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Short communication

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

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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

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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

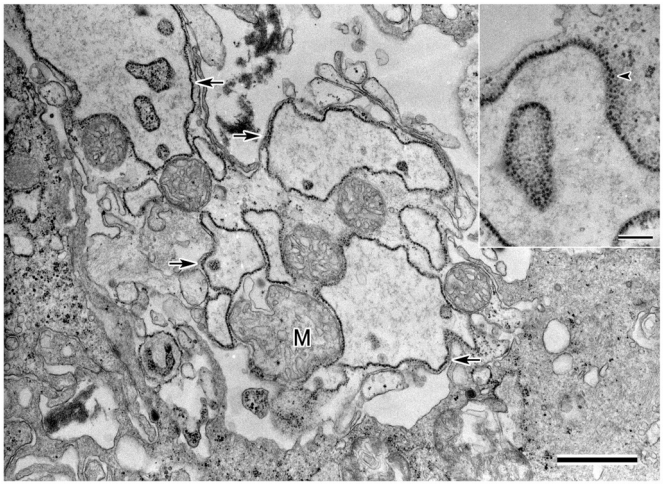

Fig. 1. Electron micrograph of fugu gonad cells 1–2 days after inoculation with kuchijirosho-affected brain extract. An increase in the number of ribosomes and dilation of the endoplasmic reticulum (ER) cisternae (arrows), containing granular material, are visible in the cytoplasm. Scale bar = 1000 nm.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

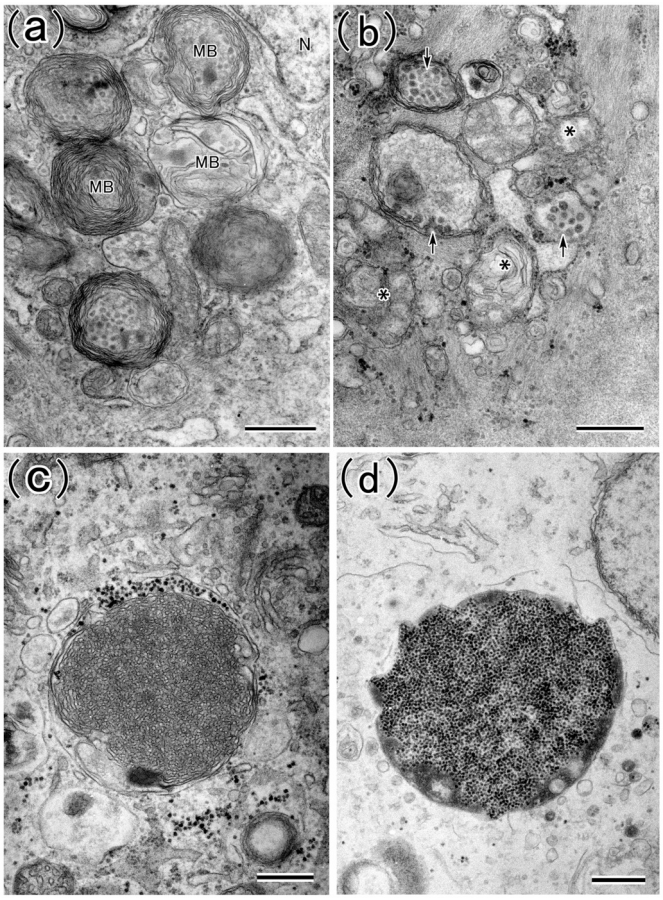

Fig. 2. Electron micrograph of fugu gonad cells 2–4 days after inoculation with kuchijirosho-affected brain extract. (a) Membranous bodies (MBs), forming myelin-like structures, have accumulated in the cytoplasm. Scale bar = 500 nm. MB, membranous body; N, nucleus. (b) MBs are being formed from invaginated endoplasmic reticulum (ER) membranes (asterisks).The occurrence of highly electron dense viral replication complex-like vesicles (arrows) is visible in the swollen ER. Scale bar = 500 nm. (c) The membranous structure of a MB becomes reticular, containing a small number of virus-like particles (VLPs). Scale bar = 500 nm. (d) A MB is replaced by VLPs, while the membranous structure has disappeared. Scale bar = 1000 nm.

