recent reports suggested a possible association of some LCVs with the regions implicated in cancer development (Iafrate et al., 2004; Sebat et al., 2004), the precise role of the LCVs detected in the current analysis in the pathogenesis of CML is still unclear and should be addressed in future studies that would include a larger number of normal subjects.

Although array CGH analysis successfully unveiled cryptic genomic aberrations in CML, we should note that it also has limitations in that the tumor content of the samples clearly affected the sensitivity of detecting copy number changes in tumor components. According to our admixture experiments, in which mixed tumor and normal DNA were tested for detection of a trisomy, the threshold of tumor content for detection of trisomies in our array CGH was estimated to be more than 20%–40% tumor components (data not shown). Thus, the trisomy 8 in AP3 and the monosomy 21 in AP4 as revealed by G-banding analysis were not expected to be detected in array CGH analysis because abnormal metaphases were found in only 2 of 20 with AP3 and 5 of 20 with AP4 (Tables 1 and 2). On the other hand, array CGH failed to detect the loss of chromosome 21 found in 17 of 20 metaphases in G-banding analysis in BC26, which was most likely a result of karyotypic overrepresentation of one or more rapidly proliferating tumor subclones in G-banding analysis. Finally, the FISH Mapped Clones V1.3 collection distributed from BACPAC Resources Center, which we used for array construction, does not cover some regions of particular interest in CML pathogenesis. For example, deletions of the 5′ region of the *ABL/BCR* junction on the der(9) chromosome, which is known to affect 10%–15% of the CML patients (Storlazzi et al., 2002), were missed in this study because our Human 1M arrays did not contain BAC clones including the *ABL* gene or the upstream *ASS* gene.

In conclusion, our array CGH analysis disclosed not only common chromosomal abnormalities, but also small, cryptic copy number alterations in CML genomes that were not detected by conventional analysis. It enabled a better description of genetic alterations in CML, which potentially could be applicable to molecular diagnostics and prediction of disease prognosis of this neoplastic disorder. The submicroscopic copy number alterations detected in this study might contribute to the identification of novel molecular targets implicated in the pathogenesis or disease progression of CML. Further studies with whole-genome tiling arrays having much higher resolutions will help to detect precisely the genes involved in the disease progression of CML.

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JAC

Pharmacokinetics of ganciclovir in haematopoietic stem cell transplantation recipients with or without renal impairment

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***Objectives*: We investigated the pharmacokinetics of ganciclovir in 12 haematopoietic stem cell transplantation (HSCT) recipients to evaluate the validity of a 50% reduction in the ganciclovir dosage for mild renal impairment.**

***Patients and methods*: Ganciclovir at 5 mg/kg/day was pre-emptively infused in patients with estimated $CL_{CR} \geq 70$ mL/min (Group A), whereas the dose was reduced to 2.5 mg/kg/day in patients with CL_{CR} between 50 and 70 mL/min (Group B).**

***Results*: The peak concentration was significantly higher in Group A ($P < 0.01$). However, the decrease in the plasma ganciclovir concentration was slower in Group B ($P = 0.09$), and the AUC of all patients in both groups was distributed within a narrow range (25.6 ± 4.77 μg·h/mL), when two patients with exceptionally high AUC values were excluded.**

***Conclusions*: A 50% reduction in ganciclovir appeared to be appropriate for patients with mild renal impairment. Measuring the ganciclovir concentration at 4 h after starting infusion may be adequate for evaluating AUC.**

Keywords: cytomegalovirus, CMV, antigenaemia, antiviral therapy

Introduction

Ganciclovir is the mainstay of antiviral agents in pre-emptive therapy against cytomegalovirus (CMV) disease after allogeneic haematopoietic stem cell transplantation (HSCT).[1] Ganciclovir is mainly excreted from the kidney and about 90% of the administered dose is recovered unchanged in the urine after intravenous (iv) administration.[2] Therefore, total body clearance correlates well with CL_{CR}.[3,4] In HSCT settings, patients frequently develop renal impairment caused by the use of nephrotoxic drugs. A 50% reduction of ganciclovir is recommended in the drug information leaflet for patients with mild renal impairment of CL_{CR} between 50 and 70 mL/min in order to achieve an unchanged AUC. However, the pharmacokinetic profiles of ganciclovir have not yet been fully evaluated in such patients. Therefore, we investigated the validity of this dose reduction by serial evaluation of the plasma ganciclovir concentration.

Patients and methods

Twelve patients (nine men and three women) aged between 23 and 61 years were enrolled in a 12 h pharmacokinetic study of intravenous ganciclovir after ethical approval. The median age and weight were 50.5 years (range 23–61) and 57.5 kg (range 36.7–80.0), respectively. All patients provided informed consent to participate in this study. The underlying disease was acute leukaemia in three patients, chronic myelogenous leukaemia in three patients, myelodysplastic syndrome in two patients and pancreatic cancer in four patients. Five patients received a graft from an HLA-matched relative and seven received a graft from an alternative donor defined as an HLA-mismatched relative or a matched unrelated donor. We calculated CL_{CR} weekly, based on a 24 h urine collection. Patients were classified into two groups according to CL_{CR} evaluated within 1 week before the initiation of ganciclovir administration: Group A included seven patients with $CL_{CR} \geq 70$ mL/min (mean 98.1 mL/min, range 74.9–142.0 mL/min) and Group B included five patients

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Table 1. Pharmacokinetic parameters of ganciclovir in Groups A and B

	Group A (CL_{CR} ≥ 70 mL/min)	Group B (CL_{CR} 50–70 mL/min)	*P* value
C0.5	6.56 (4.39–11.33) μg/mL	4.92 (2.90–10.80) μg/mL	0.37
C1	9.20 (5.50–19.03) μg/mL	4.75 (3.32–6.61) μg/mL	<0.01
C2	4.76 (2.72–12.09) μg/mL	2.38 (2.30–2.73) μg/mL	<0.01
C4	2.58 (1.25–6.30) μg/mL	1.57 (1.37–1.80) μg/mL	0.17
C6	1.69 (0.79–4.89) μg/mL	1.15 (0.90–1.30) μg/mL	0.29
C8	1.22 (0.40–3.99) μg/mL	0.91 (0.64–1.09) μg/mL	0.57
C12	0.62 (0.23–2.88) μg/mL	0.58 (0.39–0.81) μg/mL	0.94
LogC4/C1	–0.66 (–0.73– –0.48)	–0.42 (–0.68– –0.33)	0.09
AUC	29.8 (20.2–111.0) μg·h/mL	24.6 (22.5–28.3) μg·h/mL	0.57
$t_{1/2}$	3.57 (3.36–7.94) h	5.76 (5.05–8.87) h	0.03
CL_{TOT}	3.04 (0.73–4.31) mL/min/kg	1.66 (1.50–1.81) mL/min/kg	0.12

CL_{CR}, creatinine clearance; AUC, area under the concentration curve; $t_{1/2}$, elimination half-life; CL_{TOT}, total body clearance.
C0.5–C12 represent plasma ganciclovir concentrations at 30 min, and 1, 2, 4, 6, 8 and 12 h after start of infusion, respectively.
The values of each parameter are reported as the median and range.

with CL_{CR} between 50 and 70 mL/min (mean 59.1 mL/min, range 51.3–67.4 mL/min).

Antigenaemia assay for CMV infection was performed weekly after engraftment as described previously.[5] Ganciclovir was pre-emptively started when 20 or more positive cells were detected per two slides in patients who received a graft from an HLA-matched relative, whereas it was started when three or more positive cells were detected per two slides in patients who received a graft from an alternative donor. The starting dose of ganciclovir was once daily at 5 and 2.5 mg/kg/day in Groups A and B, respectively, which was infused at a constant rate over 1 h.[6] Venous blood samples were obtained before infusion (C0), 30 min (C0.5) and 1 (C1), 2 (C2), 4 (C4), 6 (C6), 8 (C8) and 12 (C12) h after starting the first-dose infusion. After the blood sample was centrifuged, the plasma was separated and stored at –20°C until measurement of the ganciclovir concentration.

