Biochemical and Biophysical Research Communications 409 (2011) 717–722

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Paradoxical effects of chondroitin sulfate-E on Japanese encephalitis viral infection

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ARTICLE INFO

Article history:
Received 2 May 2011
Available online 20 May 2011

Keywords:
Chondroitin sulfate E
Japanese encephalitis virus
Neural cell

ABSTRACT

Glycosaminoglycans (GAGs) have diverse functions in the body and are involved in viral infection. The purpose of this study was to evaluate the possible roles of the E-disaccharide units GlcAβ1–3GalNAc(4,6-*O*-disulfate) of chondroitin sulfate (CS), a GAG involved in neuritogenesis and neuronal migration, in Japanese encephalitis virus (JEV) infection. Soluble CS-E (sCS-E) derived from squid cartilage inhibited JEV infection in African green monkey kidney-derived Vero cells and baby hamster kidney-derived BHK cells by interfering with viral attachment. In contrast, sCS-E enhanced viral infection in the mouse neuroblastoma cell line Neuro-2a, despite the fact that viral attachment to Neuro-2a cells was inhibited by sCS-E. This enhancement effect in Neuro-2a cells seemed to be related to increased viral RNA replication and was also observed in a rat infection model in which intracerebral coadministration of sCS-E with JEV in 17-day-old rats resulted in higher brain viral loads than in rats infected without sCS-E administration. These results show the paradoxical effects of sCS-E on JEV infection in different cell types and indicate that potential use of sCS-E as an antiviral agent against JEV infection should be approached with caution considering its effects in the neuron, the major target of JEV.

1. Introduction

Japanese encephalitis virus (JEV) is a member of the Flaviviridae family that is transmitted primarily by *Culex tritaeniorhynchus* mosquitoes. Although most JEV infections in humans are asymptomatic, clinical cases tend to manifest as severe, often fatal encephalitis. Neurons are the major target of JEV in the brain [1].

Proteoglycans (PGs), which are composed of glycosaminoglycans (GAGs) and core proteins, are major components of the cell surface and the extracellular matrix. Chondroitin sulfate (CS)-PG constitutes the major population of GAGs in the central nervous system (CNS). CS-PG plays important roles in the developing and adult CNS with respect to the regulation of cell proliferation, differentiation, neuronal migration, neurite extension, neural plasticity, and axonal regeneration [2].

Recently, many researchers have been interested in the relationships between GAGs and viral infection. Some viruses use cell-surface PGs as their receptors [3–7], and virus clearance involves extracellular and cell surface PGs in the bloodstream [8]. However, the role of CS in flavivirus infection, especially within the CNS, remains uncertain.

The purpose of this study was to investigate the influence of soluble CS-E rich in GlcAβ1–3GalNAc(4,6-*O*-disulfate) unit, on JEV infection in a neural cell line and in animal brains.

2. Material and methods

2.1. Cells, viruses, and reagents

Neuro-2a (mouse neuroblastoma) cells were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS). Baby hamster kidney (BHK-21) cells were grown in DMEM supplemented with 10% FCS. African green monkey kidney (Vero) cells were grown in Minimum Essential Medium (Nissui, Tokyo, Japan) supplemented with 5% FCS. The JEV Nakayama strain was kindly provided by Dr. Ikuo Takashima (Hokkaido University, Sapporo, Japan) and was cloned by three rounds of plaque purification. Soluble CS (sCS)-A, -B, -C, -D, and -E were purchased from Seikagaku Corp. (Tokyo, Japan). Heparin was purchased from Sigma (St. Louis, MO).

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doi:10.1016/j.bbrc.2011.05.072

2.2. Immunofluorescence

Cells were fixed with methanol–acetone (1:1, v/v) for 30 min, washed with phosphate-buffered saline (PBS), and blocked with 2% bovine serum albumin (BSA)–PBS. Cells were incubated with rabbit anti-JEV serum in 1% BSA–PBS at room temperature for 30 min, followed by incubation with Alexa-594-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA). The cell nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). Fluorescence was observed with an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan). The number of JEV antigen-positive cells was counted by using MetaMorph software (MDS Analytical Technologies, Toronto, Canada).

2.3. Focus reduction assay

A focus reduction assay was performed as described by Aoki et al. [9] with modifications. Briefly, 40 focus-forming units (FFUs) of JEV were premixed with 10-fold serial dilutions of sCS-E and incubated at 4 °C for 30 min. The virus–sCS-E mixtures were then inoculated into BHK cells seeded in 96-well plates. After 2 h of incubation at 37 °C, overlay medium (DMEM containing 1% FBS and 0.5% methyl cellulose) was added to each well. The overlay medium was removed after 24 h of incubation at 37 °C, and the cells were washed with PBS. The cells were fixed with methanol–acetone (1:1, v/v) for 30 min, washed with PBS, and blocked with 1% BSA–PBS. Cells were incubated with rabbit anti-JEV serum in 2% BSA–PBS at room temperature for 30 min, followed by incubation with HRP-conjugated anti-rabbit IgG antibody (Nichirei, Tokyo, Japan). Infectious foci were detected with 3,3′-diaminobenzidine (DAB; Nichirei).

