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LYMPHOCYTE SUBPOPULATIONS IN CHILDREN AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

SUBPOPULACJE LIMFOCYTÓW PO PRZESZCZEPIENIU ALLOGENEICZNYCH KOMÓREK MACIERZYSTYCH

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Summary

Background. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a method of treatment in a variety of diseases, which involves replacing the recipient's bone marrow by healthy donor stem cells.

Objective. The aim of the study was the analysis of immune reconstitution in children and adolescents after allo-HSCT.

Patients and methods. We analyzed lymphocyte subpopulations in 67 children after allo-HSCT for hematological malignancies and non-malignant diseases. B-cells, T-cells, NK (natural killer cells) and CD4/CD8 ratio were assayed in peripheral blood by flow cytometry using a set of antibodies CD2/CD19, CD3/CD4/CD8, CD3/CD16/CD56 labeled with different fluorescent dyes. The tests were performed at 1, 3, 6, 9, 12, 15, 18 and 24 months after the HSCT.

Results. Normalization of the number of lymphocytes occurred rapidly in the population of NK cells; however, significant decrease in the proportion of NK cells was observed at 6 months after HSCT ($p=0.028$). The recovery of T cells occurred after 3 months for CD2 and after 6 months for CD3 cells. Within the B-cell population, a significant delay of reconstitution was observed: CD19 cell percentage exceeded 5% ($p<0.001$) in the ninth month after HSCT, while it was above 10% afterwards. The normalization of CD4/CD8 ratio was very slow and delayed and did not achieve normal values within 24 months after allo-HSCT.

Conclusions. Reconstitution of the immune system in children after allo-HSCT is the fastest for NK cells. Normal values of T- and B-lymphocytes were reached after 6 and 9 months, respectively. CD4/CD8 ratio is a parameter the normalization of which requires at least two years.

Streszczenie

Wstęp. Przeszczepienie allogenicznych komórek krwiotwórczych (allo-HSCT) to metoda terapeutyczna polegająca na zastąpieniu szpiku kostnego biorcy przez komórki hematopoetyczne pochodzące od zdrowego dawcy.

Cel. Analiza subpopulacji limfocytów u dzieci po przeszczepieniu allogenicznych komórek krwiotwórczych.

Pacjenci i metodyka. Badaniu poddanych zostało 67 dzieci, u których przeprowadzono allo-HSCT z powodu chorób nowotworowych i nie-nowotworowych. We krwi obwodowej badano limfocyty B, T i NK oraz

stosunek CD4/CD8 metodą cytometrii przepływową, stosując zestawy przeciwciał CD2/CD19, CD3/CD4/CD8, CD3/CD16/CD56 wyznakowane różnymi barwnikami fluorescencyjnymi. Badania wykonano w 1, 3, 6, 9, 12, 15, 18 i 24 miesiącu po HSCT.

Wyniki. Normalizacja liczby limfocytów nastąpiła najszybciej w obrębie populacji komórek NK, jednak po 6 miesiącach obserwowano znaczne zmniejszanie odsetka tych komórek ($p=0,028$). Normalizacja limfocytów T wystąpiła po 3 miesiącach dla CD2 i po 6 miesiącach dla

CD3. W obrębie limfocytów B stwierdzono istotne opóźnienie rekonstrukcji: dopiero w 9 miesiącu odsetek komórek CD19+ przekroczył 5% ($p < 0,001$), a w kolejnych punktach czasowych wynosił powyżej 10%. Rekonstrukcja odsetka CD4/CD8 była powolna i opóźniona i nie osiągnęła normalizacji w przeciągu 24 miesięcy po allo-HSCT.

Key words: lymphocyte subpopulation, allogeneic stem cell transplantation, children

Słowa kluczowe: subpopulacje limfocytów, allogeniczny przeszczep komórek macierzystych, dzieci

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is an established approach to treat patients with numerous hematological, oncological, immunological, metabolic and autoimmune disorders [1, 2]. The goal of hematopoietic stem cell transplantation is a transfer of a functional hematopoietic system from donor to host preceded by conditioning regimen with high-dose chemotherapy, sometimes in combination with total body irradiation [3, 4]. The clinical efficacy of HSCT is limited by delayed immune recovery resulting in high relapse rate and infectious complications [5]. A normal reconstitution of the immune system is an important issue for the control of infection, establishment of tolerance, and mediation of graft-versus-leukemia (GVL) effects [6]. The reconstitution of different immune cell subsets after an HSCT occurs at different tempos [7]. Immune deficiencies, including cellular and antibody immunity can last for more than a year, resulting in increased susceptibility to infections [2]. Immune reconstitution after HSCT is influenced by many host-, donor- and transplantation-related factors [2].