The plasma ganciclovir concentration was measured after solid-phase extraction (SPE) and dilution in mobile phase by reversed-phase HPLC. In brief, plasma samples were heated at 58°C for 30 min to inactivate the virus prior to handling. These samples were then diluted with 0.1 M phosphate buffer (pH 8.0) and applied to disposable C_{18} SPE columns (Bond Elut C18-OH; Varian, Palo Alto, CA, USA) conditioned with methanol and water. The column was washed with 0.1 M phosphate buffer (pH 8.0) and water, and ganciclovir was then eluted by 1.5 mL of 15% methanol. After 0.1 mL of 10 μg/mL guanosine was added as an internal standard, the eluent was injected into the HPLC system (C_{18} column, CAPELL PAK C18 SG 120; Shiseido, Tokyo, Japan; mobile phase: a mixture of 20 mM KH_2PO_4 (pH 2.6) containing 5 mM sodium 1-octanesulfonate and acetonitrile (95 : 5, v/v)]. The flow rate of the mobile phase and the column temperature were 0.8 mL/min and 40°C, respectively. The HPLC was equipped with a photo diode array detector (SPD-M10A vp, Shimadzu, Kyoto, Japan) set at a detection wavelength of 254 nm. This quantitative assay provided a high selectivity for determining a compound in biological samples. It was available for 0.02–5 μg/mL of an analyte in plasma samples. The precision expressed as a coefficient of variation was less than 2.5%, and the accuracy expressed as an error per cent was <±3%. Endogenous sources of interference were not detected from blank plasma.

Pharmacokinetic parameters were calculated by non-compartment modelling using WinNonlin software (version 4.0; Pharsight Corporation). CL_{CR} was normalized to 1.73 m^2 body surface area and AUC was calculated using the linear trapezoidal rules with extrapolation to infinity by standard techniques. The decline ratio was calculated as Log C4/C1 for the evaluation of the decrease in plasma ganciclovir concentration in the distribution phase and early elimination phase, whereas the elimination half-life was calculated from the terminal portion of the slope after C4. The differences between groups were compared using the Wilcoxon (Mann–Whitney)-test. *P* values of less than 0.05 were considered statistically significant. The relationship between the total AUC and plasma ganciclovir concentration at each point after starting infusion was investigated by calculating correlation coefficients r^2 using linear regression analysis after logarithmic transformation because they did not fit a normal distribution.

Results

The median pharmacokinetic parameters and the concentration versus time profile are shown in Table 1 and Figure 1(a). The peak plasma concentration (C_{max}) ranged from 3.32 to 19.03 μg/mL. The C_{max} in Group A was significantly higher than that in Group B (9.20 versus 4.75 μg/mL, $P < 0.01$). There was a borderline significance in the decline ratio between the two groups (–0.66 versus –0.42, $P = 0.09$). Total body clearance in Group B was lower than that in Group A (1.66 versus 3.04 mL/min/kg, $P = 0.12$). Also, the elimination half-life in Group B was significantly longer than that in Group A (5.76 versus 3.57 h, $P = 0.03$). There was no significant difference in AUC between the two groups (29.8 versus 24.6 μg·h/mL, $P = 0.57$). The AUCs of the patients in both groups were distributed within a narrow range (25.6 ± 4.77 μg·h/mL, Figure 1b), when we excluded two patients with exceptionally high AUC values (48.18 and 110.99 μg·h/mL). The CL_{CR} values of these two patients were 74.9 and 87.2 mL/min, respectively. Among the serial ganciclovir concentration measurements, C4 most strongly correlated with AUC ($r^2 = 0.95$, Figure 1c).

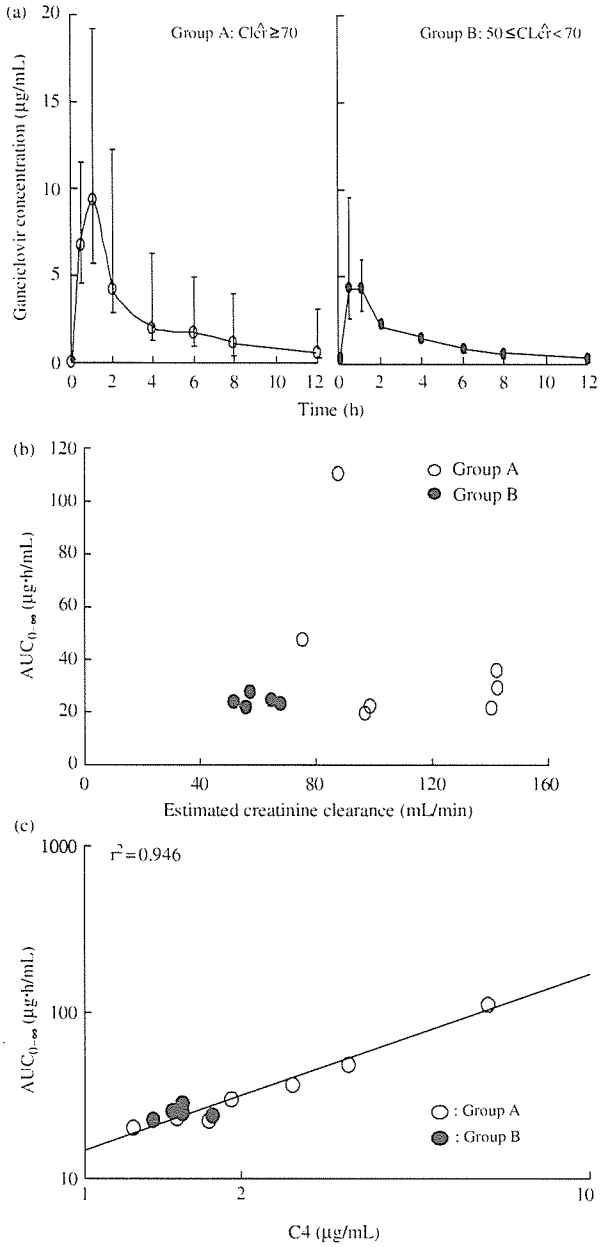

Figure 1. (a) Median concentrations of ganciclovir after 1 h iv infusion of 5 mg/kg ganciclovir in Group A and of 2.5 mg/kg ganciclovir in Group B. Open and filled circles represent each median concentration point in Groups A and B, respectively. (b) The AUC in each patient. Open and filled circles represent individual measurements in Groups A and B, respectively. (c) Correlation between the AUC and the plasma concentration at 4 h after starting infusion (C4). Open and filled circles represent individual measurements in Groups A and B, respectively. The solid line represents the orthogonal regression line described by the equation AUC = 17.666 × C4 − 4.4555.

Discussion

The results demonstrated that a 50% reduction in the ganciclovir dosage was appropriate for HSCT recipients with mild renal impairment of CL_{CR} between 50 and 70 mL/min. In addition to the significant difference in the elimination half-life, we observed a difference in the decline ratio (Log C4/C1) between the two groups with a borderline significance, which might indicate that renal excretion had started within 4 h of infusion. AUC was not significantly different from that in patients with normal renal function, probably due to the prolonged elimination in patients with mild renal impairment, although the small sample size might be responsible for the lack of significant difference. When we excluded two patients whose AUC values were exceptionally high, the AUC ranged within 25.6 ± 4.77 μg·h/mL, which was similar to the values reported previously.[4] An exceptionally high AUC was observed in two patients with CL_{CR} values between 70 and 90 mL/min. The reason for the high AUC is not clear, but it may suggest that the dose of ganciclovir should be reduced in patients with CL_{CR} values between 70 and 90 mL/min after confirming that the AUC is significantly high in such patients. Drug interaction is also a possible explanation for the high AUC, but these two patients were not being given drugs that are known to interact with ganciclovir. Also, the exceptionally high AUC might result from a transient renal dysfunction, which could not be detected even by a weekly CL_{CR} examination.

The role of clinical pharmacokinetic monitoring in solid organ transplantation as well as in HSCT is unclear.[7] Previous studies failed to show a significant correlation between the ganciclovir concentration and its efficacy or toxicity.[7,8] A possible explanation for this lack of correlation is the small number of patients in these studies, since a significant correlation between the cumulative dose of ganciclovir and the incidence of neutropenia has been shown in large-scale clinical studies.[9,10] However, it is difficult to perform a large-scale study with pharmacokinetic monitoring because of the need for repeated blood sampling from patients. In this study, C4 most strongly correlated with AUC, with r^2 values of 0.95, although we should confirm this in a larger study. Another limitation of pharmacokinetic monitoring of ganciclovir is that only the intracellular phosphorylated ganciclovir is active and it is not known how its concentration relates to the plasma concentrations. Nevertheless, a prospective study with monitoring of C4 is warranted to evaluate the role of pharmacokinetic monitoring in HSCT.

