Original Article
Aberrant expression of epstein-barr virus genes in Juvenile systemic lupus erythematosus

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Abstract: Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder which causes more severe clinical manifestations in juveniles than in adults. Many studies have shown the contribution of Epstein-Barr Virus (EBV) infection to the development of SLE. In this study, we investigated the role of EBV genes in the pathogenesis of SLE in juvenile patients. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from 20 patients with SLE and 12 healthy donors. Supernatant of EBV secreting cells was obtained from JSLE patients who were diagnosed as EBV-IgG/IgM positive by ELISA. PBMCs and the cell supernatant were co-cultured, followed by extracting RNAs from the PBMCs before and after the culturing, and RT-PCR was performed to determine the expression of EBV genes. Among all the EBV latent and lytic genes, LMP1 is the only gene showed a significant higher expression rate and expression level for the juvenile SLE patients. Co-culturing the PBMCs with EBV secreting cell supernatant reactivated the infected EBV by initiating the expression of EBNA1 and BLLF1 gene. These data indicated the aberrant expression of EBV genes in JSLE patients may be involved in the pathogenesis of JSLE, and that the EBNA1 and BLLF1 genes may be the initiation of EBV entering the lytic phase.

Keywords: Lupus erythematosus, systemic, epstein-barr virus infections, gene expression

Introduction
Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder which involves the production of autoantibodies targeting double-stranded DNAs and abnormalities of lymphocytes, leading to organ damages from immune complex deposition [1]. SLE usually causes more severe clinical manifestations in juveniles than in adults, and is one of the childhood diseases with poor prognosis [2]. The relationship between Epstein-Barr virus (EBV) and SLE has been studied for many years, and increasing evidence supports that the EBV infection contributes to the etiology of SLE [3]. Human infection with gamma-herpesvirus EBV usually occurs during childhood, and progresses to latency, whereas rare adulthood infections could lead to autoimmune diseases, such as SLE [4]. It has been reported that SLE patients have an increased EBV viral load, as well as high frequencies of infected blood cells, including B cells [5]. Such autoimmune diseases also lead to B cell activation, which then activates the EBV lytic cycle. In response, T cells are activated to attenuate the replication of EBV, but may also target self-antigens, leading to the amplification of auto-immune symptoms [6].

Herein, we demonstrated the role of EBV in the pathogenesis of JSLE by examining the expression of EBV genes in the PBMCs from JSLE patients and the PBMCs co-cultured with EBV-secreting cell supernatant.

Materials and methods

Subjects
20 patients with JSLE (average age of 8.2±2.9) including eight males and twelve females were recruited from inpatient ward at the Maternal
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and Child Health Hospital of Hainan Province and the Department of Nephropathy, Children’s Medical Center of the Second Xiangya Hospital. All patients met the revised American College of Rheumatology classification criteria for SLE. 12 healthy controls (average age of 8.7±2.4) including five males and seven females were recruited from medical staff at the Maternal and Child Health Hospital of Hainan Province. Patients and controls were age- and sex-matched in all experiments. This study was approved by the Human Ethics Committee of the Maternal and Child Health Hospital of Hainan Province, and written informed consent was obtained from all subjects.

Cells and cell culture

A total of 10 mL of venous peripheral blood was collected from each patient and healthy control, and stored with heparin added. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (Shanghai Hengxin Chemical Reagent Co., Ltd, Shanghai, China). Total RNAs and the genomic DNAs were extracted from PBMCs using TRIZOL ® reagent (Invitrogen, USA), as per the manual operation. Supernatant of EBV secreting cells was obtained from JSLE patients who were detected as EBV-IgG/IgM positive by ELISA, and then co-cultured with the isolated PBMCs for 12 days.

Real-time quantitative RT-PCR

cDNA synthesis

cDNAs were synthesized by the Reverse Transcription kit (Pmmega company, USA). 1μg of total RNAs was added to the amount of ddH₂O without the reverse transcriptase, and incubated for 70°C, followed by a rapid cooling step on ice for 10 min. dNTPs and the reverse transcriptase were added to the RNAs, and the mixture was placed into a thermocycler, incubating at 42°C for 45min, 99°C for 5 min, and stored at -20°C.

