이학석사 학위논문

CD21-independent Epstein-Barr virus entry into NK cells

Epstein-Barr 바이러스의 자연살해 세포로의 CD21 수용체 비의존적 감염경로에 관한 연구

2015 년 08 월

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이 정 후

A thesis of the Degree of Master of Science

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CD21-independent Epstein-Barr virus entry into NK cells

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Approved by Thesis Committee:

Professor _____ **Chairman**
Professor _____ **Vice chairman**
Professor _____

ABSTRACT

Purpose: Extranodal NK/T-cell lymphoma is an aggressive malignant disease which is associated with Epstein-Barr virus. Although several lines of evidence point at EBV as a key player in the pathogenesis of ENKTCL, the viral mechanism of entry into NK/T cells remains uncertain. Therefore, this study was conducted to identify the entry mechanism of EBV into NK/T cells.

Methods: NKL cells were used as EBV-negative NK cell lymphoma cell line and Raji cells were used as EBV-positive B cell lymphoma cell line which is not producible for viral particle. Viral supernatant was obtained from B95-8 cell line and exosomes were isolated by serial centrifugation from Raji cell culture supernatant. Viral gene was detected by RT-PCR/PCR.

Results: Viral mRNAs were detected in Raji-derived exosomes, and also in NKL cells co-cultured with Raji-derived exosomes. To evaluate the effect of Raji-derived exosomes on the EBV infection, viral supernatant which was obtained from B95-8 cells, treated to NKL cells after Raji-derived exosomes. Although Raji-derived exosomes transferred viral mRNAs, viral entry was not detected in NKL cells. The CD21 receptor intensity was increased after that NKL cells were co-cultured with Raji cells. To demonstrate the effect of CD21 trogocytosis on the infection, EBV treated to NKL cells which were co-cultured with Raji cells. Although CD21 trogocytosis has occurred, viral genes were not detected in NKL cells. Interestingly, viral DNA was found in all EBV treated NKL groups and primary NK cells.

Conclusions: CD21 mRNA transfer via EBV-positive B cell-derived exosomes and CD21 receptor trogocytosis from EBV-positive B cells are not key factors of viral entry mechanism to NK cells. EBV enters NKL cells without any interactions with EBV-positive B cells.

Keywords: Epstein-Barr virus, Extranodal NK/T cell lymphoma, CD21 receptor, Exosomes, Trogocytosis

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INTRODUCTION

Epstein-Barr virus (EBV), a member of the γ-herpesvirus family, is one of the most common viruses and persists in the vast majority of humans as a life-long, asymptomatic infection within the B lymphocyte [1]. Extranodal NK/T-cell lymphoma, nasal type (ENKTCL) is the major group of natural killer (NK) cell neoplasms or more rarely, T cell neoplasms [2]. ENKTCL is a rare EBV-associated non-Hodgkin lymphoma (NHL) which affects the nasal cavity [3] and upper aerodigestive tract [4]. The transforming properties of EBV and its role in pathogenesis of a range of B cell malignancies are well established. By contrast, the association of this virus with malignancies of NK and T cell origin was entirely unexpected. Although a number of mature NK and T cell lymphoma subtypes have been related to EBV, evidence for a viral/tumor relationship is largely limited.

EBV is usually not detected in NK and T cells from the blood of healthy individuals but is reported to occur infrequently in tonsillar tissue infrequently following primary infection [5, 6]. EBV infects resting B cells via the CD21 and MHC class II co-receptor, and activated NK cells also express HLA-DR, but these cells do not express CD21. For this reason, the mechanism of EBV entry into NK cells remains uncertain [1].

Exosomes-mediated transfer of mRNA from EBV-infected cells was proposed as a possible mechanism of viral entry into other cells [7, 8]. Exosomes are nanoparticles that are released from a wide variety of normal cells and malignant cells [9, 10]. Exosomes contain a wide variety of functional RNAs

including mRNA and miRNA [11]. Functional mRNAs also can be transferred to target cells where they are translated into proteins that modulate cellular signaling such as transcriptional factors, protein kinases, or metabolic enzymes [12]. Therefore we hypothesized that EBV-infected B cells-derived exosomes may transfer viral mRNA or CD21 mRNA into NK cells. We also investigated a possibility of transient membrane CD21 protein transfer (trogocytosis) which was also proposed as a possible mechanism of EBV entry into NK cells [13].

