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Ex Vivo-Expanded Donor CD4$^+$ Lymphocyte Infusion Against Relapsing Neuroblastoma: A Transient Graft-Versus-Tumor Effect

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High-risk neuroblastoma has a poor prognosis despite multimodal treatment including high-dose chemotherapy. A 7-year-old male with neuroblastoma received ex vivo-expanded donor CD4$^+$ T lymphocyte infusion (CD4$^+$ DLI) after recurrence in the bone marrow following allogeneic hematopoietic stem cell transplantation from his HLA-identical mother. The disease transiently responded to CD4$^+$ DLI with reduction of tumor cells and a decrease of serum neuron-specific enolase. The response was associated with development of continued high fever and an increase of cytotoxic T lymphocytes in peripheral blood. This case suggests a possibility of a graft-versus-tumor effect against neuroblastoma. Pediatr Blood Cancer 2009;52:895–897.

Key words: CD4$^+$ donor lymphocyte infusion; graft-versus-tumor effect; neuroblastoma

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can exert an immune graft-versus-tumor (GVT) effect mediated by donor lymphocytes, which plays a therapeutic role in the treatment of hematologic malignancies. The GVT effect was directly confirmed by the observation that donor lymphocyte infusion (DLI) can successfully induce remission of chronic myelogenous leukemia, which relapse after allo-HSCT [1]. Several small studies have also suggested GVT effects following allo-HSCT in patients with solid tumors [2–6]. Although allo-HSCT has been applied in a considerable number of patients with neuroblastoma (NBL) [6], there are few reports describing a GVT effect against this malignancy. Here, we describe a patient with relapsing NBL showing transient tumor regression after ex vivo-expanded donor CD4$^+$ lymphocyte infusion (CD4$^+$ DLI).

CASE REPORT

A 4-year-old male was diagnosed with stage 4 NBL (International NBL Staging System: INSS) who developed as a retroperitoneal mass with metastases to the bone marrow (BM), cervical lymph nodes and bone (orbit). Pathological studies showed poorly differentiated NBL (International NBL Pathology Classification: INPC) with Shimada's unfavorable histology without amplified N-myc expression. He was initially treated with combination chemotherapy consisting of cyclophosphamide, vincristine, pirarubicin (THP-adriamycin), cisplatin, and etoposide. He then received high-dose chemotherapy (HDC) consisting of thio-TEPA and melphalan with autologous peripheral blood stem cell transplantation (auto-PBSCT), followed by surgical removal of primary tumor [7,8].

The disease recurred in the BM, right mandible, bilateral cervical lymph nodes, and right iliac and inguinal lymph nodes at 6 years of age, 13 months after HDC with auto-PBSCT. Following combination chemotherapy consisting of topotecan, cyclophosphamide, and cisplatin, he received an allogeneic bone marrow transplantation (allo-BMT) from his HLA-identical mother. The conditioning regimen consisted of busulfan (16 mg/kg) and fludarabine (180 mg/m^2) preceded by topotecan (30 mg/m^2). Prophylaxis for graft-versus-host disease (GVHD) was short-term methotrexate and tacrolimus. Engraftment was prompt and no acute GVHD was observed. He was also treated with radiotherapy to lymph nodes of the neck and pelvis after allo-BMT, which led to successful renewed remission. However, he developed a recurrence in BM with elevation of serum neuron-specific enolase (NSE) 1 month after completion of radiotherapy, for which he received two courses of conventional DLI [1–5 × 10^6/kg CD3$^+$ T-lymphocytes] from his mother (Fig. 1). However, tumor cells in BM increased and

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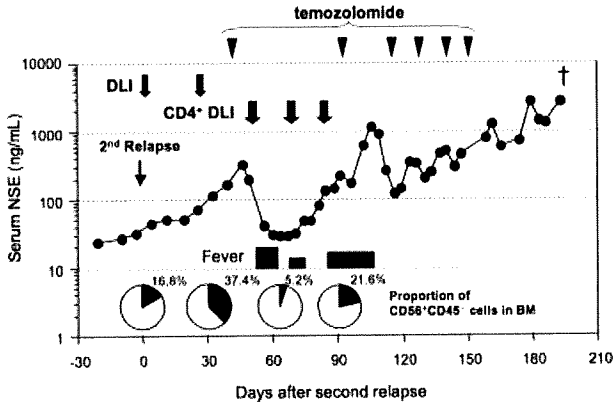

Fig. 1. Clinical course and changes in serum NSE. NSE, neuron-specific enolase; BM, bone marrow. DLI indicates donor lymphocyte infusion: 1st dose, 1×10^6/kg and 2nd dose, 5×10^6/kg $CD3^+$ T lymphocytes. $CD4^+$ DLI indicates ex vivo-expanded donor $CD4^+$ lymphocyte infusion: 1st and 2nd dose, 1×10^7/kg; and 3rd dose, 5×10^7/kg. The purity of CD4-single positive cells was 93.4%, 95.6%, and 90.9%, respectively. The majority of contaminating cells were $CD4^+CD8^+$. Temozolomide was administered at 150 mg/m^2 daily for five consecutive days for each cycle.

