Biomaterials 32 (2011) 6804–6814

Contents lists available at ScienceDirect

Biomaterials

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

Modulation of the immune-related gene responses to protect mice against Japanese encephalitis virus using the antimicrobial peptide, tilapia hepcidin 1-5

Han-Ning Huang [a], Venugopal Rajanbabu [b], Chieh-Yu Pan [b], Yi-Lin Chan [c], Cho-Fat Hui [d], Jyh-Yih Chen [b,*], Chang-Jer Wu [a,**]

[a] Department of Food Science, National Taiwan Ocean University, 2 Pei Ning Road, Keelung 202, Taiwan
[b] Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, 23-10 Dahuen Road, Jiaushi, Ilan 262, Taiwan
[c] Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan
[d] Institute of Cellular and Organismic Biology, Academia Sinica, Nankang, Taipei 115, Taiwan

ARTICLE INFO

Article history:
Received 14 March 2011
Accepted 17 May 2011
Available online 2 July 2011

Keywords:
Tilapia hepcidin 1-5
Antimicrobial peptides
Japanese encephalitis virus
Antiviral agent
Proinflammatory genes

ABSTRACT

Japanese encephalitis virus (JEV), a neurotropic flavivirus, is one of the major causes of acute encephalitis in humans. After infection, it is commonly associated with inflammatory reactions and neurological disease. There is still no effective antiviral drug available against Japanese encephalitis virus infection. Recently, a number of investigators found that antimicrobial peptide (AMPs) present a broad range of biological activities including antimicrobial and immunomodulatory activities. In this study, we found that an AMP, tilapia hepcidin (TH)1-5, caused no harm to either cells or test animals during the test course and could control JEV viral infection in BHK-21 cells. Mice co-injected with TH1-5/JEV and subsequently subjected to JEV re-challenge survived and behaved normally. The neuroprotective effects were associated with marked decreases in: (i) the viral load and viral replication within the brain, (ii) neuronal death, and (iii) secondary inflammation resulting from microglial activation. TH1-5 was also determined to enhance adaptive immunity by elevating levels of anti-JEV-neutralizing antibodies in the serum. The microarray data also showed that TH1-5 modulated Socs-6, interleukin (IL)-6, Toll-like receptor (TLR)-1, TLR-7, caspase-4, interferon (IFN)-β1, ATF-3, and several immune-responsive genes to protect mice against JEV infection. In addition, TH1-5 was confirmed to modulate the expressions of several proinflammatory and immune-responsive genes, such as IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)-α, IFN-γ and monocyte chemoattractant protein (MCP)-1 at both the transcriptional and translational levels in JEV-infected mice. In conclusion, our findings provide mechanistic insights into the actions of TH1-5 against JEV. Results from our *in vivo* and *in vitro* experiments clearly indicate that TH1-5 has antiviral, neuroprotective, anti-inflammatory, and immunomodulatory activities. Furthermore, TH1-5 successfully reduced the severity of disease induced by JEV. Our results point out that TH1-5 is a promising candidate for further development as an antiviral agent against JEV infection.

1. Introduction

Japanese encephalitis virus (JEV), a positively polarized single-stranded RNA virus, belongs to the Flaviviridae family and causes epidemics of Japanese encephalitis in human and animals [1,2]. Global warming and rapid travel among countries of the world increase the possibility of JEV becoming a global pathogen and causing a worldwide pandemic [3,4]. Although the majority of JEV infections are subclinical, the fatality rate in patients with clinical symptoms ranges 10%–50%. Both inactivated [5] and live-attenuated [6] JEV vaccines have been used in Asian countries with measurable success. However, there are no effective medicines for Japanese encephalitis therapy so far. Hence, there is a necessity to develop a cheap, easily available treatment with no or tolerable side effects against JEV infection.

