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INFECTIOUS DISEASE

Morphological Changes in Amphibian and Fish Cell Lines Infected with *Andrias davidianus* Ranavirus

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Summary

Andrias davidianus ranavirus (ADRV) is an emerging viral pathogen that causes severe disease in Chinese giant salamanders, the largest extant amphibian in the world. A fish cell line, *Epithelioma papulosum cyprinid* (EPC), and a new amphibian cell line, Chinese giant salamander spleen cell (GSSC), were infected with ADRV and observed by light and electron microscopy. The morphological changes in these two cell lines infected with ADRV were compared. Cytopathic effect (CPE) began with rounding of the cells, progressing to cell detachment in the cell monolayer, followed by cell lysis. Significant CPE was visualized as early as 24 h post infection (hpi) in EPC cells and at 36 hpi in GSSC cells. Microscopical examination showed clear and significant CPE in EPC cells, while less extensive and irregular CPE with some adherent cells remaining was observed in GSSC cells. Following ADRV infection, CPE became more extensive. Transmission electron micrographs showed many virus particles around cytoplasmic vacuoles, formed as crystalline arrays or scattered in the cytoplasm of infected cells. Infected cells showed alteration in nuclear morphology, with condensed and marginalized nuclear chromatin on the inner aspect of the nuclear membrane and formation of a cytoplasmic viromatrix adjacent to the nucleus in both cell lines. Some virus particles were also detected in the nucleus of infected GSSC cells. Both cell lines are able to support replication of ADRV and can therefore be used to investigate amphibian ranaviruses.

Keywords: *Andrias davidianus* ranavirus; Chinese giant salamander spleen cell; electron microscopy; viromatrix

Chinese giant salamanders (*Andrias davidianus*) are the largest amphibians in the world and are a critically endangered species in China. Viral epidemic diseases with a high mortality rate have occurred in farmed Chinese giant salamanders and have posed a significant threat to wild Chinese giant salamanders since 2010 (Dong *et al.*, 2011; Geng *et al.*, 2011). Fish cell lines are used widely for identifying and isolating aquatic viruses and for research on the morphogenesis of aquatic viruses and their molecular genetics and immune evasion strategies (Chinchar *et al.*, 2009; Huang *et al.*, 2009; Zhang and Gui, 2012, 2015; Ma *et al.*, 2014). A lethal ranavirus, *Andrias davidianus* ranavirus (ADRV), was isolated recently from diseased Chinese giant salamanders (Chen *et al.*, 2013). The aim of this study was to develop cell lines that could support the in-vitro growth of this virus, in order to better understand the pathogenesis of the infection.

Approximately 1×10^5 *Epithelioma papulosum cyprinid* (EPC) cells or Chinese giant salamander spleen cells (GSSC) were grown in TC199 medium supplemented with 10% fetal bovine serum at 25°C. ADRV was inoculated at a multiplicity of infection (MOI) of 0.1 onto the cultured EPC or GSSC cells in 96-well plates (Zhang *et al.*, 1999). Mock-infected controls or cells infected for different times (12, 24, 36 and 48 h) were stained with crystal violet solution and observed under a Leica DM IRB microscope (Leica, Wetzlar, Germany).

Obvious cytopathic effect (CPE) was first observed at 24 h post infection (hpi) in the EPC cells and at 36 hpi in GSSC cells. Microscopically, the cells became rounded before the monolayer of cells

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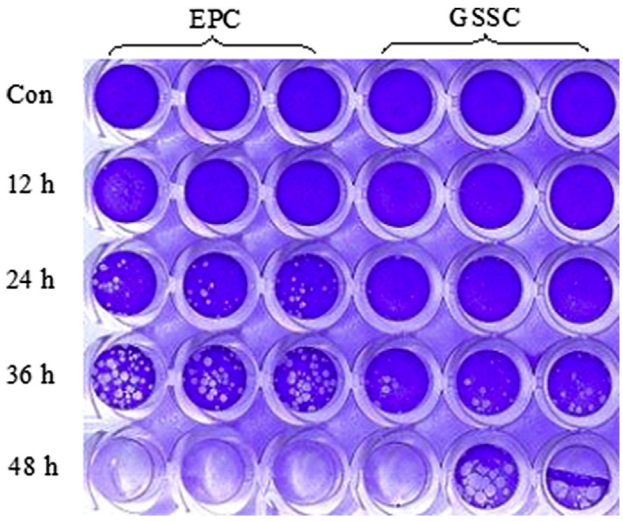

Fig. 1. Monolayers of ADRV-infected EPC and GSSC cells at 12, 24, 36 and 48 hpi. Mock-infected cells were used as controls (Con).

detached, and then finally there was lysis of the detached cells. The percentage of the monolayer showing CPE in the EPC cells was approximately 25% at 24 hpi and this increased to >50% at 36 hpi. For GSSC cells, there was minimal CPE at 24 hpi and this increased to affect 25% of the monolayer at 36 hpi. At 48 hpi, the entire EPC monolayer in triplicate wells had been completely detached, while for GSSC cells at this time point only one half of the monolayer had detached in two wells, with the entire monolayer detaching in the third well (Figs. 1 and 2).

