Morphological changes in human neural cells following tick-borne encephalitis virus infection

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Tick-borne encephalitis (TBE) is one of the leading and most dangerous human viral neuroinfections in Europe and north-eastern Asia. The clinical manifestations include asymptomatic infections, fevers and debilitating encephalitis that might progress into chronic disease or fatal infection. To understand TBE pathology further in host nervous systems, three human neural cell lines, neuroblastoma, medulloblastoma and glioblastoma, were infected with TBE virus (TBEV). The susceptibility and virus-mediated cytopathic effect, including ultrastructural and apoptotic changes of the cells, were examined. All the neural cell lines tested were susceptible to TBEV infection. Interestingly, the neural cells produced about 100- to 10 000-fold higher virus titres than the conventional cell lines of extraneural origin, indicating the highly susceptible nature of neural cells to TBEV infection. The infection of medulloblastoma and glioblastoma cells was associated with a number of major morphological changes, including proliferation of membranes of the rough endoplasmic reticulum and extensive rearrangement of cytoskeletal structures. The TBEV-infected cells exhibited either necrotic or apoptotic morphological features. We observed ultrastructural apoptotic signs (condensation, margination and fragmentation of chromatin) and other alterations, such as vacuolation of the cytoplasm, dilatation of the endoplasmic reticulum cisternae and shrinkage of cells, accompanied by a high density of the cytoplasm. On the other hand, infected neuroblastoma cells did not exhibit proliferation of membranous structures. The virions were present in both the endoplasmic reticulum and the cytoplasm. Cells were dying preferentially by necrotic mechanisms rather than apoptosis. The neuropathological significance of these observations is discussed.

INTRODUCTION

Tick-borne encephalitis virus (TBEV) is the most medically important member of the tick-borne group of the genus Flavivirus within the family Flaviviridae (Thiel et al., 2005). The virus represents a causative agent of tick-borne encephalitis (TBE), a severe infection of the central nervous system (CNS) (Gritsun et al., 2003), which is prevalent throughout wide areas in Europe and Asia.

The nature of the host cell response to TBEV infection remains undefined. Characteristic, but not disease-specific neuropathologic changes in the CNS include meningitis and polioencephalomyelitis predominantly in the spinal cord, brainstem and cerebellum associated with inflammatory cell infiltration. These pathological changes have been described in mice (Osetowska & Wróblewska-Mularczyk, 1966; Růžek et al., 2009), hamsters (Simon et al., 1966), monkeys (Simon et al., 1967) and humans (Seitelberger & Jellinger, 1966; Környey, 1978; Beer et al., 1999; Schellinger et al., 2000; Gelpi et al., 2005, 2006). Although cytopathic effects (CPEs) have been observed primarily in virus-infected neurons, other cells can also exhibit pathological changes, presumably through bystander injury.

To clarify the interaction of TBEV with neural cells, we examined the TBEV infection in three human cell lines: neuroblastosma (neuronal cells arising from neural crest element of the sympathetic nervous system), glioblastoma (glial cells with the function to support and protect the surrounding neurons) and medulloblastoma (tumour cells from posterior fossa formed from poorly developed cells originating from immature or embryonal cells at their earliest stage of development). TBEV is known to be highly neurotropic; however, this is the first time that human cells of neural origin have been used in TBE research.

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Neuroblastoma, glioblastoma or primary cultures from embryonic or neonatal mice or rats have been used as valuable models for research on many other neurotropic viruses, e.g. poliovirus, herpes simplex virus type 1, Japanese encephalitis virus (JEV), a neurovirulent variant of dengue virus (DENV), rabies virus, West Nile virus (WNV), cytomegalovirus, Sindbis virus, etc. (Ubol & Griffin, 1991; Liao et al., 1997; Despres et al., 1998; Couderc et al., 2002; Yang et al., 2004; Hoever et al., 2005; Chhabra et al., 2007; Luo & Fortunato, 2007). Due to the inherent difficulty of obtaining and working with primary human neural cultures, much work is being done with the established cell lines that are considered to be good models for the investigation of viral neuropathogenesis (Yang et al., 2004).