MATERIALS AND METHODS

Cell culture

EBV-negative NK cell leukemia cell line NKL and EBV-positive Burkitt lymphoma cell line Raji were purchased from American Type Culture Collection (ATCC). B95-8 cell lines were purchased from European Collections of Cell Culture (ECACC). Human peripheral blood mononuclear cell (PBMC) was prepared from healthy donors with leukoreduction system chambers by Ficoll density gradient centrifugation. Primary NK cells were further isolated by two-step immunomagnetic separation system using anti-CD3 and anti-CD56 antibodies labeled with magnetic microbeads (Miltenyi Biotec). All cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2mM L-glutamine and 1% gentamycin solution (Gibco-Life Technologies). Use of human cells in the study was approved by Institutional Review Board.

Exosome purification

Exosomes were isolated from supernatant of Raji cells by serial centrifugation and analyzed by Western blotting for protein staining or RT-PCR for viral gene detection. For large scale preparation of exosomes, cells were grown for 3 days in serum-free medium. 300ml of cell supernatant was collected and centrifuged at 1,500g for 10 min to sediment the cells and subsequently at 12,000g for 30min to remove the cellular debris. Cell supernatant was then

filtered over 0.22 μm and ultracentrifuged at 100,000g for 70 min. The exosome pellet was washed once in a large volume of PBS and resuspended in PBS.

Flow cytometry

Flow cytometry analysis was performed using FACSCalibur (BD) to observe the trogocytosis of NKL cell line that co-cultured with Raji cell line, and the purity of NK cell populations isolated from PBMCs. All antibodies were from BD.

Virus production and purification

Epstein-Barr viruses were isolated from supernatant of B95-8 cell line by serial centrifugation. For large scale preparation of viruses, cells were grown for 4 then 300ml of cell supernatant was collected and centrifuged at 1,500g for 10min to sediment the cells and subsequently at 12,000g for 30min to remove the cellular debris. Cell supernatant then filtered and stored at -80℃.

RT-PCR

Total RNA was isolated from the cells using an RNA Mini kit (Invitrogen) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). PCR amplification was performed using protocols with specific primers (Table 1).

RESULTS

Epstein-Barr virus did not infect NKL cells by exosomes

To elucidate the role of exosomes from EBV-positive B cells, we purified exosomes from Raji cell culture supernatant. Purity of isolated exosomes was confirmed by western blotting using organelle markers as shown in Fig 1A. Viral mRNAs (LMP1, EBNA1, and EBER1) were detected in Raji-derived exosomes (Fig 1B, Rexo) as well as Raji cells (Raji). These EBV mRNAs were also detected in EBV-negative NK leukemia cell line NKL when the cells were treated with Raji-derived exosomes (Fig 1B, +Rexo). However, expression of the EBV genes transferred from the Raji exosomes in NKL cells were diminished after 48hr (data not shown and Fig 1C). As the CD21 mRNA in Raji exosomes was also transferred into NKL cells (Fig 1B), we next tested the possibility that EBV infects NK cells via exosomes-derived CD21. As Raji cells are not producible its own virus, we treated viral supernatant from B95-8 cells to NKL cells with or without Raji exosomes. As shown in Fig 1C, however, after culture for 48hr, we did not see any viral mRNAs in NKL cells. These data showed that mRNAs of EBV or viral entry receptor CD21 were transferred into NK cells, these are not sufficient to EBV persistency or EBV entry in NK cells.

