Establishment of Technique to Grow EBV-transformed B Cells by Autologous Epstein Barr Virus

การสร้างเทคนิคในการเลี้ยง EBV-transformed B cells โดยใช้เชื้อ Epstein Barr virus สายพันธุ์ที่อยู่ในร่างกาย

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ABSTRACT

EBV infection is associated with EBV-associated malignancy. Transfer of antigen-specific T cells can be a new and safer therapeutic approach for various forms of cancer. The principle of this therapy is to stimulate EBV-specific CTLs with EBV-transformed B cells as lymphoblastoid cell lines (EBV-BLCL), which is usually established by infecting B lymphocyte with EBV lab strain (B95.8). Genetic variations of EBV in Thai population are somewhat different from B95.8 EBV and hence not having similar antigenic sequence. We develop technique to use autologous EBV for EBV-BLCL transformation. After successfully established, as many as 87% of EBV-BLCL expressed CD19 and 80% of them expressed CD86 which is antigen presenting cells. These EBV-BLCL may present EBV antigens to CTL. Our result could potentially lead to better outcome of adoptive T cell therapy, particularly Thai patients.

บทคัดย่อ

การติดเชื้อ Epstein-Barr Virus (EBV) มีความสัมพันธ์กับโรคมะเร็ง การให้ antigen-specific T cells กับผู้ป่วยโรคมะเร็งจะเป็นการรักษาแบบใหม่และปลอดภัย หลักการของการรักษานี้ คือ การกระตุ้น EBV-specific CTLs ด้วย EBV-transformed B cell หรือ lymphoblastoid cell lines (EBV-BLCL) โดยทำให้ B lymphocyte ติดเชื้อ EBV lab strain (B95.8) อย่างไรก็ตามยีนของ EBV ในประชากรของไทยมีความแตกต่างจาก B95.8 EBV ดังนั้นจึงมีความแตกต่างของ antigenic sequence ด้วย เราจึงได้พัฒนาเทคนิคการใช้ autologous EBV สำหรับ BLCL transformation ซึ่งพบว่าประสบความสำเร็จในการสร้างเซลล์เหล่านี้ อีกทั้ง EBV-BLCL มีการแสดงออกของ CD19 มากถึง 87% และ CD86 ซึ่งเป็น antigen presenting cells เท่ากับ 80% ซึ่ง EBV-BLCL น่าจะมีคุณสมบัติในการนำเสนอ EBV antigen ให้กับ T cells ดังนั้นผลวิจัยของเราสามารถนำไปสู่การพัฒนาการรักษาด้วย adoptive T cells ได้ดีโดยเฉพาะในผู้ป่วยชาวไทย

Key Words: EBV, EBV-transformed B cell, EBV-specific T cell

คำสำคัญ: เชื้อไวรัสเอปไตน์บาร์ บีเซลล์ที่ถูกทำให้เป็นมะเร็งด้วยเชื้อไวรัสเอปไตน์บาร์ ทีเซลล์ที่จำเพาะกับเชื้อไวรัสเอปไตน์บาร์

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Introduction

EBV a gamma herpesvirus infects over 90% of human population (Hsu&Glaser, 2000). The infection route is via oral secretion (Odumade et al., 2011). In the primary infection, EBV infects epithelial cell of pharynx and release infectious virions into oral secretion. EBV becomes subsequently lifelong latent infection in B lymphocytes. In latent stage, the virus express latent proteins (Epstein Barr Nuclear antigen (EBNA) 1, 2, 3A, 3B, 3C, leader protein (LP) and latent membrane protein (LMP) 1, 2 (Cohen, 2000). Malignancies which are associated with EBV have been shown to express 3 latency antigen patterns. Latency I as seen in Burkitt's lymphoma, EBV express only EBNA-1. EBV gene expressions in latency II are EBNA-1, LMP-1 and LMP-2. This is observed in latency program Hodgkin's disease, Nasopharyngeal carcinoma and T/NK cell lymphoma. EBV in latency III express all latent proteins: EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, LMP-1 and LMP-2. This pattern is seen in Post-transplant lymphoproliferative disease as well as *in vitro* EBV-transformed B cell lines as lymphoblastoid cell lines (LCLs)

