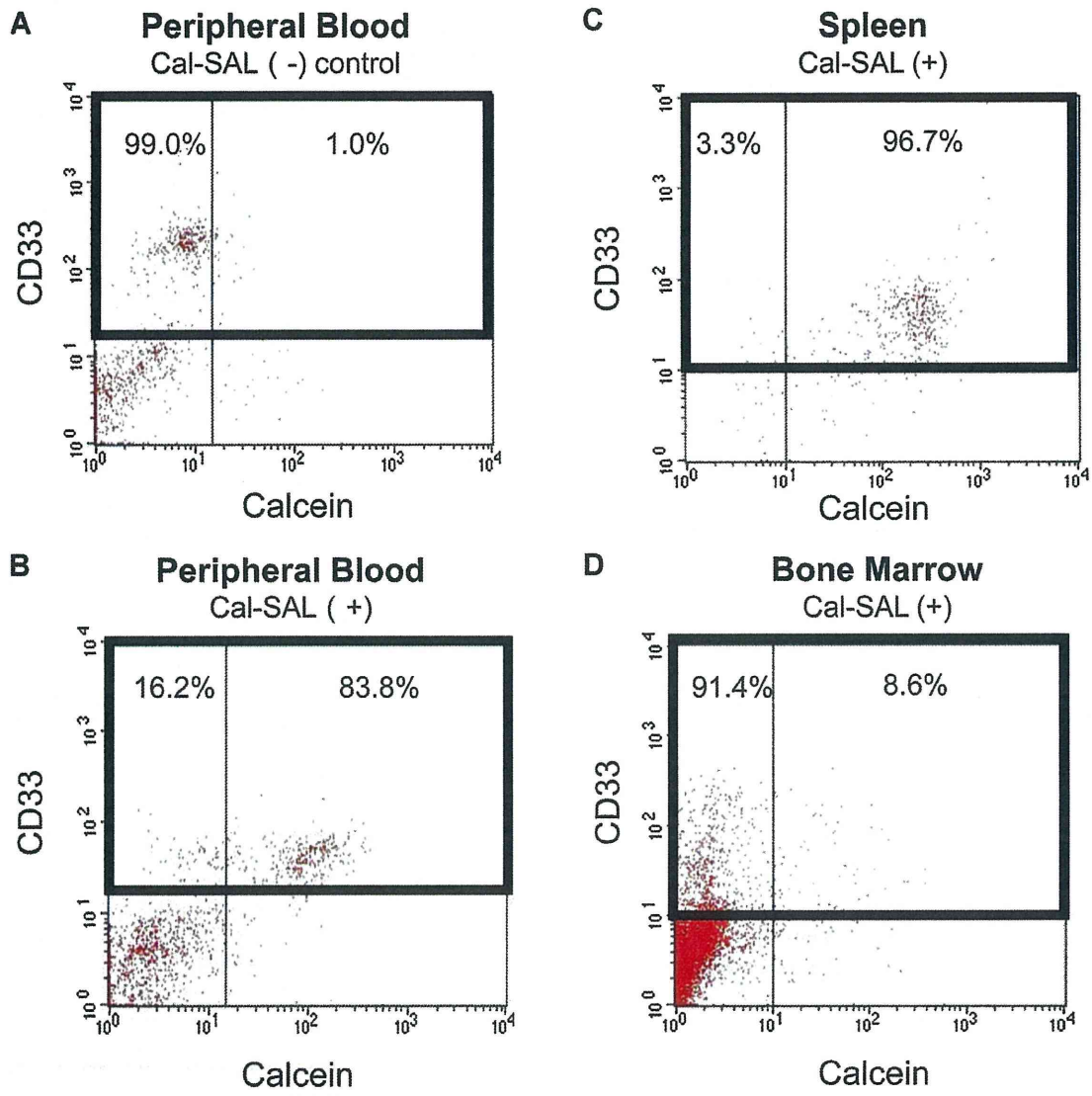

Fig. 4. Binding of Cal-SALs to leukemia cells in a mouse xenograft model. Bi-anti-CD33 MAb was injected to IMS-2-xenografted mice intravenously followed by intravenous injection of Cal-SALs. After 1 h, mice were euthanatized and peripheral blood (A, B), spleen (C) and bone marrow (D) were sampled. The obtained samples were stained with APC-labeled anti-human CD33 MAb to detect grafted IMS-M2 cells, and the cells were examined by two-color FCM. Most human $CD33^+$ cells in peripheral blood (B) or spleen (C) and some human $CD33^+$ cells in bone marrow were positive for Calcein fluorescence.

