Acta Tropica 131 (2014) 98–103

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Dexamethasone downregulated the expression of CSF 14-3-3β protein in mice with eosinophilic meningitis caused by *Angiostrongylus cantonensis* infection

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ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 20 October 2013
Accepted 9 December 2013
Available online 18 December 2013

Keywords:
Angiostrongylus cantonensis
Blood brain barrier
Corticosteroid
Eosinophilic meningitis
14-3-3β protein
Mice

ABSTRACT

Angiostrongylus cantonensis is the main causative agent of human eosinophilic meningitis in Southeast Asia and the Pacific Islands. A previous study demonstrated that the 14-3-3β protein is a neuropathological marker in monitoring neuronal damage in meningitis. Steroids are commonly used in patients with eosinophilic meningitis caused by *A. cantonensis* infection. However, the mechanism by which steroids act in eosinophilic meningitis is unknown. We hypothesized that the beneficial effect of steroids on eosinophilic meningitis is partially mediated by the down-regulation of 14-3-3β protein expression in the cerebrospinal fluid (CSF). In this animal study, we determined the dynamic changes of 14-3-3β protein in mice with eosinophilic meningitis. The 14-3-3β protein in serum and CSF was increased in week 2 and 3 after infections. Dexamethasone administration significantly decreased the amounts of CSF 14-3-3β protein. By developing an in-house ELISA to measure 14-3-3β protein, it was found that the amounts of 14-3-3β protein in the CSF and serum increased over a three-week period after infection. There was a remarkable reduction of 14-3-3β protein in the CSF after 2 weeks of dexamethasone treatment. In conclusion, the administration of corticosteroids in mice with eosinophilic meningitis decreased the expression of 14-3-3β protein in the CSF.

1. Introduction

Angiostrongylus cantonensis, also known as rat lungworm, is the main causative agent of human eosinophilic meningitis or meningoencephalitis in Southeast Asia and the Pacific Islands (Beaver and Rosen, 1964; Punyagupta et al., 1975; Rosen et al., 1962; Yii, 1976; Wang et al., 2012). Humans are infected with *A. cantonensis* by ingesting freshwater, terrestrial snails and slugs (Wen, 1973; Nishimura et al., 1986; Richards and Merritt, 1967; Yen et al., 1990). The major intermediate hosts for *A. cantonensis* in Taiwan are the African giant snail (*Achutina fulica*) and the golden apple snail (*Pomacea canaliculata*) (Wen, 1973; Yen et al., 1990). When the infection occurs in non-permissive hosts, including humans and mice, the development of the parasites will terminate at the young-adult worm stage in the brain and induce eosinophilia in the blood, and especially in the cerebrospinal fluid (CSF) (Lee et al., 2006).

A previous study demonstrated that the 14-3-3β protein is a neuropathological marker in monitoring neuronal damage in purulent bacterial meningitis (Bonora et al., 2003). Although its lack of specificity clearly implies substantial limitations in the use of the 14-3-3 β protein as a specific disease marker, its value as an indicator of neurological damage could be used to monitor the evolution of different neurological disorders with etiologies that can be otherwise established, such as eosinophilic meningitis.

Steroids have been proposed as an adjunctive treatment for eosinophilic meningitis. In the clinical observations of Chotmongkol et al. (2000), a 2-week course of prednisolone was found to be beneficial in relieving headaches in patients with eosinophilic meningitis. In addition, in the study of Sawanyawisuth et al. (2004), a 1-week course of corticosteroid treatment was also found to be effective in treating eosinophilic meningitis. However,

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http://dx.doi.org/10.1016/j.actatropica.2013.12.006

the mechanism by which steroids act in eosinophilic meningitis is unknown. We hypothesized that the beneficial effect of steroids on eosinophilic meningitis caused by *A. cantonensis* infection is related to the down-regulation of 14-3-3β protein expression in the CSF and improvement of blood brain barrier dysfunction. Based on this hypothesis, we studied the effect of steroids on the dynamic changes of 14-3-3β protein expression in mice with eosinophilic meningitis, correlated the findings with Evans blue test findings, and reviewed the pertinent literature.

2. Material and methods

2.1. Ethics statement

Animal studies were carried out in strict accordance with the recommendations from Taiwan's Animal Protection Act. The protocol was approved by the Animal Committee of the Kaohsiung Veterans General Hospital and performed according to their guidelines.

2.2. Infection of BALB/c mice and intraperitoneal steroid injections

Forty BALB/c mice, aged 6–7 weeks, were purchased from the National Laboratory Animal Breeding Research Centre. They were raised and maintained in an air-conditioned animal facility (25 ± 2 °C and 50 ± 10% relative humidity). Third-stage larvae of *A. cantonensis* were harvested from infected *Biomphalaria glabrata* after treatment with artificial gastric juice. Briefly, the snail shells were digested in 0.6% pepsin-HCl solution (pH 2–3, 500 I.U. pepsin/g tissue) according to a method previously described with modifications (Wallace and Rosen, 1969). The mice were orally infected with 50 *A. cantonensis* L3 via an orogastric tube after ketamine anesthesia, and five to seven were then sacrificed every week for 3 consecutive weeks after infection until the end of the study. Dexamethasone at a dose of 500 μg/kg/day was injected intraperitoneally from the seventh day of infection until the end of the study (21 days post infection). The total treatment duration for dexamethasone was 2 weeks.

