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Novel effect of methionine enkephalin against influenza A virus infection through inhibiting TLR7-MyD88-TRAF6-NF-κB p65 signaling pathway

Jing Tian[a], Xue Jiao[b], Xiaonan Wang[a], Jin Geng[c], Reizhe Wang[d], Ning Liu[e], Xinhua Gao[f], Noreen Griffin[g], Fengping Shan[a,*]

[a] Department of Immunology, School of Basic Medical Science, China Medical University, Shenyang 110122, China
[b] Department of Translational Medicine, No.4 Teaching Hospital, China Medical University, Shenyang 110032, China
[c] Department of Ophthalmology, No.1 Teaching Hospital, China Medical University, Shenyang 110001, China
[d] Department of Gynecology, No.1 Teaching Hospital, China Medical University, Shenyang 110001, China
[e] Department of Gynecologic Oncology, Shengjing Hospital, China Medical University, Shenyang 110016, China
[f] Department of Dermatology, No.1 Teaching Hospital, China Medical University, Shenyang 110001, China
[g] Immune Therapeutics, Inc., 37 North Orange Avenue, Suite 607, Orlando, FL 32801, USA

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ABSTRACT

The morbidity and mortality associated with influenza A virus infections, have stimulated the search for novel prophylactic and therapeutic drugs. The purpose of this study was to investigate the prophylactic and therapeutic effect of synthetic methionine enkephalin (MENK) on mice infected by A/PR/8/34 influenza virus (H1N1) in vivo. The results showed that MENK could exert both prophylactic and therapeutic influences on infected mice, significantly improve the survival rate, relieve acute lung injury and decrease cytokine (IFN-α, IFN-β, TNF-α, IL-6, and IL-1β) levels. MENK also inhibited virus replication on day 4 post infection (p.i.) through upregulating opioid receptors (MOR, DOR) and suppressing TLR7-MyD88-TRAF6-NF-κB p65 signaling pathways. These results suggest that MENK, given via intranasal administration, could provide a novel drug with a new mode of action as a nonspecific anti-influenza agent or vaccine adjuvant.

1. Introduction

Influenza A virus (H1N1) is a major respiratory pathogen, but avian-origin influenza viruses, with their high lethality and potential to cause epidemics, is a bigger problem, and has captured the world's attention [1–3]. The mechanism underlying the recurrent epidemics is the evolution of the virus to escape the immunity induced by prior infections and vaccination [4]. The emergence of a new H7N9 avian influenza virus in 2013 has shown the limitation of current vaccines [5]. In addition, the current therapeutic drugs for influenza infection are limited, and some isolated influenza A virus subtypes, are resistant to available drugs [6–9]. Therefore, novel vaccine strategies not affected by viral adaptation or mutation, which would complement the vaccines and antiviral drugs are needed for better influenza control.

Although cytokine and chemokine secretion following infection could contribute to the elimination of influenza virus, excessive inflammatory response(s) can also result in serious pathological damage and mortality [10,11]. Studies have shown that an early recruitment of inflammatory cells, including macrophages, DC, and NK cells, followed by an inflammatory response, is the dominant factor resulting in acute respiratory disease after influenza A virus infection [12]. Toll-like receptors (TLRs) are the mediators in innate and adaptive immunity, controlling infections and activating inflammatory responses against pathogens. TLRs accomplish this by recognizing the molecular patterns specific to microorganisms [13,14]. TLR7 is a nucleotide-sensing TLR, activated by single-stranded RNA. It acts via MyD88 and TRAF, triggering NF-κB p65 activation, cytokine secretion and inflammatory responses [15].