PATIENTS AND METHODS

Patients. A total number of 67 children, including 45 males and 22 females, aged 8 months – 20 years (median 10 years) were included into the study. All patients underwent allogeneic HSCT for hematological malignancies ($n=54$) or non-malignant diseases ($n=13$). Patients were diagnosed for acute lymphoblastic leukemia ($n=31$), acute myeloid leukemia ($n=19$), chronic myeloid leukemia ($n=1$), myelodysplastic syndrome ($n=1$), lymphomas ($n=2$), or non-malignant diseases ($n=13$). HSCT were performed from matched family donor ($n=25$), matched unrelated donor ($n=38$), or mismatched unrelated donor ($n=4$). The stem cell source was bone marrow ($n=30$) or peripheral blood ($n=37$).

Wnioski. Rekonstrukcja układu immunologicznego u dzieci po allo-HSCT najszybciej przebiega dla komórek NK. Prawidłowe wartości limfocytów T i B osiągane są odpowiednio, po około 6 i 9 miesiącach. Stosunek CD4/CD8 jest parametrem, którego normalizacja wymaga co najmniej 2 lat.

Study design. Following lymphocyte subpopulations were tested: lymphocytes B, T, NK (natural killer) and CD4/CD8 ratio. Flow cytometry analysis was performed at 1, 3, 6, 9, 12, 15, 18 and 24 months after HSCT. Not all children were examined at each time point.

Flow cytometry analysis. The absolute numbers of B-, T-, NK and CD4/CD8 lymphocytes were calculated from a total number of PB lymphocytes, the proportion of side scatter/forward scatter lymphocyte region gate and the percentage of positive cells within the gate. The panel of monoclonal antibodies combinations used included: CD2/CD19 or CD5/CD19; CD3/CD4/CD8; CD3-/CD16CD56+. Antibodies were purchased from BD Biosciences (San Jose, CA, USA) and DAKO (Glostrup, Denmark). Briefly, 5 μ l of appropriate fluorochrome conjugated monoclonal antibodies were added to 50 μ l of whole EDTA blood and vortexed. The samples were then incubated in the dark room temperature for 15 minutes. Erythrocyte lysis was performed with the use of Erythrocyte-Lysing Reagent Uti-Lyse DAKO (Glostrup, Denmark) after first incubation, 50 μ l of Erythrocyte-Lysing Reagent A was added to each sample, vortexed and incubated for 10 minutes in dark. The same procedure was repeated with 500 μ l of Erythrocyte-Lysing Reagent B. Expression of all antigens was analyzed by flow cytometer FACSCanto II BD Biosciences (San Jose, CA, USA) and data was analyzed using FACSDiva Software BD Biosciences (San Jose, CA, USA).

Reference values. Results of lymphocyte B, T and NK percentages and CD4/CD8 ratio were compared with the reference values [8]. Following values were regarded as normal: 12-24% for B-lymphocytes, 63.2-77.8% for T-lymphocytes, 5.4-18.6% for NK cells, and 1.18-2.65 for CD4/CD8 ratio.

Statistical analysis. Median values and range were shown. Kruskal-Wallis and Mann-Whitney tests were used to compare differences between groups. A p-value < 0.05 was considered to be significant.

RESULTS

Normalization of the number of lymphocytes occurred rapidly in the population of NK cells, which was observed from the first month after allo-HSCT (Figure 1). However, a significant decrease in the proportion of NK cells was observed from 17% after the first month to 2.8% at 24 months after HSCT ($p=0.028$). In the meantime, reconstitution of other lymphocyte subpopulations was observed. The normalization of T cells occurred after 3 months for CD2 and 6 months for CD3 cells. The percentages of T cells remained above 60% with no significant differences in the consecutive time points ($p>0.05$). A significant delay in reconstitution was found within the B cell population: CD19 cell percentage exceeded 5% in the 9th month after HSCT ($p<0.001$); while in the subsequent time points it was above 10%. A significant

delay in reconstitution of CD3+CD4+ has also been observed ($p<0.001$), as late as after the 12th months their proportion exceeded only 20%. Normalization of the percentage of CD3+CD8+ cells appeared with CD2 cell reconstitution in the third month after allo-HSCT. A steady increase of the percentage of CD3+CD8+ cells was observed in the following time points up to 47% at 18 months after HSCT. As a result, reconstitution of CD4/CD8 ratio was slow and delayed ($p=0.072$) and did not achieve normal values within 24 months after allo-HSCT (Figure 1).

DISCUSSION

Allogeneic HSCT has proved to be an effective treatment in a variety of diseases. However, this procedure is associated with the risk of serious side effects, due to the severe impairment of the immune

system induced by this treatment. Conditioning regimen with high-dose chemotherapy or total body irradiation is used to help eradicate the patient disease prior to the infusion of HSC; however, this therapy usually severely impairs immune system. First 100 days after HSCT are characterized by cellular immune deficiency with a reduced value of each subset of lymphocytes [7].

Immunocompromised patients after HSCT are more vulnerable to opportunistic and typical infections and have a higher risk of relapse. The reconstitution of the immune system is usually determined by white blood cell count with differential diagnosis, as well as lymphocyte subpopulations and immunoglobulin concentration including antibody levels.

