Original Article

Epstein-Barr virus (EBV) encoded latent membrane protein 1 (LMP-1) triggered inflammatory response through NFκB p65 signaling pathway in tonsils

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Abstract: Tonsils, which exposed continually to antigens, are the important site for recurrent inflammation in the pediatric population. The purpose of this study was to investigate the presence of Epstein-Barr virus (EBV) in children’s tonsils, and to reveal the potential infection mechanism correlation with latent membrane protein 1 (LMP-1) encoded by EBV in human tonsil epithelial cell (HTEpiC). Polymerase chain reaction (PCR) was used to examine the presence of EBV in tonsil tissues which from children aged between 2 and 14 years diagnosed with recurrent tonsillitis. Then the LMP-1 encoded by EBV was overexpressed in HTEpiCs. The mRNA and protein levels of LMP-1 were detected after transfecting for 48 h. Western blot and ELISA were adopted to detect the expression of NF-κB p65, TNF alpha, IL-1 beta and IL-8 in LMP-1 overexpressed HTEpiCs. Of all the 30 patients, PCR products amplified from EBV DNA were detected in the tonsils from the 17 patients (56.67%). And there were no direct relationship between EBV infection and age (P>0.05), gender (P>0.05) or nationality (P>0.05). Overexpression of LMP-1 gene enhanced the expression of inflammatory factors such as TNF alpha, IL-1 beta and IL-8 in HTEpiCs. The level of NF-κB p65 also showed a statistically significant increase in this model. These results suggested that EBV was associated with recurrent tonsils of children. The LMP-1 gene which encoded by EBV could trigger inflammatory response through NF-κB p65 signaling pathway.

Keywords: Epstein-Barr virus (EBV), latent membrane protein 1 (LMP1), tonsillitis, human tonsil epithelial cell (HTEpiCs), NF-κB p65

Introduction

Palatine tonsil is the first line of the mucosa associated lymphoid tissue system (MALT), which belong to Waldeyer’s lymphatic ring. Palatine tonsil, torus tubarius tonsils, pharyngeal tonsil and lingual tonsil compose of the Waldeyer’s lymphatic ring, with the function of immune acquisition and immune defense. In some situations, the immunological reaction can detrimentally affect many children, particularly there is hypertrophy with breathing disorders and/or recurrent infections [1]. Stradling et al reported that, when compared with the health children, the children who had recurrent tonsillitis showed abnormal sleep hypoxemia, excessive sleep disturbance, hyperactivity, aggression and learning difficulties [2]. In recent years, it is reported that several pathogens and microorganisms may infect these lymphatic tissues in the case of recurrent tonsillitis. But the responsible germs are still unclear and have changed, as the better detection methods are increasing. Several hypotheses have been proposed to elucidate the recurrence of tonsillitis in children, including many different bacterial pathogens such as Haemophilus influenzae, Neisseria species, and Streptococcus pneumonia which almost exclusively present in children and Obligate anaerobes like Porphyromonas, Prevotella, and Fusobacterium were massively detected in chil-
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dren [3, 4]. With the widespread use of various antibiotics, the rate of bacterial infection is decreasing, and the viral infection rate is relatively prominent which is easy to result in secondary bacterial infection with subsequently seriously clinical manifestations.

It has been suggested that the infection of virus was correlated with recurrent tonsillitis [5]. Epstein-Barr virus (EBV) is one of the virus involved in recurrent tonsillitis, which is the first identified DNA virus that is associated with human malignancies, such as Burkitt’s lymphoma, Hodgkin’s disease, nasopharyngeal carcinoma (NPC), lung cancer, gastric carcinoma and breast cancer [6-11]. EBV is a herpes virus which was found in human B lymphocytes by Epstein and Barr in 1964. It exists in the form of a linear double-stranded DNA, and its genome is approximately 172 kilobase [12]. It is estimated that EB virus has infected more than the world’s population 90% [13]. EBV can cause both lymphocyte proliferation in vitro, and correlate with the human head, neck and lymphoid tissue diseases [14, 15]. As an important part of pharyngeal lymphoid ring, whether the pathogenesis of recurrent tonsillitis is associated with EBV virus worth studies.

