Pre-transplantation thymic function is associated with the risk of acute graft versus host disease and cytomegalovirus viremia after allogeneic hematopoietic stem cell transplantation

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Abstract

Objectives: To analyze the kinetics of T-cell subsets and thymic function reconstitution after allogeneic hematopoietic stem cell transplantation (AHSCT); to determine whether sjTREC (signal joint TCR rearrangement excision circle) and CD31-positive recent thymic emigrant (CD31 + RTE) are correlated with acute graft versus host disease (aGVHD) or CMV (cytomegalovirus) viremia after AHSCT.

Methods: Forty-nine patients who underwent AHSCT in our institution were prospectively enrolled. Periphery blood samples were collected before conditioning and at 1, 2, 3 months after AHSCT. T-cell subsets were analyzed with flow cytometry. Genomic DNA was purified from peripheral blood mononuclear cells (PBMCs), and sjTREC was quantified by real-time PCR. Impact of sjTREC and CD31 + RTE on aGVHD and CMV viremia was evaluated by univariate and multivariate Cox regression analyses.

Results: The analyzed T-cell subsets and sjTREC of patients before AHSCT were all significantly lower than those of healthy donors (p < 0.05). sjTREC and CD31 + RTE were remarkably decreased in 3 months after AHSCT (p < 0.05). Patients with lower pre-transplantation sjTREC and CD31 + RTE level had higher incidence of CMV viremia after AHSCT (p < 0.05). sjTREC/10^6 PBMCs was negatively correlated with aGVHD (p = 0.024).

Conclusion: Thymic function was impaired before transplantation, and was consistently decreased in 3 months after AHSCT. Patients who had lower pre-transplantation sjTREC level were at high risk of aGVHD and CMV viremia after AHSCT, low pre-transplantation CD31 + RTE was correlated with CMV viremia after AHSCT.

Keywords: Thymic function; signal joint TCR rearrangement excision circle; CD31-positive recent thymic emigrant; acute graft versus host disease; cytomegalovirus viremia

Introduction

Allogeneic hematopoietic stem cell transplantation (AHSCT) has been widely used in the treatment of malignant and nonmalignant disorders. However, the efficacy of HSCT is still limited by the subsequent complications including graft versus host disease and opportunistic infections [1]. Previous studies have indicated that T-cell reconstitution after HSCT is correlated with acute graft versus host disease (aGVHD), CMV (cytomegalovirus) infection [2–4].

Peripheral T-cell reconstitution after AHSCT is accomplished through two mechanisms [5,6]: thymus-independent and thymus-dependent pathways. In the thymic-independent pathway, graft-derived mature donor T-cells and host T-cells having survived the conditioning regimen were triggered by antigen or cytokine, leading to cell expansion and skewed T-cell receptor (TCR) cell repertoire. On the other hand, the thymic-dependent pathway depends on the recruitment of donor-derived naïve T-cells into the thymus. It is slower and generates a more diverse TCR repertoire. Accordingly, thymic function is essential for the reconstitution of a fully functional TCR repertoire.

There are several methods that have been used as markers of thymic function. Among them, the sjTREC (signal joint TCR rearrangement excision circle) has been widely used to quantify the thymic output of de novo T cells [7–9]. sjTRECs are formed as extrachromosomal circular DNA by-product after the deletion of TCR-d locus [10,11]; they are not duplicated during cell proliferation and diluted out during cell division, therefore, can be used as an indirect measurement of thymic output [12]. Additionally, CD4 + CD45RA + CD31+T-cells are also used as a marker of recent thymic emigrant (CD31 + RTE), and contain high...
concentration of sjTREC [13,14]. sjTREC and CD31 + RTE were analyzed in many recent studies to evaluate thymic function in patients with different disorders. Increasing amount of clinical researches indicate that monitoring sjTREC and CD31 + RTE is of great value in terms of predicting and diagnosing clinical events in AHSCT [2,15–20].

CMV infection and aGVHD are major life-threatening complications after AHSCT; hence, studies which focus on screening for risk factors of aGVHD and CMV infection are of important value in clinical practice. Clave et al. [16] reported that sjTREC was of prognostic value in AHSCT, indicating pre-transplantation thymic function is inversely correlated with GVHD and CMV infection; however, in that study, they only included HLA-identical sibling HSCT patients.

