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Full Length Research Paper

The possible role of Epstein–Barr virus latent membrane protein 1 in inducing or promoting systemic lupus erythematosus

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To investigate the role of latent membrane protein 1 (LMP-1) in the Epstein-Barr virus (EBV) induced systemic lupus erythematosus (SLE) and its possible mechanism, fluorescence quantitative polymerase chain reaction (PCR) was employed to detect the mRNA expressions of LMP1, BcL-2 and Bax in the peripheral blood mononuclear cells (PBMCs) of SLE patients and healthy controls, and ELISA was performed to measure the serum B-cell activating factor (BAFF). Results showed: 1) The positive rate of LMP1 expression in 67 cases of SLE patient was 25.4%, significantly higher than the 10.8% in 65 cases of healthy control (P<0.05). The $2^{-\Delta Ct}$ value of LMP1 mRNA expression level of SLE patients was 0.0000008349, 1.59 times to that (0.0000005241) of healthy controls, with statistical significance. 2) The expression of BcL-2 in SLE patients was 1.41 times higher than that in healthy controls with statistical significance. However, there was no significant difference in the Bax expression between two groups; 3) Among these SLE patients, the BcL-2 expression in LMP1 positive patients was 1.98 times higher than that in LMP1 negative patients with statistical significance. While there was no marked difference in the Bax expression between LMP1 positive and LMP1 negative SLE patients. Moreover, the expressions of BcL-2 and Bax were similar between LMP1 positive and LMP1 negative healthy controls; 4) The serum BAFF level in LMP1 positive SLE patients, LMP1 negative SLE patients and healthy controls was 105.50±14.67, 82.42±18.64 and 65.19±17.68 µg/L respectively, showing significant difference between any two groups (P<0.05). EBV can affect the Bcl-2 expression through LMP1 and induce the production of BAFF resulting in prolonged survival of autoreactive B lymphycytes and subsequent occurrence and development of SLE.

Key words: Systemic lupus erythematosus, Epstein-Barr virus, latent membrane protein 1, Bcl-2, B-cell activating factor.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease but its pathogenesis is still poorly understood. Some researchers postulate that the imbalance between cell survival and apoptosis involves in the pathogenesis of SLE, and the compromised apoptosis of auto-reactive immune competent cells has been regarded as a main cause of SLE (Mevorach, 2003). Numerous factors have been found to affect the apoptosis. Increasing evidence has demonstrated Epstein-Barr virus (EBV) infection plays an important role in the pathogenesis of SLE (Harley et al., 2006; Poole et al., 2008). However, whether EBV can affect the apoptosis of immune competent cells is still unclear. Studies have shown that latent membrane protein 1 (LMP-1) is a predominant factor inducing the transformation of B lymphocytes (Middeldorp and Pegtel, 2008). It can promote the proliferation of B lymphocytes and enhance the anti-apoptosis of B lymphocytes. Thus, EBV might affect the apoptosis of B lymphocytes through LMP-1 which plays a critical role in the pathogenesis of SLE. Therefore, in the present study, the mRNA

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Table 1. Primers for target genes and internal reference gene.

Name	Sequence	Base pair
LMP1 F	5-CCCTTTGTATACTCCTACTGATGATCAC-3	28
LMP1 R	5-ACCCGAAGATGAACAGCACAAT-3	22
Bcl-2 F	5-TTGTGGCCTTCTTTGAGTTCGGTG-3	24
Bcl-2 R	5-GGTGCCGGTTCAGGTACTCAGTCA-3	24
Bax F	5-CCTGTGCACCAAGGTGCCGGAACT-3	24
Bax R	5-CCACCCTGGTCTTGGATCCAGCCC-3	24
GAPDH F	5-TCATGGGTGTGAACCATGAGAA-3	22
GAPDH R	5-GGCATGGACTGTGGTCATGAG-3	21

expressions of LMP1 and apoptosis related genes (BcL-2 and Bax) in the peripheral blood mononuclear cells (PBMCs) were detected, and the serum level of B cell activating factor belonging to TNF family measured by ELISA. Our study aimed to explore the role of EBV LMP-1 in the pathogenesis of SLE.

MATERIALS AND METHODS

Subjects

A total of 67 SLE patients were recruited from the Department of Rheumatology of the Affiliated Hospital of Qingdao University Medical College from May 2010 to October 2010. There were 6 males and 61 females with a mean age of 34±13 years (range: 18~52 years). The mean course of disease was 4.4 years (range: 0.1~15 years). In addition, 65 healthy volunteers were also enrolled as controls. There were 6 males and 59 females with a mean age of 34±8 years (range: 26~44 years). There were no marked differences in the gender and age between both groups (P>0.05).

RNA extraction and cDNA synthesis

In brief, 3 ml of peripheral blood were collected from SLE patients and healthy controls, and PBMCs were isolated by using lymphocyte separation medium. Total RNA was extracted from the PBMCs with Trizol Regent according to the manufacturer's instructions. The extracted RNA was subjected to 1.5% agarose gel electrophoresis to determine the integrity of RNA. Ultraviolet spectrophotometry was carried out to measure the purity of extracted RNA followed by RNA quantification. Then, 1 μg of RNA was reverse transcribed into cDNA according to the manufacturer's instructions (TaKaRa Biotechnology Co., Ltd), which was used as template for PCR.

