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Chemokine receptor antagonist block inflammation and therapy Japanese encephalitis virus infection in mouse model

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ABSTRACT

Japanese encephalitis (JE) is a viral encephalitis disease caused by infection with the Japanese encephalitis virus (JEV). The virus can cross the blood-brain barrier and cause death or long-term sequela in infected humans or animals. In this study, we first investigated the distribution of JEV infection in brain and further analyzed the dynamic change in inflammation related genes, chemokines, as well as pathological characteristics. Results demonstrated that CCR2 and CCR5 antagonist could significantly inhibit the inflammation. The mice treated with CCR2 and CCR5 antagonists had a higher survival rate between 60% and 70%, respectively. In summary, our study thoroughly illustrated the characteristics of the dynamic change in inflammation related genes and chemokines induced by JEV infection. We further indicated that CCR5 and CCR2 are potential targets for treatment of JE.

1. Introduction

Japanese encephalitis is a viral encephalitis disease caused by infection with the Japanese encephalitis virus (JEV). JEV is spread by mosquitoes and commonly infects humans, birds, horses, pigs, and other mammals [1]. The disease is mainly prevalent in tropical and subtropical environments in Asia-Pacific region [2]. According to the World Health Organization (WHO) survey (2016), there are about 3 billion people worldwide who are exposed to the risk of JEV infection [3,4]. JEV genome replicates following the virion entry into host tissues and cells. Once across the blood–brain barrier (BBB), JEV enters into central nervous system (CNS) and induces excessive inflammation, which leads to long-term sequelae or even host death. At the cellular level, JEV can infect both neuronal and glial cells. Microglial plays an essential role during JEV infection both in virus tropism and inflammatory respond. In addition to this, JEV can also infect the vascular endothelial cells as PIEC, organ endothelial cells as BHK-21 and other cells as A549. JEV has a wide tropism on different cells [5]. Vaccination is an available approach to control the disease [6,7]; however, due to some unfavorable factors, such as the low quality of some vaccines, insufficient immunization, and global warming, the annual incidence of JE is still on the rise [4,8].

Inflammation related genes and chemokines are important factors mediating inflammation in humans [9,10]. Inflammation related genes mainly include Retinoic Acid Inducible Gene-I (RIG-I), Tumor Necrosis Factor α (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Leukocyte Common Antigen (CD45), and Interferon (IFN), which are involved in the initiation and regulation of inflammation [11]. Chemokines are classified into four families, and two of them are related in the inflammatory response. The CCL families, including CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, and CCL26, and CXC families, including CXCL1, CXCL2, CXCL3, CXCL5, PPBP, CXCL9, CXCL10, CXCL11, and CXCL16, belong to inflammatory chemokines, which participate in the regulation of leukocyte migration [12]. The signature of these cytokines or genes can be used as indicators for evaluating JEV infection in the clinic. Furthermore, the cytokines and their receptors are targets for anti-inflammatory drugs [13,14].

Chemokines mediate the migration of immune cells into infection or

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injury sites, and are the key mediators in inducing inflammation. Viral inflammation is dependent on the participation of various chemokines [12]. For example, infection with (Hepatitis C virus) HCV causes a significant increase in CCL5 and MIP-1β in the liver [15]; infection with influenza virus upregulates CXCR3 and CCR5 in the lung [16,17]; infection with (West Nile Virus) WNV and (Semliki Forest virus) SFV also significantly upregulate CXCR3 and CCR2 related chemokines [11]. Uncontrolled inflammatory response is often the main cause of pathologies, secondary infection and death associated with viral infection. Therefore, receptors of proinflammatory cytokines and chemokines are potential targets for the development of novel anti-inflammatory drugs. For example, CCR2 and CCR5 specific, or dual specific antagonists are important therapies in hepatitis [18].

A few studies have investigated the correlation between JEV infection and inflammation related genes and chemokines in cells. The inflammation related genes and chemokines signature in living animals following JEV infection has not deeply investigated yet is not well known [19,20]. In the present study, we used C57BL/6 mice to study and characterize the dynamic changes in inflammation related genes and chemokines following JEV infection. We further used CCR2 and CCR5 antagonists to treat JE infection.

2. Materials and methods

2.1. Ethics statement

Animal experiments were designed according to the Guide for the Care and Use of Laboratory Animals. All animal experiments were reviewed and approved by the Animal Care and Use Committee of Nanjing Agricultural University (approval no. 200709005).

2.2. Virus and mice

JEV NJ2008 strain (Genbank No. GQ918133) was used for in study, which was propagated in BHK-21 cells and titrated in Vero cells. BHK-21 and Vero cells were cultured in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS).

