Vol. 57: 27–34, 2003 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published December 3

Infection and propagation of lymphocystis virus isolated from the cultured flounder *Paralichthys olivaceus* in grass carp cell lines

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ABSTRACT: The causative agent of lymphocystis disease that frequently occurs in cultured flounder *Paralichthys olivaceus* in China is lymphocystis virus (LV). In this study, 13 fish cell lines were tested for their susceptibility to LV. Of these, 2 cell lines derived from the freshwater grass carp *Ctenopharyngodon idellus* proved susceptible to the LV, and 1 cell line, GCO (grass carp ovary), was therefore used to replicate and propagate the virus. An obvious cytopathic effect (CPE) was first observed in cell monolayers at 1 d post-inoculation, and at 3 d this had extended to about 75% of the cell monolayer. However, no further CPE extension was observed after 4 d. Cytopathic characteristics induced by the LV were detected by Giemsa staining and fluorescence microscopic observation with Hoechst 33258 staining. The propagated virus particles were also observed by electron microscopy. Ultrastructure analysis revealed several distinct cellular changes, such as chromatin compaction and margination, vesicle formation, cell-surface convolution, nuclear fragmentation and the occurrence of characteristic 'blebs' and cell fusion. This study provides a detailed report of LV infection and propagation in a freshwater fish cell line, and presents direct electron microscopy evidence for propagation of the virus in infected cells. A possible process by which the CPEs are controlled is suggested.

KEY WORDS: Lymphocystis virus · LV · Iridovirus · Viral disease · Freshwater fish · Cell line · Apoptosis

INDRODUCTION

Lymphocystis has recently been reported to occur frequently in cultured flounder *Paralichthys olivaceus* in China, and the causative agent has been identified as a lymphocystis virus, LV (Sun et al. 2000, Xu et al. 2000), that has been extensively described worldwide (Tidona & Darai 1999). Lymphocystis disease was diagnosed early in 1874, but the viral agent was not determined by direct electron microscopic observations until 1962 (Walker 1962). LV is an iridovirus (Flugel et al. 1982, Wolf 1988: p 268–291), and a series of studies, including morphological and ultrastructural descriptions (Zwillenberg & Wolf 1968, Berthiaume et al. 1984, Samalecos 1986, Heppell & Berthiaume 1992), molecular characterization and genome sequence identification (Flugel et al. 1982, Darai et al. 1983, Schnitzler & Darai 1993, Tidona & Darai 1997), have been carried out. The complete genomic DNA sequence of LV-1 (isolated in Germany) has been determined to be 102 653 bp, and to contain 110 largely non-overlapping putative viral genes (Tidona & Darai 1997).

Although great advances have been made in LV studies, the molecular mechanism underlying LV infection, replication and pathogenesis is not clearly known. The major obstacle to progress is the lack of an efficient cell-culture system for propagation of this virus (Tidona & Darai 1999). In the past, many attempts have been made to propagate LV *in vitro*, but with limited success (Tidona & Darai 1999). For example, Wolf et al. (1966) successfully assayed and quantified an LV strain by $TCID_{50}$ and ID_{50} methods

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using the bluegill cell line BF-2. However, the titer yielded at 21 to 28 d post-infection was usually very low, and a complete replication of the virus has not been achieved for this cell line (Walker & Hill 1980). Recently, Perez-Prieto et al. (1999) evaluated the susceptibility of a new continuous cell line, SAF-1, derived from the fins of the gilt-head seabream *Sparus aurata* L., to several viruses, including LV. A cytopathic effect (CPE) was first recorded at 3 d post-inoculation, but only 25% of the cells were affected, and only 50% of the cell monolayer displayed by CPE at 6 d post-inoculation. Currently, much interest is being focused on the de-velopment of a susceptible cell-culture system for LV diagnosis, isolation, propagation and pathogenesis.