is expressed in lymphoid cells including T-ALL cells and myeloid leukemia cells with a poor prognosis (Jung and Fu, 1989; Stong et al., 1985), while both CD33 and CD7 are not expressed in uncommitted HSCs (Forraz et al., 2004). We also have successfully demonstrated that the SALs specifically and efficiently bound and internalized into the $CD33^+$ or $CD7^+$ leukemia cells with the corresponding Bi-MAb. Second, the target antigen or receptors should be internalizable after binding to immunoliposomes. G-CSFR, CD33, CD7 were known to internalize into cytoplasm rapidly after binding by the corresponding antibodies (Preijers et al., 1988; van Der Velden et al., 2001). Our study has shown that the SALs can specifically and efficiently bind and internalize into target cells with Bi-biomaterials. Third, drug release from immunoliposomes inside target cells should accumulate to therapeutic concentration. In this study, we have shown that AraC-encapsulating SALs can kill targeted cells much more effectively than bare AraC, as was expected. All these findings were indicating that our newly developed SAL-mediated DDS fulfilled the requirements for tumor-specific DDS.

In addition to the specific delivery of the SALs *in vitro*, the *in vivo* study using IMS-M2 cell-transplanted NOD-SCID mice showed specific delivery of SALs into leukemia cells with B-CD33-MAb, and the delivery was very efficient at least in spleen and peripheral blood. Although previous reports describing the efficient penetration of PEG-liposomes to bone marrow cells (Awasthi et al., 1998; Sadzuka, 2000), our SALs did not deliver so efficiently in bone marrow cells as these reports (Fig. 4). This lower efficiency would be due to lower dose and shorter time after administration to allow the SALs to reach bone marrow leukemia cells, therefore higher dose of Cal-SALs and longer incubation time may improve the efficiency. In this study, we adapted an indirect administration system in which SALs were administrated 15 min after Bi-biomaterials because the indirect method easily allows unlimited combination of the Bi-biomaterials with the SALs without pre-coupling of these materials followed by purification, which is needed in the direct method and would impair the integrity of these materials, in advance of the administration. In addition, the indirect method of Bi-biomaterials and SA-tagged radioisotopes has been shown to enhance

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anti-tumor effect and reduce adverse effect compared to the direct method, suggesting predominance of the indirect method in our SAL-based DDS (Lesch et al., 2010). Another important issue in the indirect method is time period between the Bi-biomaterials and the SALs that would affect the delivery efficiency. Therefore, optimization of the time period between these reagents may improve the delivery efficiency of bone marrow delivery. Besides, trapping of liposomes by reticuloendothelial system (RES) is another problem for efficient delivery (Maruyama, 2002). To avoid rapid clearance of liposomes from circulation by the RES, we used PEG-tagged SALs, but it is possible that SALs were trapped by bone marrow endothelial or stromal cells, resulting in the low delivery efficiency. However, the Cal-SALs actually did not bind to host mouse spleen cells, which are human CD33-negative (Fig. 4), suggesting that CaL-SALs was not trapped by RES. The SALs also did not bind to host mouse cells without proper Bi-ligands, suggesting *in vivo* specificity of the SAL-based DDS. To further address specificity of this DDS, more details in distribution of the SALs will be investigated in future.

In conclusion, we successfully developed a new liposomal-mediated drug delivery system that is easily applicable to various cell types depending on the choice of surface markers. This system allows us to use chemically fragile ligands such as G-CSF in a cell-targeting DDS. Further study to optimize administration conditions and evaluate the safety of this system will be needed before it can be put to practical use.

Role of the funding source

None.

Authors' contributions

M-H. C. and Y.S. conducted the experiments, analyzed data, wrote the manuscript, and contributed equally to this work; K.I. and S.K. analyzed data; K.M. prepared liposomes; K.T., A.T. supervised the experiments and the project; K.M. and S.A. supervised the experiments and the project and wrote the manuscript.

Conflict of interest

There are no conflicts of interest and no financial disclosures from any authors.

