The role and mechanism of NF-κB in viral encephalitis of children

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Abstract. The aim of this study was to analyze the concentration changes of nuclear factor-κB (NF-κB) and the related inflammatory factors of pre-treatment and post-treatment in children with viral encephalitis, to examine the mechanism of NF-κB in the pathogenesis of child viral encephalitis. Twenty-two children with severe viral encephalitis, 13 children with mild viral encephalitis and 12 normal children, who were treated in our hospital, were randomly selected. Before and after treatment, the concentrations of inflammation-related factors in serum including interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) were detected by ELISA and comparative analysis were performed. The expression of NF-κB in peripheral blood and cerebrospinal fluid (CSF) before and after treatment was detected by RT-PCR and western blotting, while the difference of NF-κB expressions between viral encephalitis children and normal children was analyzed. The concentrations of inflammation-related factors in serum of children with viral encephalitis, including IL-1, IL-6 and TNF-α were significantly higher than those of normal children (P<0.01), and after treatment, the concentrations of IL-1, IL-6 and TNF-α were distinctly lower than those of pre-treatment (P<0.01). The concentrations of NF-κB in peripheral blood and CSF of children with viral encephalitis in the mild group and severe group were evidently increased compared to those of pre-treatment, while the degree of increase in the severe group was higher than that in mild group, which was higher than that in the control group (P<0.01). After treatment, the concentrations of serum NF-κB of children in the severe and mild groups were distinctly lower than those of pre-treatment (P<0.01), with statistically significant difference. In conclusion, the NF-κB level in serum and CSF of children with viral encephalitis was positively related to the severity of the disease. The higher the concentration of pre-treatment was, the more serious the disease would be. Our results indicate that NF-κB plays an important role in the occurrence and development of viral encephalitis in children.

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Introduction

Viral encephalitis of children is a common disease of the central nervous system (CNS) infection in children (1). In the course of the disease, the nerve cells are damaged by the direct effect of a variety of different types of viruses or the indirect effect of other multiple cytokines, thus leading to the release of cerebrospinal fluid (CSF) (2,3). In this pathological process, a large number of inflammation-related factors are produced, including nuclear factor-κB (NF-κB), interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) (4). Among them, NF-κB is an important nuclear factor, which has the role of regulating inflammatory response and inflammation-related tumor diseases (5). NF-κB can induce the occurrence of inflammatory reaction and further affect its development, such as the unlimited proliferation of inflammatory cells, cell immortalization, vascular tissue invasion and metastasis (6).

In the present study, the expression levels of NF-κB, IL-1, IL-6 and TNF-α in peripheral blood, CSF and serum of viral encephalitis children were detected in order to investigate the pathogenesis of viral encephalitis in children and further discuss the role of NF-κB in children with viral encephalitis, so as to provide new perspectives and methods for the detection, diagnosis and treatment of viral encephalitis in children.

Patients and methods

Patient data. Twenty-two children with severe viral encephalitis and 13 children with mild viral encephalitis, who were treated in our hospital, were selected. This study was approved by the Ethics Committee of Xuzhou Children's Hospital. Signed written informed consents were obtained from the guardians of all participants before the study. The diagnosis was confirmed in accordance with the viral encephalitis inspection criteria, while other types of CNS diseases were excluded. Twelve cases in the normal control group were children with non-infection and with no other neurological diseases, also in the same period. There was no significant difference in age or gender between the children with viral

encephalitis and the children in the normal control group. Before taking CSF, informed consent form was signed by the parents. Children with viral encephalitis received lumbar puncture within 12 h of admission. CSF was removed under sterile conditions, and subsequent examinations and related experiments were performed.

Main reagent. The enzyme-linked immunosorbent assay (ELISA) kit of IL-1, IL-6 and TNF-α (Boshide, Wuhan, China), TRIzol reagent and reverse transcription kit (both from Tiangen Biotech Co., Ltd., Beijing, China), BCA protein quantitative kit (Biyuntian Biotechnology Co., Ltd., Shanghai, China), as well as anti-actin (β-actin), and anti-NF-κB monoclonal antibodies (CST, Boston, MA, USA) were prepared.

