Induction of apoptosis in a carp leucocyte cell line infected with turbot (Scophthalmus maximus L.) rhabdovirus

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Abstract

A rhabdovirus was observed from the diseased turbot (Scophthalmus maximus L.) with lethal syndrome. In this study, a carp leucocyte (CLC) cell line was used to investigate the infection process and cell death mechanism occurring during the virus infection. Strong cytopathogenic effect (CPE) and the morphological changes, such as extreme chromatin condensation, nucleus fragmentation, and apoptotic body formation, were observed under fluorescence microscopy after DAPI staining in the infected CLC cells. Transmission electron microscopy analysis showed cell shrinkage, plasma membrane blebbing, cytoplasm vacuolization, chromatin condensation, nuclear breakdown and formation of discrete apoptotic bodies. The bullet-shaped nucleocapsids were measured and ranged in size from 110 to 150 nm in length and 40 to 60 nm in diameter. And therefore the virus is called Scophthalmus maximus rhabdovirus (SMRV). Agarose gel electrophoresis analysis of the DNA extracted from infected cells showed typical DNA ladder in the course of SMRV infection. Flow cytometry analysis of SMRV infected CLC cells detected apoptotic peak in the virus infected CLC cells. Virus titre analysis and electron microscopic observation revealed that the virus replication fastigium was earlier than that of the apoptosis occurrence. No apoptosis was observed in the CLC infected with UV-inactivated SMRV. All these supported that SMRV infected CLC cells undergo apoptosis and the virus replication is necessary for apoptosis induction of CLC cells.

Keywords: Scophthalmus maximus rhabdovirus (SMRV); Apoptosis; Carp leucocyte (CLC) cells; Flow cytometry; Apoptotic peak

1. Introduction

Rhabdoviruses are significant virus pathogens in aquaculture, and more than 10 strains of fish rhabdoviruses, such as viral haemorrhagic septicemia virus (Jensen, 1963), infectious haematopoietic necrosis virus (Amend et al., 1969), pike fry rhabdovirus (de Kinkelin et al., 1973), eel virus A (Sano, 1976), Rio Grande perch rhabdovirus (Malsberger and Lautenslager, 1980), rhabdovirus salmonis (Osadchaya and Nakornchayha, 1981), hirame rhabdovirus (Kimura et al., 1986), snakehead rhabdovirus (Kasornchandra et al., 1991), viral hemorhagic septicemia virus (Ahne et al., 2002), Siniperca chaotica rhabdovirus (Zhang and Li, 1999), and Chinese sucker rhabdovirus (Zhang et al., 2000), have been isolated from cultured fishes. Most of them have been well characterized and confirmed to be pathogens that are associated with serious systemic diseases in aquaculture. They cause high mortality among the infected fishes including some important freshwater and marine species such as rainbow trout, common carp and Japanese flounder. Moreover, some of the fish rhabdoviruses also infect other species in aquaculture (Ahne et al., 2002). These rhabdoviruses have caused great economic losses and became factors restricting aquaculture development of special fishery in China (Zhang, 1997) and other parts of the world (Meyers et al., 1994; Marty et al., 1998). To explore this problem, a lot of studies on fish rhabdoviruses have been focused on their pathological, morphological and molecular characteristics (Morunov et al., 1995; Schütze et al., 1999). However, solutions to the problem will depend on the clear understanding of virus pathogenic mechanism and the interaction between the virus and host cells.

The turbot, Scophthalmus maximus L., has been used as an important aquaculture species in the world, and has been cultivated in Northern China. Recently, a pathogenic virus has been observed from the diseased turbort with lethal syn-
drome, and suggested that the pathogen might be a rhabdo-
virus (Zhang et al., unpublished data). In this report, we
adopt several methods that are known to be specific for the
identification of apoptotic cells to determine the process im-
licated in cell death induced by the virus agent infection,
and demonstrate that the major mechanism of cell death oc-
curring during the virus infection in vitro is associated with
the induction of apoptosis.

2. Materials and methods

2.1. Cell culture

The CLC (carp leucocyte) cell line (Faisal and Ahne,
1990) was used in this study. Cell cultures were grown at
25°C in TC-199 (Gibco) medium supplemented with 10%
heat-inactivated bovine serum, buffered with Na₂CO₃ to pH
7.2–7.4.

