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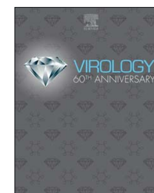

Outer nuclear membrane fusion of adjacent nuclei in varicella-zoster virus-induced syncytia

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ABSTRACT

Syncytia formation has been considered important for cell-to-cell spread and pathogenesis of many viruses. As a syncytium forms, individual nuclei often congregate together, allowing close contact of nuclear membranes and possibly fusion to occur. However, there is currently no reported evidence of nuclear membrane fusion between adjacent nuclei in wild-type virus-induced syncytia. Varicella-zoster virus (VZV) is one typical syncytia-inducing virus that causes chickenpox and shingles in humans. Here, we report, for the first time, an interesting observation of apparent fusion of the outer nuclear membranes from juxtaposed nuclei that comprise VZV syncytia both in ARPE-19 human epithelial cells *in vitro* and in human skin xenografts in the SCID-hu mouse model *in vivo*. This work reveals a novel aspect of VZV-related cytopathic effect in the context of multinucleated syncytia. Additionally, the information provided by this study could be helpful for future studies on interactions of viruses with host cell nuclei.

1. Introduction

Syncytia formation is a hallmark of infection *in vitro* and/or *in vivo* by numerous viral pathogens including varicella-zoster virus (VZV) (Cheatham et al., 1956; Esiri and Tomlinson, 1972; Nikkels et al., 1995; Tyzzer, 1906; Weigle and Grose, 1983), human cytomegalovirus (HCMV) (Gerna et al., 2016), respiratory syncytial virus (RSV) (Sarmiento et al., 2007; Bitko et al., 2003) and human immunodeficiency virus (HIV) (Compton and Schwartz, 2017; Frankel et al., 1996; Koenig et al., 1986; Pumarola-Sune et al., 1987; Rinfret et al., 1991), and may play an important role in virus spreading and pathogenesis.

Varicella-zoster virus (VZV) is one typical syncytia-inducing virus that causes varicella (chickenpox) during primary infection and may reactivate in later life to cause herpes zoster (shingles) (Cohen et al., 2007; Gilden et al., 2000). Induction of syncytia formation by VZV is commonly seen in skin lesions and in trigeminal ganglia upon VZV reactivation from latency (Cheatham et al., 1956; Cohen et al., 2007; Esiri and Tomlinson, 1972; Gilden et al., 2000; Nikkels et al., 1995; Tyzzer, 1906; Weigle and Grose, 1983). In recent years, the role of syncytia formation in VZV pathogenesis have been investigated using the SCID-hu mouse model grafted with human skin or dorsal root ganglia (DRG) (Reichelt et al., 2008; Yang et al., 2014). It has been shown that canonical regulation of syncytia formation is critical to support the skin pathogenesis of VZV *in vivo* (Yang et al., 2014). Moreover, syncytia formation between neurons and satellite cells can lead to extensive neuronal damage, which may be a potential factor for post herpetic neuralgia (PHN) (Reichelt et al., 2008). Overall, the results from these studies suggest that syncytia formation is crucial for the pathogenesis of VZV-associated skin and neurological diseases.

Syncytia formation typically occurs at a late stage of infection (Gerna et al., 2016; Yang et al., 2014). As the cell membranes fuse post-infection, individual nuclei often gather together with the cytoplasm at the exterior of the nuclear cluster (Harson and Grose, 1995), thus bringing nuclear membranes of adjacent nuclei in close proximity, maybe as a step leading to fusion. Recently, replication of some syncytia-inducing viruses has been reported in association with distinct changes in the nuclei of host cells, including dilation of nuclear pores and nuclear envelope breakdown (Klupp et al., 2011; Leuzinger et al., 2005; Maric et al., 2014). To date, however, there is still no report of nuclear membrane fusion between adjacent nuclei in wild-type virus-induced syncytia.

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Here, we report, for the first time, an interesting observation of apparent fusion of the outer nuclear membranes (ONMs) between adjacent nuclei in VZV-induced multi-nucleated syncytia both in ARPE-19 cells *in vitro* and in human skin xenografts in the SCID-hu mouse model *in vivo*.

2. Materials and methods

2.1. Ethics statement

Human fetal skin tissues were obtained from spontaneous pregnancy losses (18–20 gestational weeks [g. w.]) at the Affiliated Zhongshan Hospital of Xiamen University. The study protocol was approved by the Institutional Review Board of the hospital and the Research Ethics Committee of Xiamen University. The methods were carried out in accordance with the guidelines and regulations of Xiamen University. Informed consent for use of the fetal tissues for research was obtained from the donating parents in accordance with the institutional guidelines.

2.2. Cells and viruses

ARPE-19 cells were grown in DMEM with 10% fetal calf serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). The wild-type parental Oka strain (pOka; GenBank Accession No. AB097933) of VZV was propagated in ARPE-19 cells. Cell-free virus of pOka was prepared as described previously (Selariu et al., 2012) with slight modifications. Briefly, pOka-infected ARPE-19 cells were harvested when the cytopathic effect (CPE) reached > 80% and were subsequently resuspended in cryoprotective solution (Beijing Wantai Co., LTD, Beijing, China) and stored in −80 °C. Cell suspensions were then slowly thawed with vigorous shaking. Next, the obtained viral samples were frozen for 2 h at −40 °C, lyophilized for 16 h at −25 °C, brought to 25 °C at a speed of 10 °C per hour and dried at room temperature for 6 h. The obtained lyophilized powder of cell-free pOka was resuspended in phosphate buffered saline (PBS) and stored in aliquots in liquid nitrogen. Titers were determined by infectious focus assay in ARPE-19 cells before use.

