Fish & Shellfish Immunology (2008) **24**, 436–449

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Zebrafish anti-apoptotic protein zfBcl-x_L can block betanodavirus protein α-induced mitochondria-mediated secondary necrosis cell death

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Received 10 April 2007; revised 18 December 2007; accepted 2 January 2008
Available online 11 January 2008

KEYWORDS
Nervous necrosis virus;
Protein α;
Mitochondria;
zfBcl-x_L;
Apoptosis;
Secondary necrosis cell death

Abstract Betanodavirus protein α induces cell apoptosis or secondary necrosis by a poorly understood process. In the present work, red spotted grouper nervous necrosis virus (RGNNV) RNA 2 was cloned and transfected into tissue culture cells (GF-1) which then underwent apoptosis or post-apoptotic necrosis. In the early apoptotic stage, progressive phosphatidylserine externalization was evident at 24 h post-transfection (p.t.) by Annexin V-FLUOS staining. TUNEL assay revealed apoptotic cells at 24–72 h p.t, after which post-apoptotic necrotic cells were identified by acridine orange/ethidium bromide dual dye staining from 48 to 72 h p.t. Protein α induced progressive loss of mitochondrial membrane potential (MMP) which was detected in RNA2-transfected GF-1 cells at 24, 48, and 72 h p.t., which correlated with cytochrome *c* release, especially at 72 h p.t. To assess the effect of zfBcl-x_L on cell death, RNA2-transfected cells were co-transfected with *zfBcl-x_L*. Co-transfection of GF-1 cells prevented loss of MMP at 24 h and 48 h p.t. and blocked initiator caspase-8 and effector caspase-3 activation at 48 h p.t. We conclude that RGNNV protein α induces apoptosis followed by secondary necrotic cell death through a mitochondria-mediated death pathway and activation of caspases-8 and -3.
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doi:10.1016/j.fsi.2008.01.001

Introduction

Apoptosis is a suicidal process triggered by a wide variety of stimuli in individual cells of multicellular organisms [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 [2,3]. Apoptosis and necrosis are two stereotyped mechanisms by which nucleated eukaryotic cells die [1,4—6]. Understanding these mechanisms may ultimately lead to novel therapeutic strategies.

Mitochondria function as central integrators of pro-death stimuli [7] by sequestering apoptogenic proteins such as cytochrome *c*, Smac/DIABLO, apoptosis inducing factor, and endonuclease G in the intermembrane space, and releasing these factors into the cytosol on exposure to proapoptotic signals [8,9]. Loss of mitochondrial membrane potential (MMP) leads to cytosolic binding of caspase-9, the downstream activator of apoptosis [8,10]. 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 [7]. Hence, mitochondria integrate pro-death stimuli by joining together various types of proapoptotic signals into a common caspase-dependent pathway [10].

In contrast, little is known of cell death induced by aquatic viruses. Nodaviruses are small, non-enveloped, spherical viruses with bipartite positive-sense RNA genomes that are capped but not polyadenylated [11]. The family Nodaviridae contains two genera. Betanodaviruses predominantly infect fish, while alphanodaviruses mostly infect insects [12—14]. The betanodavirus is the causative agent of viral nervous necrosis (VNN), an infectious neuropathological disease characterized by necrosis of the central nervous system and retina. Its clinical symptoms include abnormal swimming behavior and, very often, darkening of the fish [14]. VNN kills massive numbers of larvae and juveniles of several marine teleost species globally [15].

The genome of fish nodavirus is bipartite, comprising two single-stranded molecules of positive polarity RNA (RNA1 and RNA2; about 3.1 and 1.4 kb in length, respectively) and no poly(A) extension at the 3′ end [16,17]. RNA1 encodes RNA-dependent RNA polymerase (RdRP; approximately 100 kDa), which is also named protein A, an enzyme that replicates the viral genome. RNA2 encodes the capsid protein of about 42 kDa [16,18]. According to the RNA1 sequences, betanodavirus isolates should be classed within four main groups named I, II, III and IV, which correspond to greasy grouper nervous necrosis virus (RGNNV), barfin flounder NNV (BFNNV), tiger puffer NNV (TPNNV), and striped jack nervous necrosis virus (SJNNV) [19]. These groups may in fact represent independent genotypes [19].

Despite their severe economic impact on the aquaculture industry, betanodaviruses have not been well studied. Characterization of the viral molecular regulation processes should help clarify the mechanism(s) of viral pathogenesis and infection. In a previous study, we demonstrated that RGNNV TN1 strain induces apoptosis followed by secondary necrosis in GL-av cells and loss of mitochondrial membrane potential (MMP) in the mid-apoptotic stage [20], and demonstrated that this death process is prevented by Bcl-2 member proteins zfBcl-x_L and zfMcl-1a [21,22]. In addition, the GGNNV strain protein α is an apoptosis inducer [23]. Presently, we demonstrate that RGNNV protein α induces apoptotic loss of MMP for cytochrome *c* release and an increase in caspase-8 and -3 activation, and that zfBcl-x_L can block these post-apoptotic necrosis processes thereby rescuing virus-infected cells.

Materials and methods

Cell culture and reagents

The grouper fin cell line GF-1 was grown at 28 °C in Leibovitz's L-15 medium (GibcoBRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum and 25 μg/ml of gentamycin. The annexin V-FLUOS Kit and In Situ Cell Death Detection Kit were purchased from Roche GmbH (Mannheim, Germany). Anti-NNV particle polyclonal antibody and the plasmids encoding zfBcl-x_L and infectious pancreatic necrosis virus (IPNV) VP5 [24] were gifts from Dr Jen-Leih Wu. The ECL Western blotting detection system kit was purchased from Amersham (Piscataway, NJ, USA). The apoptosis detection, mitochondria bioassay kit was purchased from USBiological (Jomar Diagnostics Pty. Ltd, Stepney, SA, Australia). PhiPhiLux-G_2D_2 cell-permeable fluorogenic substrate was from OncoImmunin (Gaithersburg, MD, USA).

Cloning of RNA2 and construction of expression vector

Synthesis and amplification of cDNA was carried out using the SuperScript One-Step™ step reverse transcriptase-polymerase chain reaction (RT-PCR) system kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RGNNV RNA2 primers P1 and P2 were each added to a final concentration of 0.2 μM. PCR cycling conditions were 54 °C for 30 min, 2 min at 94 °C (to inactivate the reverse transcriptase), 95 °C for 30 s (DNA denaturation), 57 °C for 30 s (annealing), and 72 °C for 45 s (extension) for a total of 35—40 cycles. The RT-PCR primers NNV RNA2 P1 (5′-ACA ATg gTA CgC AAA ggt gAg-3′) and NNV RNA2 P2 (5′-TTA gTT TCC CgA gTC AAC CCT-3′) were used to amplify a fragment covering the variable region of *RNA2*. The purity and size of the amplified product was checked by 1.5% agarose gel electrophoresis after staining with ethidium bromide as described previously [24]. The 1017 bp, double-stranded cDNA was purified using the QIAquick™ gel extraction system (Invitrogen) and RGNNV RNA 2 subcloned using a pCDNA3.1 cloning system (Promega, Madison, WI, USA). The cloned PCR products were sequenced by the dye termination method using an ABI PRISM 477 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and scanned against the GenBank database BLAST (http://www4.ncbi.nlm.nih.gov/) and PROSITE (http://psort.ims.u-tokyo.ac.jp/) programs.

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