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1 Necroptosis of neuronal cells is related to the neuropathology of tick-borne encephalitis

2

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17

18 Keywords

19 tick-borne encephalitis virus, programmed cell death, necroptosis

20

21 Abstract

22 Tick-borne encephalitis virus (TBEV) is a zoonotic virus that causes tick-borne encephalitis (TBE)

23 in humans. Infections of Sapporo-17-Io1 (Sapporo) and Oshima 5-10 (Oshima) TBEV strains

24 showed different pathogenic effects in mice. However, the differences between the two strains are

25 unknown. In this study, we examined neuronal degeneration and death, and activation of glial cells

26 in mice inoculated with each strain to investigate the pathogenesis of TBE. Viral growth was similar

27 between Sapporo and Oshima, but neuronal degeneration and death, and activation of glial cells, was

28 more prominent with Oshima. In human neuroblastoma cells, apoptosis and pyroptosis were not

29 observed after TBEV infection. However, the expression of the necroptosis marker, mixed lineage

30 kinase domain-like (MLKL) protein, was upregulated by TBEV infection, and this upregulation was

1 more pronounced in Oshima than Sapporo infections. As necroptosis is a pro-inflammatory type of
2 cell death, differences in necroptosis induction might be involved in the differences in
3 neuropathogenicity of TBE.
4
5 Tick-borne encephalitis virus (TBEV), a member of the genus *Flavivirus* and the family
6 *Flaviviridae*, causes tick-borne encephalitis (TBE) and long-term neurological sequelae (Gritsun et
7 al., 2003). TBEV is prevalent throughout northern Eurasia, including Europe, Russia, Far East Asia,
8 and Japan (Gritsun et al., 2003; Takahashi et al., 2020; Takashima et al., 1997). The global incidence
9 of TBE exceeds 10,000 patients per year (Takahashi et al., 2020). TBEV causes central nervous
10 system (CNS) infection with clinical manifestations ranging from mild biphasic fever to severe
11 encephalitis and meningoencephalitis (Dumpis et al., 1999; Ecker et al., 1999). It has a mortality rate
12 of 0.5–30% (Gritsun et al., 2003). Although there are several effective vaccines, there is no specific
13 therapeutic treatment for TBE (Andersson et al., 2010; Morozova et al., 2014; Petry et al., 2021).
14
15 TBEV is primarily transmitted to humans by the *Ixodes* sp., but is also occasionally acquired by the
16 consumption of unpasteurized dairy products from infected livestock (Balogh et al., 2010;
17 Brockmann et al., 2018). The virus causes encephalitis, along with neuronal cell degeneration and
18 death, glial cell activation, and immune cell infiltration (Pokorna Formanova et al., 2019). TBEV
19 mainly targets the CNS and causes neuronal cell death through two pathways: direct induction of
20 programmed cell death or neuronal injury through an uncontrolled inflammatory response involving
21 glial cytokines and chemokines (Hayasaka et al., 2009; Ruzek et al., 2009a; Ye et al., 2013).
22 However, the details of these pathways and underlying molecular mechanisms are still largely
23 unknown.
24

1 Programmed cell death, including apoptosis, pyroptosis, and necroptosis, plays a vital role in
2 maintaining homeostasis, and in anti-viral responses (Okamoto et al., 2017; Wen et al., 2021).
3 Apoptosis is tightly controlled cell death designed to avoid inflammation (Fricker et al., 2018;
4 Galluzzi et al., 2018). Pyroptosis and necroptosis, which are programmed forms of necrosis, cause
5 membrane disruption and allow the release of pro-inflammatory and immunogenic cellular contents
6 (Frank and Vince, 2019; Fricker et al., 2018; Galluzzi et al., 2018; McKenzie et al., 2020; Mocarski
7 et al., 2015; Vanden Berghe et al., 2014). It has been reported that TBEV infection triggers apoptosis
8 and pyroptosis, while other flavivirus infection, such as West Nile virus or Japanese encephalitis
9 virus infection, induces apoptosis, pyroptosis, and necroptosis (Bian et al., 2017; Cheung et al.,
10 2018; Fares et al., 2020; Fares et al., 2021; He et al., 2020; Kobayashi et al., 2012; Lim et al., 2017;
11 Okamoto et al., 2017; Ruzek et al., 2009b; Wang et al., 2020; Wen et al., 2021). It is important to
12 elucidate the relationship between programmed cell death and TBEV infection to understand the
13 neuropathogenesis of TBE.