6–8 weeks specific pathogen free (SPF) female C57BL/6 mice were used in this study, which were purchased from Shanghai SLAC Laboratory Animal Co., Ltd and maintained under pathogen-free conditions. Animal experiments were designed according to the Guide for the Care and Use of Laboratory Animals. All animal experiments were reviewed and approved by the Animal Care and Use Committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Science (Approval number 2016-0010).

2.3. Virus infection

C57BL/6 mice between 6 and 8 weeks were injected intraperitoneally (i.p.) with $4x10^6$ PFU of JEV suspended in phosphate buffered saline. All infected or mock infected mice were maintained under pathogen-free conditions in animal central of Shanghai Veterinary Research Institute.

2.4. Real-time PCR

The mice were sacrificed 1, 3, 6, 9, and 10 days after 4×10^6 PFU JEV was inoculated intraperitoneally and the brain tissues were collected. Briefly, the entire brain was removed and transferred into a precooled grinding bag or a 4% paraformaldehyde solution. And then 1 ml of PBS (containing protease and RNAse inhibitor) was added into each bag which made brain tissues quickly homogenized. The homogenate was divided into five parts, transferred into 1.5 ml centrifuge tubes with RNase and DNase free and stored at −80 °C.

For mRNA expression analysis, RNA was extracted from mice brain using TRIzol Reagent (Life Technologies). cDNA was immediately reverse-transcribed using RT Master Mix (TaKaRa, Tokyo, Japan). JEV or gene mRNA levels were detected by a SYBR real-time PCR. PCR was performed using an ABI Prism 7900 sequence-detection system (Applied Biosystems, Foster City, CA, USA), using SYBR Green PCR Master Mix. The amount of target gene expression was calculated from the respective standard curves and normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and displayed as fold change. Briefly, mock infected mice collected at the same time points were measured and set as 1. Then, JEV infected mice were calculated referenced to mock and presented as fold change. Relative gene expression to the control was determined by the standard $2^{-\Delta\Delta Ct}$ method. Mice chemokine primer was a kindly gift from Dr. Clive S. McKimmie and Dr. Gerard J. Graham.

2.5. Immunohistochemistry

Half mouse brains were removed from the skull and immediately fixed in a 4% paraformaldehyde (PFA) solution for 24 h. The brains were then transferred to 70% ethanol and embedded in paraffin. After that, sections were cut and rehydrated. Sections were then blocked with 20% horse serum and endogenous peroxidase blocked with 0.5% hydrogen peroxidase. Next, slides contained sections were incubated with the primary antibody and the secondary antibody. Slides were visualized by scanner at last. At least six mice in each group were detected; one representative mouse in each group was selected for figure presentation.

2.6. Chemokine antagonists administration

For comparison, we chose oral antagonist in the experiment. Chemokine receptor antagonists that target CCR2 and CCR5 were administered to JEV-infected mice. The CCR2 antagonist RS504393 (MedChemExpress) was given to mice orally twice daily at a concentration of 5 mg/kg of body weight/day in 300 µl PBS with 3.6% dimethyl sulfoxide (DMSO). RS504393 is a CCR2 antagonist with an IC50 of 98 nM. Chemical Name is: 6-Methyl-1′-[2-(5-methyl-2-phenyl-4-oxazolyl)ethyl]-spiro [4H-3,1-benzoxazine-4,4′-piperidin]-2(1H)-one. RS504393 selective inhibit CCR2b with IC50 concentration 98 nM and inhibit CCR1 with IC50 concentration > 100 µM. The CCR5 antagonist Maraviroc (MVC, MedChemExpress) was given to mice orally once daily at a concentration of 30 mg/kg of body weight/day in 300 µl PBS with 0.67% DMSO. MVC is a selective CCR5 antagonist with an IC50 of 6.4 nM. Chemical Name is: 4,4-Difluoro-N-[(1S)-3-[(3-exo)-3-[3-methyl-5-(1-methylethyl)-4H-1,2,4 -triazol-4-yl]-8-azabicyclo[3.2.1]oct-8-yl]-1-phenylpropyl]cyclohexanecarboxamide. MVC inhibits the downstream event of chemokine-induced intracellular calcium redistribution. The treatment of mice with all the antagonists started on dpi (days post infection) 2 and was administered until dpi 10 or until mice became ill. Mice infected with JEV were euthanized on PID 10.

Mice survival rate was also measured. Three groups of mice were used to analysis the role of RS504393 and MVC. 10 mice were randomly divided into each group. Mice were injected intraperitoneally (i.p.) with 4×10^6 PFU of JEV NJ2008. RS504393, MVC or DMSO control were administered from PID 2 to PID 10. Death amount was recorded.

2.7. Statistical analysis

All the measurements were conducted in triplicate in at least three independent experiments. Mean values ± standard deviation (SD) was calculated using Microsoft Excel. Statistical analysis was done by Student's t test or log rank test and values were considered significant when $p < 0.05$. Figures were made using the GraphPad™ Prism 5.0 software.