



Aquatic birnavirus induces necrotic cell death via the mitochondria-mediated caspase pathway

Po-Chun Chen^a, Jen-Leih Wu^b, Guor Mour Her^c, Jiann-Ruey Hong^{a,*}

^a Laboratory of Molecular Virology and Biotechnology, Institute of Biotechnology, National Cheng Kung University, Tainan 701, Taiwan

^b Laboratory of Marine Molecular Biology and Biotechnology, Institute of Cellular and Organismic Biology, Academia Sinica, Nankang, Taipei 115, Taiwan

^c Institute of Bioscience and Biotechnology, National Taiwan Ocean University, No. 2, Pei-Ning Road, Keelung 202-24, Taiwan

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ABSTRACT

Aquatic birnavirus induces necrotic cell death by an ill-understood process. Presently, we demonstrate that infectious pancreatic necrosis virus (IPNV) induces post-apoptotic necrotic cell death through loss of mitochondrial membrane potential (MMP) followed by caspase-3 activation in CHSE-214 cells. Progressive phosphatidylserine externalization was observed at 6 h post-infection (p.i.). This was followed by the development of bulb-like vesicles (bleb formation) at 8 h p.i. Progressive loss of MMP was also observed in IPNV-infected CHSE-214 cells beginning at 6 h p.i. At 8 h and 12 h p.i., IPNV-infected cells demonstrated a dramatic increase in MMP loss, rapid entry into necrotic cell death, and activation of caspase-9 and -3. Additionally, treatment with an inhibitor of MMP loss, bongkreikic acid, an adenine nucleotide translocase inhibitor, blocked IPNV-induced PS exposure and MMP loss, as well as reduced the activation of caspase-3. Taken together, our results suggest that IPNV induces apoptotic cell death via loss of MMP, thereby triggering secondary necrosis and caspases-3 activation. Furthermore, this death-signaling pathway is disrupted by bongkreikic acid in fish cells, indicating that this drug may serve to modulate IPNV-induced pathogenesis.

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1. Introduction

Apoptosis is a process of systematic self-destruction of individual cells in response to a wide variety of stimuli [1]. The process is genetically controlled and preprogrammed to eliminate redundant cells during development and is used as an emergency response following radiation damage, viral infection, or aberrant cell growth induced by oncogenes [2–4]. Apoptosis and necrosis are two stereotyped mechanisms by which nucleated eukaryotic cells die [1,5–7]. Understanding these mechanisms may ultimately lead to novel therapeutic strategies.

The mitochondrion functions as a central integrator of pro-death stimuli [8] by sequestering apoptogenic proteins that include cytochrome c, Smac/DIABLO, apoptosis inducing factor (AIF), and endonuclease G in the intermembrane space and releasing these factors into the cytosol for further induction of pro-apoptotic signals [9,10]. The loss of mitochondrial membrane potential (MMP) leads to activation of caspase-9, the downstream activator of apoptosis [9,10]. MMP loss can affect both the inner and outer mitochondrial membranes and precedes the signs of necrotic or apoptotic cell

death, including the apoptosis-specific activation of caspases [8]. Hence, the mitochondrion functions as a central integrator of pro-death stimuli, joining together various types of pro-apoptotic signals into a common caspase-dependent pathway [11].

Infectious pancreatic necrosis virus (IPNV) is a fish pathogen and the prototype (aquatic birnavirus) of *Birnaviridae* virus family [12]. Birnaviruses possess a bi-segmented, double-stranded RNA genome contained within a medium-sized, unenveloped, icosahedral capsid. Gene expression involves the production of four unrelated major genes, which undergo various post-translational cleavage processes to generate three to five different structural proteins [13]. The largest of these proteins (VP1; 90–110 kDa) is encoded by the smaller segment B RNA [14]. Within the larger genome segment A, a large open reading frame (ORF) encodes VP3 (submajor capsid protein; 32 kDa), VP4 (28 kDa), and VP2 (major capsid protein; 46 kDa) [15]. Also within this segment, a small ORF encodes the small non-structural VP5 protein (17 kDa), which performs an anti-apoptotic function [16].