14

15 We previously reported differences in pathogenicity among TBEV strains with high homology
16 (Takahashi et al., 2020), which is important for understanding TBE pathogenesis. Therefore, in this
17 study, we performed histopathological analysis of mouse brains infected with different strains, and
18 found differences in neuronal degeneration and death, as well as glial activation. We found that the
19 induction of necroptosis in cultured cells was different between the two strains.

20

21 Recombinant TBEV Oshima 5-10 and Sapporo-17-Io1 strains (GenBank accession nos. AB062063.2
22 and LC440459.1, respectively) (Takahashi et al., 2020; Takashima et al., 1997), which belong to
23 different sub-clusters of the Far-eastern subtype, were recovered from infectious cDNA clones as
24 previously described (Takano et al., 2011; Takashima et al., 1997). Working stocks of the virus were

1 propagated once in BHK-21 cells. Experiments using live TBEV were conducted in Biosafety Level
2 3 (BSL-3) facilities located at the Graduate School of Veterinary Medicine, Hokkaido University.
3
4 The mice were intracerebrally inoculated with TBEV using a previously described method
5 (Takahashi et al., 2020). Either 10 μ L of virus stock containing 50 plaque forming units (pfu) of
6 TBEV or the same volume of PBS was injected intracerebrally into female BALB/c mice (5 weeks
7 old; Japan SLC, Shizuoka, Japan). All animal experiments were performed at the Animal Biosafety
8 Level 3 (ABSL-3) facility of the Graduate School of Veterinary Medicine, Hokkaido University in
9 accordance with institutional guidelines. Ethical approval was obtained from the Hokkaido
10 University Animal Care and Use Committee (19-0142). The mice were sacrificed at 1, 4, or 7 days
11 post inoculation (dpi), and viral titers in the brain were measured using plaque assays, as described
12 previously (Takahashi et al., 2020). The virus was not detected in the brains at 1 dpi and the viral
13 titers were similar between brains inoculated with the Sapporo and Oshima strains at both 4 and 7
14 dpi (Fig. 1a). The left brains' halves of TBEV-inoculated mice were collected in formalin, fixed, and
15 then embedded in paraffin. The sections were stained with hematoxylin and eosin for
16 histopathological analysis. For immunohistochemical (IHC) analysis, the sections were subjected to
17 antigen retrieval using a pressure cooker in the presence of 10 mM sodium citrate buffer. The
18 histopathological analysis of inoculated brains showed cell shrinkage and neuronophagia in regions
19 close to the TBEV-antigen-positive cells in the cerebral cortices (Fig. 1b). Although the number of
20 viral antigen-positive cells was almost the same, cell shrinkage was more frequent in Oshima-
21 compared to Sapporo-inoculated mice (Fig. 1b). This suggested that mice infected with the Oshima
22 strain were more susceptible to cell degeneration and death. As the viral growth properties of the two
23 strains were not different, the difference in cell degeneration and death was not related to viral
24 growth.

1
2 Next, we analysed the morphologies of TBEV-infected neurons. It has been reported that a decrease
3 in fluorescence intensity of neuronal cell markers and cell shrinkage are associated with neuronal
4 cell degeneration and death (Clarke et al., 2021). The neurons were visualized using the Alexa Fluor
5 647 Anti-MAP2 antibody (Abcam, Cambridge, UK) and evaluated for cell morphology and
6 fluorescence intensity using ZEN 3.1.0 software (Carl Zeiss AG, Oberkochen, Germany). Based on a
7 previous study, MAP2-positive cells in PBS-inoculated mice were used as controls to obtain the
8 morphological scores of TBEV-inoculated mice (range: 1–4; 1, severe shrinkage; 2, moderate
9 shrinkage; 3, mild shrinkage; 4, almost the same as control) (Petry et al., 2021). Fluorescence
10 intensity and morphological scores were lower in viral antigen-positive cells compared to controls,
11 and the reduction was more marked in Oshima-inoculated cells (Fig. 1c–e). This suggested that
12 Oshima inoculation induced a greater degree of neuronal cell degeneration and death than Sapporo
13 inoculation.

