Fish & Shellfish Immunology 45 (2015) 848–857

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Giant seaperch iridovirus infection upregulates Bas and Bak expression, leading to apoptotic death of fish cells

Xin-Yu Chen [a], Chiu-Ming Wen [b], Cho-Fat Hui [c], Ming-Chyuan Chen [d], Jen-Leih Wu [c], Tsai-Ching Hsueh [a], Wei-Han Lei [a], Jiann-Ruey Hong [a, *]

[a] Laboratory of Molecular Virology and Biotechnology, Institute of Biotechnology, National Cheng-Kung University, Tainan 701, Taiwan
[b] Department of Life Sciences, National University of Kaohsiung, Kaohsiung 811, Taiwan
[c] Laboratory of Marine Molecular Biology and Biotechnology, Institute of Cellular and Organismic Biology, Academia Sinica, Nankang, Taipei 115, Taiwan
[d] Department of Marine Biotechnology, National Kaohsiung Marine University, Kaohsiung 811, Taiwan

ARTICLE INFO

Article history:
Received 5 January 2015
Received in revised form
29 May 2015
Accepted 3 June 2015
Available online 8 June 2015

Keywords:
Iridovirus
Pro-apoptotic gene
Bax
Bak
Bcl-xL
Upregulation
Overexpression

ABSTRACT

The giant seaperch iridovirus (GSIV) induces host cell apoptosis by a poorly-understood process. In this study, GSIV is shown to upregulate the pro-apoptotic death genes *Bax* and *Bak* at the middle replication stage, and factors in the grouper fin cell line (GF-1) are shown to modulate this process. Studying the mechanism of cell death, we found that upregulated, *de novo*-synthesized Bax and Bak proteins formed heterodimers. This up-regulation process correlated with mitochondrial membrane potential (MMP) loss, increased caspase-3 activity, and increased apoptotic cell death. All effects were diminished by treatment of infected GF-1 cells with the protein synthesis inhibitor cycloheximide. Interestingly, over-expression of the anti-apoptotic gene *Bcl-xL* also diminished GSIV-induced mitochondria-mediated cell death, increasing host cell viability and decreasing MMP loss at the early replication stage. Our data suggest that GSIV induces GF-1 apoptotic cell death through up-regulation of the pro-apoptotic genes *Bax* and *Bak*, which are regulated by Bcl-xL overexpression on mitochondria in GF-1 cells.

1. Introduction

Apoptosis is the suicidal response of individual cells of a multicellular organism to a wide variety of stimuli [1]. This genetically controlled, pre-programmed process eliminates cells during development to prevent redundancy. Apoptosis is also an emergency response to radiation damage, viral infection, or aberrant cell growth induced by oncogenes [2]. The cell suicide program removes damaged, infected, or superfluous cells. In most circumstances, a cell's decision to live or die rests largely with the Bcl-2 family of interacting proteins [3,4].

Members of the Bcl-2 protein family, which includes both anti- and pro-apoptotic molecules, act at a critical decision point in the common death pathway [5]. The ratio of antagonists (Bcl-2, Bcl-xL, Mcl-1, Bcl-W, and A1) to agonists (Bax, Bak, Bcl-xS, Bid, Bik, Bad, PUMA, and NOXA) dictates whether a cell responds to proximal apoptotic stimuli [5,6]. Cell death is mediated by intrinsic and extrinsic pathways. The intrinsic pathway (also called the Bcl-2 regulated or mitochondrial pathway) is activated by various developmental cues and cytotoxic insults such as viral infection, DNA damage, and growth factor deprivation. This pathway is strictly controlled by the Bcl-2 family of proteins and ends in caspase-9 activation. The extrinsic or death-receptor pathway is triggered by ligation of death receptors such as Fas and TNF receptor-1. These receptor proteins contain an intracellular death domain that recruits and activates caspase-8 through the adaptor protein Fas-associated death domain at the cell surface [6] and also interact with mitochondria to regulate the mitochondrial membrane potential (MMP) [7]. A change in the MMP results in permeabilization of both the inner and outer membranes, followed by other signs of necrotic or apoptotic cell death such as apoptosis-specific activation of caspases [8]. Hence, the mitochondrion is considered a central integrator of pro-death stimuli, channeling various types of pro-apoptotic signals into a common caspase-dependent pathway [9]. Cytochrome *c* release from mitochondria into the cytosol is initiated by the interaction of mitochondria with one or more of the Bcl-2 family members. Thus, Bcl-2 proteins, which critically regulate apoptosis, function before cellular