There has been an emerged use of the EBV-specific T cells or adoptive T cell therapy for prophylaxis and treatment of EBV-associated malignancies (Merlo et al., 2008) for example, PTLD (Helen E. Heslop et al., 2010), NPC (Comoli et al., 2005) and Hodgkin's lymphoma (Bollard et al., 2004). The standard method to activate EBV-specific T cells by stimulating them with EBV-transformed B cell lines These cell lines are infected with lab strain EBV B95.8, which is propagated in lymphoma cell line of marmoset. Therefore, they present EBV latent antigen of B95.8 origin. Among Thai population, EBV B95.8 is rare and various strains of EBV are noted throughout regions of Thailand (Saechan et al., 2010). Thus, use of EBV B95.8 as an EBV antigen may not be appropriate for Thai population.

Objectives of the study

To develop the technique of culturing spontaneous autologous EBV-transformed B cell lines

Methodology

Peripheral blood was obtained from 5 EBV-seropositive healthy subjects. The informed consent was obtained and the experimental protocol was approved by the Ethics committee Faculty of Medicine, Chulalongkorn University. The Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-hypaque. The isolated PBMC were resuspended with R10 (10% Fetal Bovine serum in 90% RPMI 1640).

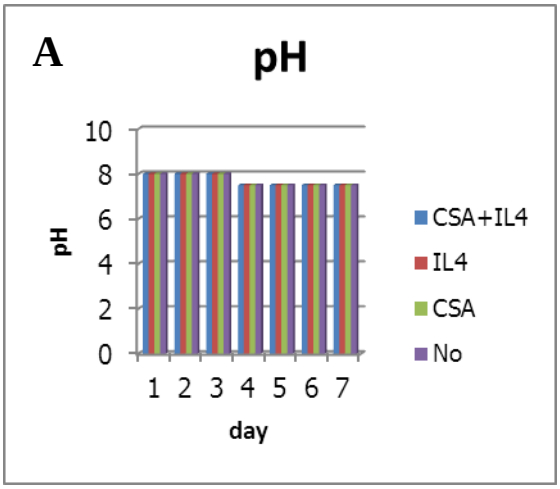
CD8+ T cells were depleted from PBMC using Dynabeads® CD8 T Cells (Invitrogen, Norway). CD8-depleted PBMC were cultured in the B cell lines setup solutions which supplement with Interleukine-4 (IL-4) and/or Cyclosporin A (CSA). Culture medium was changed once a week until the aggregation and proliferation was observed. Culture medium was collected to analyse pH, glucose and ketone by strip test (Standard Diagnositics, Korea).

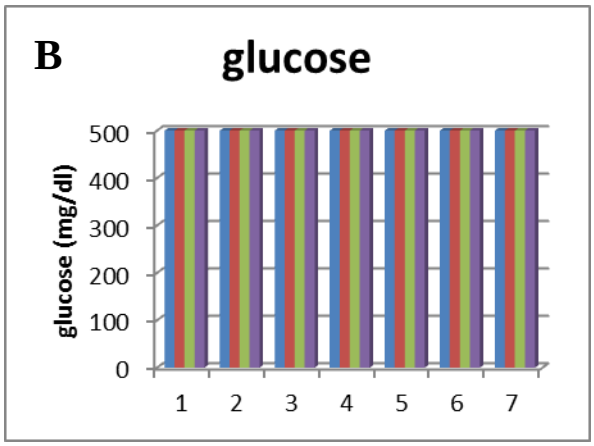
To analyse the property of antigen presenting cell of the EBV-transformed B cell lines, the EBV-transformed B cell lines were stained with Pacific Blue anti-human CD19 antibody (Biolegend), PE anti-human CD80 antibody and FITC anti-human CD86 antibody. The stained cells were incubated for 30 minutes at 37 °C and washed with phosphate buffer saline (PBS) then centrifuged at 1500 rpm for 5

minutes. The cells were fixed with paraformaldehyde and analysed using FACS Aria II.

Results

Establishment of EBV-transformed B cell lines that were transformed by autologous EBV strain: Depletion of $CD8^+$ T cells from PBMC rendered reactivation of latent EBV in the infected B cells leading to B cell transformation. CD8-depleted PBMC was cultured in 4 different B cell lines setup solutions; condition1 (IL-4 and CSA), condition2 (IL-4), condition3 (CSA) and condition4 (without supplement). Culture medium was collected everyday analyze for pH, ketone and glucose. pH of culture medium of all condition are 7.5-8 (fig.1A). Glucose level was 500 mg/dl since day 1 to 7 before replacing half of the culture medium and adding new medium (fig.1B). However, no ketone was detected in all condition of the culture media(fig.1C). This result was not different in all condition at 30th and 45th day of the glucose and ketone level, but the pH was decreased to 7. Similar results were achieved from 4 other EBV-seropositive healthy subjects (data not shown).