14
15 Astrocytes and microglia contribute to immunity and inflammation in the CNS (Barres, 2008; Nayak
16 et al., 2014; Nedergaard et al., 2003; Sofroniew and Vinters, 2010). We examined the activation of
17 astrocytes and microglia after inoculation with each strain to determine the differences in
18 encephalitis caused by Sapporo and Oshima infections. We counted the number of glial fibrillary
19 acidic protein (GFAP, astrocyte marker)-positive cells and ionized calcium-binding adapter molecule
20 1 (Iba-1, microglia marker)-positive cells in the cerebral cortices in five fields of three different
21 mice, inoculated with TBEV or PBS. The number of GFAP- and Iba-1-positive cells was greater in
22 TBEV- compared to PBS-inoculated mice (Fig. 2a, b, d, and e). The increase was more marked in
23 Oshima- than Sapporo-inoculated cells (Fig. 2b and e). As astrocyte hypertrophy implies activation
24 (Sticozzi et al., 2013; Zhou et al., 2001), the area of GFAP-positive cells in the cerebral cortices of

1 TBEV-inoculated mice was measured and compared to that of PBS mice using ImageJ software
2 (<https://imagej.nih.gov/ij/index.html>). The size of GFAP-positive cells in Oshima-inoculated brains
3 was larger compared to Sapporo-inoculated brains (Fig. 2c). Activated microglia have an amoeboid
4 morphology, with larger cell bodies and fewer dendrites compared to normal microglia (Nayak et al.,
5 2014; Subramanyam et al., 2019). We counted the Iba-1-positive amoeboid cells. Few amoeboid
6 Iba-1-positive cells were observed in PBS-inoculated brains. However, the number of these cells was
7 increased significantly in Oshima- compared to Sapporo-inoculated brains (Fig. 2f). This suggested
8 that the Oshima strain induced more neuroinflammation than the Sapporo strain.

9 We previously reported that Oshima infection was more pathogenic for mice than Sapporo infection
10 (Takahashi et al., 2020). It was suggested that the neuropathogenesis of TBEV infection in mice was
11 associated with neuronal cell death and neuroinflammation.

12

13 It is known that the type of cell death affects the induction of inflammation (Frank and Vince, 2019;
14 Mocarski et al., 2015). Differences in the degeneration and death of neuronal cells, and
15 inflammatory responses, in the brain were observed between Sapporo and Oshima infections.

16 Therefore, we analysed the types of programmed cell death caused by each TBEV strain. Human
17 neuroblastoma SH-SY5Y cells were inoculated with Sapporo or Oshima at a multiplicity of infection
18 of 1, and then stained with propidium iodide (PI) to explore the extents to which the TBEVs induce
19 cell death. Few PI-positive cells were detected at 24 hpi (Fig. 3a). The PI-positive rate was
20 significantly higher in the cells infected with TBEV as compared to control cells and the increase
21 was more evident in Oshima-infected cells than in Sapporo-infected cells at 48 hpi (Fig. 3a). At 72
22 hpi, many control cells were PI-positive; the PI-positive proportion of TBEV-infected cells was
23 higher than that of control cells (Fig. 3a). TBEV-infected SH-SY5Y cells were lysed at 48 hpi in
24 RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris;