In the present study, we prospectively measured thymic function before and in 100 days after AHSCT in 49 patients with sjTREC and CD31 + RTE to determine whether they are associated with aGVHD or CMV viremia and simultaneously investigate the kinetics of T-cell reconstitution after HSCT.

Methods

Patients

The study included 49 patients who underwent AHSCT in Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences, from October 2014 to March 2015. Informed consents were obtained from all patients according to the Declaration of Helsinki. Clinical characteristics of these patients are summarized in Table 1. The grafts were obtained from HLA-matched-related, HLA-matched-unrelated donors or HLA-haploidentical donors. Blood samples were obtained before conditioning regimen and at 1, 2, 3 months after transplantation and were immediately used for analysis. Diagnosis of aGVHD was based on Seattle criteria [21,22]. CMV viremia was identified by plasma CMV real-time polymerase chain reaction (RT-PCR) assay.

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, U.S.A.) from fresh heparinized patient blood samples. Cells were stained for 20 min at 4°C in dark with the following fluorochrome-conjugated anti-human monoclonal antibodies and isotype antibodies (Biolegend, San Diego, CA, U.S.A.): CD3 APC/CY7, CD4 FITC (Clone: A161A1), CD8 PE-CY7 (Clone: SK1), CD45RA PerCP-CY5 (Clone: H1100), CD62L APC (Clone: DREG-56), CD31 PE (Clone: WM59); PBMCs were analyzed by flow cytometry using Canto2 flow cytometer (BD Biosciences) supported by the FlowJo software (Tree Star Inc., Ashland, OR, U.S.A.). Absolute blood counts of lymphocyte subsets were then calculated by multiplying T-cell subset percentage with ALC, where ALC indicates the absolute lymphocyte count.

sjTREC quantification

Genomic DNA was purified from PBMCs using QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. sjTREC was quantified by RT-PCR [23]. Briefly, we prepared a plasmid containing one fragment of sjTREC and TR alpha constant gene (TRAC) DNA in each plasmid. And then we used the following primer and probes to detect sjTREC and TRAC: sjTREC forward primer (5′-CACATC CCT TTCAACCATGCT-3′), reverse primer (5′-GCCAGCTGAGGTTTAGG-3′) and the probe (5′-FAM-ACA CCT GTG GTT GTT GTA AAG GTG CCC ACT TAMRA-3′) [24], TRAC forward primer (5′-TGGCCTAAACCCTGATCCT-3′), reverse primer (5′-GGA TTTAGAGTCTCAGCTGGTAC-3′) and the probe (5′-FAM-TCC CACAGATCAGACCTCAGACCC ACT TAMRA-3′) [23]. PCR reactions were performed in PCR plate, 384-well, standard (Applied Biosystems, Foster City, CA, U.S.A.) in a final volume of 10 μl consisting of 1 μl (100–500 ng) genomic DNA, 5 μl of 2×TaqMan Universal PCR master mix containing AmpErase UNG

| Table 1. Patient characteristics. |
|----------------------------------|
| Total |
| No. patients | 49 |
| Recipient age in y, median (range) | 35 (5–56) |
| Recipient sex ratio, no. female/no. male | 27/22 |
| Diagnosis, no. | |
| AML | 22 |
| ALL | 6 |
| MDS | 9 |
| Aplastic anemia | 8 |
| Other | 4 |
| ABO, no. | |
| Compatible | 31 |
| Incompatible | 18 |
| GVHD prophylaxis, no. | |
| CSA and MTX | 20 |
| CSA and MTX and others | 6 |
| CSA | 1 |
| FK506 + MTX | 17 |
| FK506 + MTX and others | 5 |
| Conditioning regimen, no. | |
| TBI-based | 16 |
| Chemo-based | 33 |
| Donor, no. | |
| Matched sibling | 29 |
| Matched unrelated | 9 |
| HLA-haploidentical | 11 |
| Graft source, no. | |
| PBSCs | 45 |
| Bone marrow | 2 |
| PBSCs + bone marrow | 2 |
| Median CD34 + cells infused, ×10^5/kg | 2.4 (0.44–8.3) |