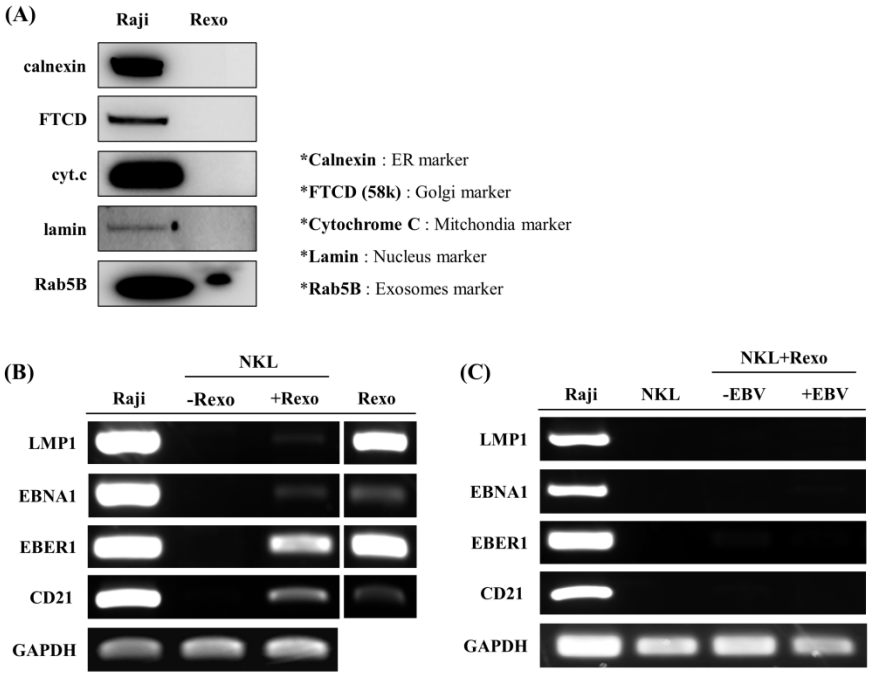

Figure 1. Exosomal transfer of CD21 and EBV mRNA into NKL cells. (A) Raji-derived exosomes (Rexo) were purified by serial centrifugation and their purity was confirmed by western blotting. (B) Rexo were treated to NKL cells for 2hr, then the cells were washed twice and mRNA was purified. RT-PCR was performed on Raji cells (positive control), Rexo, no treated NKL cells and Rexo treated NKL cells. CD21 and viral mRNA transferred to NKL cells was detected. (C) After exosomal transfer for 2hr, viral supernatant was treated to NKL cells. After 48hr incubation with EBV, RT-PCR was performed. All experiments were performed more than five times independently and representative data are shown.

Epstein-Barr virus does not infect NKL cells by CD21 trogocytosis

Trogocytosis is a process whereby lymphocytes extract surface molecules from conjugating antigen-presenting cells and express them on their own surface. During trogocytosis, lymphocytes uptake lots of different molecules from conjugating cells. Some of these molecules, which are not transcribed by lymphocytes, could influence the phenotype and function of the lymphocytes [14]. Even though resting peripheral blood NK cells do not express MHC class II, NK cells express HLA-DR when activated with cytokines such as IL-2. NK leukemia cell line NKL cells express HLA-DR that can be used as a co-receptor for EBV infection, but not CD21 (Fig 2A). CD21 is expressed in all B cells including EBV-infected cells, and can be transferred into NK cells in immune synapse (i.e. trogocytosis). When NKL cells were co-cultured with Raji cells, CD21 intensity was increased (Fig 2B), however, in further cultivated NKL cells there were no EBV mRNA (Fig 2C). Therefore, CD21 trogocytosis did not make EBV infection in NK cells in our experiments.