2.3. Immunofluorescence and confocal microscopy

ARPE-19 cells were seeded in 24-well tissue culture plates preplaced with one circular cover glass per well and then infected with pOka at an MOI of 0.1 plaque-forming unit (PFU)/cell. After incubation at 37 °C in 5% CO2 for four to seven days, cells on the cover glasses were fixed with paraformaldehyde for 30 min, permeabilized with 0.3% Triton X-100 in PBS for 10 min and blocked for 1 h with goat serum. Cells were then incubated with a mouse monoclonal antibody (mAb) against VZV glycoprotein E (gE) [clone 4A2; described in our publication (Liu et al., 2015)], or ORF7 [clone 8H3; described in our publication (Jiang et al., 2017)] for 1 h and fluorescein isothiocyanate (FITC)- or tetramethylrhodamine (TRITC)-labeled goat anti-mouse secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Cell nuclei were counterstained with 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen, Carlsbad, CA, USA), and cells on the cover glasses were observed using confocal microscopy (MRC-1024, Bio-Rad, Hercules, CA, USA).

2.4. Infection of SCID-hu mice model

Human fetal skin tissues were implanted on each side under the flank skin of 4- to 6-week-old male CB-17 SCID mice. At 2 weeks post-transplantation, the implants were surgically exposed and inoculated with ARPE-19-derived cell-free virus of pOka at 10,000 PFU. Mock-infected controls were inoculated with equal amount of uninfected ARPE-19 cell lysate. At 21 days post-infection, the implants were harvested and prepared for transmission electron microscopy (TEM) analysis. All procedures were carried out in accordance with the approved animal use protocols of Xiamen University Laboratory Animal Center.

2.5. Histopathology and immunohistochemistry

Paraffin sections (5 µm thick) of mock- and pOka-infected human skin xenografts were deparaffinized in xylene and rehydrated in graded alcohols. Then, sections were either stained with hematoxylin and eosin (H & E) or used for immunohistochemical (IHC) analysis. IHC was performed as previously described (Wang et al., 2016). Briefly, sections were subjected to heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0) and endogenous peroxide quenching in 3% hydrogen peroxide. Then, sections were blocked with 10% normal goat serum and incubated with a mouse mAb against VZV gE (clone 4A2) or ORF7 (clone 8H3). Next, immunohistochemical staining was performed using an Ultrasensitive TMS-P kit (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) and a DAB detection kit (streptavidin-biotin; Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) according to the manufacturer's instructions. Finally, sections were counterstained with hematoxylin, dehydrated and cover-slipped.

2.6. Transmission electron microscopy

Skin xenografts and ARPE-19 cells infected with pOka or not were prepared for TEM analysis as previously described (Selariu et al., 2012). Briefly, samples were fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4), postfixed with 1% osmium tetroxide, and incubated in 1% aqueous uranyl acetate overnight. After dehydration through graded ethanol, samples were embedded with an Embed 812 kit (Electron Microscopy Sciences, Fort Washington, PA). Subsequently, ultrathin sections (50–80 nm) were stained with 3.5% aqueous uranyl acetate and 0.2% lead citrate. The sections were analyzed using a Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, OR, USA).

3. Results and discussion

First, monolayer cultured ARPE-19 cells were infected at an MOI of 0.1 with the cell-free form of wild-type parental Oka strain (pOka) of VZV *in vitro*, and incubated at 37 °C in 5% CO2 for four to seven days to allow for syncytia formation. Then cells were collected and prepared for TEM analysis, when syncytia formation was observed to be ~ 50% (Fig. S1A). Using immunofluorescence analysis, we demonstrated that gE and ORF7, two VZV-encoded proteins, were abundantly expressed in pOka-induced syncytia and the surrounding infected single cells (Fig. S1B), thus indicating active VZV replication in the collected syncytia. We examined the nuclei contained in a total of 25 random syncytia formed in infected ARPE-19 monolayers from two independent experiments by TEM. Most adjacent nuclei displayed a distance between their nuclear membranes of > 50 nm. Interestingly, however, we observed fusion of ONMs of adjacent nuclei in three of these syncytia (Fig. 1). There were in total seven fusion regions seen in two of the ONM-fusion-positive syncytia. These fusion regions were all small and measured to be 200 nm to 1 µm in width (Fig. 1A, panel a3-a6). Strikingly, ONM fusion occurred over a fairly large region between two adjacent nuclei in another ONM-fusion-positive syncytium, and produced a large perinuclear “pocket” at one end of the fusion region (Fig. 1B).

To examine the nuclei in VZV-induced syncytia in a more physiological relevant system, we used the previously established SCID-hu skin xenograft mouse model (Moffat et al., 1995). Human fetal skin xenografts in SCID mice were surgically exposed and directly injected with 10,000 PFU of cell-free pOka. At 21 days post-infection, the infected skin xenografts were harvested and prepared for subsequent

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