In recent years, more than 20 fish cell lines, derived from marine and freshwater fishes, have been cultured and maintained in our laboratory for the purpose of detecting and propagating specific virus pathogens (Zhang et al. 1999, 2000, Zhang 2002). In this study, we evaluated 13 fish cell lines for their susceptibility to the LV strain isolated from cultured flounder *Paralichthys olivaceus* in Qindao, China (Sun et al. 2000), and found 2 cell lines, GCO (grass carp ovary) and GCK (grass carp kidney) derived from the freshwater grass carp *Ctenopharyngodon idellus*, that are susceptible to the LV. We therefore used 1 cell line (GCO) to replicate and propagate the virus.

MATERIALS AND METHODS

Preparation of LV isolate. Lymphocystis tissues were sampled from the tumor-like dermal lesions of diseased cultured flounder in Qindao, Shandon Province, China (kindly provided by Professor X. Q. Sun). The tissues were homogenized in phosphate-buffered saline (PBS) containing antibiotics (penicillin, 100 I.U. ml^{-1}; streptomycin, 100 µg ml^{-1}). Extracts were stored overnight at –20°C, thawed, clarified by low-speed centrifugation, and the resulting supernatants containing LV were filtrated and used to infect cell lines or stored at –80 or –20°C.

Cell lines. For viral susceptibility tests (Zhang 1997), we used a total of 13 cell lines (see Table 1) including 10 established fish cell lines: GCK (grass carp kidney; Zuo et al. 1986), FHM (fathead minnow; Gravell & Malsberger 1965), CAR (*Carassius auratus* blastula embryos; Wolf 1988; p 461), EPC (*epithelioma papulosum cyprini*; Fijan et al. 1983), EG (eel gonad; Chen & Xie 1995), CHSE (chinook salmon embryo; Wolf 1988; p 461), RL (rainbow trout liver; Ahne 1985), RTG (rainbow trout gonad; Wolf 1988; p 461), CCO (channel catfish ovary; Bowser & Plumb 1980) and PG (pike gonad; Ahne 1978); and 3 new fish cell lines, i.e. GCO (grass carp ovary; unpubl.), GCH (gibel carp heart; unpubl.) and SCE (silver carp embryo; Li & Zhang 2000). The cells were grown in Tissue Culture 199 medium supplemented with 10% fetal bovine serum and antibiotics (100 I.U. penicillin ml^{-1}; 100 µg streptomycin ml^{-1}). Culture temperature was 25°C unless otherwise stated.

Cell infection and virus propagation. The thawed LV isolates were inoculated separately onto confluent cell monolayers in 96-well plates as previously described (Zhang et al. 1996, 2000). The cytopathic effect (CPE) was observed by light microscopy.

Staining with Giemsa and Hoechst 33258. The infected and uninfected cells cultured on glass slides were fixed with 30 to 100% ethanol and stained with Giemsa; slides were examined under a light microscope. For Hoechst 33258 staining, cultures on glass slides were fixed with 4% formaldehyde in PBS, then rinsed twice (5 min each time), and stained with 5 µg

Table 1. Susceptibility to the LV of 13 fish cell lines. +, ++, +++: ~25, 50 and 75% cells, respectively, displayed cytopathic effects (CPE); –: no CPE observed

Cell line		CPE (time post-inoculation)						
		1 d	2 d	3 d	4 d	6 d	12 d	18 d
GCK	(grass carp kidney)	–	+	++	++	++	++	++
FHM	(fathead minnow)	–	–	–	–	–	–	–
CAR	(goldfish fin)	–	–	–	–	–	–	–
EPC	(*Epithelioma papulosum cyprini*)	–	–	–	–	–	–	–
EG	(eel gonad)	–	–	–	–	–	–	–
CHSE	(chinook salmon embryo)	–	–	–	–	–	–	–
RL	(rainbow trout liver)	–	–	–	–	–	–	–
RTG	(rainbow trout gonad)	–	–	–	–	–	–	–
CCO	(channel catfish ovary)	–	–	–	–	–	–	–
PG	(pike gonad)	–	–	–	–	–	–	–
GCO	(grass carp ovaries)	+	++	+++	+++	+++	+++	+++
GCH	(gibel carp heart)	–	–	–	–	–	–	–
SCE	(silver carp embryos)	–	–	–	–	–	–	–

ml^{-1} Hoechst 33258 for 15 min. The slides were rinsed twice (5 min each time) with PBS, and then mounted with 50 mMol l^{-1} Na_2HPO_4 and 50% glycerol. The Hoechst 33258-stained slides were observed under a fluorescence microscope.