We investigated the production of viral particles, the propagation of the infection in cell cultures and the strength of both the CPE and the induction of apoptosis. We described virus morphogenesis and CPEs during TBEV infection of three human neural cell lines at an ultrastructural level.

METHODS

**Viruses and cells.** The TBEV strain Hypr, a member of the European subtype, was originally isolated from the blood of a 10-year-old child diagnosed with TBE in 1953 in the Czech Republic (Pospíšil et al., 1954).

Cell lines of neural origin (kindly provided by Professor Tomáš Eckschlager, 2nd School of Medicine, Charles University, Prague, Czech Republic) comprised human neuroblastoma (UKF-NB-4), medulloblastoma (DAOY) and glioblastoma cells. The cells were grown at 37 °C in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% fetal calf serum (FCS) and 1% mixture of penicillin and streptomycin (Sigma). The neuroblastoma cell line UKF-NB-4 was established from metastasis of bone marrow cells harvested from a relapse patient with Evans stage IV neuroblastoma (Cinatl et al., 1990). The medulloblastoma cell line was derived from a cerebellar medulloblastoma in 1985 (Jacobsen et al., 1985).

Porcine kidney stable (PS) cells (Kozuch & Mayer, 1975) were grown at 37 °C in L-15 medium supplemented with 3% newborn calf serum and 1% mixture of penicillin and streptomycin (Sigma).

**Virus growth in neural and PS cells.** Monolayer cultures of the cells grown in 96-well plates were inoculated with virus diluted in small volumes of culture medium, giving a m.o.i. of 1 p.f.u. per cell. After 1 h at 37 °C, free virus was removed by serial washing with PBS and finally 0.2 ml of medium was added to each well. The virus-mediated CPE was examined using light microscopy. At 24, 48, 72, 96 and 120 h post-infection (p.i.), supernatant media from appropriate wells were collected and frozen at −70 °C. The titres were determined by plaque assay.

**Plaque assay.** Virus titres were assayed on PS cell monolayers, as described previously (De Madrid & Porterfield, 1969). Tenfold dilutions of virus were placed in 24-well tissue culture plates and PS cells were added in suspension (0.6–1.5 × 10⁶ cells per well). After incubation for 4 h, the suspension was overlaid with carboxymethylcellulose (1.5% in L-15 medium). After incubation for 5–6 days at 37 °C and 0.5% CO₂, the infected plates were washed with PBS and cell monolayers were stained with naphthalene black. Infectivity was expressed as p.f.u. ml⁻¹.

**Immunofluorescence staining.** The virus- and non-infected cells on slides were subjected to cold methanol fixation for 10 min, rinsed in PBS and blocked with 10% goat serum, 2% BSA in 0.05% (v/v) PBS and Tween 20. Cells were double-labelled with TBEV mouse monoclonal antibody 19/75 (Niedrig et al., 1994) and rat monoclonal anti-z tubulin (10 μg ml⁻¹; AbD Serotec). After washing with PBS/Tween 20, the cells were labelled with goat secondary antibodies conjugated with FITC or Cy3 (10 μg ml⁻¹; Jackson ImmunoResearch). The cells were counterstained with DAPI (1 μg ml⁻¹; Sigma) for 15 min at room temperature, mounted in 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) and examined with an Olympus BX-51 fluorescence microscope equipped with an Olympus DP-70 CCD camera.

**Apoptosis detection assay.** To determine the apoptotic DNA degradation in TBEV-infected neural cells, the method of terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) was used. Virus- (m.o.i. of 1) and mock-infected cultures grown on slides were stained using the in situ apoptosis detection kit (TaKaRa) at 48 h p.i., following the manufacturer’s protocol. Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer and left at room temperature for 30 min. Stained samples were examined by fluorescence microscopy.