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The CD3 versus CD7 Plot in Multicolor Flow Cytometry Reflects Progression of Disease Stage in Patients Infected with HTLV-I

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Abstract

Purpose: In a recent study to purify adult T-cell leukemia-lymphoma (ATL) cells from acute-type patients by flow cytometry, three subpopulations were observed in a CD3 versus CD7 plot (H: $CD3^{high}CD7^{high}$; D: $CD3^{dim}CD7^{dim}$; L: $CD3^{dim}CD7^{low}$). The majority of leukemia cells were enriched in the L subpopulation and the same clone was included in the D and L subpopulations, suggesting clonal evolution. In this study, we analyzed patients with indolent-type ATL and human T-cell leukemia virus type I (HTLV-I) asymptomatic carriers (ACs) to see whether the CD3 versus CD7 profile reflected progression in the properties of HTLV-I-infected cells.

Experimental Design: Using peripheral blood mononuclear cells from patient samples, we performed multi-color flow cytometry. Cells that underwent fluorescence-activated cell sorting were subjected to molecular analyses, including inverse long PCR.

Results: In the D(%) versus L(%) plot, patient data could largely be categorized into three groups (Group 1: AC; Group 2: smoldering- and chronic-type ATL; and Group 3: acute-type ATL). Some exceptions, however, were noted (*e.g.*, ACs in Group 2). In the follow-up of some patients, clinical disease progression correlated well with the CD3 versus CD7 profile. In clonality analysis, we clearly detected a major clone in the D and L subpopulations in ATL cases and, intriguingly, in some ACs in Group 2.

Conclusion: We propose that the CD3 versus CD7 plot reflects progression of disease stage in patients infected with HTLV-I. The CD3 versus CD7 profile will be a new indicator, along with high proviral load, for HTLV-I ACs in forecasting disease progression.

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Introduction

Human T-cell leukemia virus type I (HTLV-I) is the agent that causes HTLV-I-associated diseases, such as adult T-cell leukemia-lymphoma (ATL), HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-I uveitis (HU) [1–3]. Approximately 10–20 million people are infected with the HTLV-I virus worldwide [4]. The lifetime risk of developing ATL is estimated to be approximately 2.5–5% [5,6]. ATL includes a spectrum of diseases that are referred to as smoldering-, chronic-, lymphoma-, and acute-type [7,8]. The chronic and smoldering types of ATL are considered indolent and are usually managed with watchful waiting until the disease progresses to aggressive (lymphoma- or acute-type) ATL [9]. Because the prognosis of ATL is poor with current treatment strategies, factors to forecast progression to ATL from asymptomatic carriers (ACs) have been researched [10–13] in the hope that they will be useful for preventive therapy under development in the early malignant stage.

Various cellular dysfunctions induced by viral genes (*e.g.*, tax and HBZ), genetic and epigenetic alterations, and the host immune system are considered to cooperatively contribute to leukemogenesis in ATL [14–16]. However, the complex mechanism may hinder determination of a clear mechanism of the pathology and make discovery of risk factors difficult. In a prospective nationwide study in Japan, high proviral load (VL,

Table 1. Clinical profile of patients infected with HTLV-I and normal controls.

Clinical sutype	Number of cases	Male	Female	Age (range)	WBC(/μl) (range)	Lymphocytes(%) (range)	Abnormal lymphocytes(%) (range)
HTLV-1 AC	40	12	28	49.9 (28–70)	5525 (2680–10360)	35.9 (22.4–59.5)	0.9 (0.0–4.4)
Smoldering	7	4	3	55.3 (43–77)	5944 (3680–8710)	32.5 (13.4–47.5)	5.8 (0.7–16.5)
Chronic	7	4	3	52.7 (37–60)	9180 (4070–12790)	45.8 (35.0–61.5)	9.2 (3.4–12.7)
Acute	13	4	9	58.8 (42–74)	15328 (4450–41480)	16.3 (1.7–50.5)	40.3 (3.0–89.6)
Normal controls	10	6	4	47.4 (27–66)	ND	ND	ND

WBC: white blood cells (normal range, 3500–9100/μl).
AC: asymptomatic carrier.
ND: analysis were not performed.
Average of age, WBC, lymphocytes (%) and abnormal lymphocytes (%) are shown.
The proportion of abonormal lymphocytes in peripheral blood WBCs was evaluated by morphological exmanination.
doi:10.1371/journal.pone.0053728.t001

over 4.17 copies/100 peripheral blood mononuclear cells) was found to be a major risk factor for HTLV-I AC developing into ATL [13]. Although VL indicates the proportion of HTLV-I-infected cells, it does not indicate size or degree of malignant progression in each clone; *i.e.*, it does not directly indicate progression of disease stage in HTLV-I infection. Moreover, the majority of ACs with high VL remained intact during the study period, indicating that a more accurate indicator of progression is needed.

