Infectious pancreatic necrosis virus induces apoptosis due to down-regulation of survival factor MCL-1 protein expression in a fish cell line

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Abstract

Infectious pancreatic necrosis virus (IPNV), a member of the virus family Birnaviridae, causes an acute, contagious disease in a number of economically important fish species. CHSE-214, a Chinook salmon embryonic cell line, when infected by IPNV showed morphological and biochemical features of apoptosis, including an intense DNA laddering pattern and blebbing of the plasma membrane, followed by formation of apoptotic bodies. The Mcl-1 gene product proved to be a member of the Bcl-2 gene family, and like Bcl-2 had the capacity to promote cell viability. Here, we investigated the pattern of expression of Mcl-1 in CHSE-214 cells infected by IPNV. We found that the Mcl-1 level decreased markedly in cells undergoing apoptosis after IPNV infection. This decrease was rapid during the first 8 h postinfection and preceded cell death. Furthermore, we found that drugs including cycloheximide, genistein and EDTA either prevented the decline in Mcl-1 levels or blocked the intense DNA laddering pattern. Other drugs like serine proteinase inhibitor, 400 μg/ml aprotinin, 400 μg/ml leupeptin and 100 μg/ml tryphostin did not. The virus gene expression pattern was examined by Western blot using antivirion polyclonal antibody and was blocked during treatment with cycloheximide, genistein and EDTA but not by serine proteinase, aprotinin, leupeptin or tryphostin. Together the data showed a striking correlation between virus replication and Mcl-1 expression in CHSE-214 cells, suggesting that the virus gene expression has a possible involvement with Mcl-1 in the regulation of apoptosis in these cells. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Infectious pancreatic necrosis virus (IPNV) is an economically important fish pathogen and is the prototype of the established Birnaviridae virus family (Brown, 1986). Other members include infectious bursal disease virus (IBDV) of fowl (Do-
IPNV shows a high degree of antigenic heterogeneity. Three different serotypes, Ab, Sp and VR299 (MacDonald and Gower, 1981; Okamoto et al., 1983), and ten subgroups (Heppell et al., 1992) have been identified. IPNV is the etiological agent of a contagious, high mortality disease of young, hatchery-reared salmonids (Wolf et al., 1960; Hedrick et al., 1983; Wu et al., 1983) and other non-salmonid fishes (Adair and Ferguson, 1981; Hedrick et al., 1983; Chen et al., 1984; Ueno et al., 1984). IPNV has a two-segment dsRNA genome and four virion proteins (MacDonald and Yamamoto, 1977; MacDonald and Dobos, 1981).

Apoptosis and necrosis are the two stereotyped mechanisms by which nucleated eukaryotic cells die (Wyllie et al., 1980; Duvall and Wyllie, 1986; Kerr and Harmon, 1991; Majno and Joris, 1995). Necrosis is considered to be a pathological reaction that occurs in response to major perturbations in the cellular environment such as a lytic viral infection. On the other hand, apoptosis is considered a physiological process that takes part in homeostatic regulation, when death is part of the reactions involved in normal tissue turnover (Duvall and Wyllie, 1986; McConkey et al., 1989; Rothenberg, 1990). Recently, the mechanisms leading to apoptosis have become better understood (Jeurnissen et al., 1992; Shen and Shenk, 1995; Chen et al., 1997; Inoue et al., 1997; Jacotot et al., 1997; Rao and White, 1997) and appear to be different from one system to another.

Several other proteins exist with sequence similarity to Bcl-2 and with the ability to modulate apoptosis (White, 1996). Mcl-1 is one of the members of the Bcl-2 family. Like Bcl-2, Mcl-1 heterodimerizes with Bax, an accelerator of apoptosis in the Bcl-2 family, and neutralizes the cytotoxicity induced by Bax in yeast (Bodrug et al., 1996). Mcl-1 is also able to protect Chinese hamster ovary cells from apoptosis induced by c-myc overexpression (Reynolds et al., 1994). This protein was discovered as a novel gene induced early in the induction of differentiation of a human myeloid leukemia cell line (Kozopas et al., 1993). Expression of Mcl-1 mRNA was rapidly up-regulated with phorbol ester in those cells followed by a rapid degradation, consistent with the presence of a mRNA destabilization sequence in its 3'-untranslated region. The half-life of the Mcl-1 protein is short (Yang et al., 1995), which has been ascribed to the presence of two PEST motifs. Therefore, Mcl-1 is suggested as a rapidly inducible, short-term effector of cell viability (Yang et al., 1996).

