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T Cell Receptor Rearrangement in NKT Cells Differentiated from Cord Blood CD34+ Cells

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제대혈의 CD34양성 세포에서 증폭한 NKT 세포에서의 T 세포 수용체 유전자의 재배열

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목 적 : 제대혈에서 분리한 CD34 양성세포를 사이토카인을 이용하여 NKT 세포로 분화시키는 과정에서 T 림프구의 수용체(TCR) 유전자의 재배열이 어느 시기에 나타나는지 알아보고자 하였다.

방 법 : 제대혈에서 분리한 CD34 양성세포에 IL-15, Flt3-L와 stem cell factor를 첨가하여 액체 배양하였다. 일주일 간격으로 세포를 수거하여 DNA를 분리하고, 이것을 TCR 유전자 재조합의 결과로 생겨나는 TCR-rearrangement excision circles(TRECs)을 검출하는 시발체(primer)를 사용하여 중합효소연쇄반응을 시행하였다.

결 과 : 배양 시작 4일째부터 TREC이 검출되어 배양 후 30일까지 검출되었으나, 배양 시작 2~3주에 그 정도가 가장 높았다.

결 론 : 제대혈에서 분리한 CD34 양성세포에서 분화하는 NKT 세포의 TCR 유전자 재조합은 배양 개시 후 일주일 이내에 시작되어 배양 개시 후 2~3주에 활발히 일어난다.

중심 단어 : NKT cell · CD34+ cells · TCR gene rearrangement.

Introduction

Natural killer T (NKT) cells were originally identified as CD1d-restricted human[1] and murine T cell clones[2] that express the NK1.1 and use an invariant T cell receptor (TCR) α chain (Vα24 for human, Vα14 for mouse). Although it has found that NK1.1+TCR+cells were heterogeneous[3], the majority of NKT cells express invariant V α chain. Such invariant V α chain expressing NKT cells are also called as iNKT cells. Human NKT cells have a restricted TCR diversity, with an invariant Vα24-Jα15 and Vβ11. Mouse NKT cells have an invariant Vα14-Jα18 (formerly Jα281 or Jα15), paired with Vβ8.2, Vβ7 or Vβ2 chain. Human and mouse NKT cells recognize a glycolipid, α-galactosylceramide (α-GalCer), presented by CD1d[4]. However, other antigens that stimulate most NKT cells have not been identified.

Immune responses of NKT cells include cytolytic activity and rapid induction of various cytokines, especially IL-4 and interferon-γ, after TCR stimulation. In turn, those NKT cell derived cytokines can activate several other cell

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types, including NK cells, T cells, macrophages, and B cells[3].

The developmental processes of the NKT cells are not clearly defined. Though the finding that $CD4^+NK1.1^+$ T cells requires $CD1d^+$ cortical thymocytes favors the thymic origin of the NKT cells, NK1.1+ T cells can also be detected in congenitally athymic mouse. Previously, IL-15 in combination with Flt3-L and/or stem cell factor can induce the differentiation of NKT cells from human cord blood CD34+ cells[5] *in vitro* after 30 days of culture.

In order to detect the time of TCR gene rearrangement in differentiated NKT cell, we used TCR-rearrangement excision circles (TRECs) PCR[6]. TRECs are the episomal DNA circles that are generated during excisional rearrangement of TCR genes. TRECs are stable, and not duplicated during mitosis[7]. As a result, TRECs were diluted out with each cellular division. In $\alpha\beta$ T cells, a common requirement for productive rearrangement of the TCRA (TCR α chain) gene locus is deletion of TCRD (TCR α chain) locus, which resides in the TCRA. The two rearrangement event occur during this process : the first producing a signal-joint TREC and the second a coding-joint TREC. Thus the TRECs generated can be used to detect TCR gene rearrangement in NKT cells expanded *in vitro*.

