Original Article

Cd40 doesn't Complement The Function Of Epstein-barr Virus (Ebv) Latent Membrane Protein 1 In B Cells Transformation

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Abstact:

Objective: Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1) is known to plays important role in B cells growth and transformation. LMP1 is considered to be a functional homologue of the CD40 receptor and they can activate many overlapping signaling pathways. In this study, we compared the function of CD40 with that of LMP1 in B cells transformation. ***Materials and methods*:** Expression of CD40L was observed in infected B cells with LMP1 mutated EBV. To observe the expression reverse transcription-PCR were performed. ***Results*:** This expression of CD40L did not support proliferation and transformation of B cell. Even *in vitro* proliferation and transformation of B cell infected with LMP1 deleted EBV supplemented with CD40L were also not observed. ***Conclusion:*** Despite many similarities shared between CD40 and LMP1, CD40-CD40L interaction didn't complement on LMP1 mediated B cells transformation *in vitro*.

Key Words: Epstein-Barr virus, CD40 and CD40L, latent membrane protein, B cell transformation.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that infects the vast majority of the world's adult population [1]. This virus is well known for its association with a broad spectrum of benign and malignant cancers, including Burkitt's lymphoma, nasopharyngeal carcinoma, infectious mononucleosis, gastric carcinoma etc[1]. *In vitro* EBV infection of peripheral B-lymphocytes causes their immortalization and proliferation[1]. In immortalized lymphoblastoid cell lines (LCL), EBV expresses various proteins including nuclear antigen proteins (EBNAs), latent membrane proteins (LMP1, -2A, and -2B), a transcript from the BamHI-A region etc. It also expresses two small nuclear RNAs (EBER1 and 2) [2]. LMP1 is one of major gene products of EBV, which is an integral membrane protein, composed of a cytoplasmic amino-terminal domain, six hydrophobic transmembrane domains, and a cytoplasmic carboxy-terminal domain [3]. LMP1 has been shown to be important in EBV-mediated *in vitro* B-cell proliferation and transformation [4-6] and mimics CD40 in multiple ways [6]. CD40 and LMP1 interact with similar sets of intra-cellular signaling molecules and activate overlapping signaling pathways [7-9]. CD40 is a membrane-bound protein of the tumor necrosis factor (TNF) receptor family and is expressed on many cell types including B cells. CD40 and its ligand (CD40L) interaction is important to B cells for their proliferation, survival, immunoglobulin isotype switching, and germinal center reaction upon stimulation by activated T cells [10]. Mutations in the CD40L gene were identified in X-linked hyper IgM syndrome (XHIM), a disease characterized by drastic inhibition in T cell-dependent humoral immune responses [10,11]. CD40 or CD40L mutated mice had showed severe defects in the establishment of B cell memory and very few LCLs from XHIM B cells upon EBV infection were observed [7,10]. It was found that EBV infection of B cells induces ectopic expression of CD40L [12] and it's expression could stimulate CD40-mediated growth signals in B cells [10, 13,14], suggesting that EBV-induced CD40L facilitates B cell proliferation. The CD40L expression on LCLs and the other EBV-positive B cell lines suggests long-term expression of EBV-induced CD40L. That led us a question whether CD40L and CD40 play a role in EBV infection and subsequent B cell transformation. In our experiment, B cells were infected with LMP1 mutated EBV and observed the ectopic expression of CD40L. We also cultured the infected B cells in presence of CD40L. We found that ectopic

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expression or even LMP1 mutated EBV infected cell that was cultured in presence of supplied CD40L didn't mimic LMP1 function of *in vitro* cells proliferation and transformation. So, CD40-CD40L interaction doesn't complement *in vitro* LMP1 mediated B cells transformation.

Materials and Methods

Preparation of Human Peripheral Blood Lymphocytes

B cells were isolated from peripheral blood mononuclear cells (PBMCs) with anti-CD19 Dynabeads M-450 (Dynal, Great Neck, NY) according to the manufacturer's directions.