E1-S of IPN virus Ab strain (Wu et al., 1987) induces CHSE-214 cells apoptotic cell death that was examined by Hong et al. (1998). In the present work, we asked CHSE-214 apoptotic cell death whether via Mcl-1 dependent pathway when IPNV-infected host cells. We investigated the pattern of expression of Mcl-1 in CHSE-214 cells infected by IPNV. We found that the Mcl-1 level decreased markedly in apoptotic cells at 8 h postinfection. Furthermore, we found that drugs including cycloheximide, genistein and EDTA either prevented the decline in Mcl-1 levels or blocked the intense DNA laddering pattern. Together the data showed a striking correlation between virus replication and Mcl-1 expression in CHSE-214 cells, suggesting that the virus gene expression has a possible involvement with Mcl-1 dependent pathway in the regulation of apoptosis in CHSE-214 cells.

2. Materials and methods

2.1. CHSE-214 and viruses

Chinook salmon embryo cells (CHSE-214) were obtained from American Type Culture Collection (ATCC). Cells were grown at 18°C as monolayers in plastic tissue culture flasks (Nunc) using Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) fetal calf serum and 25 μg/ml gentamicin. E1-S of IPN virus Ab strain was isolated from Japanese eel in Taiwan (Wu et al., 1987). E1-S was propagated in CHSE-214 cell monolayers at a 0.01 multiplicity of infection (MOI) per cell. Infected cultures were incubated at 18°C until an extensive cytopathogenic effect (CPE) was observed. The cells were scraped into tissue culture medium, placed in ice, and the...
cultures were then sonicated. This virus stock ($5 \times 10^7$ to $1 \times 10^8$ pfu/ml) was dispensed into 1-ml samples and stored at $-70^\circ$C. Virus plaque assay was performed on confluent monolayers of CHSE-214 cells that were infected with virus solution for 1 h at room temperature, overlaid with 0.6% agarose containing 2.5 µg/ml of trypsin, and incubated for 3 days at 18°C. Cells were stained with 1% crystal violet in 20% ethanol (Dobos et al., 1979).

2.2. Scanning electron microscopy

About $10^6$ cells/ml were seeded on a two chambers slide for growth for more than 20 h. CHSE-214 was infected with a MOI of 1 virus and incubated for 8 h. At the end point, cells were washed twice with PBS and fixed with 2.5% glutaradehyde in 0.1 M phosphate buffer. Samples were postfixed with OsO4, dehydrated in ethanol, critical point dried, and gold sputtered.

A Philips 515 scanning electron microscope was utilized for the observations.

2.3. DNA preparation and gel electrophoresis

About $10^5$ cells/ml were seeded on a 60-mm petri dish for growth for above or more than 20 h. The monolayer of cells were infected with IPNV with a MOI of 1 virus and incubated for 8 h. Non-infected cells were used as a normal control. The two groups were also used for DNA fragmentation studies. At the end of incubation the cells were lysed with lysis buffer (10 mM Tris–HCl, 0.25% Triton X-100, 1 mM EDTA, pH 7.4). After treatment with phenol–chloroform–isoamyl alcohol (25:24:1), the DNA was precipitated in the presence of 0.3 M sodium acetate and cold absolute ethanol at $-70^\circ$C for 2 h, and then resuspended in 10 mM Tris–HCl (pH 7.4) and EDTA. Aliquots of 20 µl containing approximately 5–10 µg of DNA were then electrophoresed in 1.2% agarose gels for 2 h at 40 V. Gels were stained with ethidium bromide and photographed under UV transillumination.

