Alterations of T cell receptor Vβ repertoire of CD8 T lymphocytes in immune tolerance induction in two hemophilia A patients with inhibitors

Promene Vβ repertoara T-ćelijskog receptora CD8 T-limfocita tokom indukcije imunološke tolerancije kod dva obolela od hemofilije A sa inhibitorima

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Abstract

Background/Aim. Hemophilia A patients with inhibitors are treated effectively with immune tolerance induction (ITI) therapy. Although anti-idiotypic antibodies may play a certain role in the underlying mechanism, the detailed mechanism by which ITI produces a curative effect remains unknown. The aim of this study was to clarify the immunological aspect of ITI.

Methods. Longitudinal T-cell receptor (TCR) analysis was performed during ITI. TCR variable region α-chain and β-chain repertoires were serially analyzed for peripheral blood mononuclear cells (PBMCs), CD4 T cells, and CD8 T cells from 2 hemophilia inhibitor patients treated with ITI (Patients 1 and 2). Furthermore, to see whether skewing observed in TCR analysis resulted from clonality alterations, T-cell clonality was investigated using complementarity-determining region 3 (CDR3) size spectratyping.

Results. In the patient 1, inhibitor titer remained to be 19.6 BU/mL for 596 days after ITI commencement, and ITI was unsuccessful. In the patient 2, inhibitor titer disappeared 434 days after ITI commencement, and ITI was successful. In both cases, skewing of TCR repertoire was observed in CD8 T cell subset, whereas not in CD4 T cell subset. Conclusion. Alteration of TCR repertoires, especially TCR variable region β-chain repertoire of CD8 T cells, was distinct between successful and unsuccessful cases, suggesting that immunological response in the early phase affected the ITI outcomes.

Key words: hemophilia α; receptors, antigen, t cell, alpha-beta; factor VIII; immune tolerance.

Apstrakt

Uvod/Cilj. Oboleli od hemofilije A sa prisutnim inhibitornim antitelima uspešno se leče terapijom zasnovanom na indukciji imunološke tolerancije (ITI). Iako, antiidiotipska antitela mogu imati određenu ulogu u osnovnom mehanizmu ovog procesa, detaljni mehanizmi kojim ITI ostvaruje terapijski efekat su i dalje nepoznati. Cilj ove studije bio je da se razjasne imunološki aspekti ITI terapije.

Metode. Longitudinalna analiza repertoara T-ćelijskog receptora (TCR) izvršena je tokom ITI terapije. Repertoari promenljivog regiona α- i β-lanca TCR-a analizirani su serijski u populaciji mononuklearnih celija periferne krvi, CD4 T-ćelija i CD8 T-ćelija kod dva bolesnika sa hemofilijom A i prisutnim inhibitorima, koji su lečeni primenom ITI terapije (bolesnici 1 i 2). Takođe, ispitivana je klonalnost T-limfocita korишćenjem metode tipiziranja spektra fragmenata regiona 3 koji određuje komplementarnost (CDR3) sa ciljem da se utvrdi da li je skretanje u TCR repertoaru posleca promene klonova.

Rezultati. Kod bolesnika 1 titular inhibitori antitela zadržao je vrednost od 19,6 BU/mL tokom 596 dana od započetanja ITI terapije, na osnovu čega je zakljучeno da je ITI terapija bila bezuspešna. Kod bolesnika 2 ITI terapija je bila uspešna jer je titular inhibitori antitela isčeao posle 434 dana od početka terapije. Kod oba bolesnika skretanje TCR repertoара promenljivog regiona α- i β-lanca TCR-a u podgrupi CD8 T-ćela, ali ne i u podgrupi CD4 T-ćela.

Zaključak. Promena repertoara TCR-a, posebno promenljivog regiona β-lanca TCR-a u CD8 T-ćela bila je različita kod uspešno i neuspešno lečenih bolesnika, što ukazuje da je imunološki odgovor u ranoj fazi uticao na ishod ITI terapije.
Introduction

Approximately 25%–30% of severe type hemophilia A patients develop inhibitor antibodies that reduce or completely negate the benefits of replacement therapy. As a method of reducing inhibitor concentrations in patients with hemophilia A, immune tolerance induction to FVIII therapy (ITI) is effective with the overall successful rate to 60%–80%. Although several mechanisms by which ITI exerts its therapeutic effect have been proposed, the precise immunological mechanism remains to be elucidated.