Cell culture:

Previously established AK-BAC-GFP (recombinant EBV containing Bacterial artificial chromosome (BAC) and Green fluorescent protein (GFP) gene in their genome)[15] and d.LMP1-EBV (LMP1 knockout AK-BAC-GFP)[16] were maintained in RPMI 1640 medium (Sigma-Aldrich Fine Chemicals, St. Louis, Mo.) containing 10% fetal bovine serum at 37°C in a 5% humidified CO_2 atmosphere.

Virus production and infection:

Viruses were produce from AK-BAC-GFP and d.LMP1-EBV. Cells ($2.0X10^6$) were resuspended in 1 ml of fresh medium containing 0.5% rabbit anti-human IgG (Dako Cytomation, Carpinteria, Calif.) and incubated for 8 to 12 h. The culture medium was replaced with fresh medium, and 48 h later, the culture supernatant was harvested. The culture supernatant was then filtered through a 0.45 µm-pore-size membrane and used as a virus solution. For infection, B cells ($1.0X10^6$) isolated from PBMCs were suspended in 1 ml filtered virus solution and incubated at 37°C for 90 min with continuous gentle mixing. Infected cells were then plated at $2X10^5$ cells per well in 96-well tissue culture plates. Half of the wells containing B cell infected with d.LMP1-EBV were cultured in presence of CD40L (3 ?g/ml) and rest of the wells without CD40L. Half of the culture medium was replaced from each well with fresh medium every 5 days. After infection cells were observed under microscope for growth and LCL formation time to time up to 4 weeks of post infection.

Reverse transcription-PCR (RT-PCR):

After 2 and 5 days of post infection, total RNA was isolated using TRIzol reagent and then treated with DNase I (Invitrogen), according to the manufacturer's instructions. cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (MMLV RT) [Invitrogen] and 100 pmol of random primer (Takara) at 37°C for 60 min, followed by heat inactivation at 94°C for 10 min. The cDNA samples were then subjected to PCR as follows. For the detection of EBER1, primers 5'-AGGAC CTA CGCTGCCCTAGA-3' (sense) and 5'-AAAACATGCGGACCACCAGC-3'(anti sense) were used) [17] and the PCR conditions PCR was 20 cycles consisting of denaturation for 30 s at 94°C, annealing for 30 s at 55°C and extension for 1 min at 72°C. For the detection of CD40L and CD40 primer 5?-TGC GGC ACA TGT CAT AAG-3? (sense) and 5?-CGG AAC TGT GGG TAT TT-3? (antisense) and 5?-TGC CAG CCA GGA CAG AAA CT-3? (sense) and 5?-GGG ACC ACA GAC AAC ATC AG-3? (antisense), were used [12]. The PCR condition was 30 cycles consisting of denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 1 min at 72°C. Five ?l of the PCR product was subjected to 2% agarose gel electrophoresis and was visualized by ethidium bromide staining. The quality of the isolated RNA was verified by PCR amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The PCR products were electrophoresed on an agarose gel and visualized by staining with ethidium bromide.

Results:

d.LMP1-EBV and AK-BAC-GFP showed similar infection efficiency: At first we tested whether the LMP1-deleted EBV could efficiently infect peripheral B cells. Culture supernatants were prepared from cell clone harboring d.LMP1-EBV [16] and used to infect peripheral B cells. The culture supernatant containing AK-BAC-GFP virus [15] was also used for another infection as a control. Infection was detected by observing GFP positive cells under the fluorescent microscope at 3 days of postinfection. Almost similar percentage of the recipient cells became GFP positive after the infection of the LMP1-deleted virus as compared with control (data not shown). We also examined the expression of EBER-1 by RT-PCR to determine the efficiency of infection (Fig. 1). The EBER-1 was expressed in similar fashion in both case of d.LMP1-EBV and control. When the expression of CD40L and CD40 gene in infected B cells was determined, high expression were observed in both d.LMP1-EBV and AK-BAC-GFP virus infected B cells (Fig. 2A and B). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also performed as a control (Fig. 2C).