2.4. Immunoblotting

About $10^5$ cells/ml were seeded on a 60-mm petri dish for growth for more than 20 h. Monolayers were rinsed twice with phosphate-buffered saline (PBS). Control cells or cells that received virus at a MOI of 1 were incubated for 0, 2, 4, 6, 8, 10 and 24 h. At the end of each incubation time the culture medium was aspirated. The cells were washed with PBS and then lysed in 0.3 ml lysis buffer [10 mM Tris base, 20% glycerol, 10 mM sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol (β-ME), pH 6.8].

Proteins were separated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), electroblotted, and subjected to immunodetection as described by Kain et al. (1994). Blots were incubated with a 1:1500 dilution of anti-human Mcl-1 polyclonal antibodies (Pharmingen) or a 1:7500 dilution of a peroxidase-labeled goat anti-rabbit conjugate (Amersham). Chemiluminescent detection was performed according to the instructions provided with the Western Exposure Chemiluminescent Detection System (Amersham). Chemiluminescent signals were imaged by exposure to Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA). Primary (Mcl-1) and secondary antibodies (peroxidase-labeled goat anti-rabbit conjugate) were stripped from blots by incubation in stripping buffer containing 62.5 mM Tris–HCl (pH 6.8), 3.0% (w/v) SDS and 50 mM 1,4-dithiothreitol for 30 min at 55°C with gentle shaking. The blots were then washed four times for 10 min each time in PBS containing 0.1% (v/v) Tween 20 and reprobed with mouse actin monoclonal antibody (1:1500, Chemicon) and a 1:7500 dilution of a peroxidase-labeled sheep anti-mouse conjugate (Amersham).

The potent drugs on effect of blockage on viral protein expression experiment, the cell preparation was as described above except that extra cycloheximide (10 µg/ml), aprotinin (400 µg/ml), leupeptin (400 µg/ml), genistein (100 µg/ml), trypophostin (100 µg/ml) and EDTA (2 mM) were added to 3 ml of MEM medium on CHSE-214 cells before virus infection and incubation for 16 h. At the end of the incubation period, cells were harvested and the samples were analyzed by Western blot method as described above.
3. Results

3.1. Ultrastructure morphological changes in CHSE-214 cells with IPNV infection by scanning electron microscopy

Apoptosis induces characteristic morphological changes in cells, such as condensation and fragmentation of the nucleus as well as loss of cytoplasm (Wyllie et al., 1984). To substantiate that IPNV-infected cells had undergone apoptosis, negative control and IPNV-infected cells were harvested and processed for scanning electron microscopy. Normal negative control cells are shown in Fig. 1A. IPNV-infected CHSE-214 cells displaying detachment and blebbing of the plasma membrane are shown in Fig. 1B.

Fig. 1. Scanning electron micrographs of CHSE-214 cells. (A) Uninfected CHSE-214 cells. (B) IPNV infected CHSE-214 cells; the formation of membrane blebbing in the apoptotic cell can be seen.

Fig. 2. DNA fragment analysis of IPNV E1-S-infected CHSE-214 cells. DNA was isolated from uninfected CHSE-214 cells as a negative control cultured for 0 h (lane 2), or CHSE-214 cells infected for 4, 8 and 12 h with a MOI of 1 of E1-S (lanes 3–5), electrophoresed through 1.2% agarose gels, and visualized by staining with ethidium bromide. Lane 1 contains the molecular weight markers used in the gel (1-kb DNA ladder from MBI Fermentas Inc. USA, for sizing liner fragments ranging in size from 500 bp to 1 kb).

3.2. Induction of internucleosomal cleavage by IPNV in CHSE-214 cells

DNA fragmentation is a well-defined biochemical marker of apoptosis (Schwartzman and Cidlowski, 1993). E1-S of IPN virus Ab strain (MOI of 1) infected cells were examined for evidence of internucleosomal fragmentation. Intense internucleosomal fragmentation of DNA, a pattern highly specific to apoptosis, was observed in CHSE-214 cells infected with IPNV (Fig. 2). The IPNV induced DNA fragmentation at 8 h and 12 h postinfection was identified by gel electrophore-
sis (Fig. 2, lanes 4 and 5). The gel of the negative control at 0 h incubation and 4 h postinfection showed no DNA fragmentation (Fig. 2, lanes 2 and 3).