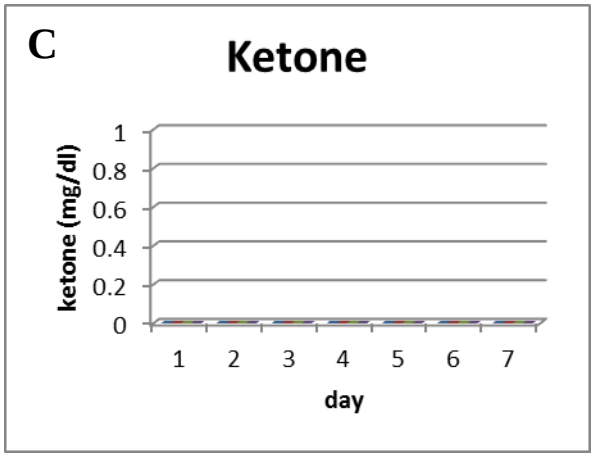
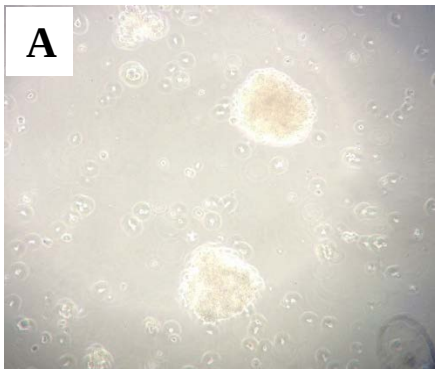
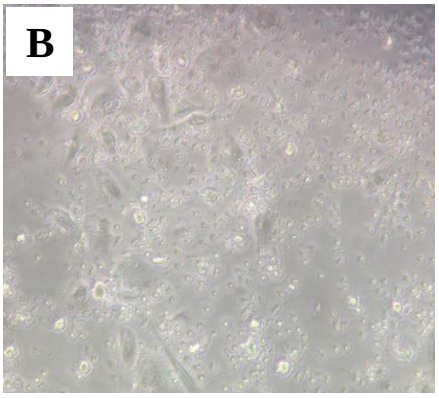


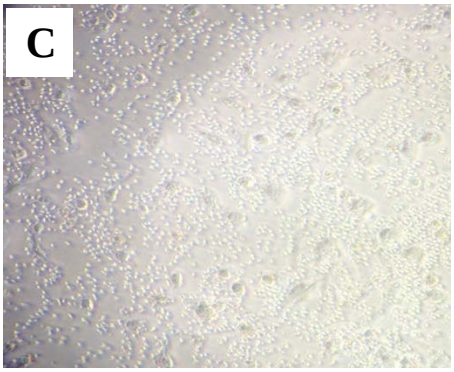
Figure 1 Analysis of pH, glucose and ketone of culture media in 4 conditions of CD8-depleted PBMC form donor 1. pH, glucose and ketone were analysed from culture media in day 1 to 7. pH was 7.5-8 (A), glucose level was 500 mg/dl without changes regarding culture time (B), no ketone was detected in the culture media (C). The culture media were added with CSA (cyclosporine) and/or IL-4 (Interleukin-4).

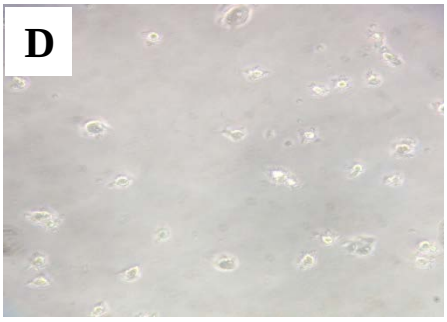
EBV-transformed B cells in condition1 (IL-4 and CSA) of culture media is successful transformation : EBV transformation process was established at 6-8 weeks which could be noted by cell aggregation and proliferation. At day 30, EBV-transformed B cell lines

which cultured with supplement of CSA and IL-4 exhibited massive cell aggregation which were not seen in other conditions (Fig2). Thus, CSA and IL4 condition was optimal for culturing EBV-transformed B cell lines. Moreover, EBV-transformed B cell lines of 5 EBV-seropositive healthy subjects all grew in the culture media contain CSA and IL-4.