Materials and Methods

1. Isolation of CD34+ cells from cord blood and liquid culture

Human cord blood (CB) was obtained from full-term deliveries with informed consent. CB was heparinized for separation of blood cells. Mononuclear cells were isolated from CB by density gradient separation over Ficoll-Paque (Amersham, UK). CD34+ cells were purified from mononuclear cells using MiniMACS system (Miltenyi Biotec, Germany) as previously described[8]. These purified CD34+ cells were cultured at a density of 1.0×10^5 cells/ml in Iscove's-modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, UT, USA) and following cytokines : stem cell factor (SCF, 50ng/ml), Flt3-L (FL, 50ng/ml), and IL-15 (50ng/ml) for NKT cell expansion[5]. SCF was provided from Kirin Brewery (Maebashi, Japan), FL was purchased from Chemicon (Temecula, CA, USA), and IL-15 was from Endogen (Woburn, MA, USA). Twice a week, cytokines were added and media were replaced. Cultured cells were harvested once a week for DNA extraction.

2. Flow cytometry

For CD34+ cell staining, fluroscein isothiocyanate (FITC) conjugated anti-CD34 antibody (BD Pharmingen, San Diago, CA, USA) was used. Cells were stained with phycoerythrin (PE) conjugated anti-human TCR V (11.1 antibody (Serotec, UK), FITC conjugated anti-human TCR V α 24 antibody (Serotec) and cychrome conjugated anti-human CD56 antibody (BD Pharmingen). Samples were collected and analyzed on FACSCalibur flow cytometer (BD) and CellQuest software (BD).

3. DNA isolation and PCR

To collect stable TREC, DNA was isolated from cultured cells with DNeasy Tissue Kit (Qiagen, Germany). DNA concentration was determined by spectrophotometric method. PCR analysis was performed using 1 μg of DNA, 10 pmole of each primer, and 1 unit of *Taq* polymerase (TaKaRa, Japan). For signal joint TREC amplification, sjTREC1 5'- AAA GAG GGC AGC CCT CTC CAA GGC AAA-3' and sjTREC2 5'- AGG CTG ATC TTG TCT GAC ATT TGC TCC G –3' were used as primers[6]. For coding joint TREC amplification, cjTREC1 5'- CCT GTT TGT TAA GGC ACA TTA GAA TCT CTC ACT G –3' and cjTREC2 5'- CTA ATA ATA AGA TCC TCA AGG GTC GAG ACT GTC –3' were used[6]. For rearranged TCR V α 24-J α 15 amplification, V α 24-forward 5'-GCA ACT GTC GAC GCA GAC AC-3' and J α Q-reverse 5'-GGA TAG AAT TCC AGA CGG TCA AC-3' were used[1]. PCR cycle was performed at 94℃ for 3 min followed by 35 cycles at 94℃ for 30 sec, 50℃ for 30 sec, and 72℃ 1 min. DNA from mononuclear cells was used as negative control.

Results

1. Isolation of CD34+ cells from cord blood and in vitro differentiation of NKT cells

The purities of the CD34+ cells isolated from CB

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were more than 95% (Fig. 1). On day 0, purified CD34+ cells were all negative of CD56, TCR V α 24, and V β 11.1 (Fig. 2A). After 30 days of culture, expanded cells expressed CD56, TCR V α 24, and V β 11.1 (Fig. 2B), showing NKT phenotypes, as previously described[5].

2. TCR recombination

In Fig. 4A, signal joint TREC appeared on day 4 (CB #3), day 8 (CB #2) and last until day 30 (CB #2). Increase of coding joint TREC was followed after signal joint TREC amplification (Fig. 4B) and mostly expressed between day 9 and day 21. In CB #4 cells, coding joint TREC was more amplified on day 20 than that of day 30 In CD #1 cells, no signal joint TREC was detected (on day 30).

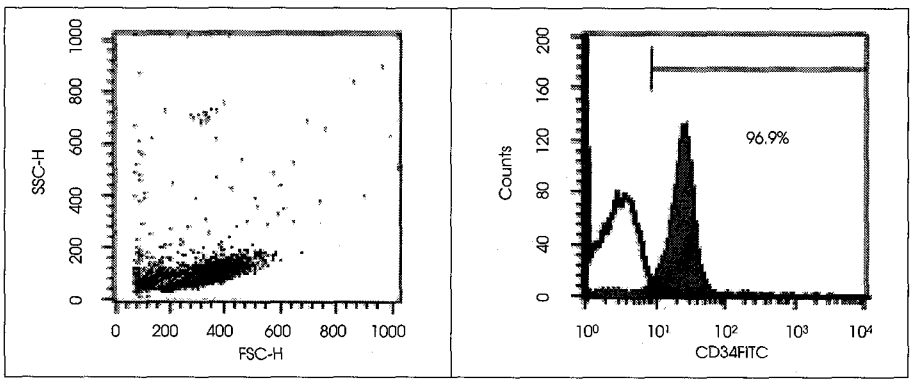

Fig. 1. The purity of CD34+ cells from human cord blood cells. Solid line represents isotypematched control antibody staining.