In conclusion, a recommended reduction of ganciclovir dosage by 50% appeared to be appropriate for HSCT recipients with mild renal impairment. Measurement of the plasma ganciclovir concentration C4 could be an accurate predictor of AUC. Further studies are necessary to validate these findings in a larger number of patients and to clarify the relationship among plasma concentrations, AUC and responses.

Transparency declarations

None to declare.

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ORIGINAL ARTICLE

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Treatment results of chemoradiation therapy for localized aggressive lymphomas: a retrospective 20-year study

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Abstract In this study we analyzed our cases of localized aggressive lymphoma treated in our institution during the last 20 years to compare the finding of this study with those of previous studies. Forty patients with Ann Arbor stage I–II aggressive lymphoma were treated with 3–6 cycles of a CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisolone) and radiation therapy (30 or 40 Gy with involved field). Between 1985 and 2003, 40 patients with stage I (N=25) or stage II (N=15) disease were treated. Chemotherapy mainly preceded radiotherapy, although the sequence of radiotherapy and chemotherapy was determined by individual physicians and patients' choice. Median and mean age was 50.5 and 48.6 years, respectively, at the time of diagnosis, with a male to female ratio of 19:21. Analyses were undertaken to determine (1) response to treatment according to age, international prognostic index (IPI), lactate dehydrogenase (LDH) value, serum interleukin 2 receptor (sIL-2R) value, cell type, stage, extent of maximum local disease, with or without mediastinal lymph nodes, number of sites, anatomic distribution, and irradiation dose, and (2) relapse patterns. Complete follow-up was obtained in all patients. The follow-up period of surviving 33 patients ranged from 24.7 to 180 months with a median of 69 and a mean of 72.7 months. A complete remission (CR) was achieved in 37 patients (93%). A study of relapse patterns after a CR showed that four patients had a first relapse within a radiation field and the other one patient had an extranodal distant relapse. Significant prognostic factors were not identified by multivariate analysis. Combined chemotherapy and radiation therapy is safe, highly effective, and probably curative for most patients with stage I–II aggressive lymphoma.

Keywords Aggressive lymphoma · Stage I–II · Combined chemotherapy and radiation therapy · CHOP regimen · Localized lymphoma · Non-Hodgkin's lymphoma

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Introduction

Throughout the past 25 years, the treatment of limited-stage diffuse aggressive non-Hodgkin's lymphoma has evolved from surgical staging and radiotherapy (RT) to primary chemotherapy (CTx) and limited RT [1–5].

Some investigators have suggested that adjunctive RT is not indicated for most patients in the management of DLCL [6–8]. Others, in an effort to reduce the incidence of local recurrence and possibly improve survival, have added RT to their CTx programs particularly for patients with bulky or other unfavorable Stage II presentations [9–16].

CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) has been used as a standard CTx for DLBCL and 3 cycles of CHOP followed by RT has also been accepted for localized DLBCL [17]. There are many reports that favor adjuvant chemotherapy with involved-field RT even for with localized lymphoma [18–21]. This article describes the usefulness of RT combined with CTx in the management of localized aggressive lymphoma.

Materials and methods

Patients

This is a retrospective study. The subjects were 40 patients with localized aggressive lymphoma and treated with combined CTx using CHOP regimen and RT for curative intent as the primary therapy. The cases in which the World

Health Organization (WHO)/Revised European-American Lymphoma (REAL) classification was known were described as it is and the cases unclassifiable with WHO/REAL classification were described according to the Working Formulation classification (Table 1) [22]. Patients with complete surgical resection of lymphoma, treated with RT alone and administered CTx other than CHOP regimen were not eligible. An overview of the patients' characteristics according to clinical parameters is given in Table 2.

Minimal staging included chest radiograph, computed tomography (CT) of the abdomen and pelvis, and single percutaneous bone marrow biopsy and blood studies. Gallium scintigram, ^{18}F 2-fluoro-2-deoxy-D-glucose–positron emission tomography (^{18}F-FDG-PET), and barium swallow were optional.

CTx

The treatment consisted of 3–6 cycles of CHOP (day 1 cyclophosphamide, 750 mg/m2; day 1 doxorubicin, 50 mg/m^2, day 1 vincristine, 1.4 mg/m^2 (capped at 2 mg); and days 1–5 oral prednisone, 100 mg) every 3 weeks. We performed 6 cycles for bulky disease more than 5 cm in diameter and 3 cycles for not bulky disease.

RT

RT was performed with a 6-MV X-ray machine. RT delivered with megavoltage equipment began 3–4 weeks after the last cycle of CHOP, confirming the recovery from the toxicity of CTx. The primary tumor and the metastatic lymph nodes were irradiated for a total dose of 30 Gy in 20 fractions over 4 weeks for cases achieving CR after CTx and 40.5 Gy in 27 fractions over 5.4 weeks for cases having a residual lymphoma.

In our institution, RT was confined to the whole neck, supraclavicular region, and Waldeyer's ring for 13 patients with cervical nodal or Waldeyer's disease (Fig. 1).

Toxicity assessment

Patients were observed weekly during the treatment to monitor toxicity. It was graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0).

Table 1 Histopathology characteristics (n=40)

Histopathology	n (%)
Diffuse large B-cell lymphoma	31 (77.5%)
NK/T-cell lymphoma	2 (5%)
Anaplastic large cell lymphoma	2 (5%)
Peripheral T-cell lymphoma	2 (5%)
Diffuse histiocystic lymphoma	1 (2.5%)
Diffuse medium-sized B-cell lymphoma	1 (2.5%)
Diffuse T-cell lymphoma	1 (2.5%)

Table 2 Patient characteristics (n=40)

Patient characteristic	Number
Age	
<60	28
≥60	12
IPI	
Low risk	29
Intermediate-low risk	11
LDH	
High	20
Normal	20
sIL-2R	
High	16
Normal	13
Cell type	
B	33
T	7
Stage	
I	24
II	16
Tumor diameter (cm)	
<5	14
5–7.5	8
7.5–10	3
≥10	4
Mediastinal LNs	
(−)	34
(+)	6
No. of sites	
<3	36
≥3	4
Anatomic distribution	
Head and neck	24
Supra-diaphragmatic	12
Infra-diaphragmatic	4

Late toxicity was graded according to the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer late radiation morbidity scoring scheme.

Follow-up evaluation

The following evaluations were performed until disease progression every 1 month for the first year after the completion of the protocol treatment, every 2 months for the second year, and every 3–4 months thereafter: physical examination, complete blood count, serum chemistries including soluble interleukin 2 receptor (sIL-2R), β2 microglobuline (β2-MG), and lactate dehydrogenase (LDH). CT scan or FDG-PET was optional.

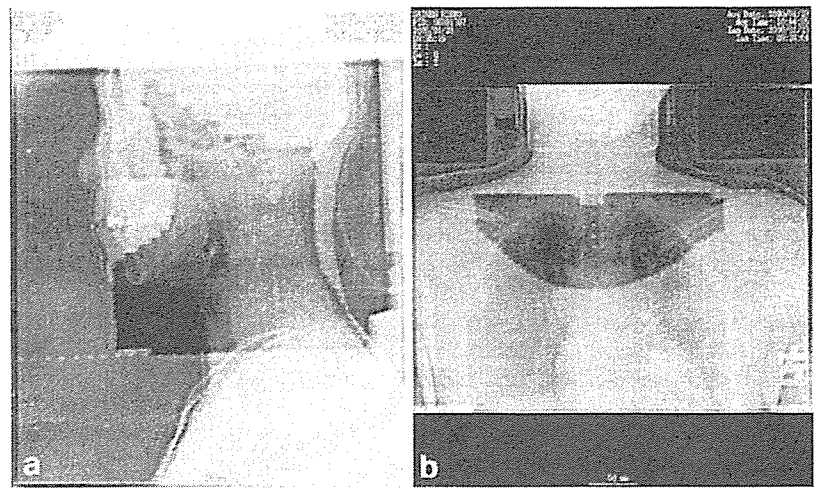

Fig. 1 Linac graphy (LG) for checking RT field for patients with cervical nodal or Waldeyer's disease. *1* upper lateral field, *2* lower anterioposterior field

Response assessment

For the primary tumor and the involved nodes, responses were judged according to the International Workshop Criteria for non-Hodgkin's lymphoma [23]. CR was defined as regression of all palpable nodes (<1×1 cm and of normal consistency) and radiographic disease. Partial response (PR) was defined as ≥50% reduction in the sum of the products of the dimensions of all measurable lesions. Progressive disease (PD) was defined as >50% increase from nadir or the appearance of new lesions. Stable disease (SD) was classified as a response less than PR but not a PD.

