Fish & Shellfish Immunology 29 (2010) 451–463

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

RGNNV induces mitochondria-mediated cell death via newly synthesized protein dependent pathway in fish cells

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ARTICLE INFO

Article history:
Received 2 January 2010
Received in revised form
17 April 2010
Accepted 7 May 2010
Available online 26 May 2010

Keywords:
Nervous necrosis virus
Necrotic cell death
Mitochondria
RNA viral genome
RNA interference
Viral death inducer

ABSTRACT

The RNA nervous necrosis virus induces necrotic cell death in fish; however, the molecular mechanism remains unknown. In this study, we demonstrated that beta-nodavirus-induced mitochondria-mediated dependent cell death is through newly synthesized protein dependent pathway in replication cycle. We determined that newly synthesized protein dependent pathway is required for red-spotted grouper nervous necrosis virus (RGNNV)-induced cell death. UV irradiation of the virus effectively blocked viral replication and cell death. Next, RGNNV RNA-dependent RNA polymerase (RdRp or protein A) was cloned and its involvement in RNA genome replication and viral protein synthesis was analyzed. Protein A was initially expressed 48 h post-infection and localized to the cytoplasm. Knockdown of protein A expression completely blocked viral genomic replication and expression of viral protein expression RNA1 small hairpin RNA (shRNA) producing cell lines, which coincided with inhibition of phosphatidylserine exposure, mitochondria-mediated death signaling, and increased cell viability 72 h post-infection. Furthermore, RGNNV-induced mitochondria-mediated caspase-3-independent necrotic cell death is dependent on viral synthesized protein dependent pathway at the middle-late replication stage. Taken together, for instance these results suggested that RGNNV induces cell death may require newly synthesized protein for triggering host mitochondria-mediated cell death. These findings may provide new insights into RNA viral pathogenesis.

1. Introduction

Beta-nodaviruses infect a wide variety of larval and juvenile marine fish worldwide [1,2], causing severe morbidity and mortality as well as significant economic losses to the aquaculture industry. Despite their economic impact, few studies have focused on beta-nodavirus pathogenesis. Characterization of the molecular regulation of beta-nodavirus may aid in elucidating the mechanisms of its pathogenesis and infection.

The Nodaviridae family of viruses contains two genera: beta-nodaviruses, which predominantly infect fish, and alpha-nodaviruses, which mostly infect insects [1,3–5]. Beta-nodaviruses are the causative agents for viral nervous necrosis (VNN), an infectious neuropathological condition characterized by necrosis of the central nervous system, including the brain and retina, and presents with clinical signs that include abnormal swimming behavior and darkening of the fish [2]. VNN is capable of causing massive mortality in the larvae and juvenile populations of several marine teleost species [6], and disease manifestation of these viruses may correlate with modulation of innate or acquired immunity [4,7]. Furthermore, beta-nodaviruses may prove useful as a model for understanding RNA virus-mediated pathogenesis and disease.

The nodavirus genome is bipartite, comprised of two single-stranded molecules of positive polarity (RNA1 and RNA2) approximately 3.1 and 1.4 kb in length, respectively, and lacking a 3′ poly (A) extension [4]. RNA1 encodes an approximate 110-kDa nonstructural protein designated RNA-dependent RNA polymerase (RdRp) or protein A. This protein is vital for replication of the viral genome. RNA2 encodes a 42-kDa capsid protein named protein alpha [4,8] from RNA1 3′ terminus, which may induce post-apoptotic necrotic cell death through a cytochrome c release-dependent pathway [9].

Alpha and beta-nodaviruses synthesize a sub-genomic RNA3 from the 3′ terminus of RNA1 during RNA replication, which encodes two small proteins, B1 and B2 [1,10,11]. In RGNNV, B1, was identified as a novel anti-necrotic cell death gene; however, the precise death mechanism it influences remains unresolved [12]. B2 has dual functions either as a host siRNA silencing suppressor in alpha [7,13,14] and beta-nodavirus [10] or inducer of necrotic cell death in fish cells [11].