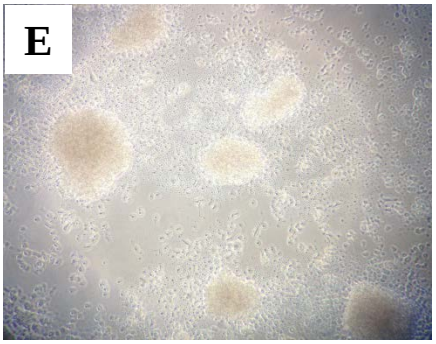


Figure 2 Visualization of cell aggregation of EBV-transformed B cell lines in 4 conditions (A-D) at the 30th day. Culture media contain with CSA and IL-4 show massive cell aggregation (A), but The cell lines do not show aggregation and non-growth with IL-4 (B), CSA (C) and no CSA and IL-4 (D). EBV-transformed B cell lines in culture media contain with CSA and IL-4 at the 45th day showed massive cell aggregation with increased numbers of clump cells (E).

Analyzing the property of antigen presenting cells of the EBV-transformed B cell lines. CD80 and CD86 are upregulated on antigen-presenting cells (APCs) and bound to CD28 on the T cell, transducing a crucial second signal for T-cell activation with the T-cell receptor. Expression of co-stimulatory molecular CD80 and CD86 were 1% and 80% respectively. (fig3.)

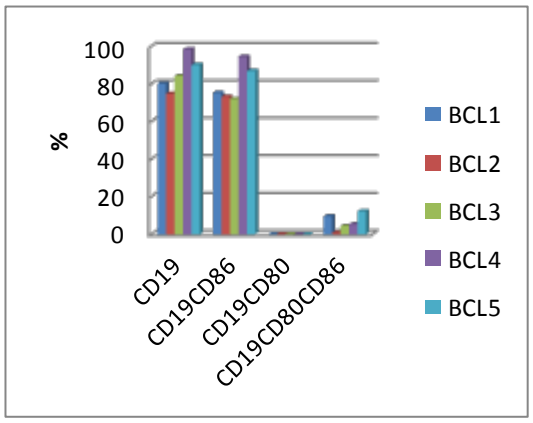


Figure 3 Flow cytometry analysis of Co-stimulatory molecule in EBV-transformed B cell lines. (n=5)

Discussion and Conclusions

We have successfully established EBV-transform B cells (EBV-BLCL) which are transformed by autologous EBV strain. We found that EBV-BLCL could be generated efficiently in culture media with supplement of Interleukin-4 (IL4) and Cyclosporin A (CSA) which showed massive cell aggregation and continuous proliferation but only IL-4, CSA and no supplement. Cyclosporin A (CSA) is a fungal metabolite effecting on T lymphocytes. It has the potential of immunosuppressive drug. The mechanism of CSA is inhibition of T cell growth factor gene expression (Krönke et al., 1984). Interleukin 4 (IL-4) is a cytokine which play an important role in activating B cells and T cells. So Establishment of that EBV-BLCL needs both supplements: IL-4 in helping proliferation of EBV-BLCL whereas suppressing T cell by CSA for EBV-BLCL not to regress. As a matter of fact, metabolism of cell proliferation resulted in glucose consumption (Brand et al., 1986) and alteration of pH and level of ketone. Nevertheless, comparison of these culture media solution in 4 conditions displays no significant difference from start till transformation. This could probably be explained by weekly changes of culture medium together with addition of new medium. Moreover, measurement method using strip might not be sensitive enough for the minute changes. Property of antigen presenting cells comprises expression co-stimulatory molecule which binds to CD28 on the T cell rendering second signal for T-cell activation. More than 89% of that EBV-BLCL expressed CD19 which is B-lymphocyte marker. Meanwhile, co-stimulatory molecule CD86 is 80% meaning that EBV-BLCL has a property of antigen presenting cells, thus might present EBV antigen to CTLs which leads to development of adoptive T cell therapy especially for Thai population.

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