Real-Time PCR

The expression of each cDNA gene was detected in the iCycler IQ real-time PCR instrument (Bio-rad company, USA). The tested genes include EBV nuclear antigen (EBNA1, EBNA2), latent membrane protein (LMP1, LMP2) and lytic genes (BZLF1, BcRF1, BLLF1). Primer sequences are listed as below:

Upstream primer 5'-TGCCCCCTCGTCAGACATGATT-3' and reverse primer 5'-AGCGTGCGCTACCGAT-3' for EBNA1. Upstream primer 5'-TTTGCTGACTACTGATGATCACC-3' and reverse primer 5'-AGTAGATCCAGATACCTAGACAGT-3' for LMP1. Upstream primer 5'-ATGACTCATCTCAACACATA-3' and reverse primer 5'-CATGTAGGCAATTGCACA-3' for LMP2. Upstream primer 5'-CGCCTGGGCATTGCAT-3' and reverse primer 5'-AAAGATCGGGTGCTTCCAGAA-3' for BZLF. Upstream primer 5'-AGGGCC-TGTTTTGATTCTTCAAGAG-3' and reverse primer 5'-TTGTCACGTGGGCGAAC-3' for BcRF. Upstream primer 5'-CATTGAGGCCGTCTCGTGATAAT-3' and reverse primer 5'-GCCAGCGATTCGGACACATTTG-3' for BLLF1.

1.5 mmol/l of MgCl₂, 0.1 mmol/l of dNTP, 0.5 μmol/l of each of the upstream and downstream primers, 1.0 U of Taq polymerase, and 3 μl of the cDNA template were mixed in an Eppendorf tube. ddH₂O was added to make the total volume of 30 μl. The PCR was running at 95°C for 3 min, then 35 cycles of 95°C for 10sec, 58°C for 30sec, and extension at 72°C for 10 min. The melting curve was analyzed with single peaks observed for each pair of primers, and no amplification was seen for the cDNAs extracted from the EBV-negative cells. The ΔΔCt method was performed to validate these primers by comparing the results with serial dilutions.

Statistical analysis

Counting data was analyzed using chi-square test or Fisher’s exact test; measurement data was analyzed by multiple group comparison, pairwise comparisons, and t test between two groups. P values of less than 0.05 were considered as a significant difference between groups. All analyses were performed with SPSS version 13.0 software (SPSS Inc., Chicago, IL, U.S.A.).

Results

The PBMCs from JSLE patients show aberrant latent and lytic gene expression

The expression of EBV genes in the PBMCs from JSLE patients was analyzed by RT-PCR.
The expression of LMP1 was detected in 10 out of 20 patients, and in 1 healthy control (P=0.023). The expression of LMP2 was detected in 4 patients and 1 healthy control, EBNA1 in 13 patients and 3 controls, BCRF1 in 3 patients and 1 control, and BLLF1 in 5 patients and 1 control (Table 1). However, the expression of these genes in patients and healthy controls did not show significant differences. LMP1 is the only EBV gene that showed significant higher expression level in JSLE than in healthy controls. These results suggest that LMP1 may play a role in the pathogenesis of JSLE (Figure 1).

The reactivation of infected EBV involves the activation of various genes, hence, we determined the EBV genes which initially responded to the circulating EBV. RT-PCR was performed for the PBMCs after 5-day co-culturing with the EBV secreting cell supernatant. The expression of EBNA1 and BLLF1 genes increased by 35% and 26% respectively (Figure 2), whereas the expression of other genes did not show a significant increase after the 5-day co-culturing. These results indicated that EBNA and BLLF genes were the first two genes responded to the stimulation of the circulating EBV or EBV proteins. Also, these two genes might be the indicators of EBV entering the lytic phase in juveniles.

Discussion

EBV can infect and reside in human B cells. The infection is maintained persistent by regulating the gene expression profile of the EBV. As aberrant expression of lytic and latent EBV proteins have been found in the blood of the patients with the autoimmune disease SLE [4], we aimed to examine the expression of specific EBV proteins in the patients with JSLE, and to investi-
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patients who were detected as EBV-IgG/IgM-positive by ELISA. After 12 days co-culturing the PBMCs with the cell supernatant, the expression of latent EBV genes and lytic genes increased in both patient and control groups. The latent EBV genes including LMP1, LMP2, EBNA-1 and the lytic genes including BCRF1, BLLF1 all exhibited significantly greater expression levels in JSLE patients than that in the controls (P < 0.05), suggesting that these genes may be involved in the pathogenesis of juvenile SLE.

We examined EBV gene expressions in PBMCs from juvenile SLE patients and healthy controls by RT-PCR. We found that latent EBV genes, including LMP1, LMP2, EBNA-1, and lytic EBV genes, including BCRF1, BLLF1, were expressed in both groups. However, compared with healthy controls, a significantly larger number of JSLE patients showed the expression of the lytic and latent EBV genes (P < 0.05). Even before co-culturing with the EBV-secreting cell supernatant, PBMCs from JSLE patients also showed a significantly higher expression of LMP1 gene. Our results were consistent with the findings in the study of adult SLE conducted by Gross AJ [6], suggesting that LMP1 may play a causative role in the pathogenesis of juvenile SLE.