Statistical analysis

Statistical analyses were performed using StatView Dataset File version 5.0J for Windows computers (North Carolina, USA). Survival periods were calculated from the start of irradiation. Statistical analysis included the Student *t* test, the Pearson 2 test, and the Fisher exact test as appropriate. The survival functions were estimated with the Kaplan–Meier method estimator, and log-rank tests were used to compare the survival distributions. Univariate Cox proportional hazards models were used to identify the independent factors that predict overall survival.

Results

Patient characteristics

From June 1985 to December 2003, 40 patients were eligible and assessable. The patient characteristics are presented in Tables 1 and 2. Thirty-eight percent of the study population had stage II disease. Extranodal involvement was present at diagnosis in 22 patients (55%), with head and neck sites constituting the vast majority. Few patients had an impaired performance status or systemic symptoms. As expected, approximately 80% of patients had diffuse large-cell lymphoma (*N*=31). A minority of patients (10%) had three or more Ann Arbor sites of disease. The tumor mass was ≥10 cm in 10%. Mediastinal lymphadenopathy was recorded in six patients (15%). CTx mainly preceded RT (*N*=35, 88%), although the sequence of RT and CTx was determined by individual physicians and patients' choice.

Total 6 cycles of CTx was performed in 18 cases (45%), 3 cycles in 19 cases (48%). In the other three cases, CTx was called off in 1 cycle (*N*=2) or 2 cycles (*N*=1) because lymphomas had a resistance to CTx.

Survival

Complete follow-up was obtained in all patients and the last follow-up was performed in December 2005. The follow-up period of surviving 33 patients ranged from 24.7 to 180 months with a median of 69 and a mean of 72.7 months. The overall survival for all 40 patients at 5 year was 81.2% (Fig. 2).

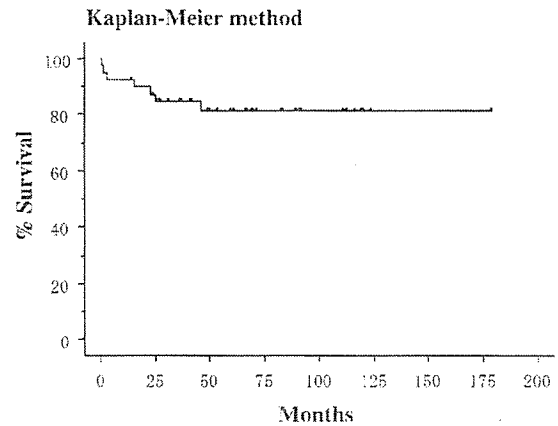

Fig. 2 Overall survival. The estimated 5-year survival rate was 81.2%

Response to the primary therapy

RT preceded CTx in five cases (12%). All the five cases achieved CR at the completion of irradiation before the start of CTx and they were all surviving at this analysis. The survival time was 72, 93, 113, 125, and 180 months respectively. CTx preceded RT in the other 35 cases (88%). In the 35 cases, CR was achieved in 28 cases, PR in two cases, SD in one case, and PD in four cases, after the completion of CTx before the start of RT. As to the seven cases without achieving CR, five cases died in 0.5, 1.2, 3.2, 15.6, and 22.9 months, respectively and the other two cases are surviving at 24.7 and 71.1 months, respectively.

Response to CRT

With application of the above criteria, CR was achieved in 37 patients (93%) and PD in three patients (7%). The three patients died in 0.5, 1.2, and 3.2 months after the completion of irradiation, respectively. All the three patients died of systemic invasion of lymphoma. The first patient died directly of liver dysfunction and sequential multiple organ failure (MOF), the second died of acute liver dysfunction, disseminated intravascular coagulation (DIC), and methicillin-resistant staphylococcus aureus (MRSA) pneumonia, and the third died of infection.

Prognostic factors

A study of relapse patterns after a CR showed that four patients had a first relapse within a radiation field and the other one patient had an extranodal distant relapse with left humeral skin. Significant prognostic factors for overall survival were not identified in age, international prognostic index (IPI), LDH value, sIL-2R value, cell type, stage (Fig. 3), gender (Fig. 4), extent of maximum local disease, with or without mediastinal lymph nodes, number of sites, anatomic distribution, and irradiation dose (Table 3) in the univariate analysis according to Cox proportional hazards models. As to the effect of CTx preceding RT (Fig. 5), CR group (n=28) was a significant better prognosis than non-CR (n=7). Overall survival curves between male and female were a significant difference according to log-rank test (p=0.0296) (Fig. 4). Female group had the significantly more proportion of patients achieving CR from CTx preceding RT (95%) than male group (63%) (p=0.0175). If using modified IPI from a South-west Oncology Group (SWOG) [17] instead of IPI, survival curves between low risk group (n=27) and not low risk group, that is either low-intermediate risk group (n=10) or intermediate-high risk group (n=3), were not of significant difference (p=0.4593). In this study, the number of patients was too small to perform multivariate analysis. The association of other variables with survival was negligible.

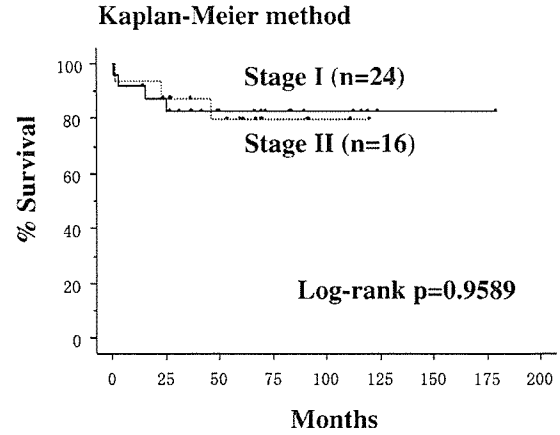

Fig. 3 Overall survival curves of patients between stages I (n=24) and II (n=16)

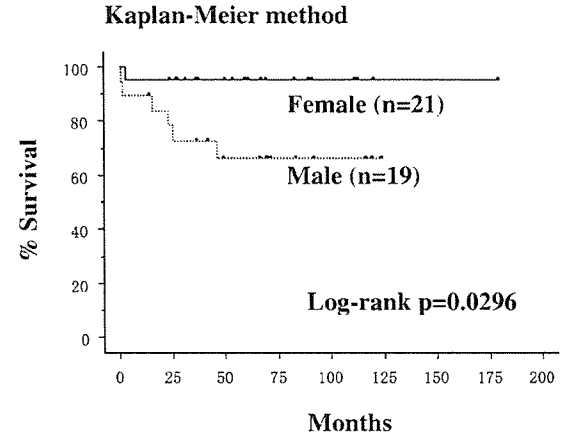

Fig. 4 Overall survival curves of patients between male (n=19) and female (n=21)

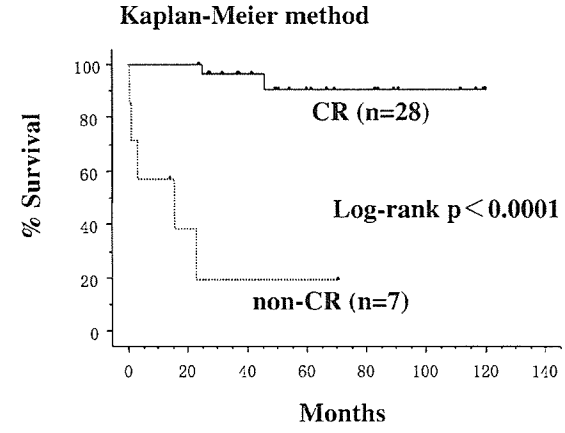

Fig. 5 Overall survival curves of patients between CR (n=28) and non-CR (n=7) of the effect of CTx in patients receiving CTx followed by RT