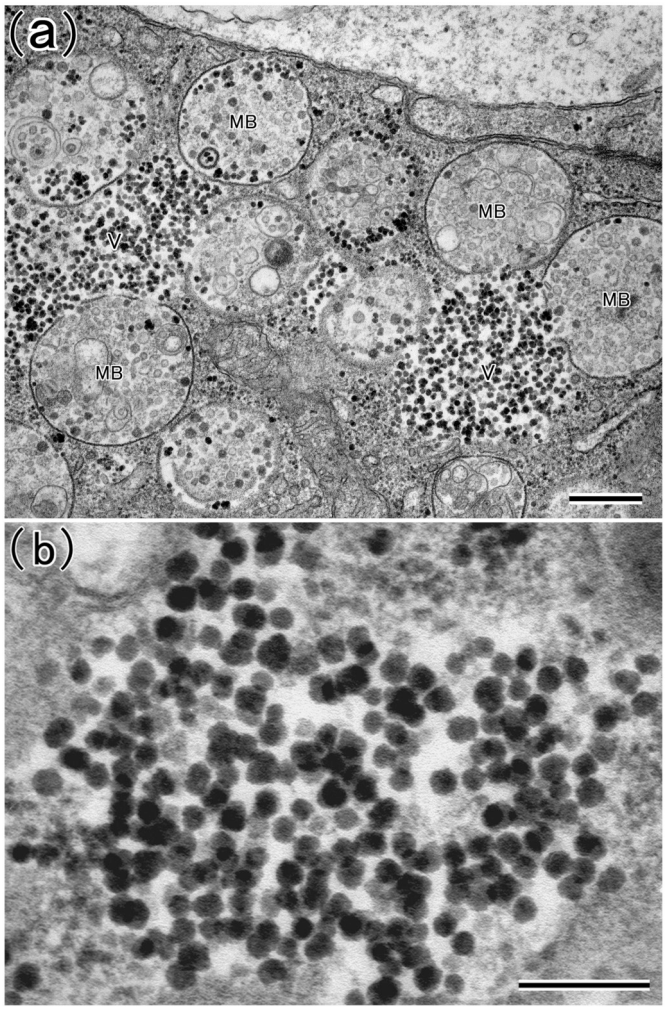

Fig. 3. Electron micrograph of fugu gonad cells 4 days after inoculation with kuchijirosho-affected brain extract. (a) Membranous bodies (MBs) consisting of fine granules, viral replication complex-like vesicles and membranous structures accumulate in the cytoplasm, and are in contact with an aggregation of virus-like particles (VLPs) within the intracellular spaces where the MBs have likely disappeared. Scale bar = 500 nm. (b) High-magnification view of VLPs. These are spherical in shape, measure approximately 40 nm in diameter, and have an electron-dense core. Scale bar = 200 nm. MB, membranous body; V, virus-like particle.

dai (Fig. 2a). The MBs were often located near the nucleus, but occasionally in the lumen of the ER. In the swollen ER around the nucleus, we observed occasional MBs forming when the ER membrane invaginated into the lumen, and the occurrence of highly electron dense viral replication complex (VRC)-like vesicles (approximately 100 nm in diameter) (Fig. 2b).

