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The effect of enterovirus 71 immunization on neuropathogenesis and protein expression profiles in the thalamus of infected rhesus neonates

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ABSTRACT

Enterovirus 71 (EV71) is a major pathogen that causes hand–foot–mouth disease (HFMD). Our previous studies have demonstrated that the complete process of pathogenesis, which may include tissue damage induced by host inflammatory responses and direct tissue damage caused by viral infection, can be observed in the central nervous system (CNS) of animals infected in the laboratory with EV71. Based on these observations, the neuropathogenesis and protein expression profiles in the thalamic tissues of EV71-infected animals were further analyzed in the present study. Changes in protein expression profiles following immunization with the inactivated EV71 vaccine followed by virus challenge were observed and evaluated, and their physiological roles in viral pathogenesis are discussed. Taken together, the results of these experiments provide evidence regarding the neuropathogenesis and molecular mechanisms associated with EV71 infection and identify several protein indicators of pathogenic changes during viral infection.

Introduction

In recent years, studies on infectious diseases in children have called attention to enterovirus 71 (EV71), which is the major pathogen that causes hand–foot–mouth disease (HFMD) throughout the world, especially in regions of Asia (McMinn, 2002; Ooi et al., 2007). This focus is not only because of the recent HFMD epidemics in mainland China and other locations in Asia (Chan et al., 2000; Shimizu et al., 1999; Yang et al., 2009) but also because of the fatal cases associated with EV71-induced neuropathogenesis during these epidemics (Chen et al., 2007; Ho et al., 1999; McMinn, 2002). At present, based on a preliminary pathological study of clinical cases of EV71 infection (Wong et al., 2008), a consensus has been reached that this viral infection might cause serious pathological changes in the central nervous system (CNS) of a small number of individuals. Infants are especially at risk for developing CNS pathology that causes neurogenic pulmonary edema, which is the major cause of death associated with HFMD (Hsueh et al., 2000; Lin et al., 2006; Wong et al., 2008). However, due to a lack of adequate human clinical pathological data, the pathological processes and mechanisms of EV71 infection remain elusive (Huang et al., 2009). Therefore, it is important to conduct further analyses of the pathogenesis of this disease, especially its neuropathogenesis, from the histopathology of infected organs to the molecular mechanisms of viral infection. In previous studies by our group and others, pathological analyses of EV71 infection in primates have revealed that the complete process of EV71 pathogenesis can be observed in the CNS of animals, such as cynomolgus monkeys and rhesus monkey infants, that are infected with the virus in the laboratory (Liu et al., 2011; Nagata et al., 2004; Zhang et al., 2011). Additionally, some pathological and etiological indicators have demonstrated that this infectious process can be validated (Liu et al., 2011; Zhang et al., 2011). These results provide an understanding of the pathological mechanisms underlying EV71 infection from a histological perspective.

Based on the data obtained in these primate studies, it might be possible to use these established animal models to evaluate EV71 vaccines or therapeutic drugs with respect to histopathology, immunology and pharmacology (Liu et al., 2011). However, the indicators obtained in previous studies are a general index and only show changes in the histopathology, etiology and immunology associated

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with EV71 infection, which may not reflect the physical changes and related molecular mechanisms involved in the process of infection. The results from recent studies of severe HFMD cases caused by EV71 infection indicate that in addition to tissue damage directly caused by viral infection of the CNS, tissue damage may also occur due to host inflammatory responses (Lin et al., 2003; Wang et al., 2003; Wong et al., 2008).

In our previous studies, experiments on EV71-infected rhesus monkey neonates demonstrated that the virus can infect animals via the respiratory tract and cause common clinical manifestations, including blister-like lesions on the oral mucosa and skin (Liu et al., 2011). Additionally, an increase in the body temperature of these animals was accompanied by the emergence of viremia and pathological changes in the CNS, among other effects (Liu et al., 2011; Zhang et al., 2011). These pathological features have been used to specifically evaluate the effectiveness of and the protection provided by the inactivated EV71 vaccine (Dong et al., 2011,2010). Based on the dynamic analysis of these features, 2D protein electrophoresis was used to evaluate the changes in protein expression in the thalamic tissues of rhesus monkey neonates that were immunized with the inactivated EV71 vaccine prior to viral challenge. Dynamic changes in protein expression were observed in these animals in comparison with the thalamic tissues from unimmunized animals that were directly infected with the virus. Based on our interpretation of the changes in the expression of these identified proteins, further evaluation of the response of immunized animals to the challenge with live virus was conducted in this study.