MENK is a naturally occurring endogenous opioid peptide, which is cleaved from pro-enkephalin [16] and found primarily in the adrenal medulla. It has a role in regulating immune and neuroendocrine systems, and modulating functions of cells related to both the innate and adaptive immune systems via binding to opioid receptors [17]. As early as 1995, Burger RA used MENK to treat influenza virus A/NWS/33 (H1N1) infections, observing that MENK had an anti-influenza effect by upregulation of NK and CTL functions [18]. Our team has demonstrated

Abbreviations: DOR, δ-opioid receptors; IAV, influenza A virus; MENK, methionine enkephalin; MOR, μ-opioid receptors; MDCK, Madin-Darby canine kidney; NS, normal saline; p.i., post infection; PR8, influenza strains A/PR/8/34; Rib, ribavirin; TLRs, toll-like receptors

* Corresponding author.
E-mail address: fpshan@cmu.edu.cn (F. Shan).

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that MENK stimulates TLR4-Myd88-mediated signal transduction in DC cells. In light of these and other previous findings, we hypothesize that MENK has antiviral activity by inhibiting inflammatory responses following binding to opioid receptors. TLR7-MyD88-TRAF6-NF-κB p65 signaling pathways may be the potential targets for MENK's mechanism of anti-influenza virus (H1N1; PR8) activity. Therefore, the focus of these studies was to elucidate the mechanisms of action by MENK.

2. Materials and methods

2.1. Mice and virus

Female C57BL/6 mice (6–8 w) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in specific pathogen-free conditions in accordance with the guide for the Care and Use of Laboratory Animals (NIH Publication) and approved by Animal Use and Care Committee of China Medical University. The mouse-adapted influenza strain A/PR/8/34 (H1N1; PR8) was kindly provided by China Center for Disease Control and Prevention (Beijing, China). The virus was amplified in 10 d-old embryo chicken eggs as described previously [19].

2.2. Reagents

MENK (≥ 99% purity) was provided by America peptide. Inc. Ribavirin was purchased from Jinan Limin Pharmaceutical Co. Ltd. RNeasy mini kit was purchased from Qiagen. One Step SYBR® Prime Script™ RT-PCR Kit was purchased from TaKaRa. The mAbs of DOR (cat.no. ab63536), TLR7 (cat.no. ab45371), MyD88 (cat.no. ab 2068), TRAF6 (cat.no. ab13853), NF-κB p65 (cat.no. ab16502), β-actin (cat.no. ab16502) were purchased from Abcam, MOR (cat.no. NB100-1620) were purchased from Novusbio. Other chemicals frequently used in our laboratory were all from Sigma or Aldrich.

2.3. Infection and treatment of mice

Female C57BL/6 were assigned to five groups (12 mice/group): normal saline (NS) group, A/PR/8/34 influenza virus (PR8) model control, pre-MENK group, MENK treated group and ribavirin (Rib) treated group. Fig. 1 illustrates an outline of the mouse model. All mice were monitored daily for weight loss and survival for 14 consecutive days post infection (p.i.). Body weight loss > 30% was considered as the critical limit of experiment.

2.4. Histological examination and immunohistochemistry

On day 4 p.i., the lungs of mice in each group were collected. The left lobes were fixed in neutrally buffered 4% formaldehyde and then dehydrated, embedded in paraffin, and cut into 4 μm-thick sections stained with hematoxylin-eosin (HE). The right lobes were stored in liquid nitrogen for hemagglutination test (HA), and total RNA extract and protein were kept for Western blot analysis.

The tissue sections were immune-stained using streptavidin-biotin-horseradish peroxidase. The sections were de-paraffinized, rehydrated and then underwent antigen retrieval using citrate buffer (pH 6.0) at 98 °C for 20 min to unmask antigen epitopes. The sections were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase, incubated with 100 μl goat serum for 30 min at 37 °C, and then incubated with antibody MOR(1:200) (Novusbio, cat.no. NB100-1620)/DOR(1:100) (Abcam, cat.no. ab63536)/TLR7(1:50) (Abcam, cat.no. ab45371)/MyD88(1:200) (Abcam, cat.no. ab 2068)/TRAF6(1:100) (Abcam,cat.no. ab13853)/NF-κB p65 (1:500) (Abcam, cat.no. ab16502) overnight at 4 °C. The slides were rinsed with PBS and incubated with HRP-labeled secondary antibody (zsbio, cat.no. PV9001) at 37 °C for 20 min, DAB staining and then hematoxylin counterstain, dehydrated, and mounted. Stained cells were calculated by the number of positive pixels per area in 3 locations of each slide by Image J 1.42 software (National Institutes of Health, Bethesda, MD).