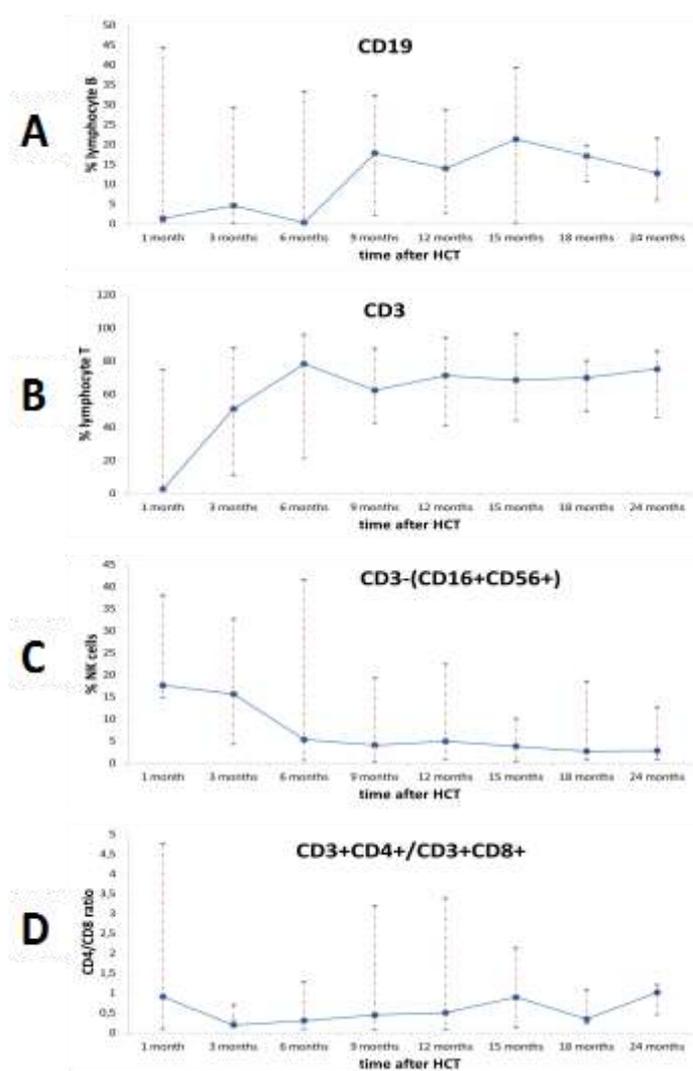


Fig. 1. Results of reconstitution of B (A), T (B), NK (C) lymphocytes and CD4/CD8 ratio (D)
Ryc. 1. Poziom rekonstrukcji limfocytów B (A), T (B), NK (C) i stosunku CD4/CD8 (D)

NK cells remains the first subset of lymphocytes to recover after autologous or allogeneic HSCT and possess the highest cytotoxic activity in early post-transplant period [9]. Rapid recovery of NK cells after HSCT – in our study one month – is based on an expansion of the cytokine-producing CD56bright NK-cell subset. This early expansion of CD56bright NK cells gradually declines; however, it may persist for a year [7]. Our study has shown that the number of NK cells significantly decreases over the time.

The count of B-lymphocytes in our group of children and adolescents has returned to normal values at 6-9 months after allo-HSCT. Bosch *et al.* also observed intermediate recovery for B-cells, which was faster for naive than memory B-cells (CD5+ vs CD5-cells) [10]. Corre *et al.* reported expansion of CD19+/CD5+ lymphocytes just after three months and a persisting defect of memory B cells in adult patients [11]. According to Heining *et al.*, CD19-positive B-cells were normalized after a year in most adult patients [12].

Long-term imbalance between recovery of two major T-cell subsets, helper T-cells (CD3+CD4+) and suppressor/cytotoxic T-cell (CD3+CD8+) remains a well-known phenomenon [5]. Initial recovery of the T-cell compartment relies on peripheral expansion of memory T cells, driven by cytokines and the presence of alloreactive antigens, before the production of naive T cells in the thymus starts. This is especially true for CD4+ T-cells that reconstitute later than CD8+ T-cells and rely more on thymic production of naive T cells after HSCT, leading to an inversion of the CD4/CD8 ratio [7]. Our results confirm this hypothesis. Although the normalization of T cell occurred after 3 months for CD2 and 6 months for CD3, a significant delay in reconstitution of helper T-lymphocytes CD3+CD4+ was shown, which resulted in slow and delayed reconstitution of CD4/CD8 ratio over 24 months.

In conclusion, on the basis of the assessment of lymphocytes subsets, we have shown that immune reconstitution in children and adolescents after allo-HSCT has occurred at different rates. Although appropriate level of NK cells was achieved by one month after HSCT, the percentages of CD19+, CD3+, CD2+ lymphocytes and CD4/CD8 ratio indicated a delayed reconstitution. Allogeneic HSCT offers the opportunity for treatment of patients with malignant and non-malignant disorders; however, delayed immune reconstitution and disease relapse remain significant limitations for its application.

Understanding the causes of hampered immune restoration provides the rationale to develop powerful immunotherapies.

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