Experimental mice were infected with JEV, and the disease severity, survival, and molecular and physiological mechanisms were studied [7]. Antigen-presenting cell (APCs), such as dendritic cells are believed to be the initial target of JEV upon introduction into a host [8]. These cells are known to induce proinflammatory

* Corresponding author. Tel.: +886 920802111; fax: +886 39871035.
** Corresponding author. Tel.: +886-224622192 ext. 5137; Fax: 886-224634203;
E-mail addresses: zoocjy@gate.sinica.edu.tw (J.-Y. Chen), cjwu@mail.ntou.edu.tw (C.-J. Wu).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.05.053

cytokines and chemokines [9]. Similar to other pathogens, JEV induces a host immune response through activation of proinflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and chemokine monocyte chemoattractant protein (MCP)-1 that promotes massive leukocyte migration and infiltration into the brain [7,9,10]. In particular, neuronal mortality caused by JEV infection causes elevated levels of proinflammatory cytokines and chemokines in the serum and cerebrospinal fluid [11–15].

Antimicrobial peptide (AMPs), positively charged molecules with short amino acid chains, isolated from various living organisms are involved in host defense mechanisms [16]. The direct antimicrobial function and immunomodulatory functions of AMPs are well documented [17–20]. Among the thousands of identified AMPs, few have a proven host defense function and succeeded in advanced clinical trials [21]. Three AMP compounds from tilapia, tilapia hepcidin (TH)1-5, TH2-2, and TH2-3, were characterized. These THs share some level of amino acid similarity with human hepcidin [22]. Among the three hepcidins, TH2-2 shows no antimicrobial functions, whereas TH1-5 and TH2-3 were, respectively, reported to have antiviral and antibacterial properties [22].

We speculated that the host-friendly TH1-5 can be developed as an antiviral agent against JEV infection. In this study, we examined the antiviral and immunomodulatory effects of TH1-5 using a JEV-infected C3H/HeN mice model. The study showed that TH1-5 effectively rescued mice from JEV-mediated death, and exhibited neuroprotective, anti-inflammatory, and antiviral activities.

2. Materials and methods

2.1. Mice, cells, and the virus

Female C3H/HeN mice were purchased and maintained as described in a previous report [23]. BHK-21 cells were purchased from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 (Sigma–Aldrich, St. Louis, MO, USA) containing 10% bovine calf serum (BCS) (Invitrogen, San Diego, CA, USA) as per the manufacturer's instructions. The Beijing-1 JEV strain was maintained in the brains of suckling mice as described previously [24], and the 50% lethal dose (LD_{50}) of JEV in female C3H/HeN mice was determined [25].

2.2. Cell proliferation assay

BHK-21 cells in 48-well plates were assayed with the CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA) following the vendor's instructions. TH1-5 was administered together with JEV as co-treatment; TH1-5 was also administered 24 h prior to (prophylaxis) or after (curative) the JEV inoculation. After blank value subtraction, the percentage survival against control cells was calculated. The percentage of JEV infection or cell inhibition was obtained by subtracting the cell viability percentage from 100.

2.3. Viral challenge and TH1-5 treatments

Six-week-old adult C3H/HeN mice were randomly divided into five groups of 10 mice each. Individuals in each group of mice were intraperitoneally (i.p.) injected with 500 μl of phosphate-buffered saline (PBS) containing 50× the LD_{50} of JEV in the presence or absence of 50, 100, or 200 μg/ml TH1-5 or PBS. After 14 days, a re-challenge was carried out on surviving mice with 50× the LD_{50} of JEV. Mouse survival and activities were monitored on a daily basis for up to 25 days after the primary injection. For serological and histology studies, serum was collected on days 4, 7, and 21 after treatment, and tissue was collected on day 7.

2.4. Plaque assay

To determine the effect of TH1-5 on the viral titer *in vivo*, BHK-21 cell were plated in six-well plates at a density of 5×10^5 cells/well in 2 mL of medium and cultured for 18 h. After 18 h in RPMI-1640 medium with 2% serum, cells were switched to serum-free media for 1 h. BHK-21 cells were then infected with inoculum isolated from JEV-infected animal brains in the presence or absence of 200 μg/ml TH1-5-treated on day 7 for 1 h with gentle shaking every 15 min. The virus was removed, and the plates were washed with 1× PBS to remove any unbound virus. The wells were then overlaid with 2 ml of 1.5% agarose (SeaPlaque, FMC, USA) in RPMI-1640 containing 2% fetal bovine serum (FBS). Plates were incubated for 3 days at 37 °C in 5% CO_2. Then cells were fixed with formalin and stained with 1% crystal violet. Plaque numbers were counted.