Transmission electron microscope observations. At Day 3 post-inoculation (CPE had extended to about 75% of the cell monolayer), the infected GCO cells were fixed with 2% glutaraldehyde, post-fixed in osmium tetroxide (OsO_4), dehydrated and embedded in Epon-812. Ultrathin sections were cut and stained with 2% uranyl acetate and lead citrate, and examined under a JEM-1230 electron microscope.

RESULTS

Susceptibility of two grass carp cell lines to LV

Of the 13 fish cell lines examined, CPE were observed in only 2 grass carp cell lines, GCO and GCK (Table 1). In GCO cells, obvious CPE was first observed in cell monolayers at 1 d post-inoculation, and had extended to about 75% of the cell monolayer at 3 d post-inoculation (Fig. 1). In GCK cells, CPE was not recorded until 2 d post-inoculation, and the frequency of the affected cells was lower than with the GCO cells. However, no further CPE extension was observed after 4 d post-inoculation for either cell line. These results indicated that the GCO and GCK cell lines were susceptible to the LV, and therefore the GCO cell line was used to propagate the virus. In a series of infection experiments, LV infection and propagation were performed for 8 passages in the GCO cell line.

Light-microscope characteristics of cytopathic effects

Cytopathic characteristics induced by the LV in GCO cells differed from those described previously for other aquatic animal viruses (Zhang et al. 1999, 2000, 2001). The infected cells showed increased microscopic refractivity and contraction. Some syncretic cell patches appeared on the monolayers, and some contracted cells were suspended in the media.

Giemsa staining revealed cell aggregation and fusion in the infected monolayers. A large number of cells aggregated in patches in affected areas, and these stained deeper red (Fig. 2A,B).