For transmission electron microscopy (TEM), ADRV-infected cells were harvested by scraping the cells into the medium. The cells were then centrifuged at 2,000 g for 3 min. Cell pellets were pre-fixed with

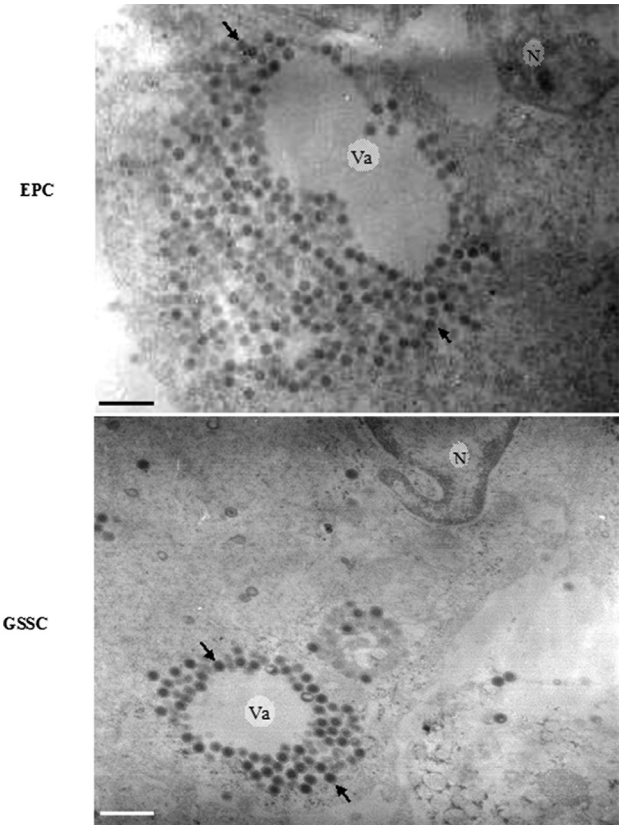

Fig. 3. ADRV-infected EPC and GSSC cells showing numerous virus particles around vacuoles in the cytoplasm of infected cells (arrows). N, nucleus; Va, vacuole. Bars, 500 nm.

2.5% glutaraldehyde, post-fixed with 1% O_SO_4, dehydrated in a graded series of ethanol and embedded in Epon-812. The blocks were sectioned on Leica Ultracut macrotome R. Ultrathin sections were double stained with 1% uranyl acetate and lead citrate and examined with a JEM-1230 electron microscope (JEOL, Tokyo, Japan) at 80 kV.

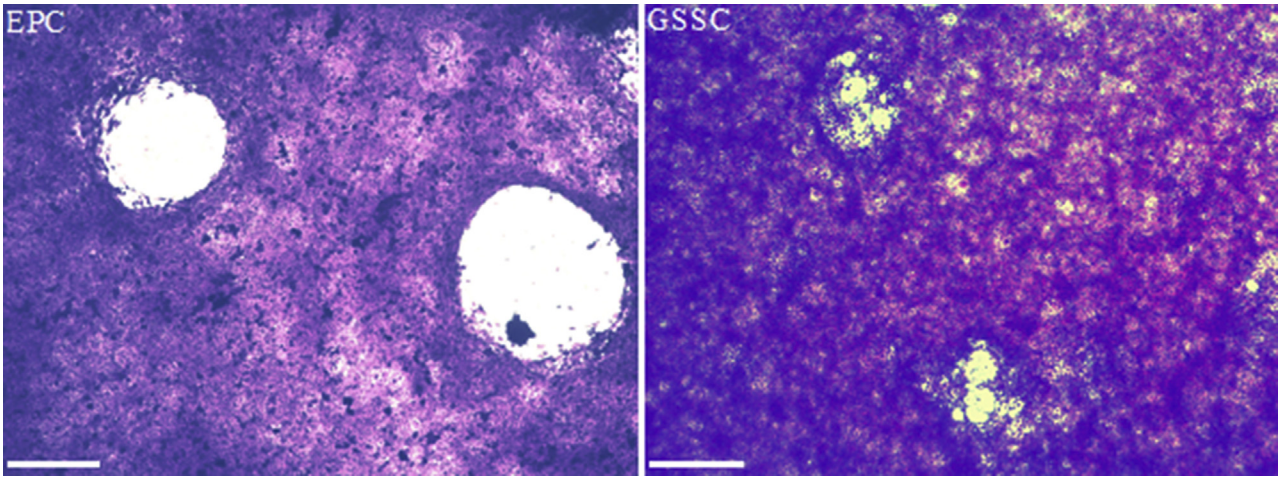

Fig. 2. CPE in EPC and GSSC monolayers at 24 hpi. Bars, 1cm.