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

http://dx.doi.org/10.1016/j.fsi.2015.06.003
1050-4648/

constituents are irreversibly damaged [10,11].

Iridoviruses are large DNA viruses that infect invertebrates and poikilothermic vertebrates such as insects, fish, amphibians, reptiles, crustaceans, and mollusks. The family Iridoviridae is comprises 5 genera: Ranavirus, Lymphocystivirus, Megalocytivirus, Iridovirus, and Chloriridovirus [12]. The megalocytiviruses infect a wide range of tropical marine and freshwater fish, including grouper, gourami, cichlid, red sea bream, angelfish, sea bass, and lamp eye, causing a similar disease in each. Megalocytiviruses are found, sometimes in large numbers, in the spleen, kidney, and gastrointestinal tract, and to a lesser extent in the liver, heart, gills, and connective tissue [13–15].

In aquatic fish, the emergence of deadly iridoviral diseases has attracted a growing interest in the mechanism of iridovirus-induced host cell death [16–18]. Most iridoviruses, including frog virus 3 (FV3), STIV, Rana grylio virus (RGV), red sea bream iridovirus (RSIV), lymphocystis disease virus (LCDV), and Chilo iridescent virus (CIV, induce typical apoptosis [16,17,19–23]. In contrast, several ranavirus isolates, including Singapore grouper iridovirus (SGIV) and grouper iridovirus (GIV), trigger non-apoptotic programmed cell death in grouper cells [16,17].

The megalocytiviruses have attracted much attention since the late 1980s because of their capacity to cause serious systemic diseases in a wide range of economically important freshwater and marine fish species in the Asia–Pacific region [24,25]. Outbreaks of iridovirus disease have been reported in cultured marine fish [23,26] in Taiwan. The phylogenetic relationships between Taiwan isolates and those from other geographic areas are unclear. Recently, Wen et al. [27] described a megalocytivirus isolate from Giant seaperch (GSIV K1) that has a major capsid protein (MCP) gene sequence similar o that of the SGIV strain. Recent evidence shows that GSIV K1 infection can induce host cell death with several features of apoptotic and post-apoptotic necrosis. Several potent drugs (such as the protein synthesis inhibitor CHX) block the death process, suggesting that death factors are involved in this death pathway (manuscript submitted for publication).

Despite the severe economic impact of megalocytiviruses on the aquaculture industry, they are not well understood. Examination of the molecular features of GSIV-induced cell death is important and may help elucidate the mechanism of viral infection and possible treatment.

2. Material and methods

2.1. Cell and virus cultures

The GF-1 cell line, which is highly permissive to NNV, was derived from the fin tissue of grouper (Epinephelus coioides) and was subcultured in Leibovitz's L-15 medium (Gibco/Life Technologies, Waltham, MA, USA) with 5% fetal bovine serum (FBS) (Gibco). Naturally-infected giant seaperch were collected in 2005 in the Kaohsiung prefecture and were the source of the GSIV Kaohsiung No. 1 (GSIV K1) strain used to infect GF-1 cells in this study. GSIV K1 is an RSIV-like strain with a comparable major capsid protein [27]. The virus was purified and stored at −80 °C until use. The viral titer was determined using the median tissue culture infective dose ($TCID_{50}$) assay according to Dobos et al. [28].