1 pH 8.0) with a protease inhibitor (Nacalai Tesque, Kyoto, Japan) and phosphatase inhibitor cocktail
2 2 and 3 (Sigma-Aldrich, St. Louis, MO, USA). The expression of the apoptosis marker, cleaved-
3 caspase 3, was undetectable in the cells infected with TBEV (Fig. 3b). Then, we examined caspase 1
4 and gasdermin D (GSDMD), which are markers of pyroptosis, and no difference was observed
5 between control and TBEV-inoculated cells (Fig. 3c). Next, the expression levels of mixed lineage
6 kinase domain-like (MLKL), phosphorylated MLKL (p-MLKL), RIP, and RIP3 were examined;
7 these proteins are markers of necroptosis. The MLKL, p-MLKL, and RIP3 levels were increased by
8 TBEV inoculation, and the increase was greater in Oshima- than Sapporo-infected cells (Fig. 3d and
9 e). Subtle RIP upregulation was observed in TBEV-inoculated cells as compared to control; a greater
10 increase was apparent in Oshima-inoculated cells (Fig. 3d). Addition of a necroptosis inhibitor
11 (necrostatin-1s; Cell Signaling Technology, Beverly, MA, USA), decreased the PI-positive
12 proportion of TBEV-infected cells (Fig. 3f). Furthermore, MLKL-positive cells were found in mouse
13 brains inoculated with the Oshima (Fig. 3g). These results suggested that necroptosis, and not
14 apoptosis or pyroptosis, was induced by TBEV infection, and that Oshima infection induced
15 necroptosis more strongly than Sapporo infection.

16

17 In this study, we found that the incidence rates of neuronal degeneration and death, and
18 inflammation with glial activation, differed between TBEV strains. Furthermore, the occurrence of
19 necroptosis also differed between strains. TBEV strains more pathogenic for mice induced
20 necroptosis more strongly than stains with lower pathogenicity. This suggested that necroptosis was
21 important for its neuropathogenesis.

22

23 Since cytopathic effects and morphological characteristics of cell death were observed in TBEV-
24 infected cultured cells, it appears that TBEV infection directly induces neuronal cell death (Gelpi et

1 al., 2005). However, previous studies demonstrated that excessive immune responses were induced
2 by TBEV and other flaviviruses, which may cause neuronal cell death (Ghoshal et al., 2007;
3 Hayasaka et al., 2009; Luo et al., 2018; Ruzek et al., 2009a). In this study, we demonstrated that
4 necroptosis was induced in cultured human neuroblastoma cells infected with TBEV, suggesting that
5 TBEV infection could induce necroptosis of neuronal cells without any influence of external factors.
6 In the neuropathogenesis of TBE, viral infection induces necroptosis directly, and necroptosis might
7 then activate glial cells, leading to encephalitis.

8
9 Elucidation of its neuropathogenesis may lead to the development of effective therapies for TBEV
10 infection. Comparison of the two TBEV strains with high amino acid homology may lead to the
11 elucidation of molecular mechanisms of necroptosis induced by TBEV, and aid identification of
12 potential therapeutic targets for TBE.

13

14 Author statements

15 Authors and contributions

16 D.T.: validation, formal analysis, investigation, data curation, write – original draft preparation,
17 visualization. K.Y.: write – review and editing, supervision, funding. M.K.: validation, formal
18 analysis, investigation, data curation. Y.T.: resources, write – review and editing. N.M.:
19 investigation, data curation. H.K.: resources, write – review and editing. S.K.: conceptualization,
20 methodology, resources, write – review and editing, supervision, funding.

21

22 Conflicts of interest

23 The authors declare that there are no conflicts of interest.

24

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12

13 Ethical approval

14 All animal experiments were performed following the basic guidelines for animal experiments of the
15 Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The President of
16 Hokkaido University approved all animal experiments after review by the Institutional Animal Care
17 and Use Committee of Hokkaido University (approval no. 19-0142).

18

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27

28 Figure legends

29 Fig. 1. Cell degeneration and death in mouse brains induced by TBEV infection. Oshima induced
30 more cell degeneration and death than Sapporo.

31 (a) Virus titers in mouse brains were determined using plaque assay. The brains were weighted and
32 homogenised (10% w/v suspension in PBS). The data are presented as mean \pm SD. The Student's t

1 test revealed no significant differences between the two strains.