In our recent study to purify monoclonal ATL cells from acute-type patients by flow cytometry, three subpopulations were observed in a CD3 versus CD7 plot of $CD4^+$ cells (H: $CD3^{high}CD7^{high}$, D: $CD3^{dim}CD7^{dim}$, L: $CD3^{dim}CD7^{low}$), and the majority of ATL cells were enriched in the L subpopulation [17]. Clonality analyses revealed that the D and L subpopulations contained the same clone, suggesting clonal evolution of HTLV-I-infected cells to ATL cells. From these findings, we speculated that the CD3 versus CD7 profile may reflect disease progression in HTLV-I infection. In this study, the CD3 versus CD7 profile by flow cytometry, combined with molecular (clonality and proviral load) characterizations, were analyzed in patients with various clinical subtypes (HTLV-I AC, and indolent and aggressive ATL). We found that the CD3 versus CD7 profile reflected disease progression of HTLV-I-infected cells to ATL cells. We also discuss the significance of this analysis as a novel risk indicator for HTLV-I ACs in forecasting progression to ATL.

Materials and Methods

Cell lines and patient samples

TL-Om1, an HTLV-I-infected cell line, established Dr. Hinuma's laboratory [18], was provided by Dr. Toshiki Watanabe (The University of Tokyo, Tokyo, Japan) and was cultured in RPMI-1640 medium containing 10% fetal bovine serum. Peripheral blood samples were collected from inpatients and outpatients at our hospital from August 2009 to November 2011. All patients with ATL were categorized according to Shimoyama's criteria [7,8]. Patients with various complications, such as autoimmune disorder and systemic infections, were excluded. Lymphoma-type patients were excluded because ATL cells are not considered to exist in peripheral blood of this clinical subtype. In patients with ATL receiving chemotherapy, blood samples were collected before treatment or during the recovery phase between chemotherapy sessions. Samples collected from 10 healthy volunteers (mean age: 47.4 years; range: 27–66 years) were used as normal controls.

The present study was approved by the research ethics committee of the institute of medical science, the university of Tokyo. Subjects provided written informed consent.

Flow cytometry and cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated whole blood by density gradient centrifugation, as described previously [17]. Cells were stained using a combination of phycoerythrin (PE)-CD7, APC-Cy7-CD3, Pacific Blue-CD4, and Pacific Orange-CD14. Pacific Orange-CD14 was purchased from Caltag-Invitrogen (Carlsbad, CA). All other antibodies were obtained from BD BioSciences (San Jose, CA). Propidium iodide (PI; Sigma, St. Louis, MO) was added to the samples to stain dead cells immediately prior to flow cytometry. A BD FACS Aria instrument (BD Immunocytometry Systems, San Jose, CA) was used for all multicolor flow cytometry and cell sorting. Data were analyzed using the FlowJo software (Treestar, San Carlos, CA).

Quantification of HTLV-I proviral load by real-time quantitative polymerase chain reaction (PCR)

The HTLV-I proviral load in FACS-sorted PBMCs was quantified by real-time quantitative polymerase chain reaction (PCR; TaqMan method) using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described previously [13,17]. Briefly, 50 ng of genomic DNA was extracted from human PBMCs using a QIAamp DNA blood Micro kit (Qiagen, Hilden, Germany). Triplicate samples of the DNA were amplified. Each PCR mixture, containing an HTLV-I pX region-specific primer pair at 0.1 μM (forward primer 5′-CGGATACCCAGTCTACGTGTT-3′ and reverse primer 5′-

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Acute kidney injury after myeloablative cord blood transplantation in adults: the efficacy of strict monitoring of vancomycin serum trough concentrations

H. Mae, J. Ooi, S. Takahashi, S. Kato, T. Kawakita, Y. Ebihara, K. Tsuji, F. Nagamura, H. Echizen, A. Tojo. Acute kidney injury after myeloablative cord blood transplantation in adults: the efficacy of strict monitoring of vancomycin serum trough concentrations. Transpl Infect Dis 2013: **15**: 181–186. All rights reserved