3.3. Western blot

The characterization of the viral protein size and Mcl-1 expression was directly quantified by Western blots from CHSE-214 cells. Fig. 3 shows the major protein expression pattern during infection of CHSE-214 cells by a MOI of 1 of E1-S. The viral proteins had a large expression after 4 h post-infection. Fig. 4 shows the Mcl-1 protein expression pattern during infection of CHSE-214 cell with a MOI of 1 of E1-S. The down-regulation of Mcl-1 expression occurred between 6 h and 8 h post-infection (as shown in Fig. 4A, lanes 4 and 5). The internal control actin is shown in Fig. 4B.

Fig. 3. Detection of major protein of IPNV E1-S strain on Western blots. Samples were electrophoresed on a SDS–polyacrylamide gel and electroblotted to a NC membrane. Antigen-specific signals were detected with a rabbit anti-E1-S virion antiserum and a goat anti-rabbit IgG conjugated to alkaline phosphatase. The chemiluminescent signal was imaged on Kodak X-OMAT film (Eastman Kodak) with a 1.5-min exposure. Lanes 1–7 correspond to a MOI of 1 infected cells for 0, 2, 4, 6, 8, 10 and 24 h post-infection.

Fig. 4. Detection of Mcl-1 protein in CHSE-214 by IPNV infection on Western blots. CHSE-214 cells were infected with IPNV (MOI of 1). Samples were electrophoresed on a SDS–polyacrylamide gel and electroblotted to a NC membrane. The NC membrane was stained with either a rabbit polyclonal antiserum directed against human MCL-1 (Pharmingen) or mouse monoclonal IgG antibodies directed against actin (Amersham) (B). The chemiluminescent signal was imaged on Kodak XAR-5 film using a 3-min (A) and 1.5-min (B) exposure. (A) Lanes 1–7, 30 µg of virus infected CHSE-214 cell lysate corresponding to 0, 2, 4, 6, 8, and 24 h post-infection, respectively. (B) The NC membrane of (A) was stripped in stripping buffer containing 62.5 mM Tris–HCl (pH 6.8), 3.0% (w/v) SDS, and 50 mM 1,4-dithiothreitol for 30 min at 55°C with gentle shaking to remove the primary (Mcl-1) and secondary antibodies (peroxidase-labeled goat anti-rabbit conjugate). The blots were then washed four times for 10 min each time in PBS containing 0.1% (v/v) Tween 20 and reprobed with mouse actin monoclonal antibody (1:1500, Chemicon) and a 1:7500 dilution of a peroxidase-labeled goat anti-mouse conjugate (Amersham).

3.4. Blocking of virus replication for prevention of down-regulation of the Mcl-1 protein by certain drugs

To confirm whether virus replication is involved in down-regulation of Mcl-1 event, virus replication in host cell was blocked by treatment with certain drugs. When the protein synthesis inhibitors, 10 µg/ml cyclohexamide, tyrosine kinase inhibitor, 100 µg/ml of genistein or the cation chelator, 2 mM of EDTA, were added to CHSE-214 cells before IPNV infection, virus replication was prevented (as shown in Fig. 5A). At the same time, these same drugs partially prevented down-regulation of Mcl-1 protein expression (as shown in Fig. 5B). However, the serine protease inhibitor aprotinin 400 µg/ml and leupeptin 400 µg/
ml (as shown in Fig. 5B, lanes 5–6) and the tyrosine kinase inhibitor tryphostin 100 µg/ml (as shown in Fig. 5B, lane 8) could not. The internal control actin protein is shown in Fig. 5C. The DNA internucleosomes were assayed under the same conditions described above. The blocking of virus replication groups consistently and strongly prevented the induction of internucleosomal cleavage by IPNV (as shown in Fig. 6), with the exception of the 2 mM EDTA treatment group which displayed minor internucleosomal cleavage (as shown in Fig. 6, lanes 14 and 15).