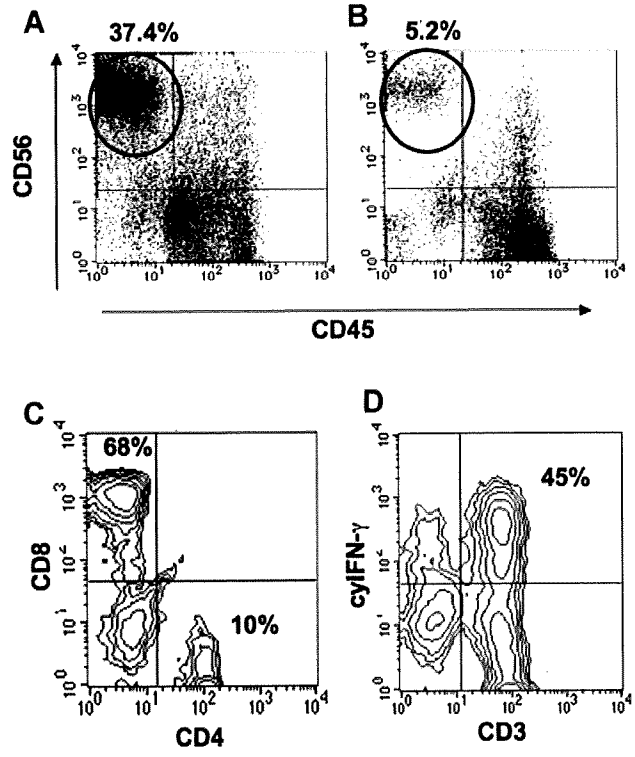

Fig. 2. Flow cytometric analysis. Tumor cells ($CD56^+CD45^-$) in iliac bone marrow before (**A**) and 12 days after (**B**) the first $CD4^+$ donor lymphocyte infusion (DLI). Proportion of $CD4^+$ or $CD8^+$ T lymphocytes (**C**) and $CD3^+$ T lymphocytes producing cytoplasmic IFN-γ (**D**) in peripheral blood mononuclear cells after $CD4^+$ DLI.

associated with increased serum NSE but without development of GVHD. We therefore infused ex vivo-expanded donor $CD4^+$ T lymphocytes ($CD4^+$ DLI) with the aim of accelerating allogeneic immunoreaction without eliciting GVHD.

Mononuclear cells were isolated from his mother. $CD4^+$ T lymphocytes were purified by CD4 monoclonal antibody (mAb)-coated magnetic beads and cultured for 1 week in the presence of recombinant IL-2 (350 IU/ml; Proleukin, Chiron BV, Amsterdam, The Netherlands) in a flask with immobilized anti-CD3 mAb, OKT3 (5 μg/ml; Jansen-Kyowa, Tokyo, Japan) [9]. This trial and culture procedure were approved by the Institutional Review Boards of Tokyo Medical and Dental University, and Osaka University Hospital. Written informed consent was obtained from the parents of the patient. The patient, then 7 years of age, was treated with $CD4^+$ DLI following administration of temozolomide (Fig. 1). Shortly after the first $CD4^+$ DLI (1×10^7/kg) with 93.4% purity of CD4-single positive cells, he developed high fever of 40°C without other GVHD signs such as skin rash, jaundice, or diarrhea. High fever continued for 2 weeks with reduction of serum NSE levels from 325.5 to 29.2 ng/ml. Iliac BM aspiration showed a decrease in the ratio of the tumor cells ($CD56^+CD45^-$ cells) from 37.4% to 5.2% (Fig. 2A,B). Twelve days after $CD4^+$ DLI, $CD8^+$ T lymphocytes with IFN-γ production predominated in peripheral blood (Fig. 2C,D). However, serum NSE increased after the second $CD4^+$ DLI. Despite the third $CD4^+$ DLI at an increased dose of 5×10^7/kg, the disease continued to progress. He then received temozolomide but without response and died 7 months after the second relapse.