Synthesis of primers for PCR

The gene sequences of LMP1, BcL-2 and Bax were obtained from the Pubmed/Nucleotide GenBank. The GAPDH served as an internal reference. Primer 5.0 was employed to design the primers according to the requirements of TaKaRa Biotechnology, and primers were synthesized in Shanghai Sangong. The primers for LMP1, BcL-2, Bax and GAPDH are shown in Table 1.

Real time fluorescence quantitative PCR

The mixture (20 μl) for PCR included: 10 μl of Premix Ex Taq (2 μM), 0.8 μl of each primer (10 μM), 2 μl of template, and 6.4 μl of DEPC treated water. The conditions for PCR were at 95℃ f or 10 s followed by 40 cycles of 95℃ for 7 s and 60℃ for 45 s. In the negative controls, the cDNA template was replaced with ddH_2O. ΔCt (mean Cttarget gene-mean CtGAPDH) and the $2^{-\Delta Ct}$ were employed to calculate the relative expression of target genes. The higher the value is, the larger the number of copies is.

Detection of serum BAFF level

The serum BAFF of SLE patients and healthy controls was determined by using ELISA according to manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0. The comparisons of positive rate were done with chi square test. The relative expression of target genes was expressed as $2^{-\Delta Ct}$ Once the ratio of $2^{-\Delta Ct}$ in SLE patients to that in healthy controls was >1, statistical significance was defined. Analysis of variance was used to compare the level of serum BAFF, and comparisons of means between two groups were done with Student-Newman-Kqeuls. A value of P<0.05 was considered statistically significant.

RESULTS

Proportion of LMP-1 positive subjects in SLE patients and healthy controls

There were 17 patients and 7 controls positive for LMP1 in SLE patients (25.37%) and healthy controls (10.77%), respectively. There was marked difference in the positive rate between two groups (χ^2=4.730, P=0.03) (Table 2).

LMP1 mRNA expression level in both groups

The $2^{-\Delta Ct}$ value of LMP1 mRNA expression level of SLE patients was 0.0000008349, 1.59 times to that (0.0000005241) of healthy controls, with statistical significance.

mRNA expressions of Bcl-2 and Bax in both groups

The expression of Bcl-2 in SLE group was 0.0257, which was 1.41 times higher than that in healthy controls

Table 2. Proportion of LMP-1 positive subjects in different groups.

	n	LMP1 expression		Positive rate (%)
		Positive	Negative	
SLE patients	67	17	50	25.37*
Healthy controls	65	7	58	10.77
Total	132	24	108	18.18

*P< 0.05 vs healthy controls.

Table 3. mRNA expressions of Bcl-2 and Bax in two groups.

		SLE group	Control group
BcL-2 mRNA	$^{\Delta Ct}$	5.28	5.77
	$2^{-\Delta Ct}$	0.0257*	0.0183
Bax mRNA	$^{\Delta Ct}$	6.68	6.54
	$2^{-\Delta Ct}$	0.0098	0.0107

*P <0.05 vs control group.

(0.00183) (P<0.05). In the SLE group, the Bax expression was 0.0098, which was similar to that in healthy controls (0.0107) Table 3.

mRNA expressions of Bcl-2 and Bax in LMP-1 positive and negative subjects in two groups

In the SLE group, the Bcl-2 expression was 0.0427 in LMP-1 positive patients, which is 1.98 times higher than that in the LMP-1 negative subjects (0.0217) showing significant difference. The Bax expression in LMP-1 positive patients was 0.64 times higher than in LMP-1 negative patients in SLE patients. In healthy controls, there were no marked differences in the expressions of Bcl-2 and Bax between LMP-1 positive controls and LMP-1 negative controls (Table 4).

Serum BAFF level in LMP-1 positive patients, LMP-1 negative patients and healthy controls

The BAFF level was 105.50±14.67, 82.42±18.64 and 65.19±17.68 μg/L in LMP-1 positive patients, LMP-1 negative patients and healthy controls (F=38.775, P=0.0000). In addition, significant difference was also noted in BAFF level between any two groups (q=6.5634, 11.8144 and 7.3131; P=0.0001, 00000 and 0.0001) Table 5.

DISCUSSION

EBV is addicted to infect B lymphocytes and susceptible to lurk in the B lymphocytes once these cells are infected. When the conditions are appropriate, replication of EBV may occur resulting in life-time stimulation by EBV antigen. Therefore, the EBV induced SLE is increasingly emphasized in clinical researches. Some studies have demonstrated that the EBV load in PBMCs of SLE patients is 10~100 times higher than that in healthy controls (James et al., 2001; Yu et al., 2005). This may be attributed to the abnormal regulation of EBV infection in subjects susceptible to SLE, which results in abnormal expression of EBV related genes (latent genes and proliferative genes) leading to the occurrence of SLE. Among these genes, LMP-1 gene plays an important role in the pathogenesis of SLE. LMP-1 can mimic the functions of CD40 and provide signals for growth and differentiation to B lymphocytes (Kilger et al., 1998). However, whether LMP-1 can affect the apoptosis of B lymphocytes through regulating apoptosis related genes and production of BAFF is still unclear in SLE.