The recent study show that B lymphocytes accounted for 90% of the total cells in tonsils. Earlier studies had suggested only the surface of B lymphocytes had virus receptor, but later researcher discover that some epithelial cells of the parotid gland, throat and cervix also have virus receptors. And the epithelial cells of the oropharynx are the first targets of infection by EBV, but the cells infected latently show no evidence of infection except when they display the gene product of EBV such as nuclear antigens (EBNA) and latent membrane protein (LMP) [16]. Latent membrane protein 1 (LMP-1) is the only one with oncogenic properties among EBV encoded proteins. Previous studies have been focused on the signaling transduction pathways mediated by LMP-1. Base on the LMP-1-inducible expression system, LMP-1 is involved in multiple biological functions, such as cell proliferation, apoptosis, invasion and metastasis, through initiating NF-κB, AP-1 and JAK/STAT signaling pathways, which affect their downstream genes [17].

The purpose of this study was to investigate the expression of the EBV in children’s recurrent tonsillitis, and then observed the EBV encoded LMP-1-induced inflammation response in human tonsil epithelial cell (HTEpiC) in vitro.

Materials and methods

Chemicals and antibodies

Dulbecco’s modified Eagle’s medium (DMEM)/nutrient mixture F12 (1:1, DMEM/F12) medium and Fetal Bovine Serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA); The monoclonal antibodies against LMP-1, NF-κB p65, GAPDH and secondary HRP-conjugated antibody were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human TNF alpha Elisa kit, human IL-1 beta Elisa kit and human IL-8 Elisa kit were all obtained from R&D Systems (Minneapolis, MN, USA). Other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) unless indicated otherwise.

Tissue samples

30 cases of tonsillectomy were randomly obtained from the surgery center at Guangzhou Women and Children’s Medical Center for the period February 2014 through January 2015. The clinical information such as age, gender and nationality were gained by reviewing all the histopathologic reports and request forms of all cases. All patients provided written informed consent for participation in the study.

Cell culture

HTEpiC cell, human tonsil epithelial cell, was obtained from the ScienCell Research Laboratories (San Diego, CA, USA). HTEpiC cells were cultured in DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). And cells were kept at 37°C in a humidified incubator supplied with 5% CO₂.

Plasmids and transfection

The full-length of LMP-1 was cloned and inserted into expression vector pcDNA3.1, the pcDNA3.1 empty vector was the negative control (NC). And the transfection of plasmid was conducted with lipofectamine 2000 (Invitrogen, U.S.A) according to the manufacturer’s instructions.
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DNA isolation and polymerase chain reaction (PCR)

The DNA used as a positive control was extracted from the EBV infected B9507 cell line; the negative control was done with sterile milli-Q water. The DNA was prepared from tonsil tissues using the Takara Universal Genomic DNA Extraction Kit (TaKaRa Bio, Shiga, Japan), according to manufacturer’s instructions. DNA detection was carried out by using PCR analysis. All reactions were run induplicate. The amplification was carried out by using TaKaRa Ex Taq kit (TaKaRa Bio, Shiga, Japan) in GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems), according to manufacturer’s instructions. PCR primers for the amplification were: forward, 5' - CCAGACAGCAGCCAATGTC-3'; and reverse, 5' - GGTAGAAGACCCCCTCTTTAC-3'. The cycling conditions for PCR were: 1 min denaturation at 94°C, 1 min annealing at 58°C and extension at 72°C; 40 cycles for repition of reaction. PCR products were analyzed with 2%-agarose gel, stained with ethidium bromide, and visualized using the Gel Doc 1000 UV Xruorescent gel documentation system (Bio-Rad Lab., CA, USA) with the accompanying Multi-Analyst software (version 1.0).