Treatment-related toxicity

In CTx setting, 32 patients (80%) experienced grade 3 or 4 neutropenia. Grade 3–4 anemia and thrombocytopenia

Table 3 Univariate of relative risks on overall survival in 40 patients

Category	*n*	Univariate analysis		
		Relative risk	95% confidence interval	*p*
Gender				
Male	19	1		
Female	21	0.135	0.016–1.125	0.0641
No. of sites				
<3	36	1		
≥3	4	3.344	0.648–17.24	0.1495
No. of CTx cycle				
3	19	1		
6	18	0.251	0.28–2.252	0.2182
Effect of CTx				
CR	28	1		
Not CR	7	25.64	4.651–142.9	0.0002
RT dose (Gy)				
30	17	1		
40	16	0.859	0.053–13.89	0.9151
Age (years)				
<60	28	1		
≥60	12	0.990	0.192–5.102	0.9909
IPI				
Low	29	1		
Intermediate low	11	1.149	0.22–5.952	0.8685
LDH				
Normal	20	1		
High	20	1.479	0.330–6.622	0.6087
sIL-2R				
Normal	13	1		
High	16	0.492	0.082–2.956	0.4384
Stage				
I	24	1		
II	16	0.784	0.130–4.694	0.9589
Cell type				
B	33	1		
T	7	2.538	0.489–13.16	0.2679
Tumor diameter (cm)				
<5	14	1		
≥5	15	0.621	0.104–3.717	0.6018
Mediastinal LNs				
(−)	34	1		
(+)	6	0.917	0.110–7.634	0.9362
Anatomic distribution				
Supra-diaphragmatic	12	1		
Head and neck	24	2.816	0.329–24.13	0.3448
Infra-diaphragmatic	4	2.713	0.169–43.57	0.4809

occurred in eight patients (5%) and eight patients (5%), respectively. Hematopoietic growth factors were not used routinely.

In RT setting, major toxicity was not seen in the study. All 13 patients, treated with RT field covering over the whole neck, supraclavicular region, and Waldeyer's ring, suffered from grade 1 (mild mouth dryness/may have slightly altered taste such as metallic taste) or grade 2 (moderate to complete dryness/markedly altered taste) acute radiation morbidity on salivary gland. Additionally, those 13 patients experienced grade 1 (slight dryness of mouth; good response on stimulation) or grade 2 (moderate dryness of mouth; poor response on stimulation) late radiation morbidity on salivary gland. No patients developed radiation-induced second malignancy.

The results and the outcome of the subset of patients with T-cell lymphoma were shown in Table 4.

Discussion

The standard therapy for patients with stage IA or contiguous stage IIA and low or low-intermediate grade is the combination of CTx (3–4 cycles of CHOP or R-CHOP) and RT. In a randomized trial by Eastern Cooperative Oncology Group (ECOG)-1484, comparing 8 cycles of CHOP with 8 cycles of CHOP plus RT (30Gy), 10-year disease-free survival was 46 and 57% (p=0.04), and the benefit of tacking on local RT to CTx was proven [24].

SWOG-8736 randomized phase III trial showed a favorable 5-year survival of 82% by 3 cycles of CHOP plus RT (40–55 Gy) compared to that of 72% by 8 cycles of CHOP alone in patients with stage I or IE and nonbulky stage II or IIE localized nodal and extranodal aggressive non-Hodgkin's lymphoma (p=0.02) [21]. The 5-year overall survival in patients with a low-risk IPI and a low-intermediate risk were 82 and 71%, respectively, including both the CHOP plus RT group and the CHOP alone group. After these results, we adopted 3 cycles of CHOP plus RT as a treatment regimen for stage I–II aggressive lymphoma in the present study.

Recently updated analysis of the SWOG trial showed an overlapping curve at 9 years for overall survival as a result of late relapses in the CHOP plus RT group, but it remained the standard treatment for stage I and nonbulky stage II patients based on survival advantages through the first 9 years and less toxicity. Late relapses also suggested that optimal treatment might include more or different systemic CTx. Recent reports suggest that rituximab plus CHOP is more effective than CHOP alone in more advanced stage DLBCL [25, 26] and the standard treatment for early stage DLBCL patients may be changed in the near future [27]. We have also changed CHOP regimen into rituximab plus CHOP (R-CHOP) since February 2004. Since the introduction of rituximab, therapy has changed the following: (1) rituximab has been administered in day 1 of every cycles and subsequently CHOP administered from day 2, and (2) both the number of cycles of CTx and the method of RT have not been changed. Nowadays, a CHOP-RT approach has been used only for patients with either T-cell lymphoma or CD20 negative B-cell lymphoma in our institution.

In the Lymphoma Non-Hodgkinien study 93-4 (LNH 93-4), randomized trial by the Groupe d'Etude des Lymphomes de l'Adulte (GELA) for elderly patients

Table 4 Results and outcome of patients with T-cell lymphoma

Pt No.	Histopathology	Gender	Age	Stage	Modified IPI	Recurrence	Survival time (months)	Outcome
1	Diffuse T cell type	M	49	1A	Low	(+)	0.5	Dead
2	NK/T-cell lymphoma	F	44	1A	Low	(−)	83.3	Alive
3	NK/T-cell lymphoma	M	59	1A	Low	(−)	72.1	Alive
4	Anaplastic large cell lymphoma	M	19	1A	Low	(+)	42.3	Alive
5	Peripheral T cell lymphoma	M	72	1A	Low-intermediate	(+)	14.7	Alive
6	Peripheral T cell lymphoma	F	44	1A	Low	(+)	3.2	Dead
7	Anaplastic large cell lymphoma	M	42	1A	Low	(−)	50.4	Alive

comparing 4 cycles of CHOP plus local radiation (40 Gy) with 4 cycles of CHOP, there was no significant difference in 5-year survival (64 vs 69%, p=0.4) [28]. In the LNH 93-1 for patients under 61 years, dose-intensified doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone (ACVBP) followed by sequential consolidation was superior to 3 cycles of CHOP plus RT for the treatment of low-risk localized lymphoma (5-year overall survival was 90 vs 81%, p=0.001) [29]. The reports, that the meaning of RT for low-risk stage I–II aggressive lymphoma was unclear, continued one after the other.

The possible benefits of a short course of chemotherapy followed by involved-field RT are the potential for eliminating microscopic sites of disease; however, RT alone is still a very important treatment modality for localized low-grade lymphomas [17].

It is well known that CR obtained after first-line treatment is associated with a longer overall survival [30]. In our study, CR obtained after the primary CTx (n=28) was highly associated with a longer overall survival (p<0.0001). A number of clinical variables, among which IPI is the standard approach, predict therapy response, but in our study, there was no significant difference between a low-risk IPI and a low-intermediate risk (p=0.8684). With regard to prognostic factors for survival, clinical variables at presentation appear to lose their predictive significance when response to therapy is taken into account [31].

Among seven cases without achieving CR in the primary CTx preceding RT, RT after CTx may be able to salvage two cases (surviving at 24.7 and 71.1 months with keeping CR, respectively).

Local and systemic-related toxicities were minimal, and patients generally tolerated treatment well [32, 33]. In most series and our experience too, long-term side effects were mild and late toxicities and second malignancies were infrequent.

Our results in the treatment of localized aggressive lymphoma are excellent. A definitive recommendation for treatment of localized aggressive lymphoma is difficult to make based on this study with such a small number of patients, albeit the excellent results.

Conclusion

Stage I–II aggressive lymphoma trend to be localized and to have good outcome. Our limited data suggest that combined CTx and RT is safe, highly effective, and probably curative, with mild and infrequent long-term side effects for the majority of patients with primary localized aggressive lymphoma.