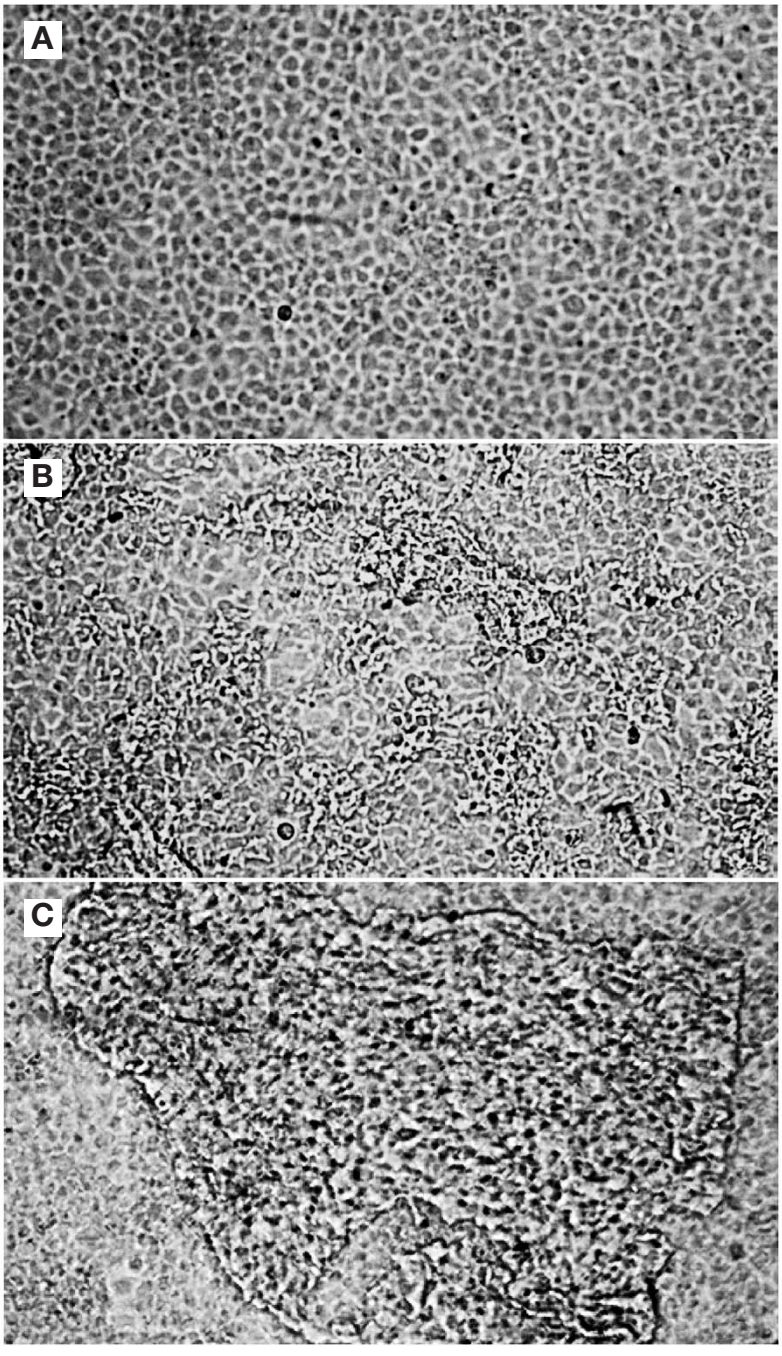

Fig. 1. Lymphocystis virus (LV)-induced cytopathogenic effect in infected GCO (grass carp ovary) cells (×160). (A) Uninfected GCO control cells; (B) infected cells at 1 d post-inoculation; (C) infected cells at 3 d post-inoculation