2.3. Collection of serum and CSF specimens

Blood samples from the experimental mice were collected by a heart puncture under ketamine anesthesia. Serum specimens separated from the blood samples after centrifugation at 3500 × *g* (Hermle, Z326K, Germany) for 5 min at 4 °C were stored at −70 °C until they were measured.

The skulls of the mice were opened after complete bleeding. Careful surgery was conducted in order to avoid blood contamination of the CSF. The brain was removed and washed with 150 μl 0.15 M phosphate buffered saline (PBS). Concurrently, the cerebral ventricles and cranial cavity were washed with 350 μl PBS. The CSF was, thus, harvested with the PBS from above, and was then centrifuged in an Eppendorf tube at 3000 × *g* (Hermle, Z326K, Germany) for 10 min at 4 °C to eliminate cells. The supernatant was stored at −70 °C until further use.

2.4. Measurement of dysfunction of the blood–brain barrier by the Evans blue method

Thirty BALB/c mice, age 6–7 weeks, six mice in each group were used for measurement of permeability of blood brain barrier. A volume of 200 μl of 2% (w/v) solution of Evans blue in PBS was injected into the tail vein of a mouse. One hour later, the brain of the mouse was removed after anesthesia with ketamine, and was then ground with 1.0 ml PBS in a glass-tissue grinder with a Teflon pestle. The extract was then centrifuged at 18,000 × *g* (Hermle, Z326K, Germany) for 10 min at room temperature. The optical density of the supernatant was read at 595 nm wavelength using a colorimeter (Thermo Scientific Multiskan FC, USA).

2.5. Measurement of 14-3-3β protein concentrations in CSF, serum and brain homogenates by Western blot analysis

CSF or serum aliquots (100 μl) and brain homogenates were mixed with 7 volumes of cold methanol, kept at −20 °C for 2 h, and then centrifuged at 20,800 × *g* for 30 min. The pellets were dissolved in 40 μl of sample buffer (3% SDS, 3% β-mercaptoethanol, 2 mM EDTA, 10% glycerol, and 62.5 mM Tris, pH 6.8) and boiled for 5 min. For each sample, 10 μL of sample buffer/well were loaded onto a 13% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). The membranes were incubated with anti 14-3-3β polyclonal rabbit IgG (Santa Cruz Biotechnology) at a 1:500 dilution and revealed with anti-rabbit horseradish peroxidase IgG (Jackson ImmunoResearch Lab, Inc.) at a 1:10,000 dilution. The blots were developed using an enhanced chemiluminescent system (Amersham). Densitometric values for each sample were obtained with a computer-assisted laser scanner (GS-710 Calibrated Imaging Densitometry; BioRad), after correction for background light. The total amount of 14-3-3β protein as quantified from each diluted and undiluted sample was expressed in arbitrary units (Bonora et al., 2003).

2.6. Generation of recombinant 14-3-3β

Recombinant human 14-3-3β protein was purified from *Escherichia coli* for antibody generation as previously described (Hu et al., 2003). Human 14-3-3β cDNA was amplified from a human fetal brain cDNA library (Stratagene, La Jolla, CA) using a polymerase chain reaction (PCR). The PCR primers used to clone the human 14-3-3β cDNA were designed based on the 14-3-3β sequence in the Gen-Bank database (accession number, NM_003404.3; forward primer, 5′-cgcggatccatgacaatggataaaagtgagctg-3′; reverse primer, 5′-ggcgaattcttagttctctccctccccagc-3′). After DNA sequencing analysis, the PCR-amplified 14-3-3β cDNA was subcloned into the *EcoR*I and *BamH*I sites of the pET28a vector (Novagen, Madison, WI) and transformed into BL-21 cells (DE3, pLysS; Novagen). After induction, the 6x-histidine-tagged 14-3-3β protein was purified on an NTA-agarose affinity column (Qiagen, Hilden, Germany) and desalted on a G25 Sephadex column (Amersham Pharmacia, Little Chalfont, United Kingdom). The recombinant protein was passed through Detoxi-Gel (Pierce Biotechnology, Rockford, IL) to minimize contamination by endotoxins. The 14-3-3β antibodies were raised by periodic injections of recombinant 14-3-3β protein into rabbits. The serum was collected from the immunized rabbits and analyzed using Western blot analysis.

2.7. Production of 14-3-3β polyclonal antisera

2.7.1. Primary immunization

All animal studies were conducted following the Guidelines for Animal Experiments by the Committee of Animal Experiments, Kaohsiung Veterans General Hospital. Purified recombinant 14-3-3β protein in PBS (500 μg in 500 μl) was mixed with complete Freunds adjuvant in a three-way stopcock until the mixture emulsified. The mixture was then transferred to a 3 ml 24-gauge syringe and injected subcutaneously into adult New Zealand white rabbits (2 to 5 kg body weight) under restraint. Two weeks after the primary immunization, the rabbits were boosted with recombinant 14-3-3β protein mixed with incomplete Freunds adjuvant at 2-week intervals for a total of nine boosts.