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described by Avirutnan et al. [10] with modifications. Maxi-Sorp microtiter plates (Nunc, Roskilde, Denmark) were adsorbed with soluble GAG preparations (500 μg/mL in PBS) at 4 °C overnight. After three washes with Tris-buffered saline containing Tween-20 (TBST), the plates were blocked with Block Ace (Yukijirushi Nyugyo Inc., Tokyo, Japan) for 2 h at 37 °C and washed three times with TBST. Purified virus (1×10^6 PFU/mL) in 10% FBS–TBST was added to each well and incubated for 1 h at 37 °C. Plates were washed three times with TBST and incubated for 1 h with rabbit anti-JEV serum in 5% BSA–PBS at 37 °C for 30 min. The plates were incubated with HRP-conjugated secondary antibody (Nichirei). After three washes with PBS, signal was detected by adding *O*-phenylenediamine dihydrochloride substrate (OPD; Sigma) in citrate buffer (pH 5.0). The enzymatic reaction was stopped by adding 1.25 M H_2SO_4. The absorbance at 490 nm was measured by using a microplate reader (BioRad, Hercules, CA).

2.5. Flow cytometry

Cells were resuspended with cell-dissociation buffer (Invitrogen, Carlsbad, CA) and washed twice with PBS. Virus and soluble CS mixtures were incubated at 4 °C for 30 min, added to dissociated cells, and incubated at 4 °C for 1 h. Cells were washed twice with PBS and fixed with 1% paraformaldehyde–PBS for 10 min. After washing three times with PBS, the cells were stained for 30 min with rabbit anti-JEV serum and incubated with Alexa-488-conjugated anti-rabbit IgG (Invitrogen). All labeling steps were performed on ice. Cells were analyzed with a FACS Canto system (BD Biosciences, San Diego, CA).

2.6. Real-time RT-PCR

Total RNA was extracted from cells with TRIzol (Invitrogen). One microgram of total RNA was reverse-transcribed by using the SuperScript® III First-Strand Synthesis system (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed with QuantiFast Probe PCR kits (QIAGEN, Valencia, CA) with the CFX96™ real-time PCR detection system (BioRad). The JEV primer and probe sequences used are as follows: JEV forward, 5′-AGAACGGAAG AYAACCATGA CTAAA-3′; JEV reverse, 5′-CCGCGTTTCA GCATATTGAT-3′; and JEV probe, FAM-5′-ACCAG-GAGGG CCCGG-3′-MGB [11]. Endogenous control primer and probe for mouse β-actin and 18S ribosomal RNA were purchased from Applied Biosystems (Foster City, CA).

2.7. Animal experiments

In all experiments, 17-day-old F344/NSIc rats (SLC Inc., Shizuoka, Japan) were used. Under anesthesia with isoflurane (Dainippon Pharmaceutical, Osaka, Japan), virus or a mixture of virus and sCS-E was administered intracerebrally to two groups of rats through the use of 27-gauge one-stop needles (Top Corp., Tokyo, Japan). At 4 days postinoculation, three rats from each group were sacrificed under deep anesthesia, and the brains were collected for virus titration. The viral titers of brain homogenates were determined through the use of Vero cells. At 6 days post-inoculation, the brains were collected for immunohistochemistry. All animal experimentation was performed in accordance with institutional guidelines, and ethical permission was obtained from the Hokkaido University Animal Care and Use Committee.

2.8. Immunohistochemistry

Brains were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, and sectioned at 3 μm. The sections were dewaxed, treated with 3% hydrogen peroxide in methanol, and blocked with 10% normal goat serum. Rabbit anti-JEV serum was added, and the sections were incubated overnight at 4 °C. The sections were washed with PBS and incubated with biotinylated goat anti-rabbit IgG (Nichirei Biosciences, Tokyo, Japan) for 10 min at room temperature. After further washing with PBS, the sections were incubated with streptavidin-conjugated peroxidase for 10 min at room temperature. The bound peroxidase was detected with DAB (Nichirei). The sections were counterstained with haematoxylin.

2.9. Statistical analysis

Data sets were compared by a 2-tailed, unpaired *t*-test. Statistical significance was achieved when the *p*-values were <0.05. Error bars indicate the standard deviation.

3. Results

3.1. sCS-E inhibits JEV infection of Vero and BHK cells

Many researchers have used soluble GAGs to study the interaction between GAGs and viruses [5–7,12]. To determine which types of CS preparations can influence JEV infection, we incubated the virus with various types of soluble CS preparations at 4 °C for 30 min and then inoculated Vero cells with each mixture. After 48 h, infected cells were detected by using an indirect immunofluorescence assay for JEV antigen. Among the soluble CS preparations examined, only sCS-E effectively inhibited JEV infection (Fig. 1A). This inhibitory effect of sCS-E was also observed in the JEV infection of BHK cells (Fig. 1B). The focus reduction assay on BHK cells