Abstract: *Background.* Acute kidney injury (AKI) is a common medical complication after myeloablative allogeneic stem cell transplantation (SCT). We have previously performed a retrospective analysis of AKI after cord blood transplantation (CBT) in adults, and found that the maximum of vancomycin (VCM) trough levels were significantly higher in patients with AKI. Following these results, we have monitored VCM serum trough concentrations more strictly, to not exceed 10.0 mg/L, since 2008.
Methods. In this report, we performed an analysis of AKI in a new group of 38 adult patients with hematological malignancies treated with unrelated CBT after myeloablative conditioning between January 2008 and July 2011.
Results. Cumulative incidence of AKI at day 100 after CBT was 34% (95% confidence interval 19–50). The median of the maximum value of VCM trough was 8.8 (4.5–12.2) mg/L. In multivariate analysis, no factor was associated with the incidence of AKI. No transplant-related mortality was observed. The probability of disease-free survival at 2 years was 83%.
Conclusion. These findings suggest that strict monitoring of VCM serum trough concentrations has a beneficial effect on outcomes of CBT.

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Key words: vancomycin; myeloablative conditioning; cord blood transplantation; acute kidney injury

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Acute kidney injury (AKI) is a common medical complication early after myeloablative allogeneic stem cell transplantation (SCT). The incidence of AKI, defined as a 2-fold rise in serum creatinine (sCr) concentration from baseline, has been reported ranging from 36% to 72% in SCT in a myeloablative setting (1–7), and about 20% required hemodialysis. We have previously reported a retrospective analysis of AKI in a group of 54 adult patients with hematological malignancies who received unrelated cord blood transplantation (CBT) after myeloablative conditioning between 2004 and 2007 (8). A statistically significant decrement of renal function from baseline was observed between days 11 and 20. Among the 54 patients, AKI occurred in 27.8% and was associated with a high mortality rate. Although no difference was seen in maximum cyclosporine (CYA) trough levels, the maximum vancomycin (VCM) trough levels were significantly higher in patients with AKI (8). Following these results, we have monitored VCM serum trough concentrations more strictly. In this report, we performed an analysis of AKI in a new group of 38 adult patients with hematological malignancies treated with unrelated CBT after myeloablative conditioning between January 2008 and

July 2011. The main purpose of this retrospective single-center study was to confirm the efficacy of strict monitoring of VCM serum trough concentrations, as well as to identify factors related to the incidence of AKI.

Patients and methods

Patients

This was a retrospective single-center analysis. Between January 2008 and July 2011, 39 consecutive adult patients with hematological malignancies were treated with unrelated CBT at The Institute of Medical Science, University of Tokyo. We excluded 1 patient who experienced primary engraftment failure. A total of 38 patients were analyzed. Patients qualified as standard risk if they were in first or second complete remission, had chronic-phase chronic myelogenous leukemia or refractory anemia of myelodysplastic syndrome, or had no high-risk cytogenetics. Patients in third complete remission, in relapse, or in refractory disease, with chronic myelogenous leukemia beyond chronic phase, or with high-risk cytogenetics were classified as high risk. Analyses of data were performed in December 2011. Written informed consent for treatment was obtained from all patients.

Conditioning

All patients received 4 fractionated 12 Gy total body irradiation on days −8 and −7, in addition to cytosine arabinoside (Ara-C) and cyclophosphamide. Ara-C was administered intravenously (IV) over 2 h at a dose of 3 g/m^2 every 12 h on day −5 and −4 (total dose 12 g/m^2). In patients with myeloid malignancies, recombinant human granulocyte colony-stimulating factor (G-CSF) was combined with Ara-C. G-CSF was administered by continuous infusion at a dose of 5 μg/kg/day. Infusion of G-CSF was started 12 h before the first dose of Ara-C and stopped at the completion of the last dose. Cyclophosphamide was administered IV over 2 h at a dose of 60 mg/kg once daily on days −3 and −2 (total dose 120 mg/kg). Two days after the completion of conditioning, patients received a CBT.