**Transmission electron microscopy (TEM).** Virus- (m.o.i. of 1; 48 h p.i.) and mock-infected cells were fixed directly in culture dishes with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4 °C and harvested 24 h later. Cells were washed in 0.1 M phosphate buffer with 4% glucose, embedded in 2% agar at 60 °C, post-fixed in 1% osmium tetroxide and dehydrated in a graded series of acetone. Samples were infiltrated and polymerized at 60 °C in Polybed 812 resin (Polysciences). Ultrathin sections were cut on a Ultracut UCT ultramicrotome (Leica Microsystems) using a diamond knife. Sections were transferred to formvar-coated grids (SPI Supplies), counterstained with uranyl acetate and lead citrate and finally carbon coated. Specimens were examined by TEM in a JEOL (Tokyo) 1010 at accelerating voltage of 80 kV, equipped with a MegaView III camera (SIS).

**Scanning electron microscopy (SEM).** Samples (cells grown on glass coverslips) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (48 h p.i. with an m.o.i. of 1), washed, post-fixed in 0.1% osmium tetroxide, then dehydrated using the graded series of aceton and dried in a critical point drier. Finally, samples were coated with gold and observed using a JEOL JSM 7401-F scanning electron microscope (Tokyo).

**RESULTS**

**CPE**

The CPE produced by TBEV on the neural cells, which includes the rounding up and aggregation of cells and cell death, was evaluated using light microscopy and SEM. For each neural cell line, there was a noticeable CPE, which was most obvious after 48 h p.i., as identified by light microscopy (Fig. 1). The most significant CPE was observed in neuroblastoma cells infected with TBEV after 48 h. In glioblastoma and medulloblastoma cell lines, the CPE was less advanced after 48 h compared with neuroblastoma cells. A clear difference between virus- and non-infected cells was observed.
SEM investigation provided a more detailed insight into the alterations of the cell morphology associated with TBEV infection. We observed clear structural alteration of the shape and surface of the cells (Fig. 1a, b). The surfaces of infected cells were contaminated by debris which originated from necrotic cells. Apoptosis was characterized by the rounding of cells (Fig. 1b).

**Virus growth in neural cells**

We evaluated and compared TBEV replication efficiency and the growth kinetics of TBEV in neuroblastoma, medulloblastoma, glioblastoma and PS cells (Fig. 1c). Virus replication was efficient in the neural cell lines, reaching titres of $10^7$ to $>10^9$ p.f.u. ml$^{-1}$, which is a 100-
to 10 000-fold higher titre than in conventional cell lines (i.e. PS) of extraneural origin. These results demonstrated the highly susceptible nature of neural cells to TBEV infection.

**Apoptosis**

To determine whether TBEV infection induces an apoptotic response in neural cells, the cells infected at an m.o.i. of 1 with strain Hypr were analysed by using the TUNEL assay to detect the presence of chromosomal DNA cleavage, an event that indicates apoptosis. Approximately, 20% of glioblastoma and medulloblastoma cells were TUNEL-positive at 48 h p.i. Apoptotic changes in the infected cells were confirmed by electron microscopy (see below). In neuroblastoma cells, approximately 20% of the cells were apoptotic; however, using electron microscopy, necrotic cells were observed more often than apoptotic ones. In uninfected neural cells, less than 1% TUNEL-positive cells were apoptotic after 48 h, which served as a negative control for apoptosis (Fig. 1d).

**Rearrangement of the cytoskeleton induced by viral replication**

We investigated the intracellular distribution of α-tubulin and E protein in infected and uninfected neural cells using indirect immunofluorescence microscopy. Most of the infected cells of neural origin underwent changes in microtubule organization and cell shape (Fig. 2).