2.5. Hemagglutinin (HA) test

Lung tissue was homogenized to a 10% (w/v) suspension with sterilized PBS, and centrifuged at 12,000 × *g* for 10 min. The supernatant was serially diluted two fold with PBS, 50 μl added to each U-bottom well, 50 μl of 1% guinea pig red blood cells, then mixed and incubated at room temperature for 30 min. The final dilution that completely agglutinated red blood cells was considered the end-point of titration.

2.6. RNA isolation and qPCR analysis

RNA was extracted from lung homogenates (200 μl) and RNA isolated and purified using RNeasy (Qiagen, cat.no. 74104), according to manufacturer's protocol. qPCR was performed using One Step SYBR Prime Script RT-PCR Kit (TaKaRa, cat.no. RRO66A) with QuantStudio 6 Flex Real-time PCR system (ABI). qPCR reactions completed as follows: 5 min at 42 °C and 10 s at 95 °C, followed by 40 cycles-3 s at 95 °C, 30 s at 60 °C-and a melting curve step. Primer sequences are detailed in Table 1. Gene expression was quantified and normalized to GAPDH RNA expression using the $2^{-\triangle\triangle CT}$ method [20].

2.7. Western blot

Lung tissue was homogenized with lysis buffer containing 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Equal amounts of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane (120 mA, 120 min). Transferred proteins were incubated overnight with specific antibodies against TLR7(1:200)(Abcam, cat.no. ab45371)/MyD88(1:1000) (Abcam, cat.no. ab 2068)/TRAF6(1:500) (Abcam, cat.no. ab13853)/NF-κB p65(1:2000)(Abcam, cat.no.

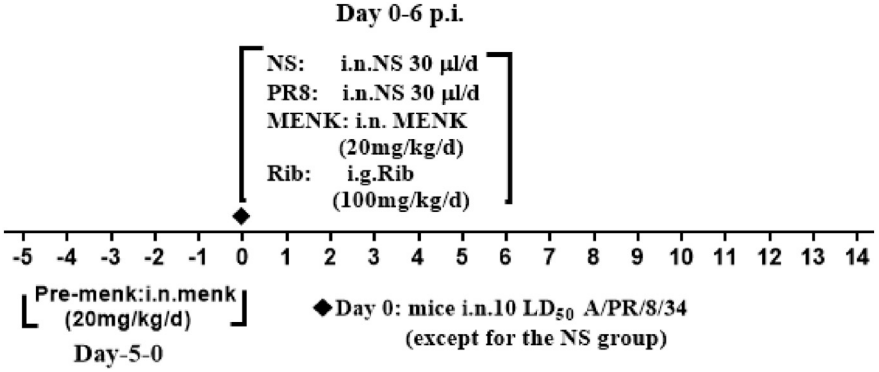

Fig. 1. Experimental design. Female C57BL/6 mice were anesthetized and infected via intranasal instillation (i.n.) with 10 LD50 of influenza A/PR/8/34 H1N1 virus (d0) except mice in NS group. The mice in pre-MENK group were treated via i.n. with 20 mg/kg MENK daily for 6 successive days (d-5-d0) prior to infection. The mice in MENK group were treated via i.n. with 20 mg/kg MENK daily for 7 successive days post infection (d0–d6 p.i.). The mice in normal control group were treated with equal volume of saline for successive 7 days, d0–d6 p.i.) and The mice in positive control group took Ribavirin (100 mg/kg/d for 7 days, d0–d6 p.i.) orally.