Graft-versus-host disease (GVHD) prophylaxis

All patients received standard CYA and methotrexate as GVHD prophylaxis. CYA was given IV every day starting on day −1 at a dose of 3 mg/kg/day. Methotrexate (15 mg/m^2 IV) was given on day 1, and 10 mg/m^2 on day 3 and 6. Once oral intake could be tolerated, patients were administered oral CYA at a dose of 1:2, in 2 divided doses per day, based on the last intravenous dose. CYA was reduced when sCr levels rose above 1.5 times baseline, or other serious agent-associated toxicities occurred. Physicians could freely modify the CYA dose for patients experiencing severe acute GVHD (aGVHD) or risk of disease relapse. Corticosteroid-based treatment was considered when grade II or higher severe aGVHD occurred (0.5–2 mg/kg).

Supportive care

All patients received G-CSF by intravenous infusion starting on day 1 until durable granulocyte recovery was achieved. The supportive care regimen, including prophylaxis for infection was the same as previously reported (8, 9).

Monitoring

All patients were monitored retrospectively 10 days before, and after the first 100 days, of CBT. Daily laboratory data collecting and the detecting method of VCM and CYA trough concentration were the same as previously reported (8). Therapeutic drug monitoring for VCM by assessing serum trough concentration was done twice in weekly, and modified to not exceed 10.0 mg/L.

End-points and definitions

AKI was defined as 2-fold rise in sCr concentration on daily laboratory results from the baseline (the average of days −10 to 0). Myeloid engraftment was defined as the first of 3 consecutive days, during which the absolute neutrophil count was at least 0.5 × 10^9/L. Platelet recovery time was achieved on the first of 3 days when the platelet count was higher than 50 × 10^9/L without transfusion support. The aGVHD was graded according to previously published criteria (10). Transplant-related mortality was defined as death from any cause except relapse. Relapse was defined by morphologic evidence of disease in peripheral blood, bone marrow, or extramedullary sites. Disease-free survival was defined as the time from CBT to relapse, death, or the last observation.

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ORIGINAL ARTICLE

Therapeutic outcome of multifocal Langerhans cell histiocytosis in adults treated with the Special C regimen formulated by the Japan LCH Study Group

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Abstract Little information is available regarding effective systemic therapies for adult Langerhans cell histiocytosis (LCH). The Japan LCH Study Group has formulated an ambulatory treatment regimen for adult patients with LCH. In total, 14 patients (median age 43 years, range 20–70 years) with multifocal LCH with biopsy-confirmed histology were enrolled. None had received cytoreductive agents for LCH previously. Four had single system (SS) and ten had multi system (MS) disease. All were treated with the Special C regimen, which consists of vinblastine/prednisolone and methotrexate with daily 6-mercaptopurine for 36 weeks. At the end of the therapeutic regimen, all SS patients achieved no active disease (NAD), and six of the ten MS patients showed a response (NAD in two, partial response in four). At the last follow-up (median 34 months), 11 patients were alive (NAD in eight and active disease in three). Of the three deceased, one died of hemorrhage during the Special C treatment, and two of infections during subsequent therapy. Although this study is limited by the small sample size, this ambulatory regimen shows signs of efficacy for adult LCH. This was particularly evident for patients with multifocal SS disease, but half of those with MS disease also benefited.

Keywords Langerhans cell histiocytosis · Adult · Chemotherapy

Introduction

Langerhans cell histiocytosis (LCH) is a rare disease that is characterized by the infiltration of clonal CD1a-positive

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dendritic cells. It mostly develops in infancy or early childhood with a childhood incidence of 2.2–8.9 cases per million; in adults, the incidence is one-third of the childhood incidence (1–2 cases per million) [1, 2]. LCH is categorized as a single system (SS) disease with multifocal or single/localized lesion(s) and as a multi system (MS) disease with or without risk organ (hematopoietic system, lung, liver, or spleen) involvement [3]. Children with multifocal SS or MS LCH are required to undergo systemic chemotherapy, but no such therapy is recommended for those with localized SS LCH [3]. Also in adults, systemic chemotherapy is required for multifocal SS or MS LCH lesions [1, 5], although adult-specific, smoking-related solitary pulmonary LCH lesions are treated differently [4]. While recent prospective, large-scale, multi-institutional trials have improved the therapeutic outcomes of multifocal childhood LCH [6, 7], only a few therapeutic trials involving a small number of cases have been performed for adult LCH [8–10].