2 (b) The serial sections were stained by hematoxylin and eosin (upper panels). Immunohistochemistry
3 was performed for the TBEV E-protein (lower panels). Arrows indicate representative degenerative
4 cells. (c) Double staining with TBEV E-protein (green) and MAP2 (red). (d) Relative MAP2
5 fluorescence intensity was measured. MAP2 fluorescence intensity was measured in eight
6 fields/mouse (n = 3 and n = 1; for Sapporo- and Oshima-inoculated mice, and a control mouse,
7 respectively). The data are presented as mean + SD; Tukey-Kramer test, **p < 0.01. (e)
8 Morphological score of TBEV-infected neurons. Scores were based on the size of the clear area
9 around TBEV-positive neurons. Scores ranged from 1 to 4 (1, < 60% of the MAP2-positive cell area
10 of controls; 2, 60–69%; 3, 70–79%; 4, ≥ 80%). Scoring was performed using a ×20 objective lens.
11 Morphological scores were derived for eight fields/mouse (n = 3 and n = 1; Sapporo- and Oshima-
12 inoculated mice, and a control mouse, respectively). The data are presented as a box plot. Tukey-
13 Kramer test, **p < 0.01.

14

15 Fig. 2. Astrocytes and microglia were activated by TBEV infection in vivo. Oshima caused greater
16 glial activation than Sapporo.

17 (a) Representative micrographs showing the immunohistochemistry of brain sections stained for
18 GFAP. Arrows indicate hypertrophic cells. (b) The number of GFAP-positive cells in cerebral
19 cortices was counted in high-power fields. (c) The relative sizes of GFAP-positive cells were
20 measured using a fluorescent secondary antibody by ImageJ. (d) Representative micrographs
21 showing the immunohistochemistry of brain sections stained for Iba-1. Arrows indicate the
22 amoeboid form of microglia. (e, f) The number of Iba-1-positive cells (e) and Iba-1-positive
23 amoeboid cells (f) in the cerebral cortices was counted in high-power fields. Data are presented as
24 mean +SD; Tukey-Kramer test, **p < 0.01.

1

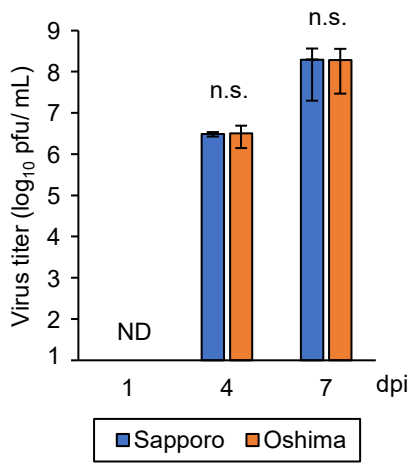
2 Fig. 3. TBEV infection induced necroptosis in the SH-SY5Y cell line rather than apoptosis or
3 pyroptosis.

4 (a) The death of the SH-SY5Y cells infected with TBEV. PI-positive cells were counted at 24, 48,
5 and 72 h. Data are presented as mean +SD; Scheffe's F test, ** $p < 0.01$ and * $p < 0.05$. (b) CASP3 (a
6 marker of apoptosis) expression was analyzed. (c) CASP1 and GSDMD (markers of pyroptosis)
7 expression were analyzed. (d) MLKL, p-MLKL, RIP and RIP3 (markers of necroptosis) expression
8 levels were analyzed. (e) The relative band intensities of MLKL normalized to that of the internal
9 control were measured ($n = 4$). Data are presented as mean +SD; Steel-Dwass test, * $p < 0.05$. (f) The
10 necroptosis inhibitor necrostatin-1s (10 or 5 mM) prevented cell death after TBEV infection. Data
11 are presented as mean +SD; Scheffe's F test, ** $p < 0.01$ and * $p < 0.05$. (g) Brains inoculated with
12 the control or either TBEV strain were immunostained for MLKL. Arrowheads indicate MLKL-
13 positive cells.

