Aquaculture 500 (2019) 141–147

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short communication

Electron microscopy of the kuchijirosho (snout ulcer disease) causative agent in cell culture derived from fugu *Takifugu rubripes*

Tadashi Isshiki[a,*], Toshiaki Miyadai[b]

[a] Laboratory of Fish Diseases, Graduate School of Bioresources, Mie University, 1577 Kurimamachiya-Chou, Tsu, Mie 514-8507, Japan
[b] Laboratory of Marine Biotechnology, Faculty of Marine Bioscience, Fukui Prefectural University, 1-1 Gakuen-Chou, Obama, Fukui 917-0003, Japan

ARTICLE INFO

Keywords:
Electron microscopy
Kuchijirosho
Snout ulcer disease
Takifugu rubripes
Fugu

ABSTRACT

Kuchijirosho (snout ulcer disease) is a fatal infectious disease of farmed fugu *Takifugu rubripes* in Japan. The causative agent of this disease appears to be an RNA virus based on its physicochemical characterization, but it has not yet been visualized. To clarify the morphology of kuchijirosho causative agent (KCA), a sequential electron microscopic analysis of KCA-infected fugu gonad cells was performed during the morphogenesis of KCA. The infection caused an advanced cytopathic effect on day 2, which continued to cell lysis on day 14. The infected cells displayed progressive cytoplasmic modifications attributable to changes in protein synthesis, followed by the formation of membranous bodies, probably derived from the endoplasmic reticulum or modified cell organelles. With the overproduction of lipid droplets, virus-like particles (VLPs) were assembled at the surface of membranes in close proximity to lipid droplets as well as that of membranous bodies, leading to the formation of spherical VLPs, approximately 40 nm in diameter with an electron-dense core, which aggregated within intracellular spaces. The VLPs were then released from the cells when the cells were destroyed. We thus conclude that the VLP represents the KCA, of which the morphogenesis resembles the mechanism considered unique to some positive-strand RNA viruses that modify cell membranes of origins to replicate their genomes and/or modulate host-cell lipid metabolism. This is the first report of the morphology of KCA during its replication in cell culture.

1. Introduction

Kuchijirosho, or “snout ulcer disease” in English, is a fatal disease of fugu *Takifugu rubripes*, and has spread epizootically through Japanese fugu farms since 1981, causing significant economic losses. The major clinical sign of the disease is the ulceration of the snout, arising when the fish furiously bite the snouts of one another in response to central nervous system dysfunction. Histopathology shows that the diseased fish also undergo degeneration and necrosis of the large nerve cells of the optic tectum, the medulla oblongata and the spinal cord, accompanied by the agglutination of the chromatin or nucleoli in the nuclei (Miyadai et al., 2001; Nakauchi et al., 1985; Wada et al., 1985, 1986). The disease is transmittable to several species of fugu (*T. niphobles*, *T. poecilonotus*, *T. pardalis*, and *Ostracion immaculatus*), red sea bream *Pagrus major*, black rockfish *Sebastes schlegeli*, and yellowtail *Seriola quinqueradiata* (Miyadai et al., 2001; Takami et al., 2007). The viral etiology of this disease was established by Inouye et al. (1986, 1992), who demonstrated microbiologically that it is not associated with any bacterium or mycoplasma, and characterized the filterability of the agent by infecting fugu with brain extracts from diseased fish. Other researchers (Hashimoto et al., 2008; Miyadai et al., 2004) have suggested that the agent is an RNA virus, based on its physicochemical characteristics. However, the morphological features of the causative virus and its genomic properties remain unknown, and the taxonomic position of the virus is not determined. Therefore, no effective preventive measures against kuchijirosho have yet been established.

In this study, we propagated the kuchijirosho causative agent (KCA) in a primary cell culture derived from the fugu gonad (FG) and observed the KCA-infected cells with electron microscopy. We provide the first description of the morphology of KCA replicating in fugu-derived cells *in vitro*. The morphogenesis of KCA appears to resemble the mechanism considered unique to some positive-strand RNA viruses.

