Early recovery of lymphocyte count after hematopoietic stem cell transplantation is a potential risk factor for chronic graft-versus-host disease

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Abbreviations	
HSCT	hematopoietic stem cell transplantation
Allo	Allogeneic
GvHD	graft-versus-host-disease
AML	acute myeloid leukemia
ALL	acute lymphoblastic leukemia
CMV	cytomegalovirus
BM	bone marrow
PBSC	peripheral blood stem cell
CsA	cyclosporin A
FK506	tacrolimus

Abstract

Background. Few studies have investigated the association between severity of lymphopenia and clinical outcome during chemotherapy or hematopoietic stem cell transplantation (HSCT). We investigated this issue by retrospectively analyzing pediatric patients who received allogeneic-HSCT (allo-HSCT) using a newly developed parameter called the LD-index that combines both the duration and the intensity of lymphopenia. ***Procedure.*** A total of 92 patients underwent allo-HSCT in our hospital from April 2007 to August 2019. The median age at HSCT was 10.3 years (range 0.4 – 28.1). ***Results.*** The median LD-index was 9,285 (range 2,217 – 36,064). A significantly high association was observed between the LD-index and the incidence of chronic graft-versus-host disease (GvHD) ($p = 0.0045$). Analysis of predictive factors for chronic GvHD was carried out using univariate analysis. Lower LD-index, donor source and duration (days) of lymphopenia were found to be significant factors associated with chronic GvHD. Multivariate analysis, however, only identified an association between lower LD-index and increased incidence of chronic GvHD ($p = 0.004$). ***Conclusion.*** The duration and the intensity of lymphopenia after allo-HSCT have an effect on the development of chronic GvHD.

Key words: LD-index, chronic GvHD, lymphopenia, allogeneic-HSCT

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is widely utilized for the treatment of relapsed or refractory malignancy, bone marrow failure, immunodeficiency, metabolic disorders and solid tumors. An important role of allogeneic HSCT (allo-HSCT) is the immune-mediated destruction of recipient tumor cells by donor lymphocytes. Repopulating lymphocytes are associated with the prevention of serious infections in the early post-transplant phase as well as the reduction of recurrence, by attacking residual tumor cells. However, these lymphocytes may also play a role in limiting treatment due to their role in the development of graft-versus-host disease (GvHD) (ref. 1-4). Natural killer cells, which mediate cytotoxicity without prior sensitization, are the first cells to recover in the early post-transplant period (ref. 5-6). Several studies have shown that delayed lymphocyte recovery after allo-HSCT is a risk factor for recurrence in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) (ref. 7-10). Previous studies indicate an association between profound lymphopenia after allo-HSCT and cytomegalovirus (CMV) reactivation, or CMV disease (ref. 11-12). Most of these studies however, evaluate lymphopenia simply by the

number of lymphocytes at a certain time point.

In this study, we developed a new parameter, the LD-index that combines the intensity and the duration of lymphopenia and analyzed its association with post-transplant related factors and events in pediatric and adolescent and young adult patients.

Materials and Methods

Patient characteristics

A total of 110 consecutive patients who underwent HSCT at our center between April 2007 and August 2019 were enrolled in this study. Among them, patients who underwent autologous-HSCT (n = 9) and who did not gain lymphocyte recovery (> 0.5×10^9/L) due to treatment-related death or death from original diseases (n = 9) were excluded. A total of 92 patients with allo-HSCT were therefore included in this study.

There were 54 male and 38 female patients with a median age at transplant of 10.3 years (range 0.4 – 28.1). There were 78 first transplants and 14 second or more transplants, and we analyzed each event for each transplant. Donor source was bone marrow (BM) (n = 36), peripheral blood stem cell (PBSC) (n = 5) and cord blood (n = 51). Thirty-one patients had

ALL and 20 patients had AML. Forty-one patients with other diseases including acute undifferentiated leukemia (n = 1), chronic myeloid leukemia (n = 3), non-Hodgkin lymphoma (n = 4), juvenile myelomonocytic leukemia (n = 2), myelodysplastic syndromes (n = 2), neuroblastoma (n = 10), hepatoblastoma (n = 2), rhabdomyosarcoma (n = 2), aplastic anemia (n = 10), X-linked severe combined immunodeficiency (n = 1), Wiskott-Aldrich syndrome (n = 1) and metabolic disorders (n = 3).