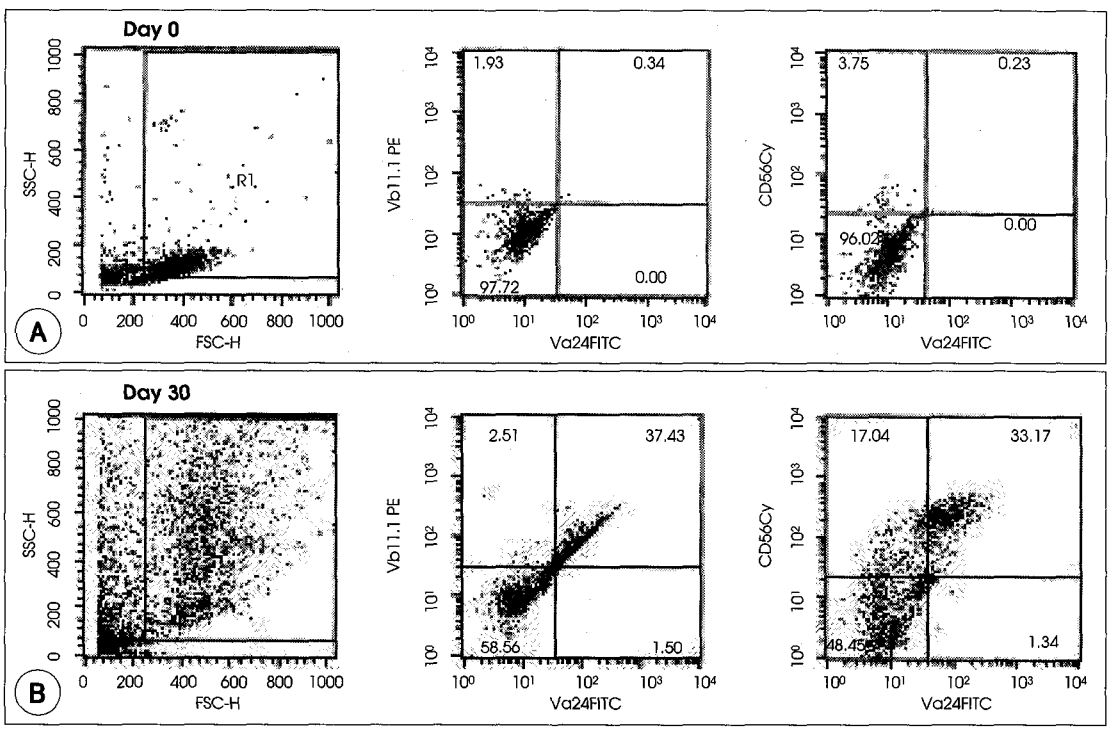

Fig. 2. CD56, V α 24 and V β 11.1 expression on differentiated NKT cells. CD34+ cells from human cord blood were cultured for 30 days with SCF (50ng/ml), FL (50ng/ml), and IL-15 (50ng/ml). On day 0, most of CD34+ cells did not express CD56, V α 24 or V β 11.1. After 30 days, 37.43% of cells showed V α 24/V β 11.1 both positives. In CD56+ cells, 66% of cells (33.17/50.21) were V α 24 positive.