To further verify the results, we collected EBV-secreting cell supernatant from the JSLE mimic the CD40 receptor on the surface of the infected B cells [7]. This mimetic CD40 receptor can interact with TNF receptor-associated factor (TRAF), activating Akt, NFκB and Stat3 pathways, which are essential for B cell survival and proliferation [8]. In the B6.Sle1 mouse model, LMP1 has been found to increase the auto-reactivity of SLE by mediating the activation of B cells, and enhance the expression of costimulatory molecules of B cells [9]. In this study, LMP1 was detected in 10 cases of juvenile SLE, and the expression of LMP1 gene was significantly increased after co-culturing the PBMCs with the EBV-secreting cell supernatant, suggesting the essential role of LMP1 in the maintenance of B cell survival and the pathogenesis of juvenile SLE.

LMP2 contains two isoforms LMP2A and LMP2B, which regulate the transformation of latent EBV into the lytic stage [10]. LMP2A was found to block B-cell receptor signaling trans-

Table 2. The expression of EBV genes in PBMCs from JSLE patients and controls after co-culturing (X ± S)

<table>
<thead>
<tr>
<th>Group</th>
<th>LMP1</th>
<th>LMP2</th>
<th>EBNA1</th>
<th>BZLF1</th>
<th>BCRF1</th>
<th>BLLF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient (n=20)</td>
<td>1.01±0.141</td>
<td>1.00±0.093</td>
<td>1.01±0.163</td>
<td>0.89±0.191</td>
<td>1.01±0.167</td>
<td>1.03±0.264</td>
</tr>
<tr>
<td>Controls (n=12)</td>
<td>0.38±0.171</td>
<td>0.20±0.082</td>
<td>0.18±0.129</td>
<td>0.71±0.278</td>
<td>0.34±0.161</td>
<td>0.11±0.081</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.069</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Figure 2. The expression levels of EBV genes after 5-day co-culturing normalized to that before the co-culturing. The expression of EBNA1 and BLLF1 were 135% and 126% of those before the 5-day co-culturing, respectively (*P < 0.05). Normalised to the expression levels before the co-culturing, LMP1, LMP2 and BCRF1 did not show significant increase in their expression levels (**P > 0.05).
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production, and to limit lytic EBV infection, whereas LMP2B can induce the activation of lytic EBV in the infected B cells, showing an opposite effect to LMP2A [8]. The balance between LMP2A and 2B might contribute to the detection of some of the EBV proteins but symptomless for the healthy controls.

EBNA-1 is encoded by EBNA-1 gene, and is the only virus-encoded protein necessary for viral replication. EBNA-1 can be recognized by virus-specific CTL, resulting in the immune response. EBNA-1 contains a region at its N-terminal homologous to the self-protein SmD, and therefore, can trigger the production of autoantibodies [11]. In this study, co-culturing the PBMCs with EBV-secreting cell supernatant could reactivate the EBV to enhance the EBNA-1 expression.

The lytic gene BCRF1 encodes vIL-10, which is 83% homologous to the human IL-10 [12], but lost the function to stimulate human immune responses [13]. Another EBV lytic gene BLLF1 encodes the membrane antigen gp350/220, which binds to CD21 expressed by B lymphocytes [14], allowing the EBV infection. In this study, the expression of EBNA1 and BLLF1 were significantly increased in the juvenile SLE group after co-culturing with the EBV-secreting cell supernatant. These results suggested the virus or the viral proteins in the cell supernatant might reactivate the EBV to enter the lytic phase through the gp350/220 mediated entrance of the virus into the B lymphocytes. Also, the higher rate of latent EBV gene expression than the lytic gene expression in the JSLE patients indicated the JSLE group may include more latent EBV infection.

BZLF1 is the immediate early gene of EBV, and is the earliest expression gene when EBVs enter the lytic state from the latent state [15]. We found that the expression of BZLF1 in PBMCs from juvenile SLE and the PBMCs co-cultured with EBV-secreting cell supernatant were both negative, suggesting that the reactivation of the EBV proliferation may be through other pathways.

In conclusion, the significant higher expression of EBV genes indicated their roles in the pathogenesis of JSLE. The resulted increased EBV gene expression by the secreted EBVs or EBV proteins suggested EBV might initially activate the expression of EBNA1 and BLLF1 genes to allow the infected EBV enter the lytic phase.

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Disclosure of conflict of interest

None.

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