A major obstacle in treating adult LCH patients is that they are often reluctant to take a leave of absence from their jobs for hospitalization, which can limit the provision of sufficient chemotherapy. Considering this adult-specific situation, the Japan LCH Study Group (JLSG) formulated Special C regimen for adult LCH patients in giving therapy safely at the outpatient clinic without hospitalization, which consisted of combinations of vinblastine (VBL)/prednisolone (PSL) and methotrexate (MTX) with daily 6-mercaptopurine (6-MP). These drugs were conventional agents and successfully employed as first-line chemotherapy for pediatric LCH patients [11]. The pilot study with the use of this regimen on adult patients with multifocal SS or MS LCH was performed. Results are reported here.

Patients and methods

This multicenter study was planned as a pilot study at the participating facilities of JLSG. The study was approved by the institutional review board (IRB). The study procedure was in accordance with the Helsinki Declaration. Eligible patients signed a detailed written informed consent statement meeting the requirements of the IRB. Patients were eligible for the study when having histologically diagnosed multifocal LCH who were at least 20 years of age. The diagnosis of LCH was confirmed by histopathology of biopsies of affected organs, which were positive for S-100 and/or CD1a antigen. Patients also needed to have adequate performance status and normal hepatic, renal, and cardiac functions. Exclusion criteria included the presence of serious infection and a history receiving cytoreductive chemotherapy for LCH. All patients were treated with the Special C regimen, which consisted of nine cycles of 6 mg/m^2 (max. 6 mg) of VBL on day 1, 2 mg/kg (max. 60 mg) of PSL on days 1–5, 20 mg/m^2 of MTX on day 15, and 1.5 mg/kg of 6-MP on days 1–28, over a period of 36 weeks. The dose of 6-MP was adjusted to white blood cell counts of 2,000–3,000 μ/L. Preventive medication of trimethoprim-sulfamethoxazole combination was recommended. At the end of treatment, the response was categorized as follows: no active disease (NAD) was defined as the disappearance of the signs or symptoms of disease, a partial response was defined as regression of >50 % of the signs or symptoms of disease without organ dysfunction and new lesions, no response was defined as regression of <50 % of the signs or symptoms of disease with or without organ dysfunction and the absence of new lesions, and progressive disease was defined as progression in the signs or symptoms of disease and/or the appearance of new lesions. Disease status at the last follow-up was defined as alive with NAD, alive with disease, or died. Common Terminology Criteria for Adverse Events v3.0 was used to grade adverse events.

Results

Fourteen adult patients with multifocal LCH (nine males and five females) were enrolled in this adult pilot study between 2002 and 2010 (Table 1). Four had a previous history of malignant disease (NK/T cell lymphoblastic lymphoma, renal cancer, diffuse large B cell lymphoma, and uterine cervical cancer). The median age at LCH onset was 34 years (range 16–69 years). In terms of prior medication other than cytoreductive agents for LCH, six patients were treated with PSL alone. Of the 14 patients with multifocal LCH, four had SS disease (skin, $n = 2$; multiple bones, $n = 2$) and ten had MS disease, of whom five had diabetes insipidus (DI) and one had central nervous system degeneration (CNSD) already at the time our treatment was initiated. The median time between disease onset to the initiation of our treatment was 2.4 years (range 0.1–32.7 years). The median age when our treatment was initiated was 40 years (range 20–70 years). Nine, three, and two of the patients were treated in the Departments of Internal Medicine, Dermatology, and Pediatrics, respectively. At the end of therapy, all SS patients attained NAD, while six of the ten MS patients had a response (NAD in two and a partial response in four) (Table 2). In terms of reactivation, two patients with SS disease in the skin had cutaneous reactivation and three patients with MS disease had reactivation in lymph node, bone, and mucosa (one in each patient). All reactivation sites were included in the primary lesions. Four of the five reactivations occurred approximately 1 year after therapy was initiated. In terms of treatment at reactivation, four patients underwent a

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Feasibility of autologous bone marrow mesenchymal stem cells cultured with autologous serum for treatment of haemophilic arthropathy

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Patients with severe haemophilia frequently experience spontaneous intra-articular haemorrhages, mainly in the ankles, knees and elbows. Over the long term, repeated episodes of haemarthrosis may cause irreversible damage to the joint, leading to haemophilic arthropathy, a polyarticular disease characterized by joint stiffness, chronic pain and a severely limited range of motion [1]. The progression from recurrent haemarthrosis to arthropathy is caused by inflammatory synovitis and cartilage destruction. Haemosiderin deposition into synovial tissues induces proliferation of the synovium and neovascularization of the subsynovial layer, which results in an inflamed, villous, synovial tissue. This friable and highly vascular synovium is more susceptible to further haemorrhage with minimal stress, which sets up a vicious cycle that is difficult to break [2]. Then, the articular cartilage defects occur and finally go into the cartilage destruction.