In the cytoplasm at 4 dai, the membranous structure of MBs became more reticular, containing a small number of virus-like particles (VLPs), and the MB was occasionally replaced by VLPs, while the membranous structure had disappeared (Fig. 2c, d). Other MBs were also observed, consisting of fine granules, VRC-like vesicles and membranes modified from the cell organelles. Large numbers of VLPs aggregated locally around the MBs, within the intracellular spaces, where the MBs had likely disappeared, involving the limiting membrane of the MBs adjacent to them (Fig. 3a). The VLPs were spherical in shape, measured approximately 40 nm in diameter, and had an electron-dense core, showing the naked capsid-like morphology (Fig. 3b). Conversely, we noted the remarkable appearance of lipid droplets (LDs), with or without a clustering morphology, frequently in the perinuclear region. The LDs occurred close to the VLP aggregations, in the areas surrounding them (Fig. 4a, b). The LDs and MBs were sometimes in contact at their membranes around the ER. When the limiting membrane of a MB contacted an LD, it became wide and highly electron dense (Fig. 4c, d). VLP assembly was clearly seen at the surfaces of membranes in close proximity to the LDs (Fig. 5a). Other assembly sites for VLPs developed on the surfaces of the electron-dense membranes of MBs (Fig. 5b). Specific intracellular compartments were referred to as VLP factories, where the establishments of cell organelle-modifications such as MBs and VRC-like vesicles, and cell component-overproductions such as LDs occurred for taking place to build VLP inclusions forming crystalline array in the cytoplasmic foci with the membrane-vesicular network (Fig. 6, as detailed in the discussion).

At 14 dai, the organelles of the KCA-infected cells were markedly fragmented and degenerate, often showing marked vacuolization and karyopyknosis (Fig. 7a). Some cell somata displayed transparent cytoplasm, resulting from the degeneration of the cell organelles, and pyknotic nuclei, accompanied by chromatin fragmentation (Fig. 7b). In other cells, many MBs had largely lost their internal membranes, vesicles, and granules, resulting in their vacuolization. Most of the VLPs aggregated around MBs and LDs that had diffused into the spaces within the fragmented cytoplasm or had been released from the

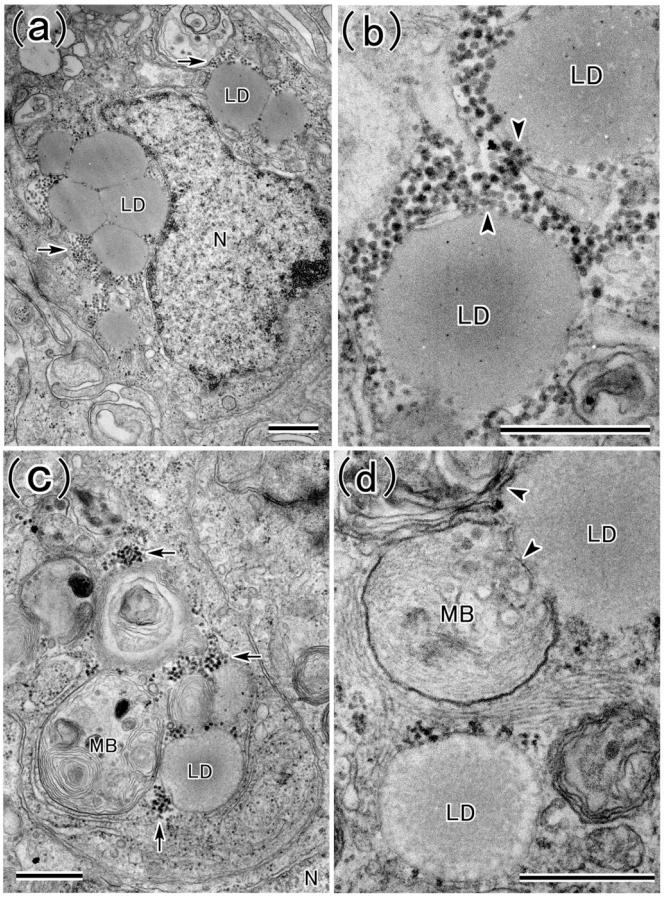

Fig. 4. Electron micrograph of fugu gonad cells 4 days after inoculation with kuchijirosho-affected brain extract. (a) Lipid droplets (LDs), with or without a clustering morphology, are present in the perinuclear region. Virus-like particles (VLPs, arrows) aggregate around the LDs. (b) High-magnification view of LDs involved in the aggregations of VLPs in the area surrounding LDs. Some of the VLPs (arrowheads) are closely associated with the LDs. (c) Accumulation of LDs, membranous bodies (MBs), and VLPs (arrows) around the endoplasmic reticulum. (d) The LD and MBs are partly in contact with each other through their membranes (arrowheads). Note that the limiting membrane of the MB contacting the LD is wide and highly electron dense. Scale bars = 500 nm. MB, membranous body; LD, lipid droplet; N, nucleus.