2. Materials and methods

2.1. KCA

The original inoculum of KCA was prepared from the brain of a

* Corresponding author.
E-mail address: isshiki@bio.mie-u.ac.jp (T. Isshiki).

https://doi.org/10.1016/j.aquaculture.2018.10.011
Received 4 July 2018; Received in revised form 3 October 2018; Accepted 7 October 2018
Available online 09 October 2018
0044-8486/

kuchijirosho-affected fugu using a modification of the method of Miyadai et al. (2004). Briefly, the frozen brain (18.6 g) was homogenized in 200 mL of phosphate-buffered saline supplemented with 0.06 M NaCl (PBS) with a rotor-stator. After centrifugation at 12,000 ×*g* for 30 min at 4 °C, the supernatant was collected. The remaining precipitate was treated again as described above, and the resulting supernatants were pooled. A 1/100 volume of 20 mg/mL protamine sulfate was added to the pooled supernatants for 30 min on ice to precipitate the fish DNA. After centrifugation at 12,000 ×*g* for 30 min at 4 °C, the supernatant was filtered through a 0.22 μm pore filter (Millipore, Billerica, MA, USA). The filtrate was stored at −80 °C until analysis.

2.2. Primary culture of FG cells

A 1-year-old fugu (approximately 200 g in bodyweight), that originated from the Fukui Prefectural Sea Farming Center, was used for cell culture. After external decontamination of the fish with 70% ethanol, its gonad tissues were excised aseptically. The tissues were then minced with a razor blade to fragments of approximately 1 mm^3. All subsequent manipulations of the tissues were made at 23 °C. The tissue fragments were suspended in a solution of 0.25% trypsin (1:250; Nacalai, Kyoto, Japan) and 0.02% EDTA (Wako, Tokyo, Japan) in PBS without calcium and magnesium cations (E/T solution), which was then slowly agitated with a magnetic stirrer in a beaker. After 1 h, the cell suspension was allowed to settle and the supernatant was decanted. Fresh E/T solution was then added to the remaining tissue fragments for further trypsinization and the supernatants were pooled. This procedure was repeated until the cell dispersion was essentially complete. The pooled cells were filtered through a lens-cleaning tissue (#105, Whatman, Little Chalfont, UK) and harvested by centrifugation at 200 ×*g* for 10 min. The cell pellet was then suspended in L-15N supplemented with 5% fetal bovine serum (FBS) in 25 cm^2 culture flasks (Sumilon, Tokyo, Japan) and incubated at 22 °C. L-15N is Leibovitz's L-15 medium (Gibco®, Invitrogen, Carlsbad, CA, USA) supplemented with 0.06 M NaCl, 100 IU/mL penicillin (Sigma, St. Louis, MO, USA), and 100 μg/mL streptomycin (Sigma). The cells were routinely subcultured in L-15N supplemented with 2.5% FBS.

2.3. Inoculation of FG cells with KCA

The KCA inoculum used in this experiment was obtained by diluting the original preparation 20 times with L-15N supplemented with 1% FBS (L-15N-1FBS). FG cells were grown overnight at 22 °C in 25 cm^2 culture flasks after they were seeded as an approximately 60% monolayer. After the growth medium was decanted from the flasks, the cells were washed once with PBS and inoculated with 3 mL of KCA. After adsorption at 22 °C overnight, the inoculated cells were washed three times and 5 mL of maintenance medium (L-15N-1FBS) was added to each flask. The flasks were incubated at 22 °C and the development of the cytopathic effect (CPE) was observed daily for up to 14 days after inoculation (dai) with an inverted light microscope. As the mock-infected culture, another sample of cells was inoculated with L-15N-1FBS and treated in the same manner. At 14 dai, when the developing CPE had caused cell lysis, a small amount of the culture medium was removed and used to infect fugu *in vivo*. For the electron microscopic analysis, the first sample was harvested after overnight adsorption, and subsequent samples at specific times (2, 4, and 14 dai), and then processed as described below.