If conservative management (analgesics, orthotics and physical therapy) for the haemophilia patients who develop chronic synovutis and arthropathy fails, surgical interventions should be considered at a relatively young age. The most common surgical procedures are synovectomy, arthrodesis and total joint arthroplasty. So far, however, the regenerative medicine aiming at the repair of articular cartilage defects has not been done in the patients with haemophilic arthropathy.

We previously performed the transplantation of mesenchymal stem cells (MSCs) for the patients with osteoarthritis (OA) to repair their articular cartilage defects. MSCs generated from autologous BM blood (BMMSCs) were transplanted to the area of the articular cartilage defects with type I collagen gel. After the transplantation, the clinical outcomes were significantly improved [3–7]. Another groups also reported the repair of the articular cartilage defects with autologous BMMSC transplantation [8]. In addition, we reported that no abnormal tumours appeared in the 45 operated joints of 41 patients who received the transplantation of autologous BMMSCs between 1998 and 2008 by our groups (follow-up duration: mean, 75 months; range 5–137 months), demonstrating that autologous BMMSC transplantation is a safe procedure [4].

Therefore, in patients with haemophilic arthropathy, repair of the articular cartilage defects with autologous BMMSCs is also expected to result in great advantage, which includes the relief of pain and swelling, and increase of joint motion, and we planed the clinical trial for the treatment of haemophilic arthropathy by the autologous BMMSC transplantation. However, there have been no reports on the potentials of BMMSCs in haemophilia patients to proliferate *in vitro* and to differentiate into chondrocytes. Then, before the clinical trial, we needed to confirm the capabilities of BM cells in haemophilia patients to produce MSCs and the potential of the BMMSCs to differentiate into chondrocytes.

For this purpose, this study was done with the approval by Ethics Committee of The Institute of Medical Science, The University of Tokyo (#19–10). We harvested 4 mL of BM samples from iliac bones of three haemophilia patients and one healthy volunteer after obtaining written informed consents. BM samples from three patients with haemophilia A, who had experienced orthopaedic surgery, were harvested during the general anaesthesia. BM sample from a healthy adult was obtained under local anaesthesia. Autologous serum from each donor of BM samples was collected in other day prior to the BM harvest.

Before 2001, we added foetal bovine serum (FBS) into the medium for BMMSC culture [3,4,9], but the issue of bovine spongiform encephalopathy changed our strategy not to use FBS in the culture of human BMMSCs for the therapeutic transplantation. Since then, considering this issue and the problem of transmitted infectious pathogen from allogeneic products, we used autologous serum instead of FBS or allogeneic human serum for the clinical application of human BMMSCs. Indeed, we experienced no cases who suffered with the infection in 41 patients who received the transplantation of autologous BMMSCs in our groups [4].

In the present culture, as described previously, 4 mL of harvested BM blood was divided into halves, and each 2 mL of BM blood was cultured in 75-cm^2 flask with culture media, which consist of Dulbecco's modified Eagle's medium and 15% volume of autologous serum [4,5]. After 3 days of culture, when attachment of cells was observed, the medium was exchanged, and red blood cells and non-adherent cells were discarded with the medium. After 10–12 days, adherent cells achieved subconfluence, and were passaged to expand in culture. When the adherent cells achieved confluence after another 10–12 days, we collected the cultured cells, which are supposed to be used in the clinical trial, and were processed for the experiments.

Morphology of the cultured cells from BM cells of three haemophilia patients was spindle-shaped like MSCs, and almost same as those from a healthy adult (Fig. 1a). Flow cytometric analysis revealed that cultured cells were positive for CD29, CD44, CD13, CD73, CD90 and CD105, but negative for CD45 and CD14 (Fig. 2). These results were identical with BMMSCs reported previously [10]. When induced to differentiate into chondrocytes using NH ChondroDiff Medium (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions, the cultured cells from BM cell of haemophilia patients and healthy adult generated toluidine blue-positive chondrocytes (Fig. 1b). This result indicated that MSCs capable of differentiating into chondrocytes were generated from BM blood in haemophilia patients, similarly to those in a healthy adult.

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