Notes: AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; MDS: myelodysplastic syndrome; CSA: cyclosporine A; MTX: methotrexate; FK506: tacrolimus; TBI: total body irradiation; PBSC: peripheral blood stem cell.
Characteristics of patients

We prospectively enrolled 49 patients who underwent AHSCT in our hematopoietic stem cell transplantation center. The median age of the patients was 35 years (range, 5–56). Bone marrow and mobilized peripheral blood was used as the stem cell source in 2 patients and 45 patients, respectively, and there were also two patients who received stem cells from both bone marrow and mobilized peripheral blood. The median number of CD34+ cells infused was 2.4 × 10^6/kg (range, 0.44–8.3). Among those patients, 29 patients underwent matched sibling AHSCT, 9 patients underwent matched-unrelated AHSCT and 11 patients underwent HLA-haploidentical AHSCT. Blood samples were collected to monitor CMV viremia by RT-PCR every week till 100 days after AHSCT and 42.8% (21/49) patients were diagnosed with aGVHD. 30.6% (15/49) patients developed CMV viremia. All the patients received myeloablative conditioning, consisting of TBI-based regimen (16/49) and chemo-based regimen (33/49).

After a median follow-up of 351 days (range, 60–592 days), 38 patients were still alive.

Thymic function recovery

sjTREC copies were analyzed before conditioning and at 1, 3 months in patients after AHSCT and also in 33 healthy donors. We simultaneously measured T-cell reconstitution by monitoring T-cell subsets with flow cytometry. T-cell subsets were significantly decreased before AHSCT, compared with healthy control (Figure 1). CD3 + T cells and CD8 + T cells were restored to pre-transplantation level as early as 1-month post AHSCT, which may be attributed to antigen-driven expansion of graft-derived T-cells through thymus-independent pathway (Figure 1(A,B)). Reconstitution of CD4 + T cells was slower than CD3 + T cells and CD8 + T cells (Figure 1(C)). However, sjTREC copies and CD31 + RTE were remarkably decreased after transplantation and were kept at very low levels in 3 months after AHSCT (Figure 1(D–F)). Both pre-transplantation CD31 + RTE and sjTREC copies were significantly lower than those of healthy control (p = 0.008, p < 0.001, respectively). As would be expected, absolute numbers of naive (CD4 + CD45RA + CD62L + T-cell) T-cells were also low in 3 months post AHSCT, showing a similar profile of recovery kinetics of sjTREC and CD31 + RTE (Figure 1(G)). sjTREC was closely related with age (r = −0.435, p < 0.001, Figure S1A), and CD31 + RTE (r = 0.744, p < 0.001, Figure S1B).

Pre-transplantation thymic function predicts aGVHD after AHSCT

Acute GVHD is a common complication after AHSCT and also a major cause of morbidity and mortality after AHSCT [25]. In 49 patients, 42.8% had acute GVHD (21/49). Patients were separated into two groups: sjTREC high and sjTREC low group, using the cut-off value from the ROC curve (Figure S2A and S2B). The cut-off value for sjTREC (per 10^6 PBMCs) and sjTREC (per ml blood) was 300/10^6 PBMCs and 370 per ml blood, respectively. Using death as a competing factor, low sjTREC level (per 10^6 PBMCs) after AHSCT was associated with higher incidence of aGVHD (p = 0.024, Figure 2(A)). When we analyzed the correlation between sjTREC level (per ml blood) and aGVHD, we also observed the same trend, although it was not statistically significant (p = 0.053, Figure 2(B)).

In the COX multivariate model (Table 2), which included recipient age, sjTREC levels, and donor types. sjTREC (per 10^6 PBMCs) had a significant impact on aGVHD incidence, independent of other two factors (HR = 3.352 95% CI 1.214–9.258, p = 0.02). As expected, HLA-mismatched donor was an independent risk factor of aGVHD (HR = 0.353, 95% CI 0.128–0.984, p = 0.046).