Experimental methods

ELISA detection. According to the ELISA kit specification, samples were washed by pre-cooled phosphate-buffered saline (PBS), and then PBS was added in accordance with the proportion of 1:9 for homogenization. After the centrifugation at 1,200 x g for 10 min of homogenate, the supernatant was detected. The blank well, standard well and the sample well were set, respectively; 100 μl sample dilution was added in the blank well, while 100 μl standard or sample to be tested was added in other corresponding wells. No air bubbles were noted. The ELISA plate was covered with membrane, for incubation at 37˚C for 90 min. The solution was discarded. Drying was performed, with no need for washing; 100 μl biotin antibody working fluid was added to each well, and the ELISA plate was covered with a membrane, for incubation for 1 h. The fluid in the wells was discarded. Drying was performed, and the plate was washed three times, with soaking for 1-2 min each time. Then 100 μl enzyme blinding working fluid was added in each well, covered with membrane, for incubation at 37˚C for 30 min. Thereafter, the fluid was discarded. Drying was performed, and the plate was washed five times. Substrate solution was added in each well, covered with a membrane, for incubation at 37˚C for 15 min. Then, 50 μl termination solution was added. Immediately, the optical density (OD) was measured by microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm wavelength, the standard curve was drawn, and the activity of sample to be tested was calculated.

Real-time PCR detection. CSF was added to the TRIzol reagent, and placed at room temperature for 5 min. When the samples were completely cracked, the centrifugation was performed at 12,000 x g, at 4˚C for 5 min, and then the supernatant was collected. Subsequently, the chloroform was added in supernatant, mixed evenly, with placing at room temperature for 5 min. After the centrifugation was performed at 12,000 x g, at 4˚C for 15 min, the supernatant was discarded. Then the same volume of avantin was added, and placed at room temperature for 10 min. Thereafter, another centrifugation was performed at 12,000 x g, at 4˚C for 10 min, the pellet was collected; 75% ethanol was added, mixed evenly, and used to wash RNA precipitation. Finally, RNas-free water was added to make it completely dissolved. Then the ratio of OD_{260}/OD_{280} and RNA concentration were detected. Finally, according to the specification and the primer sequence template as shown in Table I, the progressive amplification was performed, and the reaction products were analyzed by RT-PCR.

Table I. RT-PCR primer sequences of NF-κB and β-actin mRNA.

Gene	Primer sequences
NF-κB	5'-ATGTGCATCGGCAAGTGG-3'
	5'-CAGAAGAGTTTCGGGTAG-3'
β-actin	5'-GAGCCGGGAAATCGTGCGT-3'
	5'-GGAAGGAAGGCTGGAAGATG-3'

Western blotting detection. The protein in peripheral blood of each group was assessed, respectively. According to the molecular weight of target protein, appropriate proportion of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation gel and a certain proportion of 5% SDS-PAGE spacer gel were prepared and performed solidification for 30 min. The protein sample containing an equal amount of total protein was added into each well, and then electrophoresis was carried out under constant voltage of 220 V. When the bromophenol blue reached the bottom of the gel, electrophoresis was stopped. According to the molecular weight of target protein, gel was cut and placed into transfer buffer. PVDF membrane was cut in accordance with the size of gel, and soaked in methanol for 10 sec. Then the PVDF membrane and filter paper were placed into transfer buffer. According to the sequence of positive pole - filter paper - PVDF membrane - gel - filter paper - negative pole, it was placed into transmembrane device, with transferring membrane under constant voltage of 110 V for appropriate time, to transfer the protein on the gel onto the PVDF membrane. The PVDF membrane with protein was placed in 5% dried skimmed milk for sealing within 3 h in a table concentrator at room temperature. The sealing membrane was incubated with rabbit polyclonal NF-κB p65 antibody (dilution, 1:500; cat no. ab16502; Abcam, Cambridge, MA, USA) at 4˚C overnight. The membrane was washed with TTBS three times, 10 min each time, and was placed in the corresponding secondary goat anti-rabbit (HRP) IgG antibody (dilution, 1:2,000; cat. no. ab6721; Abcam) for incubation for 2 h in table concentrator at room temperature. The membrane was washed by TTBS three times, 10 min each time. The A and B, two kinds of reagents in ECL luminescence kit, were mixed evenly in accordance with equal volume, and added on PVDF membrane drop by drop. After the full response, the coloration was performed for 1 min avoiding light. Then it was placed in the gel imaging instrument. The dynamic integration model was used to take pictures, and the gray level analysis on protein bands was performed by Gel-Pro 4.0 software.