DISCUSSION

The prognosis of high-risk NBL, characterized by an older age, metastases, N-myc amplification, and unfavorable histologic findings, remains poor [10,11]. More than half of these high-risk patients relapse despite strategies involving HDC followed by auto-HSCT, which indicates a need for novel strategies to eradicate residual disease. Allo-HSCT has been already used for adult patients with solid tumors [4,6], in particular renal cell carcinoma [2,5] and breast cancer [3,5]. Recent trials using allo-HSCT, mostly following non-myeloablative preconditioning, showed a response rate of up to 57% against renal cell carcinoma [2,3,5].

A dramatic reduction of tumor cells was observed in our patient following $CD4^+$ DLI. The clinical response with the development of high fever immediately after $CD4^+$ DLI combined with an increase of IFN-γ-producing $CD8^+$ T lymphocytes, that is, cytotoxic T lymphocytes (CTLs), suggests a GVT effect. Moreover, we observed no increase of NK cells in peripheral blood nor increase of expression of HLA-A24 (the patient's and the donor's HLA-A type) on residual tumor cells (data not shown). Taken together, the immunoreaction against NBL cells was presumably caused by CTLs, not by NK cells. $CD8^+$ T lymphocytes (CTLs) were increased following $CD4^+$ DLI. Expanded and activated $CD4^+$ helper T lymphocytes might have produced cytokines that stimulated CTL differentiation and enhanced the ability of antigen-presenting cells to stimulate CTL differentiation through a CD40-CD40L interaction [12].

An immunological response due to lymphocytes might be attributable in our case to scattered tumor cells in BM, which were abundant in bloodstream, as is more frequently seen in leukemia. Although the administration of temozolomide shortly before $CD4^+$ DLI might have affected the clinical response, there was no response

during the second course of temozolomide during the final course of the disease, which suggests that the first course was not associated with a reduction of tumor cells.

In 1994 Matthay et al. [13] reported no advantage of allo-HSCT over auto-HSCT in patients with NBL and few reports suggest a GVT effect against NBL. Inoue et al. [14] reported a case showing the disappearance of NBL within 3 years after allo-HSCT from an HLA haploidentical donor. Although a considerable number of patients with NBL has been treated with allo-HSCT [6], detailed analysis has not been performed regarding its efficacy. Dykes et al. [15] have recently used $CD3^+$ T-cell depleted allo-PBSCT from HLA-haploidentical donor to patients with NBL.

The response in our patient suggests a transient GVT effect against NBL cells. Immunotherapy with allogeneic lymphocytes might open new avenues for overcoming the dismal prognosis of high-risk NBL.

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CASE REPORT

Successful treatment of chronic granulomatous disease with fludarabine-based reduced-intensity conditioning and unrelated bone marrow transplantation

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Abstract Allogeneic hematopoietic stem-cell transplantation (HSCT) for chronic granulomatous disease (CGD) with a reduced-intensity conditioning regimen can be expected to lead to less therapy-related mortality and late-onset impairment, whereas it has also been reported to increase the risk of unsustained mixed donor chimerism and late rejection after transplantation. Herein, we report a 4-year-old boy with CGD who was successfully treated with unrelated bone marrow transplantation with a reduced-intensity conditioning regimen (RIC). Fludarabine-based RIC, 4 Gy of total body irradiation, 120 mg/kg of cyclophosphamide, and 125 mg/m^2 of fludarabine, was adopted for transplantation, followed with 8.9×10^8/kg mononucleated donor cells infused without T-cell depletion. Although hematopoietic engraftment was rapidly obtained by day +17, he developed unstable donor chimerism. After tacrolimus withdrawal, the patient showed grade III acute graft-versus-host disease (GVHD), and subsequently reached full donor chimerism by day +61. Twelve months post-transplant, the patient has remained well with stable and durable engraftment, 100% donor chimerism, and normal superoxide production, without the requirement of donor lymphocyte infusions (DLI).

Keywords Chronic granulomatous disease · Unrelated bone marrow transplantation · Reduced intensity conditioning

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1 Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency caused by impaired phagocyte killing of intracellular pathogens, characterized by recurrent, often life-threatening bacterial and fungal infections and by granuloma formation in vital organs. It results from mutation in any one of four subunits of a nicotinamide adenine dinucleotide phosphate oxidase of phagocytic cells (gp91^{phox}, p47^{phox}, p67^{phox}, and p22^{phox}) [1]. Although the prognosis of CGD has markedly improved due to prophylactic treatment for infections, including the induction of interferon-gamma therapy, annual mortality is still between 2 and 5% [2]. Allogeneic hematopoietic stem-cell transplantation (HSCT) is an alternative to conventional treatment for CGD, but a high transplantation-related mortality rate [3] and high risk of graft rejection have lowered its therapeutic efficacy [4]. We here in report a 4-year-old boy with CGD who was successfully treated with unrelated bone marrow transplantation with a fludarabine-based reduced-intensity conditioning regimen (RIC).