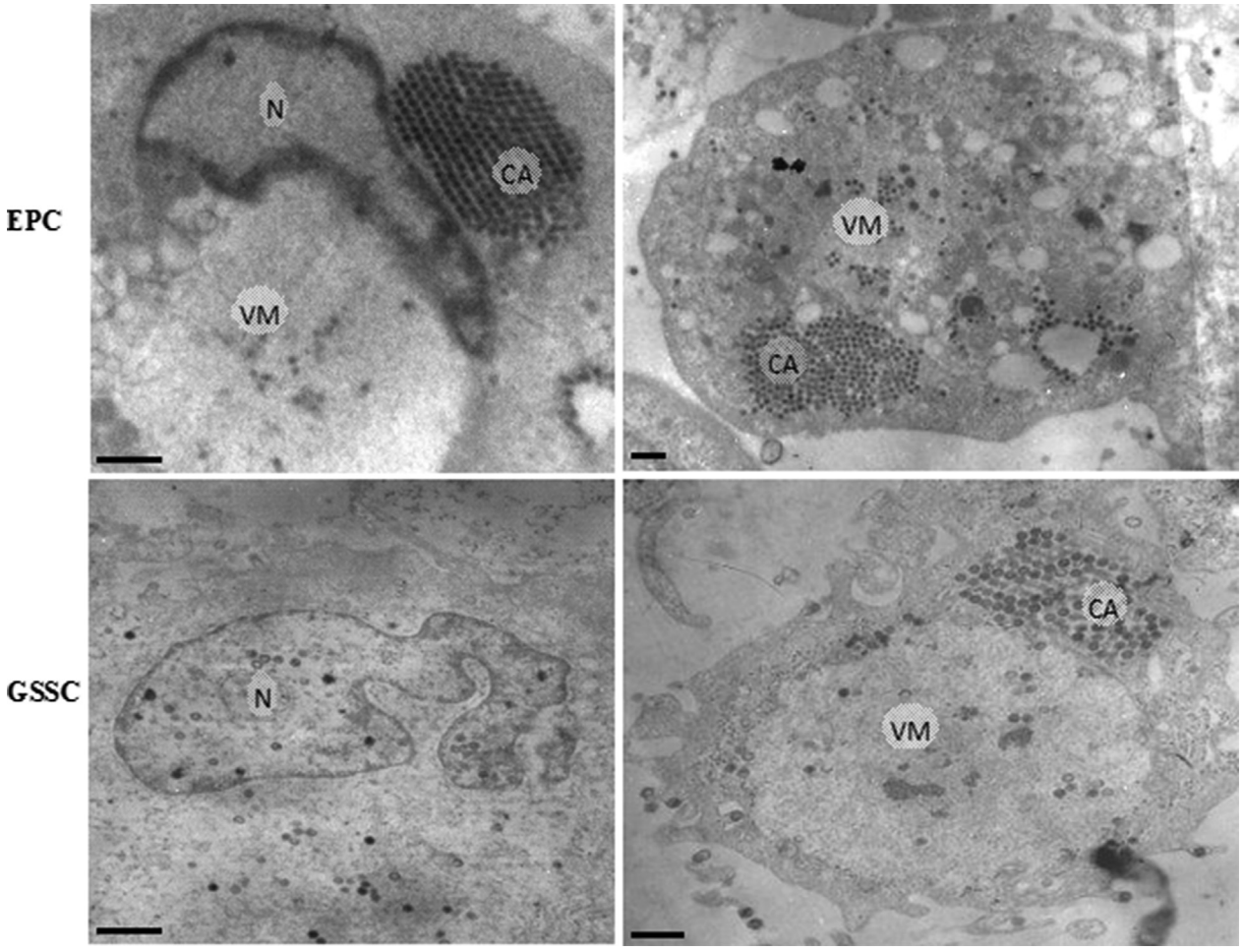

Fig. 4. ADRV-infected EPC and GSSC cells showing altered nuclei with marginalization of condensed chromatin along the nuclear membrane, viromatrix with low electron density and virus aggregation as crystalline arrays. Virus particles are present in the nucleus of GSSC cells. N, nucleus; VM, viromatrix; CA, crystalline arrays. Bars, 1 μm.

The transmission electron micrographs showed vacuolation of the cytoplasm of ADRV-infected EPC and GSSC cells, with numerous virus particles around the vacuoles (Fig. 3). Additionally, the nuclei of ADRV-infected cells were altered. The nuclear chromatin was condensed or marginalized on the inner aspect of the nuclear membrane. There was an area of viromatrix (with low electron density) adjacent to the nucleus in the cytoplasm of the infected cells. Some viruses aggregated as crystalline arrays beside the viromatrix and some were scattered as individual particles in the cytoplasm of both cell lines. Additionally, virus particles were detected in the nucleus of the GSSC cells (Fig. 4). These ultrastructural findings were similar to those described previously for other iridoviruses (Granzow *et al.*, 1997; Hyatt *et al.*, 2000; Qin *et al.*, 2001; Chao *et al.*, 2004).

CPE in ADRV-infected cells has been reported previously (Chen *et al.*, 2013); however, this is the first report of morphological changes in a Chinese giant salamander cell line infected with ADRV. The GSSC cell line may provide similar conditions for ADRV propagation as occurring in the natural host. Further studies of the morphological changes in GSSC cells could contribute to better knowledge of the mechanisms of ADRV infection *in vivo*.

Acknowledgments

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Conflict of Interest Statement

The authors declare no conflict of interest.

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