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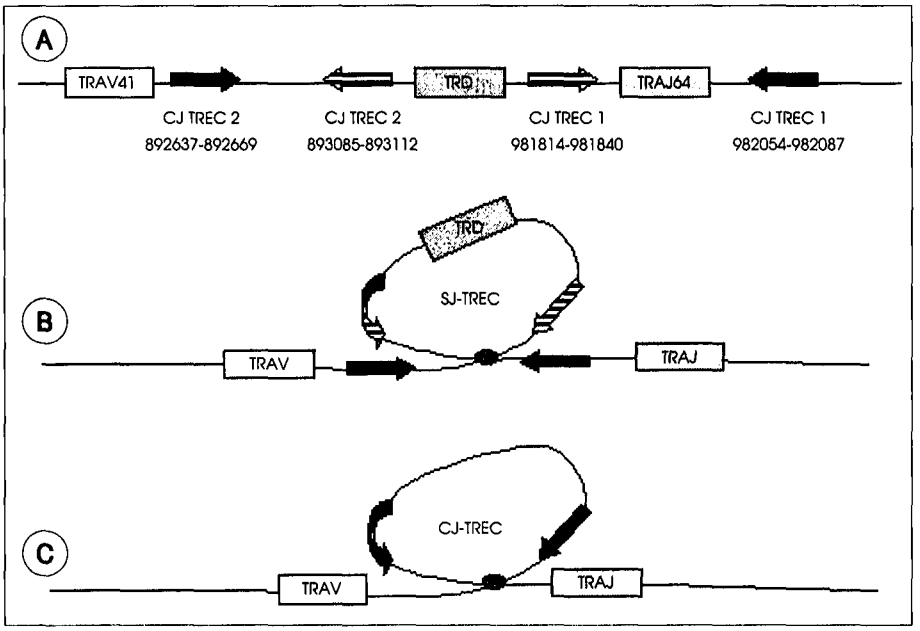

Fig. 3. Generation of signal joint TREC and coding joint TREC. A : *simplified representation of the TCR δ chain locus (TRD)* flanked by TCR α chain (TCR V α 41 and TCR J α 61). Arrows are depicted as the position of the each of the TREC primers. Numbers below the arrows indicate the sequence number from TCR α chain genome sequence (NG_001332.1) of the primers. B : End-to-end ligation of the recombination signal sequences removes most of the *TRD* gene region, forming a single TREC containing a unique signal joint sequence (signal joint TREC). C : When TCR V α and TCR J α recombination occurs, another unique sequence (coding joint) becomes the part of a second TREC.

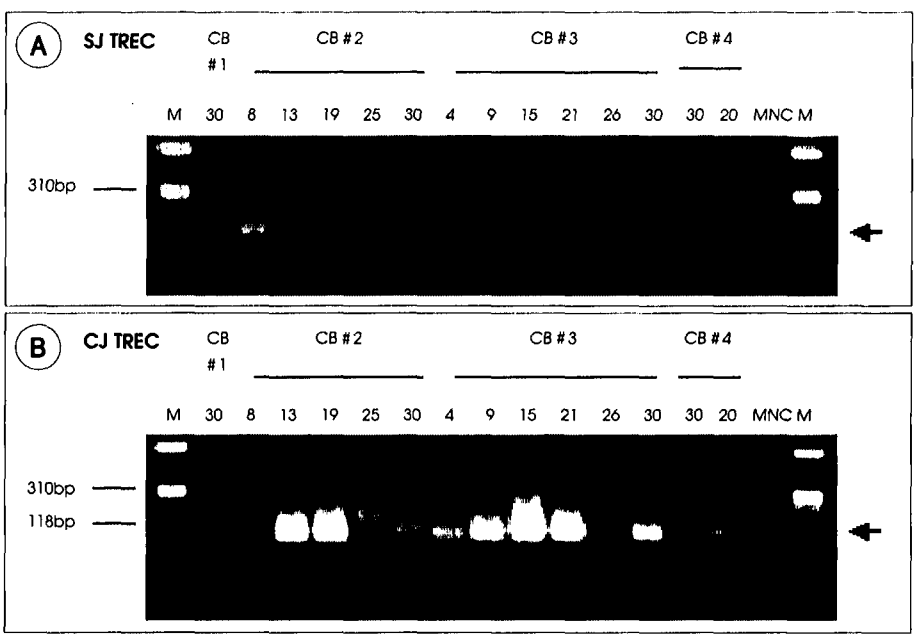

Fig. 4. PCR for TRECs. A : Amplified product of signal joint TREC (arrow) from four batches of experiment. B : Amplified product of coding TREC (arrow) from four batches of the experiment. MNC : mononuclear cells, M : molecular marker.