2 Case report

A 4-year-old boy with CGD was admitted to our hospital in August 2005. He had had recurrent bacterial and fungal infections from early infancy, and CGD was diagnosed by

reduced NADPH oxidase (0%), confirmed by $gp91^{phox}$ expression analysis when he was 1 year old. His elder brother was also diagnosed with CGD, and died of fungal pneumonia at the age of 10 years old. There was no HLA-identical HSCT donor in his family. He received anti-infectious prophylaxis consisting of itraconazole and sulfamethoxazole/trimethoprim. Diagnostic imaging at 3 years of age showed intraperitonial granulation tissue formation and hyperplasia of the intestinal tract, resulting from having intussusceptions two times. Interferon gamma therapy had been given for 6 months before transplantation, but subsequently failed. Thus, allogeneic bone marrow transplantation from an HLA-matched volunteer donor was planned.

At 4 years of age, he received allogeneic bone marrow transplantation from an HLA-matched unrelated donor in March 2006. Donor and recipient HLA matching was confirmed by serotyping and molecular typing of the HLA class I and II loci, respectively. We used a RIC for transplantation with total body irradiation at a dose of 2 Gy (days −8 and −7) without use of the gonadal shield, cyclophosphamide at a dose of 60 mg/kg (days −3 and −2) and fludarabine at a dose of 25 mg/m^2 (days −6, −5, −4, −3 and −2), because the patient had been chronically ill, showing intermittent fever and moderate elevation of CRP values, which was thought to be due to chronic enterocolitis. Repeated stool and blood cultures were negative for bacteria and fungi. Just before transplantation, laboratory findings included increased C-reactive protein (2.39 mg/dl) and a normal beta-D-glucan level. Latex agglutination test for serum *Aspergillus* and serum *Candida* antigens were negative.

A cell dose of 8.9 × 10^8/kg mononucleated cells was infused to the patient without T-cell depletion. GVHD prophylaxis consisted of tacrolimus (0.03 mg/kg/day i.v. continuous infusion from day −1) and short-term methotrexate (10 mg/m^2 i.v. on day +1, 7.5 mg/m^2 i.v. on days +3 and +6). He was also nursed in a high-efficiency, particulate-air-filtered protected environment, and underwent oral gut decontamination. He received *Peumocystis carinii* prophylaxis by sulfamethoxazole/trimethoprim, which was interrupted after transplantation until neutrophil recovery confirmed. Post-transplant regimen also included acyclovir, ursodeoxycholic acid and intravenous immunoglobulin therapy. Chimerism was studied via the analysis of informative microsatellite DNA sequences. The oxidase-positive neutrophils were detected by flow cytometry with the use of a dihydrorhodamine oxidation assay.

During the conditioning therapy for transplantation, prolonged fever rapidly resolved and C-reactive protein values also decreased to within normal ranges. A total of 300 μg/m^2 of granulocyte-colony stimulating factor was commenced on day +5 post-transplant. The patient engrafted rapidly. He achieved an absolute neutrophil count of 0.5 × 10^9/l by day +17. Chimerism analysis revealed 62.5% donor cell engraftment by day +21, and 39.3% donor cell engraftment additively decreased by day +34, respectively. To achieve complete chimerism, we stopped all immunosuppressants by day +39, because he had no GVHD confirmed at that time. Subsequently, grade III acute GVHD of his skin and gut were clinically confirmed on day +55, followed by full converted donor chimerism and normal superoxidase production by day +61. He was treated again with tacrolimus and 2 mg/kg of prednisolone for GVHD, and all GVHD symptoms disappeared by day +80. Reactivation of his Cytomegalovirus antigenemia was detected on day +65, and treated with ganciclovir with good response. Flow cytometric analysis with the use of a dihidrorhodamine