2.4. Infection experiment

To confirm the virulence against fugu of the KCA that had replicated in FG cells, an infection experiment was performed using medium removed from the cultures at 14 dai when the CPE was complete. Five fugu (average body weight, 48 g) were injected intramuscularly with 0.05 mL of the culture medium and maintained in a 50-L aquarium at 25 °C for 14 days without feeding. The clinical signs and mortality in the experimental fish were observed daily.

2.5. Electron microscopy

The cells harvested at the selected times were removed from the flasks with a rubber policeman and centrifuged at 200 ×*g* for 10 min. The resulting pellet was fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (pH 7.3), postfixed in 2% osmium tetroxide, and embedded in Epon epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed at 80 kV with a JEM1010 electron microscope (JEOL, Tokyo, Japan).

3. Results

3.1. Production of KCA in FG cells

The monolayer culture of FG cells contained epitheloid and fibroblastic cells (Suppl. Fig. 1a). In the fibroblastic cells, an advanced CPE appeared 2 dai, with cell rounding and shrinkage (Suppl. Fig. 1b), and progressed to produce refractive and degenerating cells. The CPE extended over the monolayer throughout the 4-day culture period, and cell lysis finally occurred 14 dai, resulting in the almost complete detachment of the monolayer. None of the mock-infected cultures showed any change in the cells up to 14 dai.

The infection experiment with the culture medium collected from the FG cells at 14 dai resulted in 100% mortality in the fugu, which showed typical signs of the kuchijirosho, including biting behavior and ulceration of the snout (Suppl. Fig. 2). This confirms that virulent KCA can replicate in FG cells.

3.2. Ultrastructure of KCA-infected FG cells

At 1–2 dai, increased numbers of free or membrane-bound ribosomes were observed in the cytoplasm of the KCA-infected FG cells, and large polyribosomes were often detected. The cisternae of the endoplasmic reticulum (ER) were dilated, and the lumen of the swollen ER was sometimes filled with ribosomes or granular material (Fig. 1). An accumulation of membranous bodies (MBs) that form myelin-like structures in the cytoplasm was another distinct feature of the cells at 2

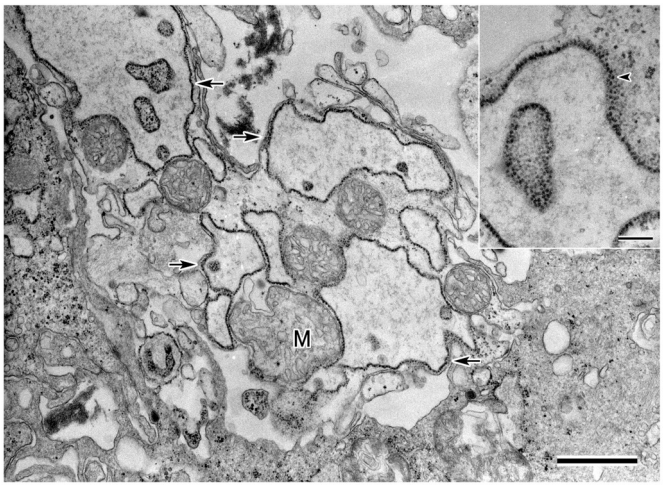

Fig. 1. Electron micrograph of fugu gonad cells 1–2 days after inoculation with kuchijirosho-affected brain extract. An increase in the number of ribosomes and dilation of the endoplasmic reticulum (ER) cisternae (arrows), containing granular material, are visible in the cytoplasm. Scale bar = 1000 nm. (inset) High-magnification view of a swollen ER with a number of polyribosomes (arrowhead). Scale bar = 200 nm. M, mitochondria.