RNA isolation and quantitative real-time PCR (qRT-PCR)

The mRNA expression of LMP-1 was detected by qRT-PCR. Briefly, total RNA was extracted using an RNA Extraction Agent (TaKaRa Bio, Shiga, Japan) following manufacturer instructions. cDNA was synthesized using a TaKaRa Bio Reverse Transcription kit (TaKaRa Bio, Shiga, Japan). Expression of LMP-1 was examined using SYBR Green kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s instructions and GAPDH was served as internal reference. The primers for LMP-1 were: sense, 5'-TGCAAGGGGGAAGGACT-3'; anti-sense, 5' - CAGGCCCGGATCTTGTTCT-3'. The primers for GAPDH were: sense, 5'-ACACCACCTCTCCACCTTT-3'; anti-sense, 5'-TTACTCCTTGGAGGCCATGT-3'.

Western blotting analysis

The cellular proteins were extracted and analyzed for protein expression. Briefly, denatured protein samples were resolved on SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocking with non-fat milk, membrane was incubated overnight at 4°C with antibodies including LMP-1 and GAPDH followed by incubation with the antirabbit HRP-conjugated secondary antibodies. Chemiluminescence detection was performed using ECL advance Western blotting detection reagents (GE healthcare, Little Chalfont, Buckinghamshire, UK). The staining intensities of the protein bands were measured, quantified, and normalized against staining of GAPDH using quantity one software (Bio-Rad Laboratories, Hercules, CA, USA).

ELISA

With the collected cellular supernatant, TNF alpha, IL-1 beta and IL-8 concentrations were
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Detection of EBV DNA in tonsils

EBV DNA was detected in 30 tonsillar tissues of children, which was amplified by PCR and analyzed using agarose-gel. As shown in Figure 1 and Table 1, of the 30 patients, amplification of the PCR products from EBV DNA were detected in 17 patients (56.67%). Of 30 tissue samples, 21 tissues were obtained from Han nationality, 9 were from Hui nationality. Of 17 tissue samples that were positive for the EBV DNA, 12 (57.14%), 5 (55.56%) tissues samples were from Han and Hui, respectively. The samples originated from 9 female and 21 male subjects, with a median age of 7.6 years (range, 2-14 years). Moreover, the prevalence of EBV genome was 61.90%, 44.44% in tissues from male and female group, respectively. And 5/12 (41.67%) and 12/18 (66.67%) positive rate of EBV genome in tissues was from preschool group (range, 2-6 years) and school-age group (range, 7-14 years), respectively. The results showed no statistically significant difference between EBV infection, age (P>0.05), gender (P>0.05) and nationality (P>0.05).

Efficiency of overexpression of the EBV encoded LMP-1 gene in HTEpiCs

In cultured HTEpiC cells under normal control conditions, When compared with the NC group (the cells were transfected with pcDNA3.1 empty vector), the mRNA level of LMP-1 which was investigated by qRT-PCR was significantly increased when transfected with LMP-1 overexpression plasmid (Figure 2A). Consistently to qRT-PCR results, the protein expression of LMP-1 which was detected by western blotting analysis was significantly enhanced when transfected with LMP-1 overexpression vector (Figure 2A).

Overexpression of LMP-1 gene triggered inflammatory response through NFκB signaling pathway

To evaluated the potential mechanism of LMP-1 overexpression vector in the HTEpiC cells. The cells were divided into two groups, then transfected with LMP-1 overexpression vector or pcDNA3.1 empty vector for 48 h, respectively. We first collected the cellular supernatant, then determined the TNF alpha, IL-1 beta and IL-8 levels by ELISA analysis. As shown in Figure 3A, the expression of TNF alpha, IL-1 beta and IL-8 levels in HTEpiC cells which was transfected with LMP-1 overexpression vector statistically significantly enhanced, as compared with NC group. Then the cellular pellet was harvested,
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Discussion

Recurrent inflammation of tonsils is one of the most ancient and common pediatric problems. It is suggested that recurrent tonsillitis could result in local and systemic complications (such as chronic nephritis, rheumatic fever, rheumatic heart disease). In recent years, the high prevalence of recurrent tonsillitis in children evoked much attention of scientists all over the world [18]. Hence, the research of pathogenesis of children’s recurrent tonsillitis has been carried out. With the development of viral molecular biology, immunohistochemistry assay, gene sequencing analysis and other new technology, scientists have moved away from bacterial field toward viral field in recurrent tonsillitis. And with the DNA and RNA detecting being a reality, viral pathogenesis theory constantly being improved, the study of the pathogenesis of children with recurrent tonsillitis has opened up new avenues.