We had performed a cross-sectional T cell receptor (TCR) repertoire analysis in hemophilia patients with or without inhibitor, and had shown that replacement therapy with plasma-derived factor VIII concentrates induced skew in T-cell receptor usage and clonal expansion of CD8 T cells in HIV-seronegative hemophilia patients. Recently, we had a chance to treat two inhibitor hemophilia A patients with ITI and perform longitudinal T cell receptor (TCR) analysis during early period of ITI. We present the alteration of TCR repertoire in this report.

Methods

Patient profiles

Two hemophilia A patients (patients 1 and 2) with inhibitors underwent ITI at Nara Medical University Hospital. The patient 1 and the patient 2 were diagnosed as hemophilia A at 5 months and 10 months old in our laboratory, respectively. Both patients were treated with recombinant factor VIII replacement after diagnosis. Inhibitor developed at 7 months old in the patient 1 and at 11 months old in the patient 2. Historical peak titer was 80 BU/mL in the patient 1 and 67 BU/mL in the patient 2. Then, ITI with recombinant FVIII concentrates (50 U/kg i.v., 3 times a week) started at 1 year 11 months old in the patient 1 and at 2 years 9 months old in the patient 2. Inhibitor titer immediately before ITI was 4.1 BU/mL in the patient 1 and 4.5 BU/mL in the patient 2. Peak inhibitor titer was 205 BU/mL in the patient 1 on day 15 after ITI started and 390 BU/mL in the patient 2 on day 28 after ITI started. Both patients were HIV-seronegative.

Leukocyte preparations

Cell separations were performed using the MACS beads technology as previously described. Briefly, the peripheral blood mononuclear cells (PBMCs) were purifed by the Ficoll-Paque method from the patients. CD4 T cells and CD8 T cells were separated from whole PBMC with CD4 Microbeads and CD8 Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. PBMCs were stained FITC-conjugated anti-CD4 antibody, FITC-conjugated anti-CD3 antibody, and PE-conjugated anti-CD8 antibody (BD Biosciences, San Jose, CA). Cell fluorescence was measured with a 2-laser FACScalibur (BD Biosciences). The purity of isolated T cells was more than 95%. RNA was extracted from whole PBMCs, and CD4 and CD8 T cell fractions as previously described. PBMCs, and CD4 and CD8 T cells were also obtained from 20 healthy donors (median age: 27 years, range: 14–51 years) in the same manner, and RNA was extracted. They were used as normal controls. All samples used in the present study were collected after written informed consent had been obtained. This study was approved by the Nara Medical University Internal Review Board.

Adaptor ligation-mediated PCR and biotinylation of PCR products

The methods of isolating RNA from PBMCs and adaptor ligation-mediated polymerase chain reaction (PCR) were previously reported.

Microplate hybridization assay

TCR α-chain variable region (TCRAV) and TCR β-chain variable region (TCRBV) repertoires were analyzed by the microplate hybridization assay (MHA) as previously reported.

Delta score

The delta score was used as an indicator to evaluate the extent of skewing of TCR repertoires. The delta score indicates the sum of absolute differences between the frequency of respective V segments in individual patients and the mean frequency in 20 healthy donors. A low delta score indicates similarity between patients and healthy donors, whereas a high score indicates dissimilarity.

CDR3 size spectratyping

PCR for CDR3 size spectratyping was performed as described previously.

Results

Outcome of ITI

In the patient 1, inhibitor titer remained to be 19.6 BU/mL for 596 days after ITI commencement, and ITI was unsuccessful. In the patient 2, inhibitor titer disappeared 434 days after ITI commencement, and ITI was successful.

TCR repertoire analysis

The usage of TCRAV and TCRBV was different between CD4 and CD8 T cells (data not shown). However, it remained less obvious whether TCR repertoire altered after ITI started.

Delta score

Figure 1 shows the fluctuation of the delta score. Before ITI was started, delta score was not elevated; i.e., marked skewing was not observed in either TCRAV or TCRBV repertoires of CD4 T cells in TCR repertoire analysis. On the other hand, increase of delta score was observed in CD8 T cells before ITI. Furthermore, the delta scores of both TCRAV and TCRBV of CD8 T cells were significantly higher than those of CD4 T cells in both patients during ITI. The delta score reached a peak around days 8 and 16 and gradually declined. In the patient 1, the delta score in CD8 T cells was high before ITI and gradually decreased in the course of ITI, nevertheless the delta score formed a peak at day 8. Furthermore, the delta scores in

CD8 T cells of TCRAV were higher than those of TCRBV in both the patients, especially in the patient 1.