Previously, we reported that IPNV infection induces apoptosis in a fish cell line [3,16,17] through activation of caspase-8 and -3 [18], and this apoptosis requires new protein synthesis [19] and may require activation of the NF- κ B transcription factor for transactivating downstream effector genes [19]. On the other hand, IPNV-induced expression of Annexin 1 could play an anti-death

* Corresponding author. Tel.: +886 6 2003082; fax: +886 6 2766505.

E-mail address: jrhong@mail.ncku.edu.tw (J.-R. Hong).

function [20]. Currently, our main interest is how IPNV induces the conversion from apoptosis to secondary necrosis in CHSE-214 cells.

In this study, we investigated how necrotic cell death is regulated in CHSE-214 cells infected by the E1-S strain of IPNV. We determined that IPNV-induced secondary necrosis might be mediated by the mitochondria-dependent pathway for caspase-3 activation. Furthermore, this process was disrupted by inhibition of MMP loss following treatment with the adenine nucleotide translocase (ANT) inhibitor, bongkreikic acid (BKA).

2. Materials and methods

2.1. Cell line and virus

Chinook salmon embryo cells (CHSE-214) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown at 18 °C in plastic tissue-culture flasks (Nalge Nunc International, Rochester, NY, USA) containing Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and gentamicin (25 µg ml⁻¹). An isolate of the Ab strain of IPNV, designated E1-S, was obtained from Japanese eels in Taiwan [14]. The virus was propagated in CHSE-214 cell monolayers at a multiplicity of infection (MOI) of 0.01 per cell. Infected cultures were monitored as described previously [21] and the TCID₅₀ assay was performed on confluent monolayers [22].

2.2. Tracing of VP3 protein by immunostaining in IPNV-infected cells

CHSE-214 cells cultured as described above were infected with IPNV E1-S at an MOI = 5 for 0 and 9 h post-infection (p.i.). At the end of RGNNV infection, cells were washed three times with PBS (phosphate-buffered saline: 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4, 2.5 mM KCl, and 135 mM NaCl) then fixed for 30 min in 4% paraformaldehyde on ice. The cells were then permeabilized for 20 min in 0.5% Tx-100 in PBS on ice and blocked in PBS containing 5% dry milk and 0.1% Tween 20. The samples were then incubated with primary antibodies (IPNV VP3 monoclonal antibody [Clone No: 8-42-E7]; 1:50 dilution) in blocking buffer followed by four consecutive 2-min washes in blocking buffer and a 60 min incubation with secondary antibodies (Fluorescein [FITC]-conjugated rabbit anti-mouse IgG; 1:100 dilution; Jackson ImmunoResearch Laboratories, INC, West Grove, PA, USA) in blocking buffer. After four additional washes in PBS, the coverslips were incubated with DAPI (0.1 µg ml⁻¹; Sigma Chemical Co.) nucleus dye for 5 min in PBST buffer, and then the staining buffer was aspirated. The samples were mounted in a solution of 1 mg ml⁻¹ *p*-phenylenediamine (Sigma Chemical Co.) in 90% glyceride, pH 8.3. The immunofluorescence was examined using an Olympus IX70 fluorescence microscopy using a 488 nm excitation and 515 nm long-pass filter for detection RGNNV B1-fluorescein [18] and a DAPI filter for detection blue fluorescence in the nucleus.

2.3. Western-blot analysis

Monolayers of CHSE-214 cells (4.0 ml, 10⁵ cells per ml) on 60 mm Petri dishes were cultivated for at least 20 h and rinsed twice with PBS. Cells were infected with virus (MOI = 5) for 0 h, 6 h, 9 h, or 12 h prior to the viral protein expression assay and for 0 h, 4 h, 8 h, or 12 h prior to the caspase-3 cleavage assay. At the end of each incubation period, the culture medium was aspirated and the cells were washed with PBS and then lysed in 0.3 ml of lysis buffer (10 mM Tris base, 20% glycerol, 10 mM sodium dodecyl sulphate, and 2% β-mercaptoethanol; pH = 6.8).