Network assemblies of microtubules were only seen in uninfected control neural cells (Fig. 2a, e and i), whereas tubulin networks in infected cells were disrupted and partially degraded. Viral replication was detected mainly in the perikaryon region of the cell cytoplasm and was also locally distributed in other parts of the cell cytoplasm (Fig. 2d, h and l). Interestingly, the most pronounced areas of viral replication were surrounded by a dense net of tubulin.

**Ultrastructural changes in TBEV-infected neural cell lines**

In infected medulloblastoma and glioblastoma cells, the early cellular alteration induced by virus development was a disorganization of the rough endoplasmic reticulum (RER). We observed virions and numerous smooth membrane structures (SMS) inside the cisternae of the RER as the first dominant morphological signs of virus development (Fig. 3b).

Several cells contained areas with a focal or generalized dilation of the RER, while some cells possessed an abundant proliferation of RER cisternae (Figs 3d, e and 4c, d), followed by ribosome degranulation. An observed example of RER alteration was a spiral formation of endoplasmic reticulum (ER) cisternae enclosing ribosomes together with virions and SMS (Fig. 3c). In the areas of prolific irregular RER (Fig. 4f), other atypical membrane modifications and cell abnormalities, such as enlargement of the Golgi apparatus (GA; Fig. 4b), the presence of irregular structures in the mitochondria and enlargement
of cristae (Figs 3d and 4d), were observed. Infected cells contained numerous phagosomes/phagolysosomes containing debris originating from necrotic cells together with virions, as well as many autophagosomes and lysosomes (Figs 3f, i and 4b).

A portion of infected medulloblastoma and glioblastoma cells displayed early features of apoptosis, such as changes in nuclear morphology (the condensation, margination and fragmentation of chromatin to the nuclear membrane; Figs 3f and 4e). High cytoplasmic density was seen in late stage apoptotic cells together with cell shrinkage and changes in cell shape (Figs 1b, 3j, 4g, j and k). Although the virus replicated very efficiently in these cells, virus particles were rarely seen and only in greatly enlarged ER cisternae (Figs 3m and 4j). In these cells, we observed numerous SMS enclosed in well-preserved and concentrically arranged cisternae of the RER (Fig. 3k), which locally lacked ribosomes. In the later stages of apoptosis, we observed various cytoplasmic alterations, such as the

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**Fig. 3.** Glioblastoma cells visualized by TEM. Uninfected cells (a) and cells infected with TBEV (b–n) are shown, which illustrate several features. (b) The presence of SMS and a virion (black arrow) inside RER cisternae with ribosomes almost detached (white arrow). (c) The RER cisternae arranged in a concentric array that encloses the SMS. (d) Glioblast cytoplasm containing lysosomes (Ly), mitochondria with enlarged inner space and proliferated, disorganized ER cisternae that contain several virions (arrows). (e) Detailed view of (d) showing the presence of SMS and many virions (black arrows) inside an irregular and dilated RER cisternae. (f) Glioblast with signs of autophagy (boxed views are shown in g–i). (g, h) Virions inside the RER (arrows). (i) A part of cytoplasm containing enlarged ER cisternae filled with virions, phagosomes and autophagic vacuoles (AV) encapsulating virions and the bulk of the cytoplasm. (j) Glioblast displaying cytoplasmic condensation and cell shrinkage, and containing phagosomes and autophagosomes. An enlarged view of the boxed region (k) shows coiled and well-preserved RER cisternae that accommodate the SMS. (l) Apoptotic rounded cell with condensed chromatin and DNA fragmentation. (m) Peripheral membrane blebbing created by enlarged RER cisternae with virion remnants. (n) Apoptotic bodies (AB) phagocytosed by neighbouring glioblasts.
presence of autophagosomes (Fig. 3i), protein aggregation in the cytoplasm (Fig. 4g), membrane blebbing (Figs 3m and 4e, h and k) and apoptotic bodies that were phagocysed by surrounding cells (Fig. 3n).