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ORIGINAL ARTICLE: RESEARCH

CD1d expression level in tumor cells is an important determinant for anti-tumor immunity by natural killer T cells

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Abstract

Invariant natural killer T (iNKT) cells are thought to regulate anti-tumor immunity. Human iNKT (i.e. Vα24$^+$ NKT) cells have been reported to recognize CD1d on target cells and show cytotoxicity directly on the target cells *in vitro*. However, the anti-tumor effect of mouse iNKT (i.e. Vα14$^+$ NKT) cells has been repeatedly reported to be dependent on the activity of natural killer (NK) cells via interferon-γ, with no evidence of direct cytotoxicity. In the present study, we report that *in vitro* cytolysis of EL-4 mouse lymphoblastic lymphoma cells by Vα24$^+$ NKT cells and *in vivo* eradication of these cells are both dependent on the level of CD1d expression on the tumor cell surface. These observations possibly suggest that direct cytotoxicity of tumor cells by iNKT cells is common to both humans and mice, and that the high expression level of CD1d may be a predictor whether the tumor is a good target of iNKT cells.

Keywords: *Anti-tumor immunity, CD1d, natural killer T cell, mouse tumor model, T cell lymphoma, immunotherapy, α-galactosylceramide*

Introduction

Natural killer T (NKT) cells are a population of T cells that have natural killer (NK) cell markers such as NK1.1 (NKR-P1C) in mice [1,2]. Sizable populations of NKT cells use an invariant T cell receptor (TCR)-α chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) paired with Vβ8, Vβ7 or Vβ2 in mice, or with Vβ11 in humans [1,2], and are called invariant NKT (iNKT) cells. iNKT cells are activated through TCR by synthetic glycolipids such as α-galactosylceramide (α-GalCer) in a CD1d-restricted manner [1,2]. iNKT cells produce both Th1 [such as interferon (IFN)-γ and tumor necrosis factor-α] and Th2 [such as interleukin (IL)-4, IL-10 and IL-13] cytokines upon TCR-mediated signaling [1,2]. It has been reported that iNKT cells control immune responses in infections, tumors, autoimmune diseases and transplantation [1–3].

In mice, iNKT cells have been demonstrated to have an anti-tumor effect *in vivo*, in which iNKT-dependent activation of NK cells and/or CD8$^+$ cytotoxic T-cell via IFN-γ is considered to be the most important mechanism [2–4]. The direct cytotoxicity of mouse iNKT cells on tumor cells has not been defined to date. A similar mechanism had been suggested for the anti-tumor effect of human iNKT cells through *in vitro* experiments [5]. However, in humans, a small number of recent reports described that iNKT cells directly recognize CD1d on target cells [6–8]. Moreover, some primary leukemia cells express CD1d and are killed by Vα24$^+$ iNKT cells in a CD1d-dependent manner [9–11].

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In the present study, we describe that the expression level of CD1d in EL-4 mouse lymphoblastic lymphoma cells correlates with α-GalCer-dependent anti-tumor activity of iNKT cells *in vitro* and tumor growth suppression *in vivo*. Our results potentially support the theory that iNKT cells directly kill the target tumor cells and that the level of CD1d expression on tumor cells is an important indicator for α-GalCer therapy or NKT immunotherapy.

Materials and methods

Mice

C57BL/6 (H-2^b) mice were purchased from Japan Clea (Tokyo, Japan). Female mice, aged 6–12 weeks, were used in all the experiments. Mouse studies were conducted according to the University of Tokyo Animal Experiment Manual.

El-4 T cells and murine CD1d transfection

The murine CD1d expression vector (pSRα neo/CD1d1) was kindly provided by Dr Exley (Beth Israel Deaconess Medical Centre, Boston, MA, USA). The murine T-lineage lymphoblastic lymphoma EL-4 cells were transfected with the plasmids using Superfect (Qiagen GmbH, Hilden, Germany) and then cultured at 3×10^4 cells/well of 24-well plates in RPMI1640 supplemented with 10% FCS (cRPMI) and 1 mg/ml of Geneticin (G418, Invitrogen, Carlsbad, CA, USA). Independent clones with various levels of CD1d expression were obtained. We calculated the growth rate of the clones by daily counting viable cell numbers for 2 weeks.

Phenotypic assay

Anti-CD1d (1B1) and isotype control (A95-1) antibodies were purchased from BD Pharmingen (San Jose, CA, USA). Immunofluorescence staining was performed according to the standard procedures. Cells were analysed by flow cytometry using a FACSCalibur (Becton Dickinson Bioscience, San Jose, CA, USA).

Cytotoxicity assay

Human iNKT cells were established as previously described [6,9]. Briefly, monocytes from healthy human donors were cultured in AIM medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FCS with recombinant human (rh) IL-4 (500 U/ml, CellGenix, Freiburg, Germany) and rhGM-CSF (500 U/ml, CellGenix) for 5 days, and used as monocyte-derived dendritic cells (Mo-DCs). Vα24$^+$ cells, which were isolated from the same donor by positive magnetic bead sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), were cultured with irradiated (50 Gy)-Mo-DCs that were pulsed for 12 h with α-GalCer (100 ng/ml, kindly provided by Kirin Brewery, Gunma, Japan). The cells were maintained in the presence of rhIL-2 (40 U/ml, kindly provided by Shionogi, Osaka, Japan) and restimulated by irradiated-Mo-DCs every 7 days. Identity of the expanded cell populations as iNKT cells was confirmed by cytometric analysis with anti-Vα24 (C15) and Vβ11 (C21) antibodies (Beckman Coulter, Fullerton, CA, USA). Five $\times 10^3$ of ^{51}Cr-labelled (Amersham, Arlington Hights, IL, USA) EL-4 sublines and the effecter iNKT cells at the indicated ratios were seeded onto 96-well round-bottomed microtiter plates in 200 μl of cRPMI with or without 100 ng/ml of α-GalCer. The co-cultured cells were incubated for 4 h, and 100 μl of supernatant was collected from each well and was counted with a Packard COBRA gamma counter (Packard Instrument, Meriden, CT, USA). The percentage of specific ^{51}Cr-release was calculated as: (c.p.m. experimental release − c.p.m. spontaneous release)/(c.p.m. maximal release − c.p.m. spontaneous release) × 100. The ratio of spontaneous release to maximal release was less than 20% in all experiments.

In vitro *iNKT stimulation and cytokine production*

Five $\times 10^4$ Vα24$^+$ iNKT cells and 5×10^4 EL-4 clones were suspended in 200 μl cRPMI, with or without 100 ng/ml of α-GalCer, and cultured in 96-well plates. After 24 h, the supernatants were collected from each well and the concentrations of IL-4 and IFN-γ were evaluated by ELISA according to the manufacturer's protocol (CytoSets; Biosource, Camarillo, CA, USA). The cultured cells were cytometrically analysed with anti-TCR Vα24, human CD25 (B1.49.9) and human CD69 (TP1.55.3) antibodies (Beckman Coulter) and analysed by FACSCalbur.

In vivo *tumor-bearing model*

EL-4 cell lines (1×10^5 cells/mouse) were injected to mice intravenously. The resulting tumors were removed from all the dead mice immediately (<24 h) after death and histopathological specimens from tumors were stained with hematoxylin and eosin.

Molecular analysis of iGb3, a natural ligand for TCR on iNKT cells

Total RNA was extracted from EL-4 lines according to the manufacturer's protocol (Tri Reagent LS; Sigma-Aldrich, St Louis, MO, USA) and reverse

transcribed (SuperScript II; Invitrogen). The resulting cDNA was subjected to polymerase chain reaction amplification using the primer pair for iGb3 (5′-ATTATTATCAGGCTCATAGAAGG-3′ and 5′-CTAGTTTCGCACCAGCGTATATTC-3′) [12] or the pair for β-actin (5′-GAGAGGGAAATC GTGCGTGA-3′ and 5′-ACATCTGCTGGAAGG TGGAC-3′).