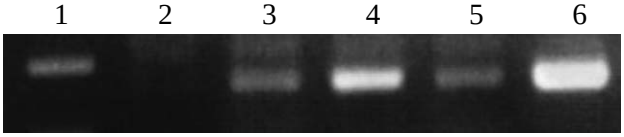

Fig. I. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of EBER-1 derived from d.LMP1-EBV and AK-BAC-GFP. Lane 1=DNA marker; Lane 2= B cell only, lane 3 and 4= 2 and 5 days of post infection with d.LMP1-EBV; Lane 4 and 5= 2 and 5 days of post infection with AK-BAC-GFP.

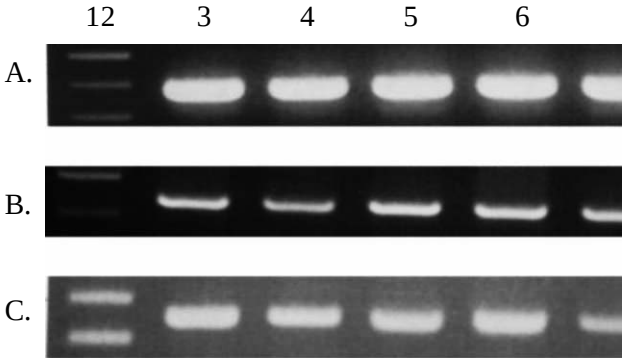

Fig. II. Expression of (A) CD40L and (B) CD40 gene in B cells infected with d.LMP1-EBV and AK-BAC-GFP. Lane 1=DNA marker; Lane 2 and 3= 2 and 5 days of post infection with d.LMP1-EBV; Lane 4 and 5= 2 and 5 days of post infection with AK-BAC-GFP; Lane 6= positive control, for CD40L-T cell line; for CD40-B cell line. (C) Expression of glycer-aldehydes-3-phosphate dehydrogenase (GAPDH) is presented as a control of RT-PCR.

d.LMP1-EBV infected B cells with or without CD40L supplement failed to survive, proliferate and LCL formation: To investigate whether CD40L contributes to LCL development in absence of LMP1 expression, isolated B cells was infected with d.LMP1-EBV with simultaneous addition of CD40L. The B cell growth and LCL formation kept under observation time to time up to 4 weeks of postinfection. No proliferation of B cells and formation of LCLs were visible in both case of d.LMP1-EBV infected B cells added with or without CD40L. However, B cells were proliferated and well-developed LCLs were formed when infection was carried out with AK-BAC-GFP viruses (Fig. 3A, B), which has functional LMP1 gene. This result demonstrated that CD40 and CD40L interaction didn't complement the role LMP1 expression of *in vitro* B cells proliferation and transformation.