2.2. Western-blot analysis

Monolayers of GF-1, enhanced green fluorescent protein (EGFP)-producing GF-1 cells, and EGFP-Bcl-xL-producing GF-1 cells (4.0 mL, 10^5 cells/mL) on 60-mm Petri dishes were cultivated for at least 20 h, rinsed twice with phosphate buffered saline (PBS), treated with the protein synthesis inhibitor cycloheximide (CHX, 0.33 μg/mL) for 0–5 d, infected with GSIV K1 strain (multiplicity of infection [m.o.i.] = 5) for 0–5 days post-infection (dpi), subjected to culture medium aspiration, washed with PBS, and then lysed in lysis buffer (0.3 mL) (10 mM Tris base, 20% glycerol, 10 mM sodium dodecyl sulfate, and 2% β-mercaptoethanol; pH 6.8).

Proteins were separated by SDS-polyacrylamide gel electrophoresis [29], electroblotted, and subjected to immunodetection as described elsewhere [30]. Blots were incubated with Bax and Bak anti-rabbit polyclonal antibodies (1:3000) and peroxidase-labeled goat anti-rabbit conjugate (1:10, 000; Amersham, Piscataway, NJ, USA); or with anti-EGFP and -actin mouse monoclonal antibodies (1:5000) and peroxidase-labeled rabbit anti-mouse conjugate (1:10,000).

Chemiluminescence was detected according to the instructions provided with the Western Exposure Chemiluminescence Kit (Amersham, Arlington Heights, IL) and visualized by exposure to Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA).

2.3. Annexin V-FLUOS staining

Analysis of PS on the outer leaflet of apoptotic cell membranes was performed using annexin V-fluorescein and propidium iodide (PI) to differentiate non-apoptotic from apoptotic cells. At the end of various incubation times, cells were removed from the medium, washed with PBS, incubated in staining solution (100 μL) (annexin V-fluorescein in an HEPES buffer containing PI; Boehringer-Mannheim, Mannheim, Germany) for 10–15 min, and evaluated by fluorescence microscopy (Olympus IX 70; Halagaya Shibuta-ku, Tokyo, Japan) using a 488-nm excitation and 515-nm long-pass filter [31].

2.4. Evaluation of mitochondrial membrane potential using a lipophilic cationic dye

To assess ΔΨm, GSIV-infected GF-1 cells were stained using a MitoCapture Mitochondrial Apoptosis Detection Kit (Jomar Diagnostics Pty. Ltd, Stepney, SA, Australia) with a lipophilic cation dye that is trapped in mitochondria when ΔΨm is normal and released into the cytoplasm when ΔΨm is abnormal. Loss of fluorescence intensity observed under fluorescence microscopy was taken as a marker of mitochondrial membrane potential disruption. GSIV-infected cells were incubated for 0–3 dpi before the medium was discarded, and 500 μL of diluted MitoCapture reagent was added. Each dish was incubated at 37 °C for 15–20 min and evaluated by fluorescence microscopy using a 488-nm excitation and 515-nm long-pass filter for detection of fluorescein and a 510-nm excitation and 590-nm long-pass filter for detection of rhodamine [32].

2.5. Caspase-3 activity assays

Cells (1×10^6) were tested for caspase-3 activity using a kit purchased from Clontech (Palo Alto, CA, USA) at each time point. After centrifugation at 2000 × *g* and removal of the supernatants, the cell pellets were frozen at −70 °C until analysis. Caspase activity (cleavage of the synthetic caspase-3 substrates DEVD-AFC and IETD-AB) was assayed in 96-well plates using a fluorescence plate reader (CytoFluor 4000, PerSeptive Biosystems, Framingham, MA, USA). Cleavage after the second Asp residue produces free AFC [33,34]. The amount of fluorescence detected is directly proportional to the amount of caspase-3 activity. Fluorogenic substrate assays were performed simultaneously. In cells infected with GSIV and treated with CHX, the caspase-3 activity profiles were the same in all experiments and are included in each figure to facilitate comparison. Results of all experiments are reported as mean ± SEM.