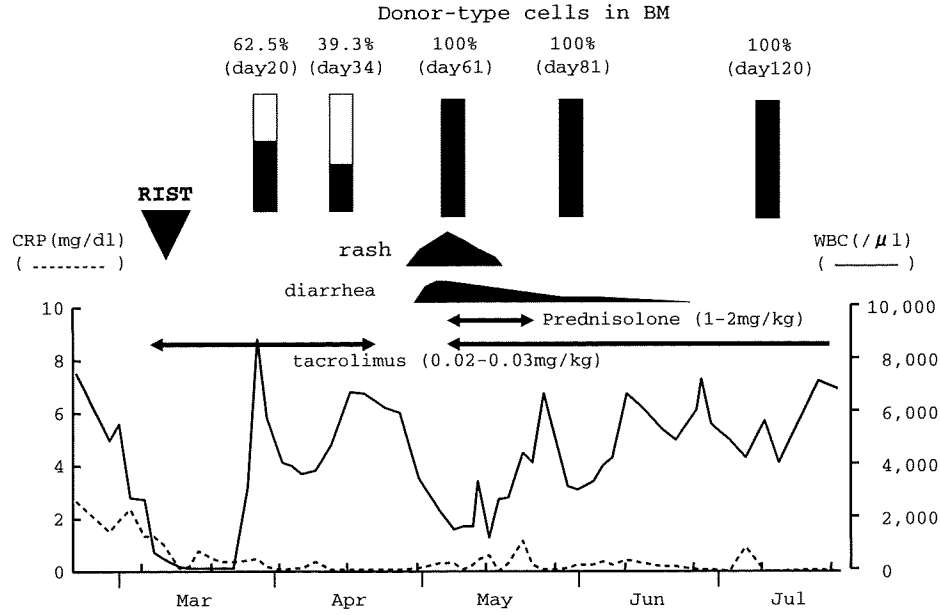

Fig. 1 Clinical course after unrelated bone marrow transplantation. RIST indicates reduced intensity stem cell transplantation. In the upper section of the figure, the donor-type cells in the bone marrow are represented as *black-lacquered*

oxidation assay showed that oxidase-positive neutrophils were detected as 100% of engrafted cells since then. Twelve months post-transplant, the patient has remained well, with stable and durable engraftment, 100% donor chimerism, normal superoxide production, without donor lymphocyte infusion (DLI) requirement (Fig. 1).

3 Discussion

Allogeneic HSCT is the curative therapy for CGD, especially in patients with no inflammatory or infectious lesions at transplant with an excellent disease-free survival rate (DFS). A survey of European Group for blood and marrow transplantation (EBMT) has advocated myeloablative regimens, mostly consisting of busulfan (16 mg/kg) and cyclophosphamide (200 mg/kg), and T-cell replete allografts from HLA-matched related donors, which provided excellent results in low-risk CGD patients (15 children and 1 adult) with no overt infectious complications at transplant and a DFS of 100% [3]. However, in the EBMT report, inadequately high rates of severe acute GVHD and pulmonary infectious complication with a transplant-related mortality of 36% (4 of 11 patients) were also observed in advanced CGD patients with active inflammation due to granulomatous colitis or active infectious disease. Thus, transplant-related mortality with standard myeloablative transplantation regimens, especially in advanced CGD, has been a major obstacle to the more widespread use of allogeneic HSCT.

Horwitz et al. recently reported promising results in the treatment of 10 advanced CGD patients with the combination of a nonmyeloablative regimen consisting of cyclophosphamide, fludarabine, and antithymocyte globulin and the use of a T-cell depleted HLA-identical allograft [5]. This US trial demonstrated that seven out of 10 patients were successfully cured of the disease, even though two patients rejected their graft and DLI led to GVHD in three patients, which was fatal in one case. There are also several reports of successful outcomes for CDG with fludarabine-based RIC [6–9], while most of them consisted of transplant from HLA-matched related donors. Furthermore, T-cell depletion could be a promising approach to reduce the incidence of GVHD, while it could be associated with an increased risk of infectious complications and graft rejection. Thus, RIC is associated with a lower toxity from the conditioning agents and may be an alternative option for CGD, while it still carries a significant risk of graft rejection and GVHD, particularly if DLI have to be used to ensure engraftment.

A national survey of HSCT for CGD in Japan has shown fairly high survival rate (22 of 28), in which the survival rate of HSCT from HLA-matched siblings were comparable to that of HSCT from HLA-matched unrelated donors, whereas that of cord blood transplantation were improperly poor (2 of 4) [10]. Recently, nonmyeloablative conditioning regimens, mostly consisting cyclophosphamide and fludarabine, have been preferred, while the myeloablative conditioning, consisted of busulfan and cyclophosphamide, have been initially performed. However, inadequately high rates of development of unsustained mixed chimerism with the requirement of DLIs were also demonstrated in the patients with RIC by cyclophosphamide and fludarabine. In current case, we adapted fludarabine-based RIC without T-cell deletion for transplantation, because it is not allowed to manipulate unrelated donor allografts for DLIs, and also increased the total body irradiation dose to 4 Gy to ensure engraftment. Taken together, although standard regimens for transplantation of advanced CGD have not been established, our present case encourages the consideration of unrelated HSCT with fludarabine-based RIC for patients with CGD, even if they have infectious complications and no suitable related donors.

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