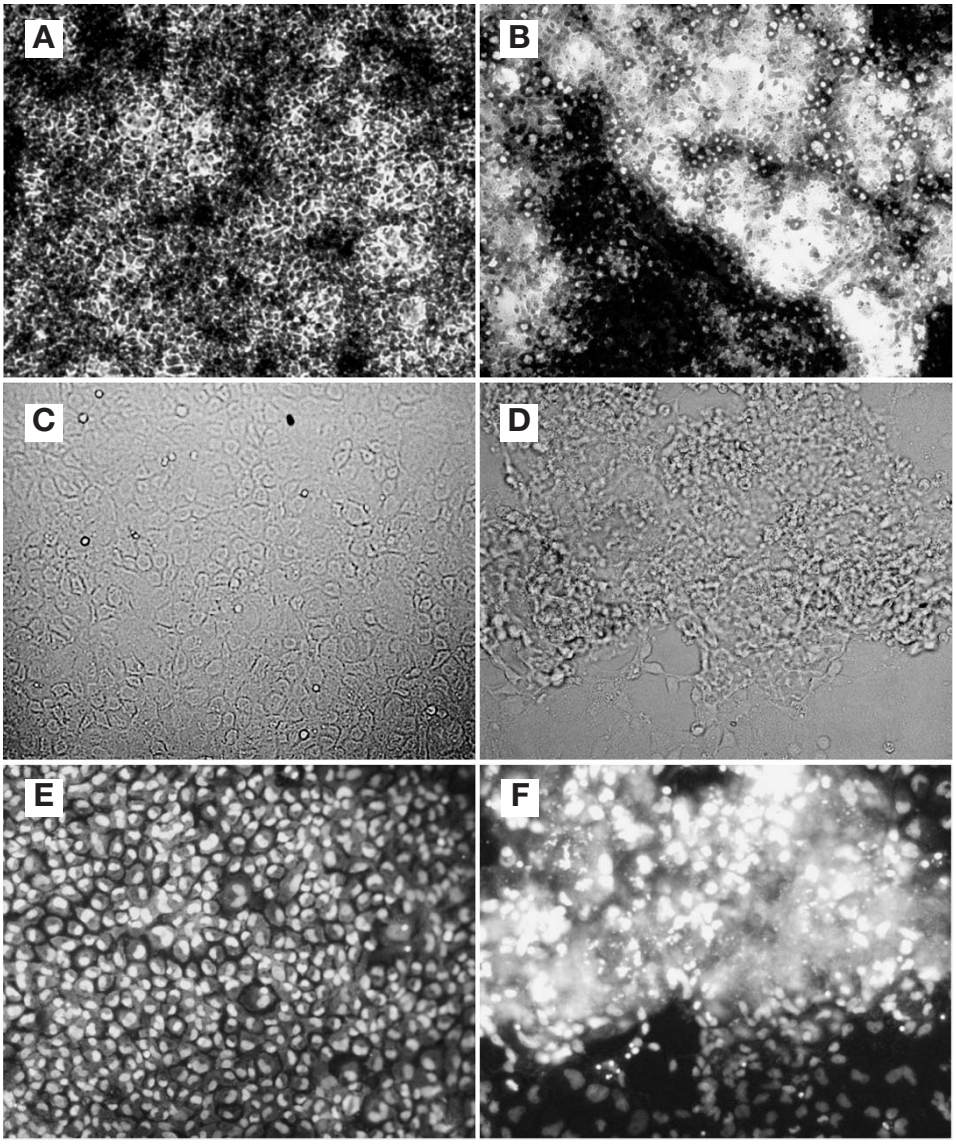

Fig. 2. Light microscopic characteristics of cytopathogenic effects in LV-infected GCO cells. Left: uninfected control cells; right: infected cells showing cytopathic effects. (A,B) Staining with Giemsa (×80); (C–F) staining with Hoechst 33258; (C,D) observed by ordinary light microscopy and (E,F) by fluorescence microscopy (×150)

Fluorescence microscopic observation revealed morphological characteristics of CPE in the infected cells. Normal GCO cell nuclei stained uniformly with Hoechst 33258, and the contour between a green-blue nucleus and cytoplasm was clear in control cells (Fig. 2C,E). In infected GCO cells, the green-blue fluorescence was diffused in the cytopathogenic patch. Affected cells became either hypertrophied or broken. Some marked changes in nucleus morphology, including fragmentation into lobular structures and nuclear lysis, were observed (Fig. 2D,F). Much of the lysed nuclear debris was present in the cytopathogenic patch.

Electron microscopic observation of propagated viruses

In the infected GCO cells that had produced CPE, the LV propagated rapidly. High concentrations of virus particles were visible in the cytoplasm and nucleus (Fig. 3A). A viromatrix composed of electron-dense fiber-like materials was also observed in the cytoplasm. The viromatrix contained various virus particles at different stages of development, including empty capsids and matured nucleocapsids (Fig. 3A); it was similar to that observed in another iridovirus, RGV (*Rana grylio* virus) (Zhang et al. 1999, 2001), and may also be the central area for the LV assembly. Abundant