2.5. Immunohistochemistry

On the 7th day post-infection, brains from animals in the JEV-infected group, the group co-injected with TH1-5 and JEV, and the control (PBS-injected) group were fixed with 3.7% PFA in PBS. The brains were then processed for cryostat sectioning. Coronal sections of the cerebral cortex were taken and stained with an anti-JEV-E primary antibody [25]. Sections were developed using a corresponding biotinylated secondary antibody and developed with 3,3′-diaminobenzidine (DAB). Sections were then mounted with distyrene plasticizer xylene (DPX) and observed under an Olympus light microscope (BX-51, Olympus, Tokyo, Japan) (40× magnification). Cryostat sections from control, JEV-infected, and TH1-5/JEV-co-injected animals were also processed for thionin staining.

2.6. Immunoblot analysis

A Western blot analysis was performed with protein isolated from brain tissues of all four groups of animals [control (injected with PBS), infected with 50× the LD_{50} JEV, and infected with 50× the LD_{50} of JEV in the presence and absence of 200 μg/ml TH1-5], on the 7th day post-infection. Briefly, 50 μg of each sample was electrophoresed and transferred onto a nitrocellulose membrane. Membranes were then blocked and probed with several primary antibodies that included anti-JEV Beijing-1

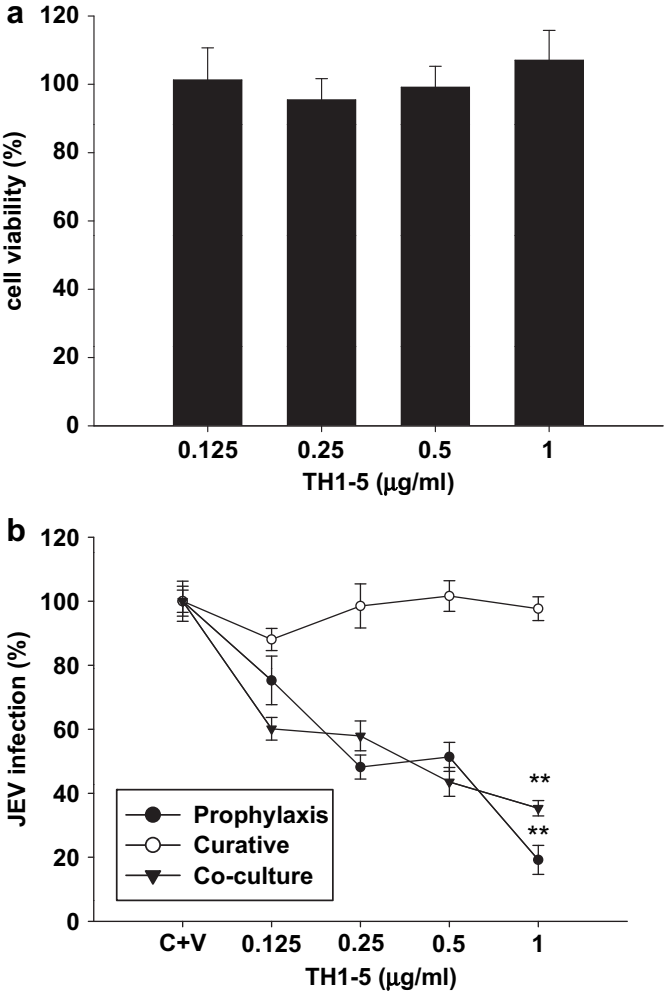

Fig. 1. Tilapia hepcidin (TH)1-5 controls Japanese encephalitis virus (JEV) infection. BHK-21 cells were infected with an MOI of 0.1 of JEV alone or JEV along with various concentrations of TH1-5. For prophylactic treatment, TH1-5 was treated first, and the mouse was infected with JEV 1 h later, and in curative treatment, the mouse was infected with JEV first and TH1-5 was administered 1 h later. JEV and TH1-5 were treated at the same time for co-treatment. At 48 h post-treatment, the percent inhibition of cell viability related to cells treated with only JEV was calculated by an MTS assay. $^*p < 0.05$. Error bars, SEM.