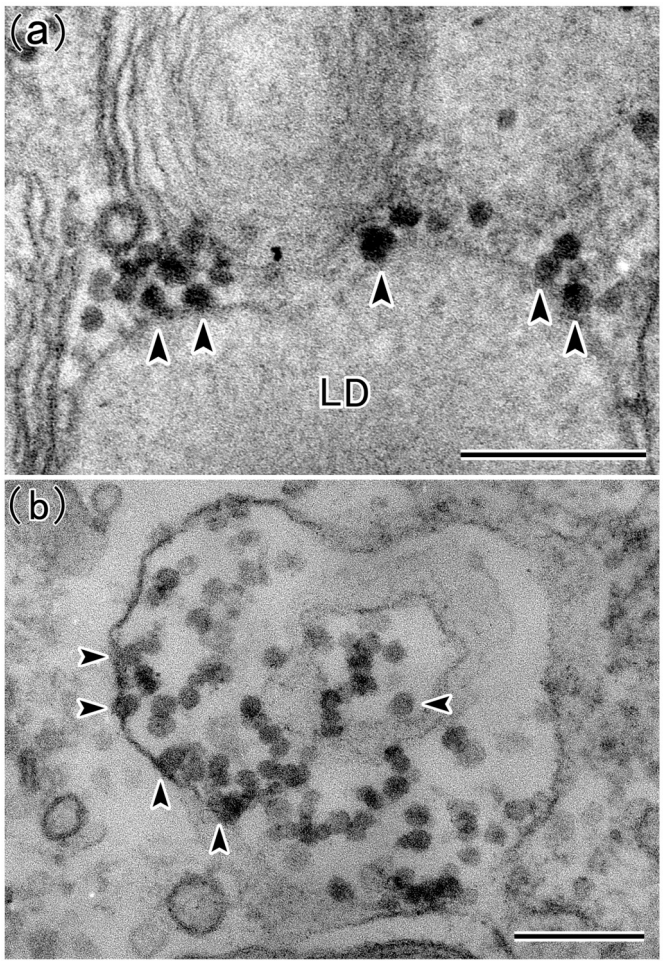

Fig. 5. Electron micrograph of fugu gonad cells 4 days after inoculation with kuchijirosho-affected brain extract. (a) Virus-like particles (VLPs, arrowheads) are assembled at the surface of a membrane in close proximity to a lipid droplet. LD, lipid droplet. (b) Similar assembly of VLPs (arrowheads) developing at the surface of the electron-dense membrane of a membranous body. Scale bars = 200 nm.

degenerate cells, whereas a small number of VLPs were still present in the residual cytoplasmic vesicles (Fig. 7c). Cell debris containing VLPs was sometimes apparent during the destruction of the cells (Fig. 7d).

None of the mock-infected cultures showed any obvious ultrastructural changes similar to those observed in the KCA-infected culture up to 14 dai. Although we observed LDs in the mock-infected culture, their frequency was low compared with that in the KCA-infected culture.

4. Discussion

Our primary culture of FG cells, used to isolate KCA and in the subsequent infection experiment, showed that FG cells allow the proliferation of KCA. This is supported by previous studies conducted in the same manner (Hashimoto et al., 2008; Inouye et al., 1986, 1992). Therefore, the fugu-derived cell culture system is a suitable tool for studying KCA production *in vitro*. We used FG cells infected with KCA and electron microscopy to visualize KCA *in vitro* and to follow the sequential events in the morphogenesis of KCA. In this study, we provided the first description of the morphology of KCA replicating in fugu-derived cells *in vitro*. The morphogenesis of KCA appeared to resemble the mechanism considered unique to some positive-strand RNA viruses.