In neuroblastoma cells, the TBE infection had the most dramatic effect on the ultrastructural organization of cells. General patterns of the ultrastructural changes were different from the medulloblastoma and glioblastoma cells. Virions were seen predominantly in dilated cisternae of the ER; however, a number of enveloped viral particles were seen in the cytoplasm, mostly associated with microtubular structures (Fig. 5c, d). SMS inside the ER, as well as concentrical disorganization of the ER, were rarely present. Several cells displayed necrotic and apoptotic features (Fig. 5b).

**DISCUSSION**

Despite the importance of TBE infection, the underlying basis for the development of encephalitis remains undefined. Although neurons are the primary target for encephalitic flaviviruses, we demonstrated that glioblastoma and medulloblastoma cells cultured in vitro are also TBEV-sensitive and the virus titre produced by these cells is similar to neuroblastoma-derived cells. Although there is scant evidence for infection of glial cells in vivo, oligodendrocytes and astrocytes are permissive to infection with other flaviviruses, e.g. WNV or JEV, under in vitro conditions (Liu et al., 1988; Suri & Banerjee, 1995; Chen et al., 2000; Jordan et al., 2000).

We have described the cell alterations caused by TBE viral replication on an ultrastructural level. In medulloblasts and...
glioblasts, we observed similar structural abnormalities formed by membrane modifications such as the formation of SMS, ER proliferation, accumulation and convolution of membranes, which are in accordance with typical cell abnormalities described for other flavivirus infections (Matsumura et al., 1971; Ko et al., 1979; Leary & Blair, 1980; Ho et al., 1987; Ng & Hong, 1989; Chu & Westaway, 1992; Ng et al., 1994b). SMS is a typical flavivirus-induced structure associated with RER (Hase, 1993; Wang et al., 1997) that is probably formed through budding from the RER.

Chu & Ng (2003) described that the mechanism of cell death for cells infected with WNV is dependent on the initial infectious dose. However, we observed cells dying by both apoptotic and necrotic mechanisms in the same culture, i.e. after the same infectious dose of TBEV (m.o.i. of 1). The ratio of necrotic to apoptotic cells differed depending on the cell lines used.

Taken together, we have described here that TBEV induces a proliferation of the membranous system in both glioblast and medulloblast cell lines but not in neuroblastoma cells. The induction of membrane rearrangement has also been described in other flavivirus infections. Similar dilation of RER with the presence of numerous virions and SMS-like particles was described in cultured mouse neurons and BHK-21 cells infected by JEV (Hase, 1993; Hase et al., 1993; Takegami et al., 1995; Wang et al. 1997). SMS and smooth convoluted membranes have been described as the site of virus replication in Kujin virus-infected Vero cells (Westaway et al., 1997; Mackenzie et al., 2001).

In all three neural cell lines, extensive rearrangement of cytoskeletal architecture was observed at 48 h p.i. There are several reports that the host microtubular structure is directly involved in the movement of both enveloped and non-enveloped DNA and RNA viruses (Greber & Way, 2006), while actin filaments are involved in budding of several viruses, including WNV (Sarafend) (Chu et al., 2003). Interestingly, we observed that there was a dense net of microtubulin in the areas where viral E protein was accumulated, suggesting that the host cytoskeleton is functionally important in the maturation process of the virus. More importantly, we observed the direct association of enveloped viral particles with microtubules in infected neuroblastoma cells (Fig. 5c, d). However, the role of the cytoskeletal network in TBEV replication still remains largely unknown and is worth investigating in the future.