Statistical analysis. SPSS 18.0 software (Chicago, IL, USA) was used for statistical analysis. The experiment data were expressed as mean ± standard deviation, and the t-test, p-test

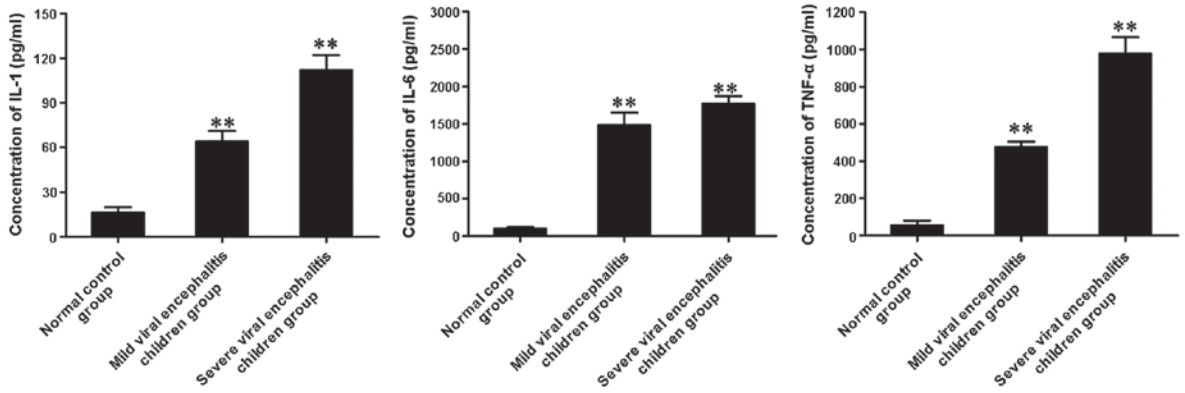

Figure 1. Levels of interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) of children in the normal control group and children with viral encephalitis in mild group and severe group. **P<0.01.

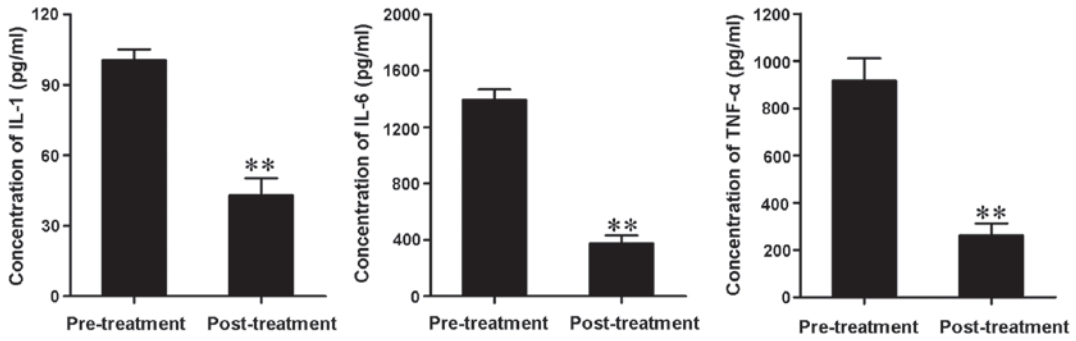

Figure 2. Levels of interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α) of pre-treatment and post-treatment in children with viral encephalitis. **P<0.01.