Using electron microscopy, ultrastructural changes were observed at the beginning of the CPE, and consisted of increased numbers of ribosomes, the formation of large polyribosomes, and the dilation of the ER cisternae. Similar changes have been reported to occur in hepatitis A virus (HAV)-infected monkey cells (Frp/3 and FRhK-4), and appear to be consistent with the translation of viral mRNAs, subsequent protein synthesis, and viral secretion (Klinger et al., 2001; Tinari et al., 1989). Thus, the protein synthesis apparatus of KCA-infected cells seems to be activated for KCA production in the early phase of infection.

We also observed changes in the cytoplasmic structures, including the occurrence of MBs that form myelin-like structures, occasionally arising when the ER membrane invaginated into the lumen. Vacuolar structures with invaginations, morphologically similar to the MBs observed in the present study, occur in positive-strand RNA virus-infected cells (Romero-Brey and Bartenschlager, 2014). Klinger et al. (2001) have assumed that the invaginated vacuoles had arisen from the ER and represented autophagocytic structures in HAV-infected cells. HAV infection has also been reported to induce the enlargement of the ER cisternae, with the occasional formation of myelin-like structures within its lumen (Asher et al., 1987; Klinger et al., 2001; Tinari et al., 1989). It is well known that the ER plays an important role in the replication of viral genome and virion formation, not only in HAV but

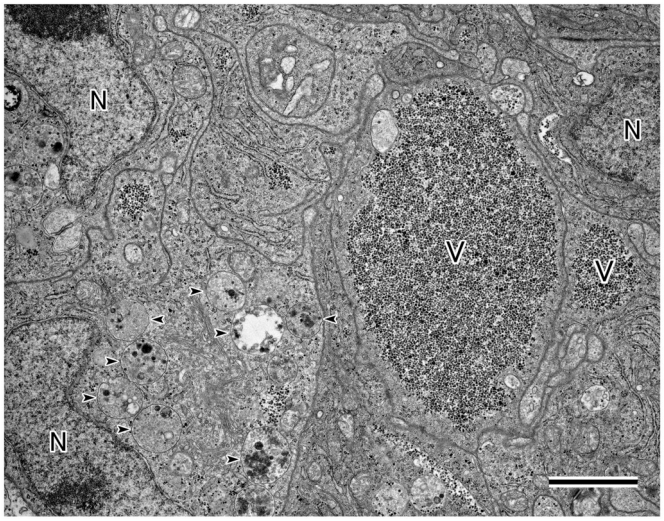

Fig. 6. Electron micrograph of fugu gonad cells 4 days after inoculation with kuchijirosho-affected brain extract. Virus-like particle (VLP) factories display the developed VLP-inclusions forming crystalline arrays, and the concentrating of modified cell-organelles including membranous bodies (arrowheads) for the subsequent steps in VLP replication in restricted area with the membrane-vesicular network. Scale bar = 2000 nm. V, virus-like particle; N, nucleus.