The conditioning regimens included a busulfan (≥ 6.4 mg/kg) based regimen (n = 23), a total body irradiation (≥ 8 Gy) based regimen (n = 24), fludarabine combined with busulfan (n = 3), fludarabine combined with melphalan (n = 32), fludarabine combined with cyclophosphamide (n = 8), and other (n = 4). Classification of the intensity of the conditioning regimen was based on the Center for International Blood and Marrow Transplant Research suggested criteria (ref. 13), and patients were conditioned either with myeloablative conditioning (n = 45) or reduced-intensity conditioning (n = 47) regimens. GvHD prophylaxis consisted of intravenous drips of either cyclosporin A (CsA) (n = 20) or continuous infusion of tacrolimus (FK506) (n = 12) combined with short-term methotrexate (MTX), CsA (n = 54) or FK506 (n = 1) combined with methylprednisolone, and MTX only (n = 5). Data were analyzed as of July 2020 (Table 1). This study was approved by the

Institutional Review Board of Sapporo Hokuyu Hospital.

Definition and calculation of LD-index

The LD-index was derived by plotting the absolute lymphocyte count (ALC) during lymphopenia (tentatively defined as < 500/μL) and calculating the area over the curve, which was the difference of the expected lymphocyte area (A_e) minus the area under the curve (A_0) (Fig. 1A). A_e was calculated as the product of 500 with the number of days of lymphopenia (< 500/μL) from day 0 to the day the ALC was more than 0.5×10^9/L. A_0 was calculated using the trapezium rule, as follows:

$$A_0=\sum_{i=2}^{n} ¿¿$$

Here, $(t_i - t_{i-1})$ is the time interval (days) between two consecutive lymphocyte counts, and L_i and L_{i-1} are the respective lymphocyte counts (per microliter) at times t_i and t_{i-1}. The LD-index is the difference of $A_e - A_0$. All areas were expressed in days · lymphocyte/μL. As an example, we show the calculation of the A_0, A_e, and the LD-index for a virtual case (Fig. 1B). This case has the following lymphocyte counts: day 0 = 200/μL, day 2 = 0/μL, day 4 = 0/μL, day 6 = 250/μL, day 8 = 450/μL, day 9 = 600/μL. We then compute the sum of the area of four trapeziums as we have five lymphocyte counts (n = 5). Hence, A_0 = [1/2 (200 + 0)×2] +

[1/2 (0 + 0)×2] + [1/2 (0 + 250)×2] + [1/2 (250 + 450)×2] + [1/2 (450 + 600)×2] = 200 + 0 + 250 + 700 + 1050 = 2,200 days · lymphocyte / μL.

The A_e of this case with 8 days of lymphopenia (see Fig. 1B) is calculated to be:

$A_e = 500 \times 8 = 4{,}000$ days · lymphocyte / μL.

Finally, the LD-index of this case is the difference $A_e - A_0 = 1{,}800$ days · lymphocyte / μL.

Definition of fungal infection

Fungal infection was defined according to the standardized definitions of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Disease Mycosis Study Group consensus group (ref. 14). In this study, cases that fit the criteria of both possible and probable were adopted as fungal infection.

Definition of acute GvHD and chronic GvHD

There are several different diagnostic criteria for acute GvHD. This study used the MAGIC criteria (ref. 15). For chronic GvHD, we used the 2014 NIH Chronic GvHD Diagnosis and Staging Consensus Recommendations for our diagnosis and severity classification (ref. 16).

