison of characteristics based on EBV-infected cell type in 108 patients with EBV+ T/NK-LPD

	Total cells (n = 108)	T cells (n = 64)	NK cells (n = 44)	P*
Sex (male/female)	50/58	27/37	23/21	NS
Age at disease onset, y	12.1 ± 10.6	12.7 ± 10.3	11.3 ± 11.0	NS
Clinical category at diagnosis, n				
CAEBV	80	47	33	NS
HLH	15	12	3	.066
Severe mosquito bite allergy	9	**1**	**8**	**.003**
Hydroa vacciniforme	4	4	0	NS
Past history of infectious mononucleosis, n (%)	37 (34)	24 (22)	13 (12)	NS
Primary infection at diagnosis, n (%)	19 (18)	**16 (15)**	**3 (3)**	**.012**
EBV DNA quantity in peripheral blood at diagnosis				
Mononuclear cells, log copies/μg DNA, mean	4.3 ± 0.9	4.2 ± 0.9	4.5 ± 0.8	NS
Plasma, log copies/mL, mean	3.3 ± 1.7	3.5 ± 1.6	3.1 ± 2.0	NS
EBV clonality, monoclonal/oligoclonal/polyclonal	64/8/4	36/4/3	28/4/1	NS
TCR rearrangement, any rearrangement/none	42/48	**36/20**	**6/28**	**< .001**
Chromosomal aberration (abnormal/normal cases)	6/84	4/50	2/34	NS
Symptoms and signs at diagnosis, n (%)				
Fever	98 (91)	59 (92)	39 (89)	NS
Liver dysfunction	83 (77)	49 (77)	34 (77)	NS
Splenomegaly	64 (59)	39 (61)	25 (57)	NS
Thrombocytopenia	47 (44)	26 (41)	21 (48)	NS
Anemia	46 (43)	29 (45)	17 (39)	NS
Lymphadenopathy	41 (38)	27 (42)	14 (32)	NS
Hemophagocytic syndrome	38 (36)	23 (36)	15 (34)	NS
Hypersensitivity to mosquito bites (HMB)	32 (30)	**3 (5)**	**29 (43)**	**< .001**
Hydroa vacciniforme-like eruption (HV-LE)	15 (14)	8 (13)	7 (16)	NS
HMB+HV−LE+	5 (5)	**0 (0)**	**5 (11)**	**.001**
HMB−HV−LE+	10 (9)	**8 (13)**	**2 (5)**	NS
Chemotherapy, n (%)	70 (65)	45 (70)	25 (57)	NS
HSCT, n (%)	59 (55)	32 (50)	27 (61)	NS
Outcome, n (%)				
Dead	47 (44)	27 (42)	20 (45)	NS
Alive	61 (57)	37 (58)	27 (61)	NS
Stable disease	11 (10)	8 (13)	3 (7)	NS
Complete remission	46 (43)	26 (41)	20 (20)	NS
Partial remission	4 (4)	3 (5)	1 (2)	NS

NS indicates not significant.

*$P < .10$ are shown; $P < .05$ (shown in bold) are statistically significant.

hepatosplenomegaly, pancytopenia, or progressive skin lesions along with an elevated EBV load in the peripheral blood. Liver dysfunction was defined as an increase in alanine transaminase levels to 2 times above the upper limit of normal on at least 2 consecutive occasions.

Analyses of EBV and determination of EBV-infected cells

DNA was extracted from 1×10^6 PBMCs or 200 μL of plasma and real-time quantitative PCR was then performed as described previously.[12,32] EBV clonality was assessed by Southern blotting with a terminal repeat probe, as described previously.[12,41] To determine which cell population harbored EBV, either immunobead sorting followed by quantitative PCR or FISH assay was performed. For the former method, PBMCs were fractionated into CD3+, CD4+, CD8+, CD16+, CD19+, CD56+, TCRαβ+, and TCRγδ+ cells using an immunobead method (IMag Cell Separation System; BD Biosciences) that resulted in 97%-99% purity.[34-35] Purified cells were analyzed by real-time quantitative PCR. The infected-cell phenotypes were determined in comparison with unfractionated (whole) PBMCs, as described previously.[34-35] For example, patients were defined as CD3+ when CD3+ cells contained higher amounts of EBV DNA than whole PBMCs. The FISH assay was performed as described previously.[36] Briefly, PBMCs were stained with fluorescence labeled mAbs against surface marker, fixed, permeabilized, and hybridized with EBER-specific PNA Probe/FITC (Y5200; Dako). After enhancing fluorescence, stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest Version 5.1.1 software (BD Biosciences). More than 0.1% of EBER+ cells was considered to be significant and such subset was designated EBV+. This frequency was chosen based on previous data using EBV+ cell lines.[36]

TCR gene rearrangement

TCR gene rearrangement was determined by multiplex PCR using the T-cell Gene Rearrangement/Clonality assay (InVivoScribe Technologies), which was developed and standardized in a European BIOMED-2 collaborative study.[42]

Histopathology

Immunostaining was performed using an avidin-biotin peroxidase complex method with mAbs against CD3 (Dako), CD56 (Novocastra Laboratories), perforin (Novocastra Laboratories), T cell–restricted intracellular Ag 1 (TIA-1; Immunotech), and granzyme B (Monosan).[43] FISH was performed using the EBER probe (Dako) as described previously.[43] Hybridization was detected using mouse monoclonal anti-FITC Ab (Dako) and a Vectastain ABC kit (Vector).

Statistical analysis

Statistical analysis was performed using SPSS for Windows Version 18.0. For univariate analysis, either the χ^2 or the Fisher exact test (single-sided) was used to compare categorical variables. The Mann-Whitney U test was used to compare quantitative variables. Logistic regression analysis was used for multivariate analysis. Comparison between quantities of EBV-DNA in PBMCs and plasma was performed by regression analysis. The Kaplan-Meier method and the log-rank test were used for survival analysis. $P < .05$ was considered statistically significant for all analyses.