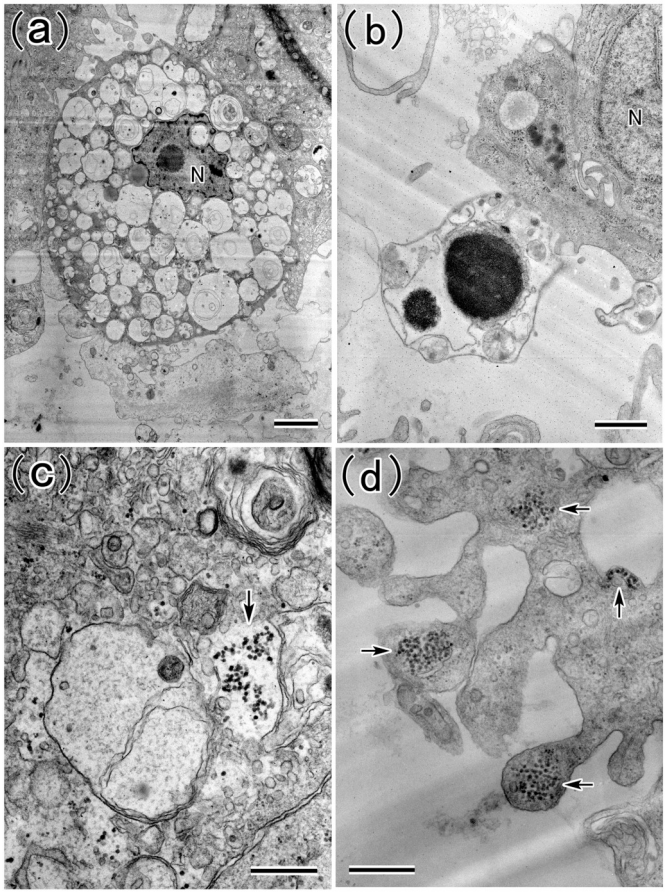

Fig. 7. Electron micrograph of fugu gonad cells 14 days after inoculation with kuchijirosho-affected brain extract. (a) An infected cell is undergoing necrosis, showing marked cytoplasmic vacuolization and karyopyknosis. Scale bar = 2000 nm. (b) A markedly degenerate cell displays transparent cytoplasm and a pyknotic nucleus with fragmented chromatin. Scale bar = 1000 nm. (c) Membranous bodies in the fragmented cytoplasm usually lose their internal membranes, vesicles, and granules, resulting in their vacuolization. A small number of virus-like particles (VLPs, arrow) are present in the residual cytoplasmic vesicles. Scale bar = 500 nm. (d) VLPs in the cell debris (arrows) are released from the destroyed cell. Scale bar = 500 nm. N, nucleus.

also in other positive-strand RNA viruses (Romero-Brey and Bartenschlager, 2014). In the present study, we observed VRC-like vesicles in the ER forming MBs. Therefore, our observations in KCA-infected cells suggest that the MB observed soon after KCA inoculation may be an autophagosome originating from the ER, which is induced by the entry of KCA into the cell and the subsequent steps in its replication.

In the KCA-infected cells harvested at the advanced stage of the CPE, VLPs (putative KCA) that aggregated within the intracellular spaces where the MBs had likely disappeared were in contact with the MBs that had accumulated, suggesting the participation of the MBs in the replication and morphogenesis of KCA, if the VLP is indeed the KCA. It is noteworthy that the morphology and size of the VLPs identified in this study are similar, at least to some extent in the immature virion, to those of members of the family *Flaviviridae* including dengue virus (Clark et al., 2012; Sriurairatna et al., 1973) or Zika virus (Barreto-Vieira et al., 2017), or the some hepatitis viruses (Mizuno et al., 2003), as are the intracellular events observed early in KCA infection. We also note that viruses belonging to the family *Flaviviridae* can activate the machinery of autophagy and benefit from autophagy to enhance their replication (Jin et al., 2013; Lee et al., 2008; Sir et al., 2008).

We have reported the marked ultrastructural changes observed in CPE-affected cells, including the overproduction of LDs and VLPs, closely associated with the membranes close to the LDs. These observations suggest the involvement of the LDs in VLP production, including in the replication and/or assembly of the VLP in the infected cells. To our knowledge, there is evidence that mainly RNA viruses rely on LDs for part of their replication and manipulate cell membrane (Camus et al., 2013). In the present study, the aggregation of MBs and the development of the ER around the LDs were clear, and a membranous association formed between the LDs, ER, and MBs, suggesting that KCA infection induces the apposition of the LDs and the ER-derived membranes (LD-associated membranes). This is followed by the processes of virus assembly, through the associations of structural and nonstructural proteins around the LDs, as reported in positive-strand RNA viruses (Camus et al., 2013). These findings prompt the hypothesis that the localization of the VLP components, possibly recruited to the LD-associated membrane, is responsible for the observed membrane changes, including the enlarged and highly electron-dense membranes in close proximity to the LDs in KCA-infected cells. These LD-related phenomena strongly suggest that the VLP should be considered the KCA or its major component, and that LDs are an important organelle in KCA production, as well as in positive-strand RNA virus production.