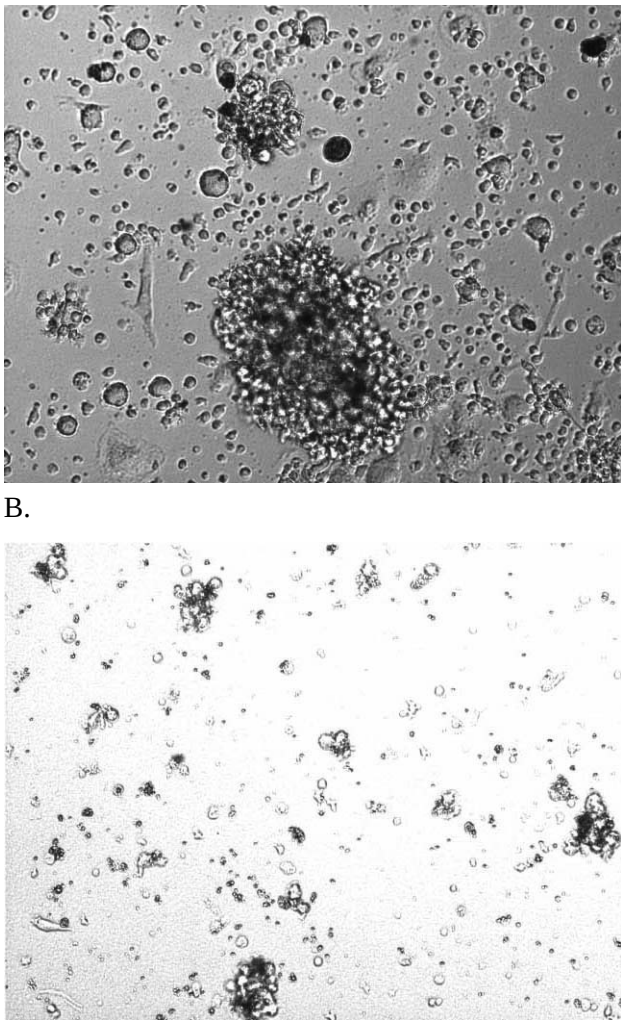

Fig. III. Lymphobalstoid cell lines (LCLs) formation by infection with (A) AK-BAC-GFP 3 weeks after infection. (B) No LCL formation in B cells by infection with d.LMP1-EBV treated with CD40L.

Discussion:

It is well known that LMP1 plays an important role in EBV infected B cell activation and transformation. This protein triggers an intracellular signaling cascade similar to that of CD40. LMP1 and CD40 interact with the same tumor necrosis receptor–associated factors (TRAFs) leading to activation of NF?B, c-Jun NH_2 terminal kinase (JNK), and p38 MAPK signaling pathways[1,18,19]. Activation of NF?B is required for EBV-induced B cell transformation [20]. LMP1 also activates phosphatidylinositol 3 kinase (PI3K)/Akt signaling which is required for the transformation of rodent fibroblasts [21]. LMP1 has been shown to stimulate IL-10 expression that constitutively activates Akt, NF?B, and Stat3 pathways, which are associated with the transformation of B

lymphomas in mice [22,23]. In our experiment we found that LMP1 expression is important for B cell transformation and LCL formation even in presence of CD40L.

We previously constructed a recombinant Akata strain EBV as AK-BAC-GFP that had a GFP gene in their genome[15] and d.LMP1-EBV by using AK-BAC-GFP as the starting material for mutagenesis [16]. This d.LMP1-EBV also expressed all viral genes except LMP1. We used these two recombinants in our study. After infection of peripheral B-lymphocytes with these recombinants, viral infection was easily observed under the microscope using UV as these recombinants contained GFP gene in their genome. We found that the infection ability of both of the recombinants was comparable. We also checked EBER-1 expression to compare the pattern of gene expression with the control infection. We found that the gene was expressed as like as control. Taken together, these data demonstrate the efficient infection of B cells with d.LMP1-EBV. We also checked the ectopic expression of CD40L after infection. We found that upon EBV infection the CD40L was expressed significantly that could stimulate CD40-mediated growth signals.

Though infection ability of these two recombinants was seemed to be similar and expression of CD40L was also evident, no LCLs formation was observed in case of B cells infected with d.LMP1-EBV even after CD40L expression or cells cultured in presence of CD40L. The presence and expression of LMP1 in B cells may activate specific signal pathways that apparently induce malignant growth of B cells. There are reports that LMP1 cannot fully compensate for a deficiency of CD40 in mice, and LMP1 and CD40 do not interact with exactly the same sets of signaling molecules[24,25]. This argued that their signaling pathways might differ in some respects. Thus, we propose that Akata stain EBV-induced CD40L/CD40 signaling together with LMP1 might be required in B cell survival and transformation.

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