**CDR3 size spectratyping**

T-cell clonality was analyzed by CDR3 size spectratyping with primers specific for the 40 AV and 37 BV segments (Figure 2). CD4 T cells showed a polyclonal pattern in any variable chains before and during ITI. On the other hand, the spectratyping pattern in CD8 T cells altered during ITI. Clonal peaks appeared in several TCRBVs (BV021, BV052, BV061, BV082 and BV151 in the patient 1, and BV062 and BV064 in the patient 2), or contrariwise skewing was reduced (BV091, BV181 and BV201 in the patient 1, and BV082 and BV091 in the patient 2).

**Discussion**

In this study, we firstly performed TCR repertoire analysis to investigate whether the usage of TCRAV and TCRBV was altered by ITI. TCRAV and TCRBV repertoires were analyzed in whole PBMCs, and CD4 and CD8 T cells from patients by MHA. The different usage of TCRAV and TCRBV between CD4 and CD8 T cells suggested that TCR analysis of PBMCs had little meaning because the result of PBMCs could have been the sum of the results of CD4 and CD8. Therefore, interpretation of results of CD4 and CD8 T cells would provide significant information. Hereinafter, we focused on the difference in TCR repertoire between CD4 and CD8 T cells of the patients.

CD4 T cell helper activity is essential for B cell production of IgG antibodies with high affinity for antigens. Since neutralizing antibodies against FVIII (inhibitors) raised by FVIII replacement therapy belong to IgG-subclass antibodies, CD4 T cells that respond to FVIII will play an important role in the production of inhibitors.

Our previous study using whole PBMCs had demonstrated that there was no association between the presence of inhibitor and clonal T cell proliferation. When CD4 TCR repertoire is perturbed in hemophilia A patients with inhibitors, one of the reasons for no association between the presence of inhibitor and clonal T cell proliferation might be that...
expanded clonal CD8 T cells masked the alteration of CD4 T cell repertoire associated with a large amount of exogenous protein FVIII exposure. Therefore, PBMCs were separated into CD4 T and CD8 T cells in this study. MHA revealed that little alteration of TCR repertoire was observed in CD4 T cells. Furthermore, TCRBV that had shown high frequent usage before ITI was started remained to be high levels and showed skewed repertoire.

We evaluated the skew in the usage of TCR repertoires by the delta scores (the sum of the differences in AV/BV frequencies) as an indicator. The higher delta score in CD8 T cells than in CD4 T cells before ITI suggested that TCR repertoire skewed more in CD8 T cells than in CD4 T cells, confirming the results of MHA. The delta score achieved a peak around 1 or 2 weeks after ITI was started. Since the skew in TCR repertoire from baseline (before ITI) was larger as the score was higher, TCR repertoires were considered to have altered at that time.

The delta scores suggested that CD8 TCRBV repertoire was highly skewed in hemophilia patients treated with ITI. Although the delta score of TCRBV was high before ITI in the patient 1, the analysis of CD8 TCRBV repertoires might be worth considering as the delta score of TCRBV was still high during ITI.

Furthermore, we tried to determine whether the skew in the usage of TCR repertoires was due to clonal T-cell expansion during ITI. T-cell clonality was analyzed by CDR3 size spectratyping. A polyclonal pattern in any variable chains region of CD4 T cells without clonal proliferation. ITI would provide us with a clue to the factor predicting the outcomes of ITI.

We are aware of limitations of our study in the small sample size and the short duration for following alterations of TCR Vβ repertoire. Nevertheless, our findings of the difference of immune response in an early phase of ITI might provide us with a clue to the factor predicting the outcomes of ITI. Further studies will elucidate whether the skew in the usage of CD8 TCR repertoires play a role as an immunomodulatory factor and determine success of ITI.

**Conclusion**

Alteration of TCR repertoires, especially TCR variable region β-chain repertoire of CD8 T cells, was distinct between successful and unsuccessful cases, suggesting that immunological response in the early phase affected the ITI outcomes.

**References**