Viral genome replication and the assembly of viral particles is believed to occur frequently in specific intracellular compartments where the viral components concentrate. This constitutes the formation of functional structures, known as viral factories (Novoa et al., 2005). Our findings 1–4 dai in the present study indicate that MBs including VRC-like vesicles, and LDs were made from the cell organelles through their modifications, which seemingly resulted from an interaction between cellular and KCA components following the entry of KCA by an inoculation of kuchijirosho-affected brain extract into FG cells. It is known that replication or assembly sites for RNA viruses contain some of the cell organelles and their modifications, *e.g.* ER, Golgi apparatus, cytoplasmic membrane, lysosomes, endosomes, or autophagosomes (Novoa et al., 2005; Netherton and Wileman, 2011). KCA morphogenesis-related structures, MBs, were involved in cytoplasmic membrane, ER, or autophagosomes, as well as cell component, LD. The MBs or LDs thus seem to act in replication and/or assembly of KCA, probably through their membranous associations, for the subsequent yield of

abundant KCA particles. The KCA-propagating process above were being developed in the cytoplasmic foci, which are considered membranous replication factories reported previously in positive-strand RNA viruses (Romero-Brey and Bartenschlager, 2014).

In the last phase of KCA infection, when the CPE was complete, there was significant degeneration of the organelles of the KCA-infected cells, which displayed necrosis accompanied by vacuolization and karyopyknosis. A previous histopathological study of fish experimentally infected with KCA showed characteristic changes, such as liquefactive necrosis and vacuolization, in the nerve cells of the medulla oblongata and the epithelial cells in the snouts of the diseased fish (Wada et al., 1986). These indicate that the *in vivo* pathological changes in the KCA-infected fish were partly reproduced in our KCA-infected cells *in vitro*. The destruction of the KCA-infected cells allows the KCA particles localized inside the vesicles or the intracellular spaces to be released from the cells. As described above, our experimental infection of fish with the culture medium removed from cells in the final stages of the CPE confirmed its virulence against fugu *in vivo*. Therefore, the KCA particles released from the infected host cells are newly infectious agents against other hosts.

We note that the morphology and size of KCA in cell culture is consistent with the only previous results obtained by electron microscopy with the tissues of kuchijirosho-affected fugu (Inouye, 1988). Despite the evidently visualized morphogenesis of KCA *in vitro*, the previous electron microscopic findings *in vivo* (Inouye, 1988) include little evidence of the replication or assembly of KCA, and these remain still to be accounted for.

In conclusion, the ultrastructural analyses undertaken in this study, together with microbiological evidence reported previously (Hashimoto et al., 2008; Inouye et al., 1986), allow us to speculate 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. Further studies on the molecular virology are required to determine the taxonomic position of KCA using genetic and biochemical approaches.

Finally, we would like to discuss the relationship between the metabolism of lipids in fugu, a host of KCA, and kuchijirosho as a KCA-related disease. Fugu maintains a markedly low muscle lipid content (up to 1%) throughout the year and accumulates lipids in the hepatopancreas to make up for the shortage in lipid nutrients. This indicates an abnormal utilization and metabolism of lipids in fugu, compared with other cultured fish species, such as the red sea bream, amberjack *Seriola dumerili*, and yellowtail (Takii et al., 1995; Takii, 2005). For this reason, fugu that are fed excess dietary lipids often display diffuse fatty degeneration of the liver, and occasionally subsequent hepatopathy in intensive culture. Data on the hematological characteristics of kuchijirosho-affected fugu showed suspected hepatopathy (Inouye, 1988), indicating that the development of the disease might depend on hepatic failure in fugu infected with KCA. These findings have led to the hypotheses that the overproduction of LDs occurs in tissues, including the hepatopancreas, in kuchijirosho-affected fugu as well as in KCA-infected cell cultures, and that lipid accumulation in the hepatopancreas in response to excess dietary lipid triggers KCA propagation in intensively cultured fugu. Cholesterol, a main component of lipids, is abundant in the nerve tissues of the brain, which is an organ targeted by kuchijirosho (Itabashi et al., 2008). Understanding the association between lipids and the outbreak of the disease in intensively cultured fish may allow us not only to determine the life cycle of KCA in host cells but also to establish control measures against kuchijirosho with dietary restriction.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.10.011.

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