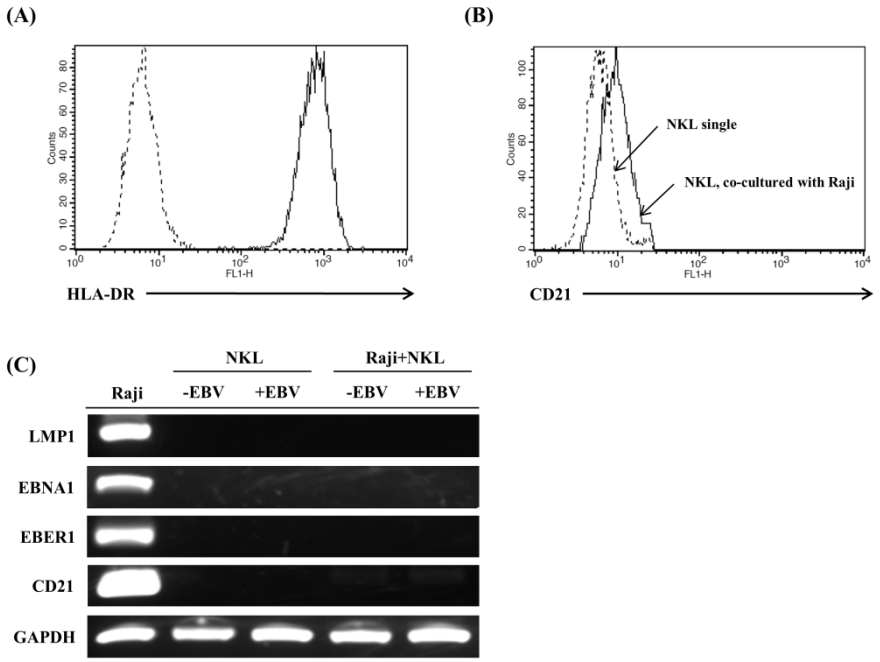

Figure 2. Trogocytosis of CD21 to NKL cells. Trogocytosis of CD21 to NKL cells. (A) HLA-DR intensity of NKL cells was measured by flow cytometry. (B) After co-culture with Raji cells for 2hr, increased CD21 intensity of NKL cells was measured by flow cytometry. CD19- CD94+ cells (NKL) were gated and shown. (C) Viral supernatant was treated to NKL cells after trogocytosis and incubated for 48hr. NKL cells were purified by depleting CD19+ cells (Raji) by MACS, and further cultured for 7 days. All experiments were performed more than five times independently and representative data are shown.

Epstein-Barr virus enters NKL cells without EBV-positive B cells

EBV is an enveloped virus that contains viral DNA. Following primary infection, EBV usually persists within memory B cells in a latent state [1]. In latency 0 state, viral gene expression is mostly suppressed [15]. To identify the type of latent state, DNA from NK cells was extracted in previous experiments. Unexpectedly, EBV genes (LMP1, EBNA1, and EBER1) were found in all EBV treated NKL cells (Fig 3). Interestingly, detection of EBV genes in NKL cells did not need B cell co-culture.

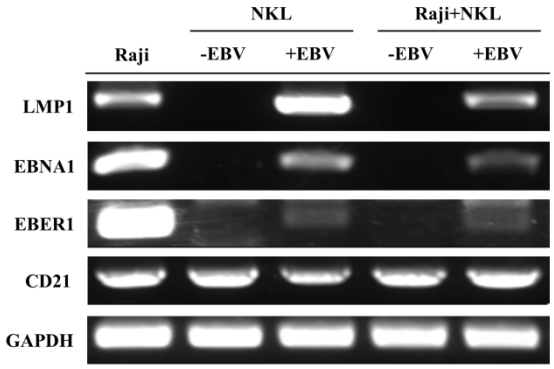

Figure 3. Detection of viral DNA in EBV treated NKL cells. As indicated, NKL cells were cocultured with Raji cells for 48hr, purified, then further cultured for 7 days. Whole DNA was extracted and PCR was performed on cells.

Epstein-Barr virus infects primary NK cells

To confirm our finding in primary human NK cells, we isolated peripheral blood NK and B cells from 5 healthy donors, and treat EBV to the cells. After 14 days of culture, DNA from the samples was purified, and PCR analysis for viral DNA was performed. As shown in Figure 4, EBER1 DNA was detected in NK cells, but LMP1 which was detected in B cells was not.