2.2. Virus infection and titre determination

The virus was isolated from the tissues of diseased tur-
bot and propagated in CLC cells. The residual virus after
adsorption was removed and virus titre of the supernatant
with cells associated was determined by limited dilutions
and scoring CLC cell line described previously (Yao et al.,
2002). In this study, cells were infected with the virus isolate
at a multiplicity of infection of 5.

2.3. Inactivation of virus

The virus isolate was inactivated by UV irradiation as de-
scribed previously (Zhang et al., 2003b). In brief, the viruses
were placed at room temperature in a lidless culture dish
and exposed to UV lamp at an intensity of 30 W from 15 cm
above the sample for 25 min and then harvested for cell
infection.

2.4. Fluorescent staining of virus-infected cells

The CLC cells, grown on glass microscope slides, were
infected with virus, at different time the cells were fixed
with 3.7% formaldehyde (20°C, 15 min), dried in acetone,
and stained with the DNA binding fluorescent dye DAPI
(4’,6’-diamidino-2-phenylindole, Sigma) at the final concen-
tration of 4 μg/ml in PBS (phosphate-buffered saline, pH
7.5) for 20 min at room temperature (Kang et al., 1999). The
samples were examined by fluorescence microscopy (Leica)
(Chejanovsky and Gershburg, 1995).

Fig. 1. Induction of plaques formation on cultured monolayer cells by SMRV infection. Control cells and cells infected with SMRV was examined by
DAPI staining at different times post-infection. The arrows indicate the plaques formed by virus infection. Magnification: 50×.
2.5. Electron microscopy

The infected CLC cells were collected at 18 hpi (hour post-infection). Cytotoxic effect (CPE) was usually observed under light microscopy at about 8 hpi. The collected cells were fixed with 2% glutaraldehyde, post-fixed in osmium tetroxide (OsO4), dehydrated and embedded as described previously (Zhang et al., 2003a). Thin sections were cut and stained with uranyl acetate and lead citrate, then examined with Hitachi H-7000 electron microscopy.

2.6. DNA preparation and gel electrophoresis

The medium of the viral-infected cells was removed and the cells were extracted with TES (10 mM Tris, pH 8.0, 1 mM EDTA, 1% SDS, to which proteinase K to a final concentration of 100 μg/ml was added). Then they were incubated for a period of 2–2.5 h at 37°C. The DNA was extracted with phenol:chloroform (1:1 v/v) and with chloroform. Finally, 100% ethanol was added to the final concentration of 70% (v/v) to precipitate the DNA at −20°C overnight and then by microcentrifugation at 15,000 × g for 15 min. The precipitation was washed with 70% (v/v) ethanol. The pellets were re-suspended in TE with RNase A (50 μg/ml) and analyzed by 1.5% agarose gel electrophoresis.

2.7. Flow cytometry analysis

The cells were harvested and washed with PBS (pH 7.2) twice and suspended in 80% ethanol at −20°C for 24 h, then thawed quickly at room temperature and centrifuged to collect the cells. The cells were washed with PBS twice again and re-suspended in the extraction buffer (0.2 M Na2HPO4, 0.1 M citric acid) for 5 min, and finally re-suspended in PBS containing RNase A (100 μg/ml) and 50 μg/ml propidium iodide for 30 min. The cell cycle distribution and quantitation of apoptotic cells were determined by the fluorescence of individual cells measured by flow cytometry (Beckman-Coulter Epics Altra).

3. Results

3.1. Morphological changes of infected cells

The virus isolate can induce strong CPE in the infected CLC cells. As shown in Fig. 1, a few of plaques resulted from

![Fig. 2. Induction of morphologic changes in SMRV infected CLC cells. Apoptosis morphology was examined by DAPI staining at different times post-infection. Cells showing characteristic morphologic modifications such as nuclear condensation and blebbing were considered as apoptotic. The arrowhead indicates the fragmented nuclei and the arrow indicates apoptotic body. Magnification: 400×.](image-url)
CPE was observed at 15 hpi, and more and more plaques were produced as the infection time increased. When the cells were infected for 24 h, the plaques have extended to the entire cell sheets.