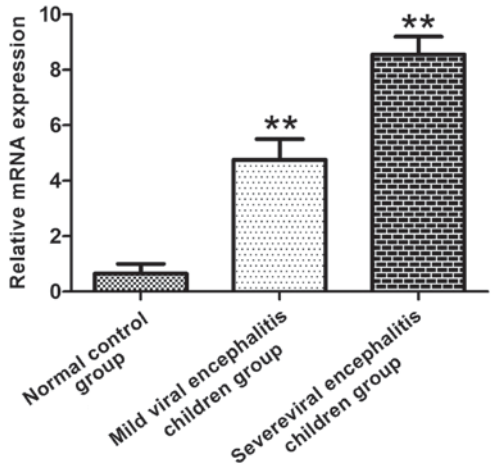

Figure 3. Expression levels of NF-κB of children in the normal control group and children with viral encephalitis in the mild and severe groups. **P<0.01.

and one-way ANOVA were applied. P<0.05 indicated that the difference was statistically significant.

Results

Detection of IL-1, IL-6 and TNF-α levels of children in normal control group and children with viral encephalitis in the mild and severe groups. As shown in Fig. 1, the concentrations of inflammation-related factors in serum of children with viral encephalitis, including IL-1, IL-6 and TNF-α, were significantly higher than those of normal children (P<0.01), while the concentrations of IL-1, IL-6 and TNF-α in children with severe viral encephalitis were distinctly higher than those of children with mild viral encephalitis (P<0.05). The result indicated that IL-1, IL-6 and TNF-α were highly expressed in viral encephalitis.

Levels of IL-1, IL-6 and TNF-α of pre-treatment and post-treatment in children with viral encephalitis. As shown in Fig. 2, the concentrations of inflammation-related factors of post-treatment in children with viral encephalitis, including IL-1, IL-6 and TNF-α were significantly lower than those of pre-treatment (P<0.01). It indicated that the inhibition of expression of inflammation-related factors including IL-1, IL-6 and TNF-α played an effective role in the treatment of viral encephalitis.

Levels of NF-κB of children in the normal control group and children with viral encephalitis in the mild and severe groups. After the total RNA was, respectively, extracted from the normal control group, mild and severe viral encephalitis groups, NF-κB expression was detected by RT-PCR. As shown in Fig. 3, the expression of NF-κB in children with severe viral encephalitis was distinctly higher than that of the normal children, while the expression level in the severe viral encephalitis group was evidently higher than that of children in the mild group. The result indicated that NF-κB plays an important role in the occurrence and development of children viral encephalitis.

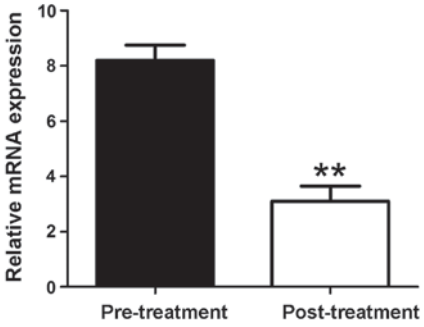

Figure 4. Expression levels of NF-κB of pre-treatment and post-treatment in children with viral encephalitis. **P<0.01.

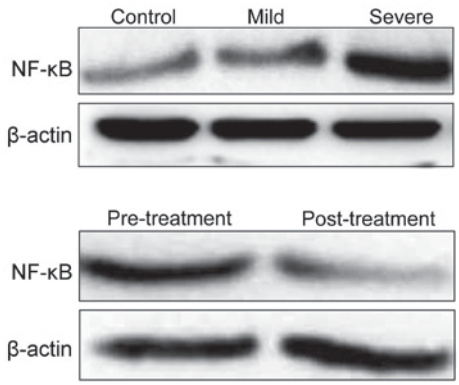

Figure 5. Expression level of NF-κB protein.

Levels of NF-κB of pre-treatment and post-treatment in children with viral encephalitis. After the total RNA of pre-treatment and post-treatment in children with viral encephalitis were respectively extracted, NF-κB expression was detected by RT-PCR. As shown in Fig. 4, the expression of NF-κB of post-treatment was significantly lower than that of pre-treatment, which indicated that the inhibition of NF-κB played an effective role in the treatment of children with viral encephalitis.