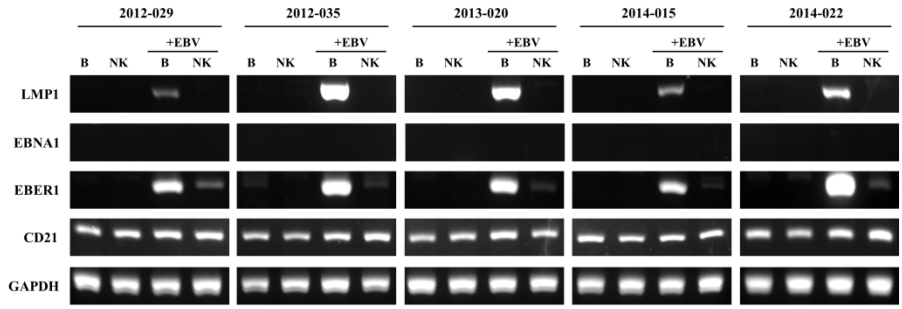

Figure 4. Detection of viral DNA in EBV treated primary NK cells. Primary B and NK cells were isolated from PBMC from healthy donors. Viral supernatant was treated to isolated primary lymphocytes for 2 weeks. PCR was performed on the cells.

Table 1. Reverse transcriptase-PCR (RT-PCR) primers and products

Transcripts	Product size	Sequence (5'-3')	Coordinates in B95-8	Annealing temp, PCR cycle
EBNA1	330	AGGCGCGGGATAGCGTGCGCTACCGGA CACCATCTCTATGTCTTGGC	62426–62452 108157–108138	48℃, 38 cycles
LMP1	324	TCCTCCTCTTGGCGCTACTG GGAGTCATCGTGGTGGTGTT	169383–169364 168721–168740	62℃, 38 cycles
EBER1	167	AGGACCTACGCTGCCCTAGA AAAACATGCGGACCACCAGC	6629–6648 6795–6776	55℃, 38 cycles
CD21	327	GTTGTTCAGGTACCTTCCGC TAGGAAGTGCTGGACACTCG		62℃, 38 cycles

DISCUSSION

Several lines of evidence point at EBV as a key player in the pathogenesis of ENKTCL. Similar to Hodgkin lymphoma, ENKTCL is thought to be in latency II phase, with an expression of EBNA1, LMP1, and EBER1. Although a role for EBV in ENKTCL pathogenesis is highly suspected, the precise oncogenic functions of EBV are not fully understood. The primary function of EBNA1 is to ensure transmission of the circularized EBV episomes to daughter cells by facilitating its replication during cell division [16]. Potential oncogenic effects of EBNA1 are considered to include promotion of EBV induced B cell immortalization [17], induction of cellular genomic instability [18], and protection from p53-mediated apoptosis [19]. LMP1 is the principal transforming protein of EBV. It functions as a classic oncogene in rodent fibroblast transformation assays and transformation of EBV-induced B cell in vitro [1]. LMP1 is also a key modulator of cell signaling, inducing expression of a number of antiapoptotic proteins, including BCL2 [20] and A20 (TNFAIP3) [21], as well as adhesion molecules and activation antigens. The EBER1 transcripts are small non-coding RNA molecules that are the most highly expressed viral products in EBV-transformed cells. Their detection by in situ hybridization is a widely adopted assay to confirm EBV infection in fresh and fixed tissues. In B cells, some evidence indicates that EBER1 expression protects against apoptosis and contributes to cell transformation and proliferation [22].

In this study, we demonstrated that EBV enters NK cells without interactions

with EBV-positive B cells. Transfer of CD21 mRNAs via EBV-positive B cell-derived exosomes and CD21 trogocytosis are not the key mechanism for the infection. After primary infection, EBV usually latently infects B cells. In latency 0 state, viral gene expression is mostly suppressed [15]. We have shown that all EBV treated NKL cells and primary NK cells were not express viral mRNAs. But from all these cells, viral DNA was detected after EBV treatment. Although we have demonstrated that EBV enters NK cells without any interactions with EBV-positive B cells, which receptor is involved in viral entry is not determined. Also, reactivation mechanism of latently infected virus is not clear. Further studies will be needed to clarify these issues to understand pathophysiology of ENKTCL.