Proteins were separated by SDS-polyacrylamide gel electrophoresis [23], electroblotted, and subjected to immunodetection as described elsewhere [24]. Blots were incubated with a 1:3000 dilution of anti-IPNV E1-S particle polyclonal antibodies and a 1:10 000 dilution of a peroxidase-labelled goat anti-rabbit conjugate (Amersham, Piscataway, NJ, USA) or with a 1:2000 dilution of anti-mouse caspase-3 monoclonal antibodies and a 1:8000 dilution of a peroxidase-labelled rabbit anti-mouse conjugate. Chemiluminescence detection was performed according to the instructions provided with the Western Exposure Chemiluminescence Kit (Amersham). Chemiluminescence was visualized by exposure to Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA).

2.4. PS exposure assay

Monolayers of CHSE-214 cells (4.0 ml, 10⁵ cells per ml) on 60 mm Petri dishes were cultivated for at least 20 h and rinsed twice with PBS. Cells were infected with virus (MOI = 5) and incubated for 0, 8 h, or 12 h p.i. The cells pre-treated with BKA (20 µg ml⁻¹) were infected with virus (MOI = 5) 2 h after pre-treatment and then incubated for 0, 8 h, or 12 h p.i. For the early apoptotic cell assays [25], exposure of PS on the outer leaflet of early apoptotic cell membranes was analyzed using Annexin V-fluorescein to differentiate apoptotic from non-apoptotic cells. At 0, 8, and 12 h p.i., cells were removed from the medium, washed with PBS, and then incubated with 100 µl of a commercially available staining solution (Annexin V-fluorescein in HEPES buffer; Boehringer-Mannheim, Mannheim, Germany) for 10–15 min. Evaluation was performed by fluorescence microscopy (Olympus IX 70; Halagaya Shibuta-ku, Tokyo, Japan) using a 488 nm excitation wavelength and 515 nm long-pass filter for detection [25]. Each group sample (two wells) was counted three times, and 200 or more cells were counted each time. The colour and structure characteristics of the cells were recorded. The mean of the three counts for each of the different cell characteristic was used to calculate the apoptotic and necrotic cell indices and their respective error bars. Each point represents the mean viability of three independent experiments ± SEM. Data were analyzed using either paired or unpaired Student's *t*-tests as appropriate. A value of *p* < 0.05 was taken to represent a statistically significant difference between the mean values of the groups.

2.5. Evaluation of mitochondrial membrane potential

CHSE-214 cells (10⁵ ml⁻¹ in a 60 mm Petri dish) were cultured as monolayers for 20 h and then rinsed twice with phosphate-buffered saline (PBS). To evaluate IPNV-induced mitochondria-mediated cell death, CHSE-214 cells were infected with the E1-S strain (MOI = 5) and then incubated for 0, 6 h, 8 h, or 12 h. By contrast, to evaluate bongkreikic acid (20 µg ml⁻¹; BKA) rescue, cells were pre-treated with BKA for 2 h and then incubated for 0, 8 h, or 12 h p.i. For assessment of MMP, cells were stained using MitoCapture reagent (500 µl per dish incubated at 37 °C for 15–20 min; Bio-Vision, Mountain View, CA, USA). This lipophilic cationic dye accumulates and aggregates in mitochondria when MMP is normal and remains in the cytoplasm when it is not. Loss of fluorescence intensity observed under a fluorescence microscopy was taken as an indicator of mitochondrial membrane disruption and reduced potential. Evaluation was performed by fluorescence microscopy using a 488 nm excitation wavelength and 515 nm long-pass filter for detection of fluorescein and using a 510 nm excitation wavelength and 590 nm long-pass filter for detection of rhodamine. Data were analyzed using either paired or unpaired Student's *t*-tests as appropriate. A value of *p* < 0.05 was taken to represent a statistically significant difference between the mean values of the groups.

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