Expression level of NF-κB protein. After the protein was, respectively, extracted from normal control group, mild and severe viral encephalitis groups, NF-κB expression was detected by western blotting. As shown in Fig. 5, the expression of NF-κB in children with mild and severe viral encephalitis was distinctly higher than that of normal children, while the expression level in children in the severe viral encephalitis group was evidently higher than that in the mild group. It was also evident that NF-κB expression of post-treatment was significantly lower than that of pre-treatment, which indicated that the expression level of NF-κB plays an important role in children with viral encephalitis.

Discussion

Viral meningitis in children is the most common clinical aseptic meningitis, which is manifested by fever, headache, vomiting and meningeal irritation sign, as well as a diffuse inflammatory syndrome of pia mater that is mainly caused by a variety of viral infections (7). The virus is mostly divided into intestinal virus, mumps virus, herpes virus and adenovirus. CSF in children with viral meningitis is colorless and transparent. Generally, the course of disease is two-week, mostly benign, and has a great prognosis (8). Viral encephalitis in children is a common infectious disease of the CNS caused by viruses. According to the different conditions of incidence, the severity of the disease is also different. As showing a rapidly progressive process, severe viral encephalitis could cause other CNS responses, and the sequelae can even lead to death. The mild viral encephalitis is commonly benign, which may have a great prognosis after reasonable treatment. Viral encephalitis of children is also related to geography and the seasons, while a different geographical environment and seasonal transformation may cause different types of infections (9).

When a child is occasionally found to have sensory disorder, ataxia and positive pathologic reflex, it should be noted whether there is a virus infection. Once symptoms of nervous system damage occur, such as lethargy and others, it means the disease has developed to a severe situation, while nervous system damage symptoms are usually rare in clinic (10). The therapy of children with viral encephalitis is symptomatic treatment in accordance with the virus. The children in severe acute phase are treated with intravenous drip of dexamethasone, of no more than two weeks. Ribavirin also can be used for antiviral therapy, while Ara-A and acyclovir can be used in malaria viral encephalitis. At present, symptomatic treatment, supportive treatment and prevention and treatment of complications are main therapies. As the first choice drug for viral meningitis, ganciclovir two times a day, has good curative effect, while infusion of nutritional brain cell drugs is helpful to promote the recovery of brain function (11).

NF-κB is a significant transcription factor, which plays an important role in immune and inflammatory responses to regulate the expression of inflammation induced enzymes (12,13). Therefore, controlling the regulation of NF-κB activation is a potential therapeutic strategy for the prevention and treatment of human inflammatory diseases associated with NF-κB (14). It can be tracked that NF-κB plays a central role in the transcriptional regulation of traumatic inflammatory reactions (15). NF-κB regulates a variety of gene expression and plays an important role in cell proliferation, tumor genesis and inflammatory process (16,17). NF-κB is a transcription factor protein family, including the subunits p65, p52, p50, cRel and RelB. Of those, p65, which contains N-terminal Rel homologous region and C-terminal transactivation domain, is responsible for the combination with DNA, dimerization and nuclear translocation. The homodimer, p50 and p52 did not activate gene transcription, but occured as a type of inhibitory molecule (18). Generally, p50 and p52 exist in forms of precursor p105 and p100 in cells, respectively. The homo- and/or hetero-dimer formed by two subunits may combine with a target gene-specific sequence to regulate gene transcription, while different NF-κB dimers may be slightly different in the selection of the binding sequence, which is a way of NF-κB to finely regulate the expression of different genes through different forms of dimers (19). The main form of NF-κB is a heterologous dimer pair, p65/p50 subunit. Many inflammatory factors, even some of the oxidative and apoptotic factors are regulated by the NF-κB (20).

In this study, the expression levels of NF-κB and inflammation-related factors (IL-1, IL-6 and TNF-α) in peripheral blood, CSF and serum of children with viral encephalitis were detected by ELISA, RT-PCR and western blotting, to investigate the pathogenesis of viral encephalitis in children and the role of NF-κB in children with viral encephalitis, to provide a new direction for the detection, diagnosis and treatment of viral encephalitis in children.

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