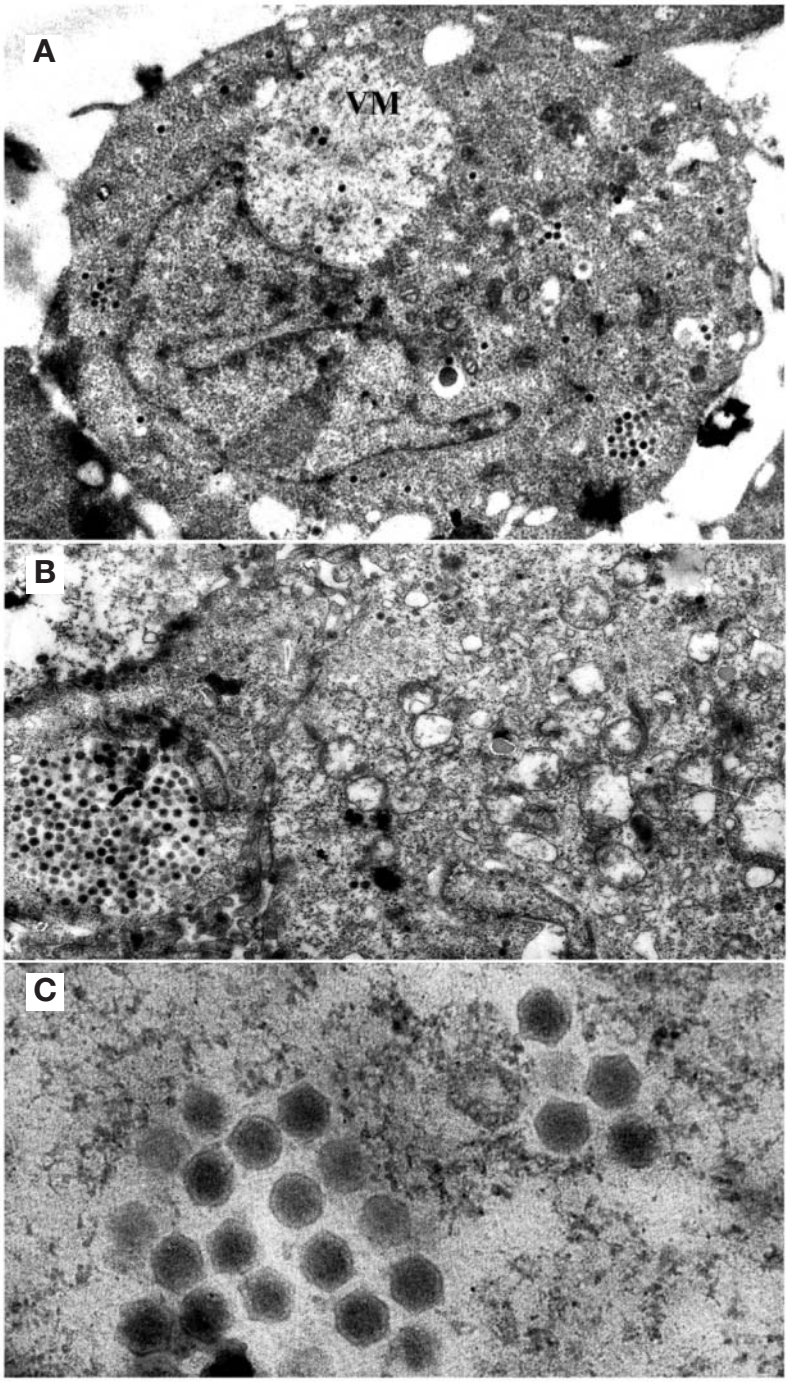

Fig. 3. Electron micrographs of infected cell and propagated virus particles. (A) Typical LV-infected cell containing 1 viromatrix (VM) and large number of propagated virus particles (×4600); (B) aggregation of propagated virus particles (×8500); (C) enlarged micrograph of propagated virus particles (×42600)

mature virus particles aggregated in the cytoplasm of some cells, but none of the crystalline arrays found in other iridoviruses (Zhang et al. 1999) were observed (Fig. 3B). Matured icosahedral virus particles in the cytoplasm were relatively uniform in size, with a diameter of approximately 170 nm (Fig. 3C).

Ultrastructure analysis of infected cells

Distinct changes, such as chromatin compaction and margination, vesicle formation, cell surface convolution and cell fusion, were revealed by ultrastructure analysis of the infected GCO cells (Fig. 4A). Most of the

