Materials and Methods

Cell culture

Baby hamster kidney (BHK) cell line was grown at 37 °C in Eagle’s minimum essential medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 8% fetal calf serum (FCS; BioWest, Nuaillé, France). SH-SY5Y cell line, derived from a human neuroblastoma, was grown at 37 °C in Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FCS.

Virus preparation

The recombinant viruses of tick-borne encephalitis virus (TBEV) Oshima 5-10 strain or Sapporo-2017 strain (GenBank accession no. AB062063.2, LC440459.1, respectively) (Takahashi et al., 2020; Takashima et al., 1997) were recovered from infectious cDNA clones (Oshima-ICs, Sapporo-ICs) as previously described (Takahashi et al., 2020; Takano et al., 2011). Working stocks of the viruses were propagated once in BHK-21 cells and stored at −80 °C. Experiments using live TBEVs were conducted in Bio-safety Level 3 (BSL-3) facilities located at the Graduate School of Veterinary Medicine at Hokkaido University.

Antibodies

Rabbit anti-TBEV antibody was prepared by immunization with recombinant E proteins expressed in the pET43 system (Novagen, Madison, WI, USA) (Yoshii et al., 2004). Alexa Fluor 647 Anti-MAP2 [EPR19691] and Rabbit anti-MLKL (phospho S358) antibodies were purchased from abcam (Cambridge, MA, USA). Rabbit anti-GFAP, anti-Caspase 1/p20/p10, anti-GSDMD, anti-MLKL antibodies were purchased from Proteintech (Tokyo, Japan). Rabbit anti-Iba-1 antibody was purchased from FUJIFILM Wako Pure Chemical Corporation. Rabbit anti-Cleaved Caspase3, anti-RIP, and anti-RIP3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-β-actin and anti-GAPDH antibodies were purchased from Medical & Biological Laboratories (Nagoya, Japan).

Mouse inoculation with TBEV

Mouse were infected intracerebrally according to a previously described method (Takahashi et al., 2020). Briefly, 50 plaque forming units (p.f.u.) of TBEV or the same volume of PBS was injected intracerebrally into female BALB/c mouse (5 weeks old; Japan SLC, Shizuoka, Japan). Mouse were sacrificed at 1, 4, or 7 days post infection (d.p.i). The left brain of these mouse was collected in formalin for fixation, and the fixed brain tissues were embedded in paraffin. To determine the virus titer in mouse brain homogenate (10% w/v), we performed plaque assays as described previously (Takahashi et al., 2020). For titration, BHK cell monolayers prepared in 12-well plates were incubated with serial dilutions of viral suspensions medium containing 2% FCS and 1.5% carboxymethyl cellulose, and then incubated for 3 days. The cells were fixed and stained with crystal violet (0.25% in 10% buffered formalin) to visualize plaques. Finally, plaques were counted, and viral titer was presented as p.f.u.

All animal experiments were performed at the Animal Bio-safety level 3 (ABSL-3) facility located at the Graduate School of Veterinary Medicine at Hokkaido University in accordance with institutional guidelines, and ethical permission was obtained from the Hokkaido University Animal Care and Use Committee (19-0142).

Histological analysis

For ordinal pathological analysis, sections were stained with hematoxylin and eosin. For immunohistochemical (IHC) analysis, sections were subjected to antigen retrieval by pressure cooker in the presence of 10 mM sodium citrate buffer. Subsequently, sections were washed in PBS, blocked with 10% normal goat serum for 10 min, and incubated with primary antibodies overnight at 4 °C. After washing with PBS, sections were incubated with rabbit biotinylated secondary antibodies for 1 hour at room temperature and visualized by Histofine diamino benzidine substrate (Nichirei, Tokyo, Japan) as described previously (Kobayashi et al., 2012).

For immunofluorescence analysis, sections were incubated with primary antibody, washed and incubated with secondary antibody (goat anti-rabbit Alexa Fluor 555; Thermo Fisher sicientific, Waltham, MA, USA). Then, incubated with Alexa Fluor 647 Anti-MAP2 antibody (abcam). Coverslips were applied using 90% Glycerol in PBS.

Histological evaluation

The slides visualized by fluorescence antibodies were evaluated for cell morphology and microtubule associated protein 2 (MAP2) fluorescence intensity. The size of MAP2 positive cells in PBS-infected mouse was set as 100%, morphological score in the mouse inoculated with TBEV was evaluated ranging from 1 to 4 (1, <60% of MAP2 positive cell size in brain of control mouse; 2, 60% ~ 70% ; 3, 70% ~ 80%; 4, 80%<). MAP2 fluorescence intensity was measured by using ZEN 3.1.0 software (Carl Zeiss, Jena, Germany).

The number of GFAP-positive cells or Iba-1-positive cells in the cerebral cortex were counted in the five fields of three different mice inoculated with TBEV or PBS. The fluorescence area of GFAP-positive cells was measured by using Image J software (https://imagej.nih.gov/ij/index.html).

Cell death assay

SH-SY5Y cells were inoculated with TBEV (1 p.f.u./cell), and the cells were incubated for 24, 48, or 72 h. The dead or live cells were stained by propidium iodide (PI) and Hoechst 33258 (Dojindo, Kumamoto, Japan), respectively. The stained cells were fixed with 4% paraformaldehyde for 10 min before washing with PBS. The number of dead cells was counted using Image J software (https://imagej.nih.gov/ij/index.html).

For inhibition of necroptosis, SH-SY5Y cells inoculated with TBEV were treated with necrostatin-1s (Cell Signaling Technology) before staining by PI and Hoechst 33258.

Immunoblotting

SH-SY5Y cell line infected with each strain of TBEV at MOI of 1 were lysed at 48 hpi in RIPA buffer (150 mM NaCl, 1% Triton, 0.1% SDS, 0.5% Sodium deoxycholate, 50 mM Tris, pH 8.0) with protease inhibitor (nacalai tesque, Kyoto, Japan) and phosphatase inhibitor cocktail 2 and 3 (Sigma, St. Louis, MO, USA). The cell lysates were then separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) filter (Merck Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in TBS-T, incubated overnight with each primary antibody at 4°C, and the immune complexes were detected with HRP-conjugated secondary antibodies and Immobilon Western Chemiluminescence HRP Substrate (Merck Millipore). Chemiluminescence was visualized using the ChemiDoc XRS+ Imager (Bio-Rad, Hercules, CA, USA), and the obtained images were analyzed using the Image Lab Software (Bio-Rad) as previously described (Kobayashi et al., 2020).

Statistical analyses

Data are expressed as means ± standard deviation. Data between two independent groups were analyzed using two-tailed Student’s *t*-test and data among multiple independent groups were analyzed via one-way ANOVA with Tukey-Kramer test, Scheffe's F-test, or Steel-Dwass test. A p-value of <0.05 was considered significant and indicated by (\*), and p-value of <0.01 was indicated by (\*\*).

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