In the present study, our results showed 25.4% of SLE patients were positive for LMP-1, which was significantly higher than that in healthy controls (10.8%).This finding was similar to previously reported (Poole et al., 2009). EBV LMP1 mRNA expression level in SLE patients was significantly higher than the control group, suggesting that EBV LMP1 may be involved in the pathogenesis of SLE. Thus, EBV may involve in the pathogenesis of SLE via LMP-1. In addition, apoptosis related proteins (Bcl-2 and Bax) were also detected in SLE patients and healthy subjects. Results showed the Bcl-2 expression in SLE patients was markedly higher than that in healthy controls, which has been noted in previous study (Liphaus et al., 2007). Moreover, the BcL-2 expression in LMP1 positive patients was significantly higher than that in LMP-1 negative patients (P<0.05). However, there was no marked difference in the Bax expression between patients and healthy control, and between LMP-1 positive patients and LMP-1 negative patients. These findings suggest LMP-1 may lead to over-expression of anti-apoptotic gene (Bcl-2), but has no effect on the expression of pro-apoptotic gene (Bax). Through inducing Bcl-2 expression, LMP-1 can interfere with the apoptosis of B lymphocytes resulting in occurrence of SLE. However, on healthy controls, no significant differences were noted in the expressions of Bcl-2 and Bax between LMP-1 positive controls and LMP-1 negative controls. This finding suggests LMP-1 has no influence on the expressions of Bcl-2 and Bax in healthy controls. Therefore, there is discrepancy in the regulation of Bcl-2 expression between healthy subjects and SLE patients, and other mechanisms may also involve in the regulation of Bcl-2 expression. In the SLE patients, the over-expression of LMP-1 leads to high expression of Bcl-2, which then affects the apoptosis of the affected B lymphocytes.

PBMCs rather than B cells are studied in this experiment, which is not accurate in principle. However, EBV was characteristics of addiction to B lymphocytes, and the presence of other mononuclear cells did not significantly affect the test results, so it still is a credibility

Table 4. mRNA expressions of Bcl-2 and Bax in in LMP-1 positive and negative subjects in two groups.

		SLE group		Control group	
		LMP1 positive	**LMP1 negative**	**LMP1 positive**	**LMP1 negative**
BcL-2 mRNA	$^{\Delta Ct}$	4.55	7.15	7.05	6.08
	$2^{-\Delta Ct}$	0.0427*	0.0217	0.0076	0.0148
Bax mRNA	$^{\Delta Ct}$	5.53	6.52	7.05	6.66
	$2^{-\Delta Ct}$	0.0070	0.0109	0.0076	0.0099

*P<0.05 vs LMP1 negative patients.

Table 5. Serum BAFF level in LMP-1 positive patients, LMP-1 negative patients and healthy controls (μg/L).

Group		**n**	**BAFF ($\bar{x} \pm s$)**
SLE group	LMP1 positive	17	105.50±14.67*
	LMP1 negative	50	82.42±18.64*
Control group		65	65.19±17.68

P<0.01 among three groups; P<0.01 between any two groups.

test result.

BAFF is a cytokine of TNF family. BAFF can activate B lymphocytes and promote the proliferation and prolong the survival of B lymphocytes. In the present study, our results showed significant difference in the serum BAFF level among LMP-1 positive patients, LMP-1 negative patients and healthy controls. Moreover, the BAFF level in LMP-1 positive SLE patients was higher than that in the other groups. Previous studies also reported SLE patients had significantly increased BAFF level when compared with healthy controls, and the increased BAFF level was closely related to the elevated ds-DNA antibody titer in the serum (Zhang et al., 2001; Thorn et al., 2010). Animal experiments showed BAFF may facilitate the escape of auto-reactive B lymphocytes from negative selection during the maturation and development leading to the survival of B lymphocytes (Batten et al., 2000). These findings suggest BAFF can prolong the survival of auto-reactive B lymphocytes which then produce a large amount of auto-antibodies resulting in occurrence of SLE. However, the increase of serum BAFF may be related to the LMP-1 expression. Previous study also revealed the LMP-1 of EBV can activate the BAFF expression through NF-κB pathway in immortalized B lymphocytes, resulting in over-expression of BAFF in B lymphocytes (He et al., 2003).

In SLE susceptible subjects, EBV latent gene LMP-1 can mimic the function of CD40 to provide the signals for growth and differentiation to the B lymphocytes. In addition, LMP-1 can also increase the Bcl-2 expression resulting in disordered apoptosis of B lymphocytes, and increase the BAFF production in B lymphocytes (He et al., 2003). Therefore, the survival of auto-reactive B lymphocytes was prolonged. These changes may finally lead to the occurrence of SLE. Therefore, we speculate that the disordered apoptosis of auto-reactive B lymphocytes induced by EBV LMP-1 expression is one of mechanisms underlying the pathogenesis of SLE.

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