Statistical analysis

Survival of mice was analysed by the log-rank test. Differences in the amount of secreted cytokines, the percentage specific lysis of EL-4 cells and the proportion of activated iNKT cells were analysed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Establishment of CD1d-transfected EL-4 sublines and cytotoxic activity of NKT cells against the individual sublines

We obtained multiple EL-4 subclones that express various levels of CD1d. Among these, five and two clones with the highest and intermediate CD1d expression levels (EL-4/CD1d^{hi} and EL-4/CD1d^{int}) were used for the following analyses. Three mock-transfected clones were used as controls. The mean ± SD growth rates were 4.0 ± 0.40 divisions per 24 h in mock-transfected clones, 3.8 ± 0.12 in El-4/CD1d^{int} clones and 3.8 ± 0.43 in EL-4/CD1d^{hi} clones, indicating that the expression levels of CD1d did not affect the growth rate. Wild-type and mock-transfected EL-4 expressed a low level of CD1d (Figure 1A). We used human iNKT cells (Figure 1B) as effector cells because *in vitro* expansion of mouse iNKT cells is generally difficult, while the method to expand human iNKT cells has been established [6,9]. Importantly, the mechanism of recognition of CD1d-bound α-GalCer by iNKT cells is conserved through mammalian species [13]. With dependence on the CD1d expression level, iNKT cells exhibited cytotoxicity to EL-4 cells only in the presence of α-GalCer (Figure 1C). We also characterized the activation of the iNKT cells by the different sublines of EL-4 *in vitro* by analysing the surface expression of activation markers (Figure 1D) and the profiles of secreted cytokines (Figure 1E). The proportions of CD69 and CD25 positive cells among iNKT cells were independent of the expression level of CD1d on co-culturing EL-4 clones. Although the amounts of IL-4 and IFN-γ secreted from iNKT cells stimulated by EL-4/CD1d^{hi} tended to be higher than those from iNKT cells stimulated by EL-4/CD1d^{low}, the difference was not always statistically significant. The cytotoxicity of iNKT cells via IFN-γ might not be a major mechanism of anti-tumor activity.

Survival of EL-4 tumor-bearing mice correlated to CD1d expression level on the surface of tumor cells

We next examined whether the CD1d expression level on the surface of tumor cells could affect the survival of the tumor-bearing mice. All the wild-type EL-4-inoculated mice died within 6 weeks, showing multiple lymph node swelling. All the enlarged lymph nodes examined were diffusely infiltrated with morphologically obvious tumor cells. Analysis of the tumor cells by flow cytometry revealed that they had characteristics of T-lineage cells and CD1d expression at levels that were the same as those in the injected EL-4 cells (Figure 2A). Survival of wild-type and mock-transfected EL-4-inoculated mice was not significantly different (data not shown). In contrast, mice inoculated with all of the five EL-4/CD1d^{hi} clones survived significantly longer than those inoculated with EL-4 expressing CD1d at lower levels (Figure 2B). These results suggest that interaction between iNKT cells and CD1d on the tumor cell surface is useful for protecting the host from tumor growth. This anti-tumor immunity should be based on natural ligands. We examined the expression of iGb3, a known natural ligand for TCR on NKT cells [12], and found that all the clones expressed iGb3 (Figure 2C). Therefore, iNKT cells acquired cytotoxic activity against CD1d-expressing EL-4 cells *in vivo* possibly by stimulation with iGb3 that is naturally expressed on EL-4.

Discussion

Anti-tumor activity is one of the attractive functions of iNKT cells. α-GalCer has been tested in phase I clinical trials, either by direct administration of the compound [14] or by administration of α-GalCer-pulsed autologous DCs [15]. *In vitro* expanded iNKT cells might also be used as an immunotherapy. However, despite such progress, very little is known about the tumor-suppressing mechanisms of iNKT cells.

iNKT cells produce both Th1 and Th2 cytokines, suggesting that they also have a certain regulatory function. Some studies reported that NKT cells suppressed tumor immunity [5,16]. Accordingly, there is no definitive information as to whether

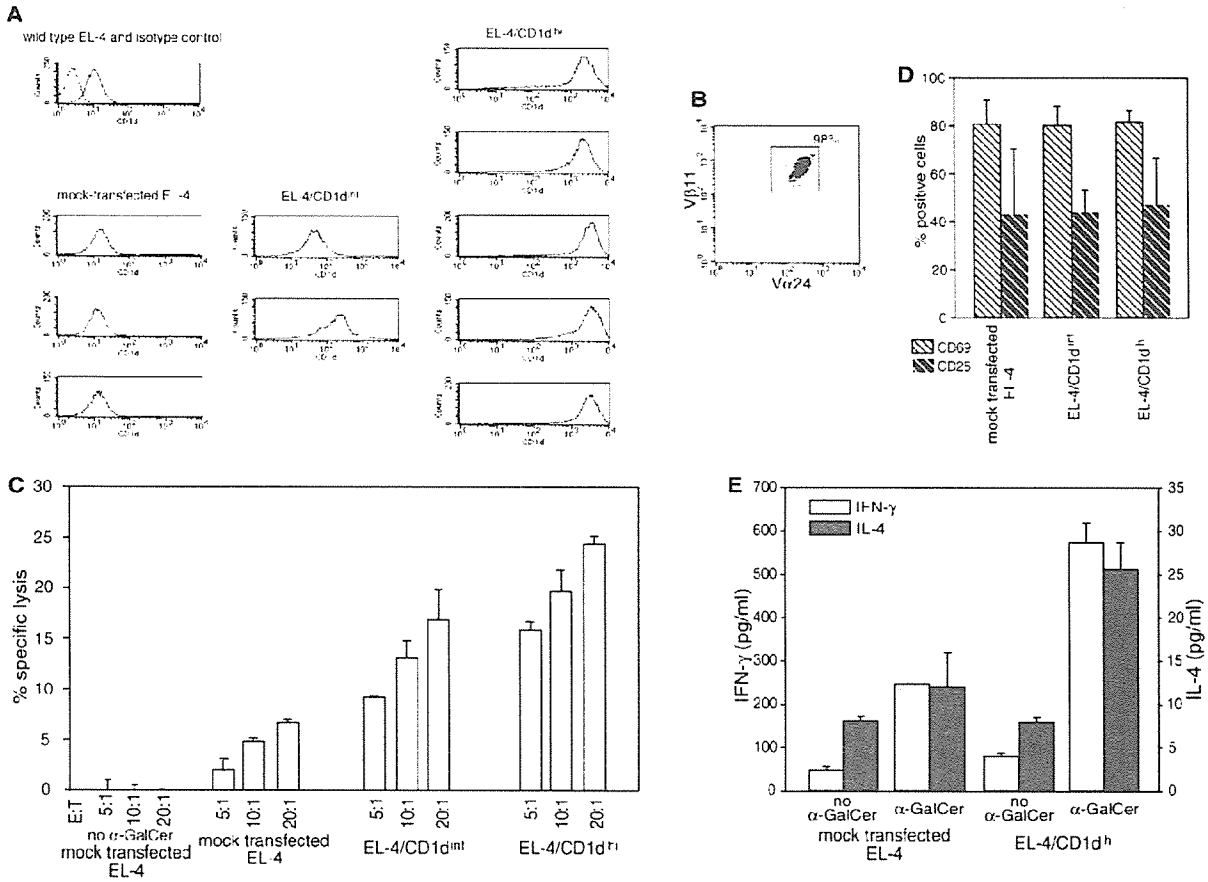

Figure 1. *In vitro* cytotoxic activity of CD1d-transfected EL-4. (A) Expression levels of CD1d in transfected EL-4 clones. The pattern of CD1d expression on the wild-type EL-4 cell surface is overlayed on an isotype-control (dashed line). EL-4/CD1d^{hi} and EL-4/CD1d^{int} are clones with the highest and intermediate CD1d expression level, respectively. (B) Flow cytometric analysis of the expanded iNKT cells. (C) Cytotoxic assay for iNKT cells. ^{51}Cr-labelled mock-transfected EL-4, EL-4/CD1d^{int} and EL-4/CD1d^{hi} were used as the target cells. Comparing each E : T ratio, there are significant differences in the percentage of specific lysis among the three groups of EL-4 sublines classified by the expression level of CD1d. Data are shown as the mean ± SD. Very similar results were obtained using other clones from each group. (D) Expression of activation markers of iNKT cells by each subline of EL-4. The results shown are the sum of seven independent experiments. (E) Cytokine profile of activated iNKT cells by each subline of EL-4. The levels of cytokines secreted by mock-transfected EL-4 and EL-4/CD1d^{hi} were not significantly different. Similar results were obtained using other clones from each group.

iNKT cells act as a cytotoxic effector or a regulatory effector for individual tumors. The results of the present study raise the possibility that the CD1d expression level is the determinant in an *in vivo* animal model and that such an effect is mainly mediated through the direct killing activity of iNKT cells rather than secretion of Th1 cytokines.