Generally, flavivirus-infected cells exhibit two modes of maturation, termed cis and trans (Hase et al., 1989). In trans mode maturation, virus particles are assembled in the RER and associated membraneous structures. Mature virions are then transported by the host secretory channel to the plasma membrane. cis mode maturation is characterized by the assembly of nucleocapsids in the cytoplasm. Either cis or trans modes of maturation have been shown in several flaviviruses (e.g. WNV, DENV-2 and TBEV), depending on the particular virus strain and cells used (Leary & Blair, 1980; Hase et al., 1987; Ng, 1987; Ng & Hong, 1989; Ng et al., 1994a, 2001; Mackenzie et al., 1999; Mackenzie & Westaway, 2001; Ng & Chu, 2002; Chu & Ng, 2002; Šenigl et al., 2006). Šenigl et al. (2006) described that TBEV exhibits a trans mode of maturation in mammalian cells, but a cis mode in tick cells. This difference in the maturation process could be associated with the different characteristics of CPEs observed in vertebrate and invertebrate (tick or mosquito) cells infected with flaviviruses (Zhang et al. 1993; Šenigl et al., 2006; Růžek et al., 2008). However, the maturation of TBEV observed in neuroblastoma cell lines cannot be defined as trans or cis. The virions were present in dilated RER, suggesting that the RER is the site of virion assembly and maturation. The enveloped viral particles were present in the cytoplasm, mostly associated with the trans-Golgi network.
with microtubuli, suggesting that the mature virions are transported independent of the secretory pathway of the cell. This interesting phenomenon will be investigated in more detail in our future studies.

It was demonstrated previously that TBEV causes apoptosis in mouse and monkey brain neurons (Isaeva et al., 1998; Kamalov et al., 1998). By contrast, no prominent signs of neuronal apoptosis were seen in post-mortem brain tissues from human TBE patients, as demonstrated by anti-caspase 3 immunohistochemistry and TUNEL assay (Gelpi et al., 2006b). Recently, it was observed that host CD8+ T cells infiltrating brain parenchyma in TBEV-infected mice significantly contribute to the fatal outcome of infection, indicating the immunopathological basis of the disease (Růžek et al., 2009). Here, we proved by TUNEL assay and direct electronmicroscopy that human neural cells infected with TBEV were dying by apoptosis, but necrosis was also observed and represented the main type of dying by TBEV-infected neuroblastoma cells. This is in correlation with our observations that a number of neurons from TBEV-infected mice brains exhibit morphological signs of necrosis (Růžek et al., 2009).

Apoptosis in all three lines occurred within 48 h p.i., which is in accordance with the kinetics observed in neuroblastoma cells and neurons infected with Langat virus and WNV or a neurovirulent variant of DENV (Despres et al., 1998). As demonstrated in studies on DENV, the apoptotic process in infected cells could be generated by the accumulation of viral proteins in the ER (Marianneau et al., 1997).

TBE seems to be a complex issue, where immunopathology as well as neuronal necrosis and/or apoptosis take place. An understanding of these phenomena are crucial for the prevention and/or treatment of neurological sequelae mediated by TBEV in humans. Thus, an effective control of TBEV-mediated cell death may be useful in treating patients with TBE. The seriousness of the infection is also determined by other factors, such as the infectious dose, age of the patient, neurovirulence and neuroinvasiveness of the particular TBEV strain (Růžek et al., 2008).

Based on the results described herein, three clinical implications may be addresed: (i) the fact that TBEV replicates more efficiently in neural cells than in cells of extraneural origin may help to explain why TBEV tends to be involved in infections of the CNS rather than other organs; (ii) ultrastructural differences observed in neuroblastoma and glioblastoma/medulloblastoma cells may help to explain why the virus prefers neurons rather than glial cells for its replication in vivo; and finally, (iii) observed apoptosis as well as necrosis of neural cells following TBEV infection indicate that these mechanisms play an important role in the development of encephalitis in addition to immunopathology. Future investigations of specific apoptosis signalling cascades activated in TBEV-infected neural cells may clarify these interpretations.

Moreover, we have shown that the human neural cell lines used in this project are a suitable and promising model for the subsequent studies of TBEV neuropathogenesis, including the study of virus–cell interactions on a molecular level, the cellular response, molecular basis of neurotropism and the neurobiology of the infection.

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