Results

Pathological analysis of CNS tissues from neonatal monkeys in the VI and UI groups

As previously demonstrated by pathological data from the fatal human cases and animals infected with EV71, the majority of the pathological lesions in the CNS induced by EV71 infection are found in the thalamus, pons and spinal cord (Liu et al., 2011; Wong et al., 2008; Zhang et al., 2011). The lesions observed in these regions could contribute to neurogenic pulmonary edema (Lum et al., 1998). Therefore, most of our work focused on the pathological features of the tissues, specifically the thalamus, pons and spinal cord, to observe the efficacy of the inactivated EV71 vaccine and to ultimately define these three sites as those potentially critical for the evaluation of candidate vaccines (Dong et al., 2011). In our comparative study, no significant pathological changes were observed in the VI group, except for a few cases of inflammatory cell infiltration in some parts of the thalamus, pons and spinal cord. However, changes were observed in the UI group, such as typical inflammatory cell aggregation, vascular cuffing and a small amount of neuronal cell degeneration and necrosis (Fig. 1). Similar pathological changes to those observed in the thalamus, pons and spinal cord were also observed during synchronized observations of other tissues of the CNS and major organs (Fig. S1). These results are in accordance with the previous work on other target organs (Dong et al., 2011). Furthermore, the pathological changes were found to be most pronounced on day 10 post-infection (p.i.) and began to disappear slightly on day 14 p.i. (Fig. 1). These pathological changes might therefore be used as defined pathological markers of encephalitis.

Virus distribution in the CNS tissues of neonatal monkeys in the VI and UI groups

Our previous studies on EV71-infected neonatal rhesus monkeys demonstrated that the highest viral loads were detectable during days 7–10 p.i. in the main organs and tissues of the monkeys (Liu et al., 2011), which might indicate that viral loads in the organs and tissues may be used as indicators of EV71 infection. In this study, the viral loads in tissues of the CNS, including cerebrospinal fluid (CSF) collected from the VI and UI groups, were measured to evaluate the ability of the inactivated EV71 vaccine to protect the CNS of infected monkeys. The qRT-PCR data and direct virus titers in tissue culture were similar. No virus was detected in the CNS tissue samples collected from the VI group (Fig. 2a). In contrast, peak viral titers were observed 10 day p.i. in most tissues of the CNS, including the cerebrum, thalamus and cerebellum, except in the CSF that was collected from the UI group. The peak viral titer in the spinal cord was observed at day 7 p.i. and was maintained until day 10 p.i. (Fig. 2a). Furthermore, qRT-PCR amplification demonstrated that the viral RNA copies in the samples from the VI group were lower than 10^0 (Fig. 2b). The medulla oblongata from the UI group had higher than 10^6 viral RNA copies (Fig. 2b), and the viral peak coincided with the viral titers that were determined in tissue culture. These results and previous reports (Liu et al., 2011; Nagata et al., 2004) indicate that the CNS is a primary target organ of EV71 viral proliferation in neonatal monkeys. Observations made using immunohistochemical detection with EV71-specific antibodies in the various CNS tissues further support these findings (Fig. S2). The viral loads detected in the CNS of the samples from the VI group, which had been immunized with the inactivated EV71 vaccine, clearly indicate that the immune response induced by vaccination has the capacity to inhibit viral proliferation in the CNS.

The detection of antibodies and inflammatory factors in the cerebrospinal fluid of VI and UI monkeys

Clinical descriptions of fatal cases of EV71 infection have suggested that the pathological lesions in the CNS can progress rapidly to pulmonary edema and respiratory failure (Liu et al., 2000; Nolan et al., 2003). This type of acute pathogenic progress might be caused by a severe inflammatory reaction mechanism, such as a "cytokine storm" (Lin et al., 2002; Wang et al., 2003). In studies evaluating the efficacy and safety of candidate EV71 vaccines, the levels of inflammatory factors are commonly considered to be one type of potential indicator, especially in the CSF (Dong et al., 2011; Liu et al., 2011). However, based on reports about antibody-dependent enhanced infection (ADE) by EV71 (Han et al., 2011; Wang et al., 2010) and antibody-mediated cytotoxicity (Ch'ng et al., 2011), levels of specific antibodies against EV71 in the cerebrospinal fluid were measured, and the titer was compared to that in peripheral blood. The results demonstrated that the levels of the IL-2, IL-4, IL-5, IL-6, TNF-α and IFN-γ in the cerebrospinal fluid of the monkeys in the VI group did not increase significantly or fluctuate and were maintained within the normal range (Fig. 3a). In contrast, the levels of the majority of these inflammatory factors, especially IL-6 and TNF-α, tended to increase in the cerebrospinal fluid of the monkeys in the UI group, which was demonstrated by higher observed levels of IL-6 during disease progression (Fig. 3). These factors were sustained at levels that were significantly higher than normal from days 7 to 14 p.i. The highest values were observed on day 10 and then gradually declined. However, measurement of anti-EV71 antibodies using the neutralizing test failed to detect antibodies in the cerebrospinal fluid of either group (Fig. S3). In contrast, an extensive increase in antibodies in the peripheral blood of the VI group and a slight increase in the UI group at the same time point were observed (Fig. S3). The higher levels of cytokines in the cerebrospinal fluid are similar to those reported from the studies of other animals infected with EV71 as