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국문 초록

서론: 결절 외 NK/T 세포 림프종은 매우 공격적인 암종으로 Epstein-Barr 바이러스와 밀접한 관련이 있는 것으로 알려져 있다. 선행연구들에 의해 Epstein-Barr 바이러스가 결절 외 NK/T 세포 림프종의 발병에도 관여하는 것이 밝혀지고 있지만, NK/T 세포로의 감염경로에 대해서는 아직도 불분명한 상태이다. 따라서 본 연구를 통해 Epstein-Barr 바이러스가 NK/T 세포에 감염되는 원리에 대해 밝히고자 한다.

방법: Epstein-Barr 바이러스 음성 NK 세포 림프종으로 NKL 세포주를 사용하였으며, 바이러스를 생산해내지는 못하는 Epstein-Barr 바이러스 양성 B 세포 림프종으로 Raji 세포주를 사용하였다. Epstein-Barr 바이러스는 B95-8 세포주로부터 획득하였으며, exosomes 은 Raji 세포주의 세포배양액으로부터 순차적 원심분리를 통해 분리하였다. 바이러스 유전자는 RT-PCR 과 PCR 을 통해 확인하였다.

결과: Raji 세포주로부터 유래한 exosomes 내에 바이러스 mRNA 가 존재함을 확인하였으며, Epstein-Barr 바이러스의 주요 감염경로로 잘 알려진 CD21 수용기의 mRNA 또한 존재함을 확인하였다. 분리한 exosomes 을 NKL 세포주와 혼합 배양하였을 때 NKL 세

포 내에서도 바이러스 mRNA 를 확인할 수 있었으며, CD21 mRNA 또한 exosomes 으로부터 NKL 세포로 옮겨감을 확인하였다. Raji 유래 exosomes 이 Epstein-Barr 바이러스의 감염에 어떤 역할을 하는지 확인하기 위해 NKL 세포주와 exosomes 을 혼합 배양한 후 바이러스를 처리해주었으나, 바이러스의 감염은 확인되지 않았다. NKL 세포의 표면에는 CD21 수용기가 존재하지 않으므로 Raji 세포주와의 혼합 배양을 통해 CD21 의 trgocytosis 를 유도하였다. FACS 를 통해 분석한 결과, Raji 세포주와 혼합 배양한 NKL 세포주에서 CD21 의 발현강도가 증가하는 것을 관찰할 수 있었다. CD21 trogocytosis 가 Epstein-Barr 바이러스의 감염에 어떤 역할을 하는지 확인하기 위해 Raji 세포주와 혼합 배양 중인 NKL 세포주에 바이러스를 처리해주었으나, 바이러스의 감염은 확인되지 않았다. 흥미롭게도, 모든 실험군에서 바이러스 mRNA 는 관찰할 수 없었으나, 바이러스를 처리해준 모든 NKL 세포와 신선 NK 세포들에서 PCR 을 수행한 결과, 바이러스 DNA 가 존재함을 확인할 수 있었다.

결론: B 세포 유래 exosomes 에 의한 CD21 수용기 mRNA 의 이동이나 trogocytosis 에 의한 CD21 수용기의 직접 이동은, Epstein-Barr 바이러스가 NK 세포에 감염되는 데에 주된 역할을 하지 않음이 확인되었다. Epstein-Barr 바이러스는, Epstein-Barr 바이러스 양성 B 세포와는 관계없이 NK 세포로 들어가며, 어떤 수

용체를 통해 감염되는지, 감염된 바이러스가 어떻게 활성화되어 림프종을 일으키는지에 대해서는 추가 연구가 필요할 것으로 보인다.

주요어 : Epstein-Barr 바이러스, 결절 외 NK/T 세포 림프종, CD21 수용체, Exosomes, Trogocytosis

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