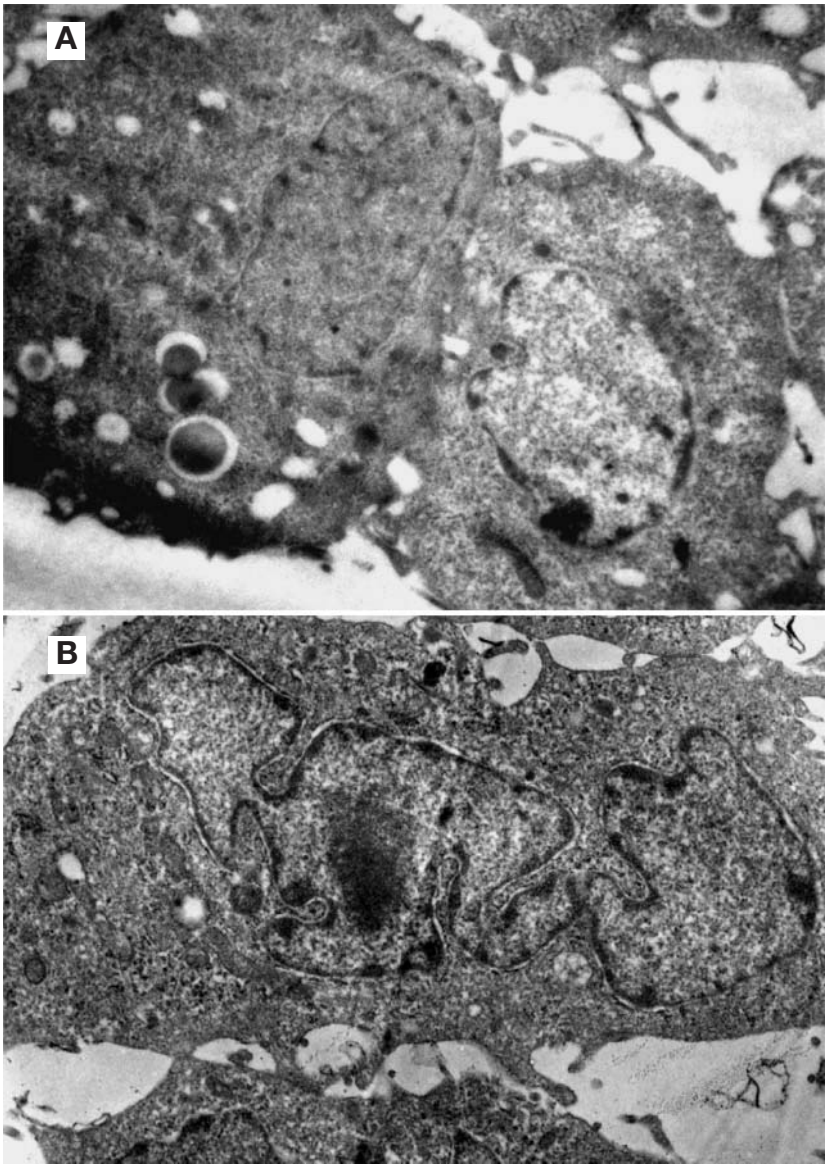

Fig. 4. Cellular changes observed by electron microscopy in infected GCO cells. (A) Chromatin compaction and margination, vesicle formation, cell-surface convolution and cell fusion in the 2 cells (×4300); (B) nuclear fragmentation and characteristic 'blebs' visible in cell (×12000)

nuclei were of eccentric size or shape, and gave rise to fragmentation and characteristic 'blebs' (Fig. 4B).

DISCUSSION

In the current study, 13 fish cell lines were used to examine virus susceptibility, and 2 grass carp cell lines, GCO and GCK, were determined to be susceptible to the LV isolated from cultured flounder *Paralichthys olivaceus* in China. In addition to the susceptibility of freshwater fish cell lines to LV, cytopathic characteristics induced by the LV in GCO cells were demonstrated by Giemsa staining and fluorescence microscopic observation using Hoechst 33258 staining. Furthermore, a large number of propagated virus particles were detected in the infected GCO cells by electron microscopy. These results indicate that the LV replicated well in the grass carp cell line, and can therefore be passed in GCO cells.