Information about the physiological ligands for the invariant TCR, which should play a significant role in anti-tumor function of iNKT cells in EL-4-bearing mice, is limited. Unfortunately, examination of tumor-infiltrating lymph nodes in the present study failed to delineate the mechanisms of tumor eradication by *in situ* iNKT cells present in the lymph nodes. The fraction of iNKT cells in the EL-4 CD1d^{hi} tumor ($0.24 \pm 0.14\%$, $n=9$) was significantly ($P=0.04$) larger than in the mock-transfected EL-4 tumor ($0.12 \pm 0.05\%$, $n=5$). However, interpretation of this finding is difficult because we could not standardize the sizes of lymph nodes, the cellularity of infiltrating EL-4 cells and various infiltrating immune cells other than iNKT cells (data not shown).

Nevertheless, CD1d expression on the tumor surface may be a predictor of whether the tumor is a good target for iNKT cells. The results of the present study raise the possibility of NKT cell therapy being used as a treatment modality for CD1d-expressing tumors. Direct evidence showing that iNKT cells kill the tumor cells *in vivo* should

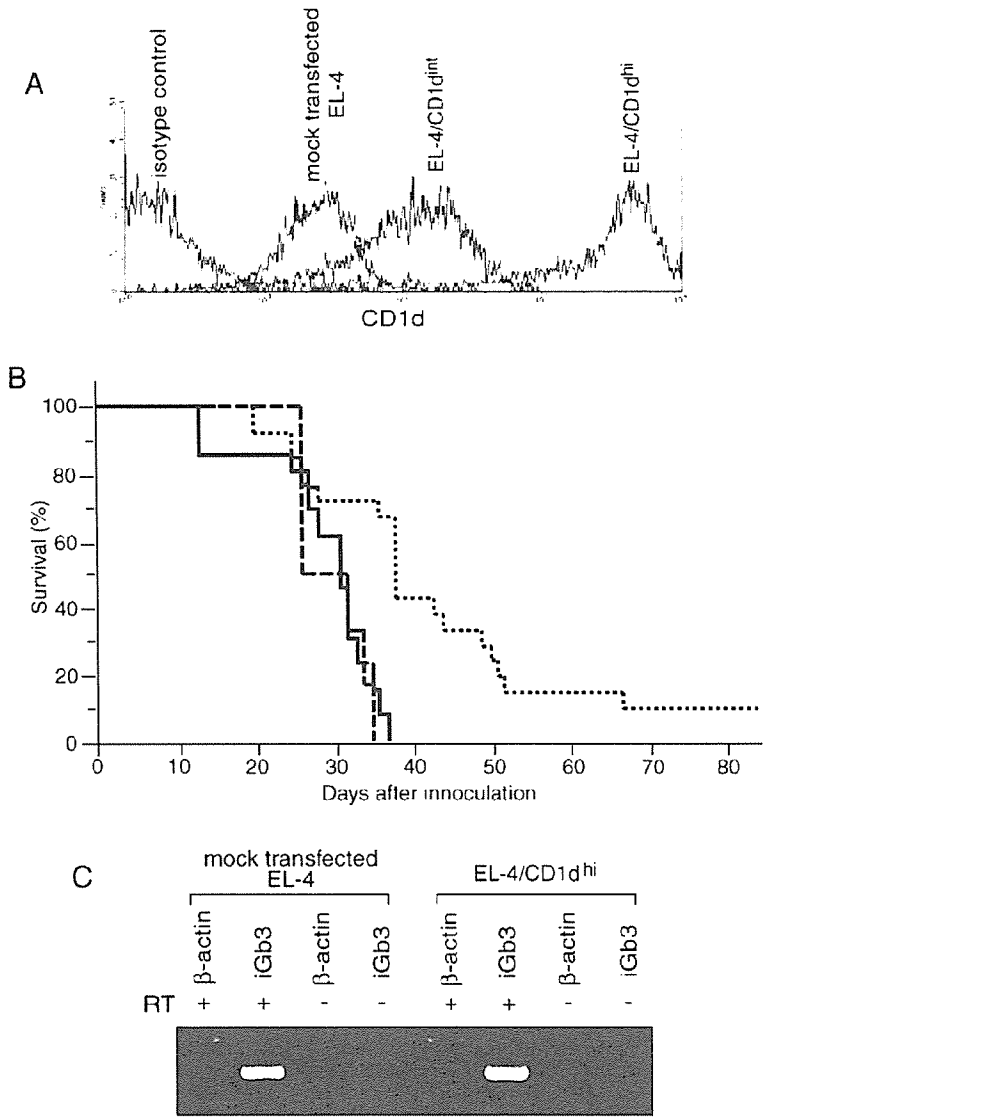

Figure 2. Survival of mice inoculated with various EL-4 sublines. (A) Flow cytometric analysis of the infiltrating lymphoma cells. (B) Survival of mock-transfected (solid line, $n = 21$) EL-4-, EL-4/CD1d^{int}-(dashed line, $n = 6$) and EL-4/CD1d^{hi}-(dotted line, $n = 13$) inoculated mice. EL-4/CD1d^{hi}-inoculated mice survived significantly longer ($P = 0.0005$). The result is the sum of six independent experiments. (C) RT-PCR assay showing that the EL-4 cell lines expressed iGb3. Very similar results were obtained using all the clones.

be warranted, which might be obtained after the technical improvements have been implemented.

Acknowledgments

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STEM CELLS
TISSUE-SPECIFIC STEM CELLS

Highly Efficient Ex Vivo Expansion of Human Hematopoietic Stem Cells Using Delta1-Fc Chimeric Protein

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Key Words. AC133 antigen • Hematopoietic stem cells • Notch • Stem cell expansion

ABSTRACT

Ex vivo expansion of hematopoietic stem cells (HSCs) has been explored in the fields of stem cell biology, gene therapy, and clinical transplantation. Here, we demonstrate efficient ex vivo expansion of HSCs measured by long-term severe combined immunodeficient (SCID) repopulating cells (SRCs) from human cord blood CD133-sorted cells using a soluble form of Delta1. After a 3-week culture on immobilized Delta1 supplemented with stem cell factor, thrombopoietin, Flt-3 ligand, interleukin (IL)-3, and IL-6/soluble IL-6 receptor chimeric protein (FP6) in a serum- and stromal cell-free condition, we achieved approximately sixfold expansion of SRCs when evaluated by limiting dilution/transplantation assays. The maintenance of full multipotency and self-renewal capacity during culture was confirmed by transplantation to nonobese diabetic/SCID/γc^{null} mice, which showed myeloid, B, T, and natural killer cells as well as $CD133^+CD34^+$ cells, and hematopoietic reconstitution in the secondary recipients. Interestingly, the CD133-sorted cells contained approximately 4.5 times more SRCs than the CD34-sorted cells. The present study provides a promising method to expand HSCs and encourages future trials on clinical transplantation. STEM CELLS *2006;24: 2456–2465*

INTRODUCTION

Umbilical cord blood (CB) is an established stem cell source for hematopoietic stem cell (HSC) transplantation. In many cases, however, CB transplantation is unavailable to patients with relatively high body weight because of the insufficient number of HSCs obtained from a single CB unit [1–3]. Recently, transplantation of multiple units of CB in adult patients was reported in an experimental attempt to infuse a higher number of HSCs or hematopoietic progenitor cells (HPCs), but the effectiveness of this novel trial needs further investigation [4–7].

Another possibility to acquire a higher number of stem cells is ex vivo expansion of HSCs. Although many reports have described potential methods to increase HSCs ex vivo, only a few of them have clearly demonstrated the expansion of long-term severe combined immunodeficient (SCID) repopulating cells (SRCs), currently the only reliable measure of HSCs [8–10]. According to one of these reports, combined use of soluble interleukin (IL)-6 receptor (sIL-6R) and IL-6 together with stem cell factor (SCF), thrombopoietin (TPO), and flt-3 ligand (FL) appeared to be helpful for the successful expansion

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