Statistical analysis

Comparison between patients and healthy donors was done with the Mann–Whitney rank-sum test. Changes of T-cell subsets and sjTREC at different time points were longitudinally evaluated by the SPSS Mixed Model. Receiver operating characteristic (ROC) curves were performed to determine the cut-off values of sjTREC and CD31 + RTE: values were selected to maximize specificity while maintaining a good sensitivity. Probability of aGVHD and CMV viremia was analyzed and plotted by the cumulative incidence estimates and compared with Gray’s test. Cox proportional-hazards multivariate regression was performed, including recipient age, donor types in all multivariate models. Hazards ratios (HR) and 95% confidence intervals (CI) were shown for this model. The effect of CD31 + RTE levels on CMV viremia was not analyzed in Cox proportional-hazards multivariate regression model, because of the small sample size. Two-sided p value of 0.05 was regarded as significant. The statistics was performed by the software SPSS17.0 (IBM Corporation, Armonk, NY, U.S.A.) and R2.15.0.
aGVHD was not associated with pre-transplantation absolute CD31 + RTE cell counts or CD31 + RTE percentage in CD4 + T cells (data not shown).

**Pre-transplantation thymic function predicts CMV viremia after AHSCT**

CMV viremia is also a major cause of morbidity and mortality after AHSCT. Among the enrolled patients, we recorded 16 patients who developed CMV viremia. A higher incidence of CMV viremia was found in patients with a low pre-transplantation sjTREC level (per $10^6$ PBMCs), as compared with those with high sjTREC level ($P = 0.004$, Figure 3(A)). Similarly, patients with low sjTREC level (per ml blood) also had a higher incidence of CMV viremia ($P = 0.021$, Figure 3(B)). The cut-off value of sjTREC (per $10^6$ PBMCs) and sjTREC (per ml blood) was determined by ROC curve (Figure S3A and S3B); and they were 256 per $10^6$ PBMCs and 370 per ml blood, respectively. We also analyzed if CD31 + RTE has predictive value in CMV viremia after AHSCT, both lower CD31 + RTE percentage in CD4 + T cells and absolute CD31 + RTE cell number could predict CMV viremia ($P = 0.022$ and 0.002, respectively,

**Figure 1.** Kinetics of T-cell subsets and sjTREC after AHSCT. In each panel, results are expressed as absolute number of cells per μl periphery blood for T-cell subsets, copies per $10^6$ PBMCs and copies per ml periphery blood for sjTREC. Results of healthy donors are also included in each panel. Different panels show the distribution of a distinct cell subset at different time points: before AHSCT, and at 1 month, 2 months, 3 months. (A) CD3+ T cells. (B) CD3+CD8+ T cells. (C) CD3+CD4+ T cells. (D) CD4+CD45RA+CD31+T cells. (E) sjTREC per ml blood. (F) sjTREC per $10^6$ PBMCs. (G) CD4+CD45RA+CD62L+ T cells. Each box shows the median, quartiles, and extreme values.
Figure 2. sjTREC level was of predictive value for aGVHD in patients after AHSCT. Cumulative incidence of aGVHD in patients who underwent AHSCT (n = 40). Gray line indicates high sjTREC level, black dashed line indicates low sjTREC level. sjTREC levels are expressed in sjTREC per 10^6 PBMCs (A) and sjTREC per ml blood (B).

Figure 3(C,D)). The cut-off values were 32% and 110 μl blood, respectively (Figure S3C and S3D).

Using the same parameters as for aGVHD, multivariate analysis was also done for CMV viremia (Table 2). sjTREC (per 10^6 PBMCs) was still related with CMV viremia (HR = 9.949 95% CI 2.281–43.386, p = 0.002). In consistent with sjTREC (per 10^6 PBMCs), lower sjTREC (per ml) was also an independent risk factor for CMV viremia (HR = 4.802 95% CI1.173–19.667, p = 0.029). Interestingly, HLA-mismatched donor was also at higher risk of CMV viremia (p < 0.05).