Statistical analysis

Data were described using median, ranges, and percentage values. All statistical analyses were conducted using EZR (Saitama Medical Center, Jichi Medical University), a graphical user interface for R commander version 2.5-1 (The R Foundation for Statistical Computing, Vienna, Austria) (ref. 17). Dichotomous variables were compared using Fisher's exact test, and continuous variables were compared using the Mann-Whitney *U*-test. We evaluated factors for continuous variables (such as the LD-index) using a receiver operating characteristic (ROC) curve analysis. A *P*-value of < 0.05 was considered to be significant. Multivariate stepwise regression was performed to explore the independent effects of variables that showed a significant influence via univariate analyses ($P < 0.10$). Results were expressed as relative risks (95% confidence intervals).

Results

The median LD-index deduced from all eligible patients was 9,285 (range 2,217 – 36,064).

As can be seen in Table 2, the age at HSCT, sex, donor source, human leukocyte antigen (HLA) matching, the number of transplantations, diseases, infectious complications including bacterial and fungal infections during HSCT, the CMV antigenemia, the incidence of acute GvHD, treatment related death and death were not significantly associated with the LD-index. Only chronic GvHD exhibited a significant association with the LD-index ($p = 0.0045$). The LD-index of patients with or without chronic GvHD was 8,298.5 (range 3,725 – 13,521) and 10,112.5 (range 2,217 – 36,044), respectively. This result indicated that chronic GvHD post-transplant was strongly associated with early recovery of lymphopenia.

We then analyzed predictive factors for chronic GvHD in our 92 patients with allo-HSCT. In this analysis, the optimal cut-off value was calculated using the ROC curve analysis for the LD-index, age at HSCT and simple duration of days of lymphopenia ($< 0.5\times10^9$/L), all of which are continuous variables. The optimal cut-off was calculated to be 9,040 for the LD-index, 10.5 years for age at HSCT, and 23 days for duration of lymphopenia (Table 3). We then analyzed the association of chronic GvHD with a number of items including the LD-index, the age at HSCT, the simple duration (days) of lymphopenia and so on (Table 3). In this univariate analysis, lower LD-index (LD-index < 9,040), graft by non-BM cells, graft by PBSC and short duration of lymphopenia (< 23 days) were extracted as factors associated

with a high incidence of chronic GvHD. In multivariate analysis, however, only low LD-index (LD-index < 9,040) appeared to be a potential risk for chronic GvHD (odds ratio = 3.90, p = 0.004) (Table 4).

Discussion

This study demonstrates that a lower LD-index combining both the intensity and the duration of post-transplant lymphopenia (< 0.5×10^9/L) was significantly associated with the development of chronic GvHD. With respect to the intensity and the duration of cytopenia after HSCT, the first item of interest was neutrophils. The D-index is a method used to evaluate the duration and intensity of neutropenia, and there have been reports regarding the D-index and the development of fungal infections (ref. 18-19).

Several studies have shown that early recovery of lymphocytes after allo-HSCT is associated with better survival, reduced relapse, and lower transplant-related mortality, especially in AML (ref. 1-4). In pediatric patients, Afzal *et al*. reported that early lymphocyte recovery was associated with significant graft-versus-leukemia without an increase in acute GvHD for ALL but was not prognostic for AML (ref. 20). Thus, the effect of lymphopenia

after allo-HSCT has not been paid much attention.

Previous reports have focused on lymphopenia at a single time point after HSCT, which is insufficient to explore the true effect of repopulated lymphocytes on the outcome of HSCT patients. We therefore developed a new parameter called the LD-index which combines the intensity of lymphopenia (< 0.5×10^9/L) with its duration, in accord with the procedure developed for the D-index (ref. 18). A previous report by Kimura *et al*. also used a parameter combining intensity and duration of lymphopenia, called the L-index, in order to evaluate lymphopenia (ref. 21). However, the L-index uses the intensity and duration of lymphopenia (< 0.7×10^9/L) from the beginning of conditioning until either day 30 or day 100 after HSCT. This is different to our LD-index in its span and depth of lymphopenia of measurements (day 0 after HSCT to the day of lymphocytes > 0.5×10^9/L). According to the report by Kimura *et al*., the L-index was significantly associated with CMV reactivation and grade II-IV acute GvHD (ref. 21-22).