Morphological changes in apoptotic cells could be obviously investigated under fluorescence microscopy after DAPI staining. When observed at high magnification, extreme chromatin condensation and nucleus fragmentation could be found from some infected cells at 9 hpi. As the infection time increased, more and more cells produced extreme chromatin condensation and nucleus fragmentation, and led to apoptotic body formation. At 24 h of infection, almost all cells had undergone the morphological changes of apoptosis (Fig. 2).

To further characterize the morphologic changes in the infected cells, we performed the electron microscopic analysis. The infected CLC cells displayed cell shrinkage, plasma membrane blebbing, cytoplasm vacuolization, chromatin condensation, nuclear breakdown and formation of discrete apoptotic bodies (Fig. 3A). All of the induced changes are the typical indications of apoptosis. At the same time, a lot of bullet-shaped virus particles were released from the breakdown cells (Fig. 3B). The bullet-shaped nucleocapsids were measured and ranged in size from 110 to 150 nm in length and 40 to 60 nm in diameter. The spikes (about 10 nm long) dispersed evenly over the entire surface of the virus (Fig. 3B). One central electron-lucent hole of 10–20 nm in diameter was observed in the cross sections of nucleo-
capsids (Fig. 3B). The isolated virus should be a member of the rhabdoviridae, and is therefore called Scophthalmus maximus rhabdovirus (SMRV).

3.2. DNA fragmentation of apoptotic cells

DNA fragmentation was also examined in the SMRV infected CLC cells. In order to observe the DNA fragmentation in the course of infection, the DNA samples were isolated from uninfected and infected CLC cells at different times after the start of infection, separated by electrophoresis in 1% agarose gel containing ethidium bromide, and visualized under UV transillumination. As shown in Fig. 4, a characteristic of oligonucleosome ladder pattern was displayed on the electrophoresed gel in the course of SMRV infection. There was no DNA fragmentation at 0 h. In the infected cells at 9 hpi, some weaker ladder bands were observed. The intensity of DNA fragmentation increased obviously in the infected cells at 12 hpi, and became stronger and stronger gradually as the infection course extended. Moreover, the proportion of fragments with lower molecular weight increased more obviously at later stage of infection, especially at 36 and 48 hpi. The data indicated that the cellular DNAs had been cleaved into an incremental manner of multiples of 180–200 bp associated with each nucleosomes in the apoptosis induced by SMRV.

3.3. Flow cytometric analysis of apoptotic cells

The above morphological changes and DNA fragmentation demonstrated that apoptosis has occurred in the SMRV infected CLC cells. Furthermore, the apoptotic cells were determined as the hypoploid sub-G0/G1 peak localized on the left of normal diploid G0/G1 peak of the cell cycle, which were measured by flow cytometry. As shown in Fig. 5, only two normal peaks, which represent diploid G0/G1 cells and

Fig. 4. DNA fragmentation in SMRV infected CLC cells as assessed by agarose gel electrophoresis. Lane 1: DNA marker (DL2000); Lane 2: DNA from uninfected CLC cells; Lanes 3–10: DNA of CLC cells 9, 12, 15, 18, 21, 24, 36 and 48 h post-infection, respectively; Lane 11: DNA marker (H9261/HindIII).

Fig. 6. Virus titre and apoptotic cells percentage at different time post-infection. The trend of virus replication (A) and cell apoptosis (B) according to the virus infection time.
tetraploid G_2/M cells, were observed in uninfected CLC cells and mock-infected cells after 24 h in culture. In contrast, an extra hypoploid sub-G_0/G_1 peak (apoptotic peak) was detected in the SMRV infected CLC cells beginning at 9 hpi. As the infection time extended, the apoptotic peak rose strongly. At 24 hpi, the apoptotic peak was significantly wider and higher than the diploid G_0/G_1 peak. The comparison on flow cytometric histogram (Fig. 5) of cell cycle distribution of the mock-infected and SMRV infected cells at different infection time indicated that the percentage of apoptotic cells increased rapidly, whereas the percentage of G_0/G_1 cells decreased quickly.