4. Discussion

IPNV is a highly contagious disease of susceptible hatchery-reared trout and Japanese eel (*Anguilla japonica*) in Taiwan (Wu et al., 1983, 1987). IPNV replicates in a variety of continuous cell lines from teleost fish at temperatures below 24°C (Wolf and Mann, 1980). The virus replicates in the cytoplasm and a single cycle of replication takes 16–20 h at 22°C resulting in a characteristic cytopathic effect (CPE) (Malsberger and Cerini, 1963). Rainbow trout gonad (RTG-2) cells infected with IPNV which yielded infectious titers of only $10^6–10^7$ pfu/ml (Malsberger, 1965), because the RTG-2 cells can produce interferon when IPNV infection and are themselves sensitive to interferon treatment, as reported by MacDonald and Kennedy (1979). On the other hand, chinook salmon embryo (CHSE-214) cells do not produce interferon, and are insensitive to interferon treatment resulting in virus yields of $2–5 \times 10^8$ pfu/ml or higher. In the present investigation, by assaying for cell ultra-morphological features (as shown in Fig. 1) and DNA fragmentation (as shown in Fig. 2) we examined the possibility that apoptosis may contribute to the death of IPNV infection cells, but these features did not happen in an IPNV persistent cell line (data not shown). Reports by Ahne (1977), and Hedrick et al. (1978) found CHSE-214 cells which survived in a lytic virus infection grew into persistently infected cultures that continued to produce low levels of infectious virus without any CPE. The maintenance of these carrier cultures was attributed to DI (defective interfering) particles (MacDonald, 1978; MacDonald and Kennedy, 1979).
Virus replication (as shown in Fig. 3) is correlated with down-regulation of Mcl-1 protein expression (as shown in Fig. 4A). This is the first study that confirms down-regulation of Mcl-1 (a member of the Bcl-2 family) protein expression by virus infection. The results from the present data are different from studies of other viruses. For example, expression of the Epstein–Barr virus transforming protein LMP1 causes a rapid and transient stimulation of Mcl-1 level in B-cell lines (Wang et al., 1996). Treating CHSE-214 cells with IPNV at a high MOI of virus (MOI of 20) quickly induce the DNA internucleosomal cleavage 30 min after infection. This finding supports the possibility of another mechanism being involved in the induction of apoptosis through a Mcl-1 independent pathway.

In view of the capacity of Mcl-1 to block or delays apoptosis (Reynolds et al., 1994; Bodrug et al., 1996) and its sequence feature as a PEST (proline, glutamic acid, serine, threonine) protein that can be degraded rapidly (Kozopas et al., 1993), one possible role of this protein is as a rapid turnover effector that controls the rate of apoptosis (Kozopas et al., 1993). However, when treatment with some drugs before IPNV-infected CHSE-214 cells was performed, the protein synthesis inhibitor, cycloheximide, or the tyrosine kinase inhibitor, genistein, and the cation chelator, EDTA, all blocked viral protein expression (as shown in Fig. 5A). And at the same time, same drugs helped to maintain Mcl-1 expression level (as shown in Fig. 5B) and blocked to the induction of DNA internucleosomal cleavage (as shown in Fig. 6) for rescue or delay of apoptotic cell death.

Our data show that viral protein synthesis or virus replication correlate to down-regulate the survival factor Mcl-1 expression level for induced host cells undergoing the apoptotic cell death at low MOIs of infection. This finding suggests that viral gene product(s) expression between 6 and 8 h after IPNV infection may be involved in direct or indirect down-regulation of the Mcl-1 pathway. However, further experiments are required to evaluate the significance of our findings regarding which protein(s) relate to inducing down-regulation of Mcl-1 for inducing post-apoptotic necrosis at the pre-late stage (Hong et al., 1998).
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