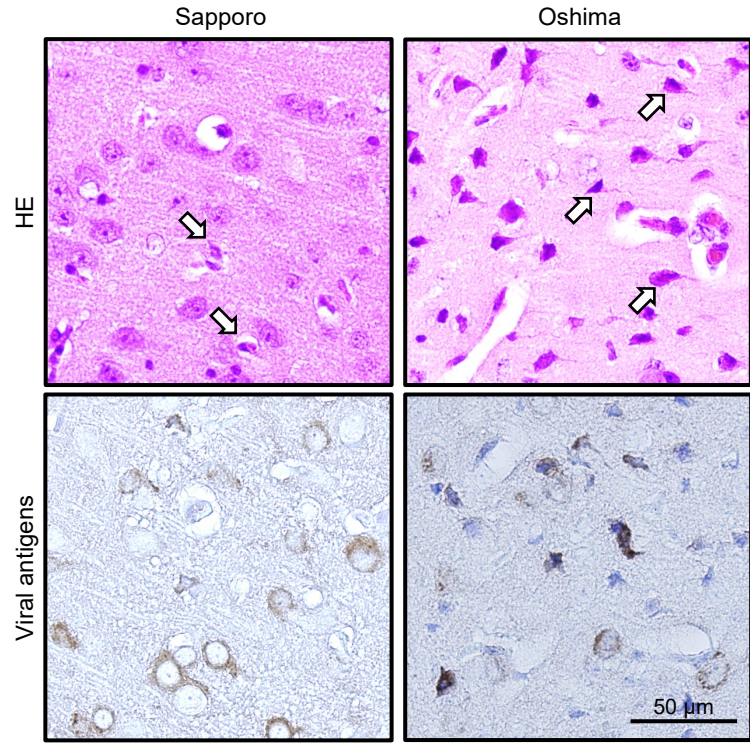
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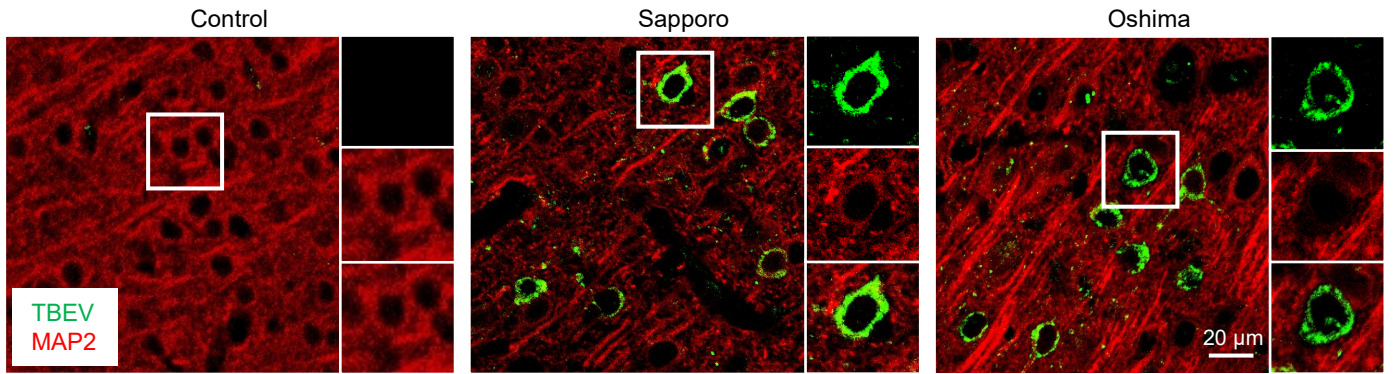
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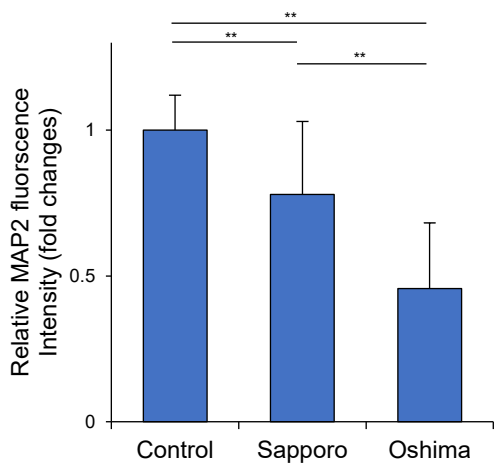
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