3.4. Quantitation of apoptotic cells and virus titre

One of advantages in flow cytometry assays is capable of quantitation of apoptotic cells from virus-infected cells (Hoff and Donis, 1997). Using this advantage, we further determined the proportion of apoptotic cells by statistical analysis on the repeated flow cytometric measurements. As shown in Fig. 6B, the proportion of apoptotic cells was very high in the SMRV infected CLC cells, and increased as the infection course extended. At 9 hpi, the percentage of apoptotic cells was about 10.5%, at 24 hpi, the percentage reached to about 51.9%. In addition, we also measured the virus titre in the course of infection (Fig. 6A). The virus titre was basically steady before 18 hpi. From 18 to 24 hpi, the virus titre rose quickly (Fig. 6A). By comparing the changes between proportion of apoptotic cells and virus titre, we observed that virus titre in infected cells was correlated with degree of the apoptosis. When the virus titre increased, the apoptotic cells also increased, while the virus replication fastigium appeared earlier than that of the apoptosis occurrence.

3.5. Effect on apoptosis of UV-inactivated SMRV

UV-inactivated SMRV infected CLC cells were also analyzed by fluorescent staining and flow cytometry. No apoptosis was induced in the UV-inactivated SMRV infected CLC cells, because no plaque formation and nuclei fragmentation were observed under fluorescence microscopy and no hypoploid sub-G_0/G_1 peak was detected by flow cytometric measurements (Fig. 7). These data suggested that virus replication is necessary for induction of apoptosis.

4. Discussion

In recent years, a lot of viruses in more than 10 different families have been shown to induce apoptosis in the infected cells (Summerfield et al., 2001; Du et al., 2002; Yao et al., 2002; Humlova et al., 2002; Ishikawa et al., 2003; Clarke and Clem, 2003), and the viral-induced apoptosis has been recognized as a common strategy and method utilized by the viruses to overcome the host cells (Licata and Harty, 2003). In fish rhabdoviruses, some typical characteristics of apoptosis have been revealed in the epithelial papilloma of carp (EPC) cells infected by the rhabdoviruses spring viremia of carp virus (SVCV), infectious hematopoietic necrosis virus, and viral hemorrhagic septicemia virus (Bjorklund et al., 1997). In this study, we have further observed these typical induction changes of apoptosis in the SMRV infected CLC cells by a series of studies, such as morphology observation, DNA fragmentation analysis and flow cytometry assays. The results indicated that the newly isolated rhabdovirus SMRV, just like other fish-pathogenic rhabdoviruses...
(Bjorklund et al., 1997), was able to induce apoptosis in the infected CLC cells, and further confirmed that apoptosis should be a general cell killing mechanism of rhabdoviruses as suggested previously (Bjorklund et al., 1997; Licata and Harty, 2003).

In order to get more details about the apoptosis, we compared the association between morphological changes and virus titre at different time after infection. The results showed that the chromatin condensation and nucleus fragmentation happened at 9 hpi and were much earlier than the occurrence of CPE which can be observed at 15 hpi (Figs. 1 and 2). At the same time the DNA ladder that was the indication of DNA fragmentation also appeared at the early stage and 2). At the same time the DNA ladder that was the indication of DNA fragmentation also appeared at the early stage during the infection procedure (Fig. 4). The data confirmed the association between morphological changes and virus titre at different time after infection. The results showed that the chromatin condensation and nucleus fragmentation happened at 9 hpi and were much earlier than the occurrence of CPE which can be observed at 15 hpi (Figs. 1 and 2). At the same time the DNA ladder that was the indication of DNA fragmentation also appeared at the early stage.

A highlight in this study is the application of flow cytometry in apoptosis detection. With the help of flow cytometry, the cells at different stages of the apoptosis process were distinguished, and the existence of an extra hypoploid sub-G0/G1 peak (apoptotic peak) was revealed. Quantitative analysis further demonstrated the association between virus titre and apoptotic degree in the SMRV infected CLC cells.

UV light is able to destroy the nucleic acid of virus, and can keep viral envelop proteins active, which permit the active virus’s binding to and entering the host cells without replication (Zhang et al., 2003b). In this report, we found that the UV-inactivated SMRV could not induce cell apoptosis. These results suggested that the active virus replication and production of progeny virus is necessary for apoptosis induction.

In summary, this study provides further evidence that apoptosis is involved in the cell death caused by the newly isolated virus in the carp leucocyte (CLC) cell line, and demonstrates that the newly isolated virus from *Scophthalmus maximus* L. is a rhabdovirus that is therefore called *Scophthalmus maximus* L. rhabdovirus (SMRV). In the course of these experiments, we have also observed the detailed process and the consequences of apoptotic induction.

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References