**Discussion**

In the current study, we monitored kinetics of sjTREC and T-cell subsets before and early after AHSCT in 49 patients who underwent AHSCT in our institution. We found that both sjTREC and CD31 + RTE cells were remarkably lower in patients before AHSCT, compared with healthy donors, and were significantly depressed till 100 days after AHSCT. sjTREC value had predictive value for aGVHD after AHSCT. Moreover, sjTREC value and CD31 + RTE cells were of predictive value for CMV viremia after AHSCT.

aGVHD is a major cause of mortality and morbidity following AHSCT and therefore limits AHSCT success [25]. There are many predictors of aGVHD, which include HLA differences between donor and recipient, recipient age, gender mismatch between donor and recipient, mHA in otherwise identical AHSCT, donor age, source and dose of stem cells, intensity of conditioning, and GVHD prophylaxis [26]. In this study, we want to investigate whether pre-transplantation sjTREC and CD31 + RTE are of predictive value for aGVHD and CMV viremia after AHSCT. Our data showed lower pre-transplantation sjTREC level (per 10^6 PBMCs) was associated with higher aGVHD incidence after AHSCT. Similarly, the same trend was found when we analyzed sjTREC (per ml blood), despite it was not statistically significant. Previous studies have associated sjTREC levels with aGVHD, but there is still no clear conclusion. Clave et al. [16] showed that there was a significant association between low pre-transplantation sjTREC counts and a higher incidence of grade II-IV aGVHD. However, when they included other parameters (age of 25 years, donor/recipient CMV serology, ABO incompatibility, sex mismatch, and disease risk) in the COX multi-parameter analysis, it was not significantly important. The disparity of this study with our results may be explained by the difference of donor types between these two studies.

The probable mechanism may involve impaired negative selection [27]. It has been long known thymic epithelial cells (TECs) play an essential role in negative selection in thymus to get rid of autoimmune T-cells [28]. The negative selection relies on the ectopic
expression of tissue-restricted peripheral self-antigens (TRA) in mature medullary thymic epithelial cells, which may be damaged in patients with impaired thymic function, thus, lead to compromised negative selection and GVHD [27,29].

Moreover, another mechanism relevant to thymic function could implicate donor-derived CD4 CD25^{high} regulatory T (Treg)-cell reconstitution [30,31]. Those cells were also selected in the thymus, a competent thymic is needed for regulatory T-cells recovery in AHSCT patients [32]. Previous studies showed the important role of Treg cells in the prevention and treatment of GVHD in murine models and humans [33–36]. Impaired thymic function due to pre-transplantation chemotherapy, conditioning regimen or aging may lead to failed negative selection and impaired regulatory T-cell recovery, which may consequently increase the risk of GVHD after AHSCT.

CMV infection is also a life-threatening complication after AHSCT. We analyzed the effect of pre-transplantation thymic function on CMV viremia, our data indicated that both sjTREC (per 10^6 PBMCs) and sjTREC (per ml blood) were correlated with CMV viremia, which confirmed the result of the previous study [16]. Additionally, pre-transplantation CD31^{+}RTE level also had the same impact on CMV viremia as sjTREC. As reported by many previous studies, CD31^{+}RTE and sjTREC are closely related with each other, they may both act as markers of thymic function [13]. The possible explanation may be that pre-transplantation thymic function may predict residual thymic function after AHSCT, which was shown in Chen’s study [15], and residual thymic function may be helpful to early reconstitution of CMV-specific T-cells and thus protect patients from CMV reactivation.

In summary, we showed pre-transplantation CD31^{+}RTE and sjTREC were both related with CMV viremia.

Figure 3. sjTREC level and CD31^{+}RTE were of predictive value for CMV viremia in patients after AHSCT. Cumulative incidence of CMV viremia in patients who underwent AHSCT. Gray line indicates high sjTREC level, black dashed line indicates low sjTREC level. sjTREC are expressed in sjTREC copies per 10^6 PBMCs (n = 40) (A) and sjTREC copies per ml blood (n = 40) (B) CD31^{+}RTE are expressed in percentage in CD4^{+}T cells (n = 32) (C) and μl blood (n = 31) (D).
after HSCT. sjTREC was of predictive value for aGVHD after AHSCT. sjTREC and CD31 + RTE may be useful in guiding individualized prophylaxis and pre-emptive therapies for aGVHD and CMV infection.

Disclosure statement

No potential conflict of interest was reported by the authors.

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