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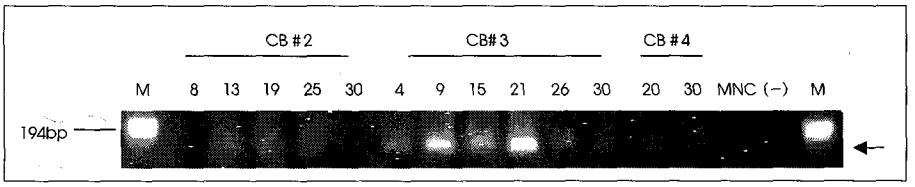

Fig. 5. PCR for V α 24-J α 15 sequence. Result showed is three batches of experiment. Arrow indicates expected V α 24-J α 15 PCR product. M : molecular marker, MNC : mononuclear cells, (−) : no template DNA included PCR product.

For confirmation of V α 24-J α 15 recombination of DNA in differentiated NKT cells, V α 24-J α 15 PCR was performed from extracted genomic DNA (Fig. 5). Though V α 24-J α 15 sequences were amplified in most of cultured cells, expression time and degree showed variation between different batches of CBs.

Discussion

The developmental process of NKT cells is not clearly defined yet, but primarily it was thought to develop and be educated in the thymus. However, extrathymic origin of the NKT cells was also suggested in athymic mice studies[9] and in other studies[10]. As previously described, NKT cells can be differentiated from CB CD34+ cells, eventually expressing CD3+, CD16+, CD56+, CD94+ and invariant V α 24/V β 11 TCR chains after 30 days of liquid culture supplemented with IL-15, FL and/or SCF. Those *ex vivo* differentiated NKT cells expressed both IFN-γ and IL-4, which was also one of typical characteristics of NKT cells[5].

We used TREC PCR to trace the time and degree of the TCR gene rearrangement during *ex vivo* NKT differentiation process. TRECs are the episomal DNA circles that are generated during excisional rearrangement of TCR genes. Because TRECs are stable, and not duplicated during mitosis, TRECs can be used as a marker of recombination of TCR gene. In Fig. 4, signal joint TREC first appeared on day 4 or day 8 and continuously existed until day 30. Between day 9 and day 21, cells showed high output of coding joint TRECs after signal joint rearrangement. After 30 days of liquid culture, most of cells expressed V α 24 and V β 11.1 as it showed in Fig. 2. In addition, percentage of the V α 24 and V β 11.1 positive cells had also increased by 30 days of culture[5]. Because TREC output was decreased as it approached to day 30, high expression of V α 24 and V β 11.1 TCR can be explained as a result of division of already differentiated NKT cells. To detect V α 24-J α 15 recombination event on genomic level, sequence amplifications were done in cultured cells (Fig. 5). It showed variation between batches of CB and culture days. It could be explained that the V α 24-J α 15 joining CDR3 base sequences had been changed or had sequence variation between batches of CBs.

Although the TCR rearrangement was indirectly showed in this study using TREC PCR, NKT cell development must be considered with the possibility of co-existing CD1d+ cells. Because NKT cells are derived from DP thymocytes and are positively selected by CD1d expressing cells[11] The existence and role of CD1d expressing cells in *ex vivo* differentiation of NKT cells remains to be elucidated.

Summary

Objectives :

To investigate the time of rearrangement of the TCR gene in the process of NKT cell differentiation from CD34+ human cord blood cells *in vitro*.

Methods :

We isolated the CD34+ human cord blood cells and induced the differentiation of NKT cells by liquid culture including IL-15, FL and SCF for 30 days. In order to detect the time of TCR gene rearrangement in differentiated NKT cell, we performed PCR for TCR-rearrangement excision circles (TRECs) with isolated DNA.

Results :

Signal joint TREC first appeared on day 4 or day 8 and continuously existed until day 30. Between day 9 and

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day 21, cells showed high output of coding joint TRECs after signal joint rearrangement.

Conclusion :

In differentiated NKT cells, TCR gene rearrangement started within a week after culture started and mostly occurred in 2 to 3 weeks after culture started.

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