Endo et al used the in situ hybridization (ISH) to detect the tonsils which were obtained from 85 patients who underwent tonsils and adenoid (T&A) removal because of recurrent tonsillitis (RT) or T&A hypertrophy (TH). The prevalence of EBV infection was 29.4% (15 RT and 33 TH) [19]. Sohaila et al determined the prevalence of EBV in the tonsils of the United Arab Emirates (UAE) nationals by using ISH analysis, and found that EBV was prevalent in 43% of tonsillectomy specimens. Then, they supposed ISH analysis was the gold standard for the determination of EBV in tissue [20]. In 2014, Faith et al published a study in which a new Taq Man-based quantitative real-time PCR assay was used to verify the presence of viruses in the 56 recurrent tonsillitis tissue samples. Of the 56 tissue samples, EBV was the most frequently detected virus, being found in 53.6% (30/56) [21]. Dogan et al reported that EBV infection usually occurred in early childhood. These authors used real-time PCR and ELISA analysis to examine the presence of EBV in palatine tonsils and relationship between EBV-DNA quantity in tonsil tissues and VCA-IgG quantity in autologous sera. They found that the positive rate of EBV-DNA reaction was 75% [22]. Eliane et al used PCR methods to investigate the presence of EBV and immunohistochemistry to detect the expression of EBV encoded LMP-1 protein in the tissue samples which removed from children aged between 2 and 12 years diagnosed with recurrent tonsillitis. Of the 24 patients, 13 (54.1%) of 24 cases were EBV-positive while 9 (37.5%) were LMP-1-positive [23]. These results suggested that various methods such as in situ hybridization, PCR and immunohistochemistry have been used to study the prevalence of the EBV, and the infection by EBV and its reactivation could be an important mechanism involved in recurrent tonsillitis. In the present study, we also concerned whether
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the regions or the ethnic played an important role in recurrent tonsillitis associated with EBV infection. Our results showed that the EBV-positive cases accounted for 17 of 30 cases. However, there was no statistically significant difference between EBV infection including age, gender and nationality, probably because of the small sample size and the single detection method. Therefore, the sample size of the recurrent tonsillitis will be expanded, and a variety of reasonable detection methods will be used to investigate the association between the EBV infection and recurrent tonsillitis in the future study.

LMP-1 is an important protein in the incubation period of EBV infection. It is reported that the epithelial cells of the oropharynx were highly susceptible to EBV, and were the first targets of infection by EBV [24]. Since the epithelium of respiratory tract, urinary tract and gastrointestinal tract constitute the interface between the organism and the environment. Thus, the epithelium is the most likely part by which the EBV enters host cells and the organism releases the virus through body fluids. In this study, we chosen human tonsil epithelial cells which transfected with LMP-1 overexpression vector to investigate the inflammation response induced by EBV encoded LMP-1 protein and the potential signaling pathway. We found that The LMP-1 protein which encoded by EBV genome could enhance inflammatory factors such as TNF alpha, IL-1 beta and IL-8 through NF-κB p65 signaling pathway. The results established an epithelial culture system for a hypothesized target of EBV infection in tonsils in vivo. However, this observation and other infection mechanism of EBV are needed to elucidate in the future studies.

Conclusion

The sophistication of the metastasis process is probably the most challenging part of investigations to determine the most useful immunohistochemical agent for predicting cervical lymph node metastasis in patients with head and neck SCC. Different agents acting at different steps of the metastasis mechanism and interactions between them necessitate further research. In addition, methodological differences, patient selection criteria and differences in interpretation of expression values lead to the emergence of conflicting results. Although studies focused on this issue have not provided a clear diagnostic agent to date, multifunctional and multidisciplinary immunohistochemical studies conducted with standardized methodology and also with more patient participation may help to obtain more specific results.

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Disclosure of conflict of interest

None.

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