Since the detection of LV by direct electron microscopy in 1962 (Walker 1962), many attempts have been made to propagate LV *in vitro*, but no efficient propagation of the virus in cultured cells has been docu-

mented to date: 3 fish cell lines—the bluegill fry cell line BF2 (Wolf et al. 1966, Walker & Hill 1980), the gilthead seabream (*Sparus aurata* L.) fin cell line SAF-1 (Perez-Prieto et al. 1999), and the Asian seabass fry cell line SF (Chang et al. 2001)—were used to evaluate their susceptibility to LV, and in all cases CPE was recorded after inoculation, but a complete replication of the virus was not achieved. The present study has demonstrated the susceptibility of 2 grass carp cell lines to LV, and has also demonstrated the propagation of virus particles in the infected GCO cells under the electron microscope.

Iridoviruses of the genus *Ranavirus* are associated with high mortality in lower vertebrate animals (Zhang et al. 2001, Chinchar 2002), and we observed some similar characteristics in the LV virus in the present study (e.g. an aggregated distribution of mature LV particles in the cytoplasm; Fig. 3B); however, the replication efficiency of the LV in the culture cells was much lower. For example, iridovirus RGV infectivity has a $TCID_{50}$ 10^6 at 25°C, whereas maximum LV infectivity is achieved with only $TCID_{50}$ 10^3. This may be related to the virus properties and pathogenetic mechanism. Lymphocystis disease is characterized by the development of nodules or tumor-like clusters on the skin and fins of infected fishes that are provoked by abnormal enlargement of cells (Wolf 1988). Cytopathological characteristics induced by the LV in culture cells were also obviously different from those described previously for other aquatic animal viruses (Zhang et al. 1999, 2000, 2001). Several distinct cellular changes, such as chromatin compaction and margination, vesicle formation, cell-surface convolution, nuclear fragmentation and the occurrence of characteristic 'blebs' and cell fusion, were revealed by ultrastructure analysis of the infected GCO cells. These changes, identical to the typical medial steps of apoptosis (Flint et al. 2000), indicated the presence of a possible apoptotic mechanism in LV pathogenesis. In nature, LV-infected fishes develop clusters of lymphocystis cells, and the individual cells may undergo a 50 000- to 100 000-fold increase in size (Tidona & Darai 1999). However, although infected fishes are unsightly, the disease rarely causes death. During the cell infection process, we observed that obvious CPEs first appeared in GCO cell monolayers at 1 d post-inoculation, and had extended to about 75% of the cell monolayer at 3 d post-inoculation (Fig. 1). However, no further CPEs were observed after 4 d post-inoculation. In combination with these cellular changes, we believe that LV infection initiates rapid cell death, and thus prevents further infection and virus propagation. There were large numbers of LV particles present in the medium after 4 d of infection, and neither infected nor non-infected cells were able to survive. This could be the reason why no further CPE extension was observed after 4 d post-inoculation.

The lymphocystis virus is distributed worldwide, and the resultant lymphocystis disease has been reported to occur in over 100 different fish species in marine and freshwaters. According to a recent report (Grinwis et al. 2001), a series of experiments have been set up to examine the effects of pollution on marine and estuarine fish health, since the European flounder has shown a relatively high prevalence of lymphocystis disease in Dutch coastal and estuarine waters. The major impact of LV on infected fishes is the production of unsightly tumor-like external lesions that appear as white or grey nodules on fins and skin, and result in significant economic losses. Therefore, studies on the molecular mechanism underlying LV infection, replication and pathogenesis are important.

In summary, this study has provided a detailed report of LV infection and propagation in a freshwater fish cell line, and clear electron microscopy evidence of the propagated virus in infected cells. A possible process by which the CPE is controlled has been suggested. These data will be useful in understanding the interaction between the LV and host cells and the mechanism underlying LV infection and propagation.

Acknowledgements. This study was supported by grants from the National 863 High Technology Research program (2001AA626030), the National Natural Science Foundation of China (30170726), the Project of Chinese Academy of Sciences (STZ-00-13, KSCX2-SW-302) and the Frontier Science Projects Program of the Institute of Hydrobiology, Chinese Academy of Sciences (220301).

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Editorial responsibility: Jo-Ann Leong, Kaneohe, Hawaii, USA

Submitted: September 8, 2002; Accepted: April 24, 2003
Proofs received from author(s): September 29, 2003