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Elevated cerebrospinal fluid endothelin 1 associated with neurogenic pulmonary edema in children with enterovirus 71 encephalitis

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SUMMARY

Objectives: Neurogenic pulmonary edema (NPE) is a fatal complication in children with enterovirus 71 (EV71) encephalitis. Endothelin 1 (ET-1), a potent vasoconstrictor, can induce pulmonary edema in rats via intrathecal injections. Thus, it was hypothesized that ET-1 in the central nervous system may correlate with NPE in children with EV71 encephalitis.
Methods: Clinical data and ET-1 in the cerebrospinal fluid (CSF) were compared between three groups: (1) EV71 encephalitis with NPE; (2) EV71 encephalitis without NPE; and (3) non-EV71 aseptic meningitis. ET-1 immunostaining was performed on the brainstem of autopsy patients.
Results: The EV71 with NPE group showed significantly increased CSF levels of ET-1 compared to the EV71 without NPE and the non-EV71 aseptic meningitis groups (both $p < 0.01$). The optimum cut-off point of ET-1 to predict NPE in EV71 patients, based on the receiver operating characteristic curve, was 0.5 pg/ml (sensitivity 83%, specificity 100%). Immunostaining in the brainstem showed increased ET-1 expression, mainly in the oligodendrocytes, in EV71 with NPE patients compared with control patients.
Conclusion: ET-1 in the central nervous system may play a role in the development of NPE in children with EV71 infection and could be used as a biomarker or therapeutic target for NPE in EV71 encephalitis.

1. Introduction

Enterovirus 71 (EV71) belongs to the human *Enterovirus A* species within the family *Picornaviridae*. EV71 is a frequent cause of hand, foot and mouth disease, which can be complicated by severe neurological involvement in children, especially those younger than 5 years of age.[1] Most of the fatal cases initially present with minor neurological symptoms, rapidly become complicated with autonomic neurological dysfunction and neurogenic pulmonary edema (NPE), and die of cardiopulmonary failure soon after admission. The mortality from this condition ranges from 80% to 90%.[1] The presence of NPE is usually the main cause of fatal EV71 during epidemics, but its pathogenesis is not completely understood. Brain magnetic resonance imaging (MRI) studies have shown a propensity of EV71 infection for the tegmentum of the pons and medulla oblongata. Post-mortem studies have demonstrated that patients who died from EV71-induced NPE had acute inflammation and EV71 antigens within neurons in the brainstem.[2,3] Thus, brainstem encephalitis is speculated to be the cause of acute fatality with NPE after EV71 infection.[4]

Endothelin 1 (ET-1), a 21-amino acid peptide, is a potent vasoconstrictor.[5] Because its mRNA and receptor binding sites are expressed in several regions of the brain, ET-1 can also work as a neurotransmitter to regulate neurological functions. [6,7] Several animal studies have shown that intrathecal injection of ET-1 elicits a transient increase in arterial blood pressure, heart rate, and

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sympathetic nerve activity.[8] Intrathecal injection of ET-1 also enhances pulmonary vascular permeability and causes pulmonary edema.[7] It was concluded that pulmonary edema was due to intense pulmonary vasoconstriction mediated by α-adrenoceptors following the release of catecholamines in response to the activation of endothelin receptors in the central nervous system.[7] Thus, ET-1 plays a role in inducing NPE.

It was hypothesized that the occurrence of NPE in children with EV71 infection is correlated with an increase in ET-1 in the brainstem. Cerebrospinal fluid (CSF) samples and autopsy brain specimens from patients with EV71 infection were used to investigate this issue.

2. Materials and methods

2.1. Patients

The cases of 16 patients with EV71 encephalitis who had been admitted to the study hospital during the EV71 epidemic from 1998 to 2000 in southern Taiwan were reviewed. The inclusion criteria for the patients with EV71 encephalitis were the following: (1) defined EV71 infection by viral isolation, (2) presence of neurological manifestations including headache, altered consciousness, myoclonic jerks, ataxia, tremor, acute flaccid paralysis, bulbar palsy, or NPE, and (3) CSF leukocyte count $>10 \times 10^6$ cells/l.[1] NPE was defined as the occurrence of respiratory distress, tachycardia, tachypnea, and copious frothy sputum, with chest radiological findings of bilateral pulmonary infiltrates without cardiomegaly. The 16 patients who had EV71 encephalitis were further stratified into two groups: EV71 encephalitis without NPE ($n = 10$) and EV71 encephalitis with NPE ($n = 6$).