In this study, we set the standard of lymphopenia to 0.5×10^9/L. Ninety-two out of 101 patients with allo-HSCT in our hospital recovered > 0.5×10^9/L lymphocytes. Nine patients did not recover: 8 patients had treatment-related death and 1 patient died as a result of the original disease. All 9 of these patients died early after HSCT (median 26 days; range 1 – 47

days). Although these patients were excluded from our study, we did not consider lymphocyte depletion to affect the cause of death in these patients.

Our study shows that the patients with chronic GvHD had a significantly lower LD-index compared to those without it. Contrary to the L-index, no association was observed between the LD-index, and CMV reactivation or the onset of acute GvHD. Several reports assess the relationship between lymphocyte recovery after HSCT and the relapse of hematological malignancies (ref. 7-10). We therefore performed a subgroup analysis to assess the association between the LD-index and relapse of ALL (n = 31) or AML (n = 19), however no significant association was found.

Chronic GvHD develops in 30 – 50% of patients with allo-HSCT. Chronic GvHD is a syndrome of variable clinical features resembling autoimmune and other immunologic disorders, such as scleroderma, Sjögren's syndrome, primary biliary cirrhosis, wasting syndrome, bronchiolitis obliterans (ref. 23-24). It affects the risk of late morbidity and mortality and is one of the major causes of poor quality of life (QOL). Chronic GvHD was previously defined as symptoms that occurred 100 days post HSCT, but is now defined by the 2005 NIH Working Group as characteristic clinical symptoms at any time (ref. 24-25). Although the NIH has reported on the biology of chronic GvHD (ref. 26), much about its

pathology remains unclear. A number of reports have studied the factors that increase the risk for chronic GvHD such as an unrelated and HLA mismatched donor, history of acute GvHD, older donors, older recipients, HSCT from female to male and donor source by PBSC (ref. 23, 27-29). Few studies addressing the association between lymphopenia and the development of chronic GvHD have however been carried out.

To our knowledge, this is the first study that demonstrates the duration and the intensity of lymphopenia after allo-HSCT is associated with the risk of developing chronic GvHD. One study by Savani *et al*. found that an ALC > 0.45×10^9 /L at day 30 after T cell-depleted allogeneic HSCT was independently associated with an increased incidence of chronic GvHD (ref. 30). Although the report by Savani *et al.* differed from our study in the stem cells used for HSCT, it also suggests that recovery from lymphopenia after HSCT might have some effect on chronic GvHD.

There were limitations to this study. First, the subset of repopulated lymphocytes after HSCT was unknown as lymphocyte subset analysis was not routinely performed at our center. Therefore, it was not possible to evaluate which lymphocyte affected chronic GvHD. Second, the number of cases was small and their diseases were heterogeneous, as was their conditioning regimen and GvHD prophylaxis.

In conclusion, the LD-index that combines both the duration and the intensity of lymphopenia following allo-HSCT, was found to be a powerful predictor of chronic GvHD. Patients with chronic GvHD suffer significant morbidity with reduced quality of life and functional defects, as well as increased risk of mortality. The frequency and severity of chronic GvHD in long-term survivors is an issue that will increase in significance as procedures in stem cell transplantation become more complex. Further analyses are being carried out in order to further explore the mechanism of the association between lymphopenia and the development of chronic GvHD.

Conflicts of interest

The authors have no conflicts of interest or sources of funding to disclose.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends:

Figure 1A ; Calculation of the LD-index

Figure 1B ; Calculation of the LD-index (virtual case)