An additional control group comprised nine patients with non-EV71 aseptic meningitis who were admitted during the same period. The diagnosis of non-EV71 aseptic meningitis was based on the following criteria: (1) CSF leukocyte count $>10 \times 10^6$ cells/l with a predominance of mononuclear cells, (2) negative bacterial studies, including CSF and blood cultures, and (3) self-limited clinical course without clinical evidence of encephalopathy, such as seizures, disturbances of consciousness, or focal neurological signs.

All of the medical charts, laboratory results, and MRI scans were reviewed retrospectively. The study was approved by the institutional review board of the National Cheng Kung University Hospital.

2.2. Virology studies

EV71 was confirmed by virus isolation as described previously.[1] Briefly, specimens were inoculated onto monolayers of A549, Vero, and green monkey kidney cells within 24 h and then incubated and evaluated for evidence of a viral cytopathic effect. Isolates were further typed using an immunofluorescence assay with EV71 monoclonal antibodies 3323 and 3324 (Chemicon International, Temecula, CA, USA). EV71 was identified by positive staining of both antibodies. These isolates were further confirmed by neutralization testing using polyclonal antiserum (American Type Culture Collection, Rockville, MD, USA).

2.3. Analysis of ET-1

Archived CSF specimens had been stored in cryogenic vials in −80 °C freezers; these specimens had been placed in the freezers within 4–6 h after collection. ET-1 concentrations were determined using the QuantiGlo Human ET-1 Chemiluminescent Immunoassay (R&D Systems, Minneapolis, MN, USA). According to the manufacturer, the sensitivity of the ET-1 assay is 0.102 pg/ml. In brief, a monoclonal antibody specific for ET-1 was pre-coated onto a microplate to bind ET-1 in standards and samples. After washing, an enzyme-linked monoclonal antibody specific for ET-1 was added to the wells. Following a wash, an enhanced luminol/hydrogen peroxide substrate solution was added and light was produced in proportion to the amount of ET-1 bound in the initial step. A microplate luminometer (Luminoskan Ascent; Thermo Scientific, Waltham, MA, USA) was used to measure the intensity of the light emitted. The standard curve was modified from the R&D Systems recommendations to include 0.04, 0.2, 1, and 5 pg/ml standards. Raw data were transferred to a computer in which a log concentration–log RLU (relative light unit) equation was defined and sample concentrations were calculated.

2.4. Immunohistochemistry and double-immunofluorescence staining

ET-1 immunohistochemistry was performed on selected blocks of nervous tissue from a patient who had died in the EV71 encephalitis with NPE group. To compare the differences in ET-1 expression in the brainstem, another age-matched patient who had died of severe neuroblastoma without brainstem metastasis was used as a control. Tissue sections (10 μm in thickness) were deparaffinized with xylene and graded ethanol solutions. The antigen was retrieved by microwaving for 10 min in sodium citrate buffer, pH 6.0. For immunohistochemistry, tissue sections were blocked with 2% normal goat serum and 0.1% Triton X-100, and probed with primary antibodies to ET-1 (1:200, Abcam) at 4 °C overnight, followed by a 2-h incubation with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies at room temperature. Biotin-peroxidase signals were detected using 0.5 mg/ml 3,3′-diaminobenzidine/0.003% H_2O_2 as a substrate. For double-immunofluorescence staining, tissue sections were probed with primary antibodies anti-microtubular-associated protein 2 (MAP-2) (1:50, Cell Signaling), anti-CD68 (1:50, Santa Cruz), anti-Iba1 (1:500, Wako), anti-glial fibrillary acidic protein (GFAP) (1:500, Abcam), anti-Oligo2 (1:500, Abcam), or anti-ET-1 (1:200, Abcam) at 4 °C overnight after blocking. The sections were then incubated with Alexa Fluor 488 goat IgG and Alexa Fluor 594 goat IgG secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature after washing. Images were acquired on a Nikon E400 fluorescence microscope (Tokyo, Japan). Digitally captured images were analyzed using Imaging Software NIS-Elements (Nikon, Tokyo, Japan).

2.5. Statistical analysis

Significant differences between the study groups were assessed using a commercial software program (SPSS version 17.0; SPSS Inc., Chicago, IL, USA). One of the authors (CHL), who was blinded to the study design, performed all the statistical analyses. Continuous data are presented as the mean ± standard deviation (SD) and were tested by non-parametric Mann–Whitney *U*-test. Categorical variables were tested by Chi-square test or Fisher's exact test. A receiver operating characteristic (ROC) curve was constructed to assess the sensitivity and specificity of ET-1. A p-value of <0.05 was considered statistically significant, and all probabilities were two-tailed.

3. Results

3.1. Study patients

The demographic information and clinical manifestations of the three groups are shown in Table 1. Although the EV71 encephalitis with NPE group was younger at disease onset, the age at onset was comparable between the three groups. On admission, the EV71