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1050-4648/$ – see front matter
doi:10.1016/j.fsi.2010.05.003

Apoptosis is controlled at the mitochondrial level by sequestration of a series of apoptogenic proteins, including cytochrome c, Smac/DIABLO, apoptosis-inducing factor, and endonuclease G, in the mitochondrial intermembrane space, and cytosolic release of these factors on exposure to proapoptotic signals [15]. Mitochondria membrane permeabilization (MMP),which can include both inner and outer membrane permeabilization, precedes the signs of necrotic or apoptotic cell death, such as apoptosis-specific activation of caspases [16]. Hence, the mitochondrion is appreciated as a central integrator of pro-death stimuli, combining various types of proapoptic signals into a common caspase-dependent pathway [17], which depends on cytochrome c release from mitochondria to the cytosol. Cytochrome c release is initiated by the interaction of mitochondria with one or more members of the Bcl-2 family of proteins. Thus, Bcl-2 proteins, which critically regulate apoptosis, function prior to irreversible damage of cellular constituents [18,19].

Necrosis is considered a pathological reaction to major perturbations in the cellular environment such as anoxia [20]; necrosis has long been described as a consequence of physicochemical stress and, thus, accidental and uncontrolled. Recently, it has become clear that necrotic cell death is as tightly-controlled as caspase-dependent apoptosis, and it may be an important cell death mode that is both pathologically and physiologically relevant [18,21–23]. The mechanism by which beta-nodavirus infection induces necrotic cell death remains largely uncharacterized at the molecular level.

The RGNNV TN1 strain induces host apoptosis, which precedes the onset of necrosis in a grouper liver cell line, GL-av cells [24]. Apoptosis can activate the mitochondrial permeability transition pore and can be blocked by the adenine nucleotide translocase (ANT) inhibitor, BKA [24], and the mitochondria membrane stabilizer, zfBcl-xL, a member of the Bcl-2 protein family [24,25]. Here, we show that necrotic cell death induction by RGNNV requires viral genome replication and subsequent encoding of viral protein, thereby inducing mitochondria-mediated caspase-3-independent cell death.

2. Materials and methods

2.1. Cell line and virus

The grouper-fin cell line, GF-1 cells, was obtained from Dr. Chi of the Institute of Zoology and Development of Life Science, Taiwan, ROC. GF-1 cells were grown at 28 °C in Leibovitz's L-15 medium (Gibco BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum and 25 μg/ml of gentamycin. Naturally-infected red grouper larvae were collected in 2002 in the Tainan prefecture and were the source of the RGNNV Tainan No. 1 (RGNNV TN1) used to infect GF-1 cells in this study. The virus was purified as described by Mori et al. [6] and was stored at −80 °C until use. The vial titer was determined using the $TCID_{50}$, assay, according to Dobos et al. [26].

2.2. UV-irradiated virus

Two-milliliter aliquots of virus were dispersed in a 6-cm tissue culture dish (Nalge Nunc international, Rochester, NY, USA), followed by UV irradiation to inactivate the virus. A compact UV lamp (Vilber Lourmat, Cedex, France) was placed directly on top of the dish (on ice) for 2 h. The remaining titer of the inactivated virus was determined using a plaque assay [26].

2.3. Cloning and sequence analysis of NNV RNA1

Synthesis and amplification of cDNA was carried out using the SuperScript One-Step™ reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. NNV RNA1 primers, P1 and P2, were each added to a final concentration of 0.2 μM. PCR cycling conditions were as follows: 54 °C for 30 min, 94 °C for 2 min, and 35–40 cycles of 30 s at 95 °C, 57 °C for 30 s, and 72 °C for 45 s. The RT-PCR primers including NNV RNA1 P1 (5′- GCGGATCCATGCGTCGCTTTGAGTTTGC -3′), which contained an BamHI restriction site and translational start codon (underlined) and NNV RNA1 P2 (5′- GGAATTCCACTTGAGTGC-GACGTCGCT -3′), which contained a EcoRI restriction site (underlined) were used to amplify a fragment covering the coding region of *RNA1*. The purity and size of the amplified product was verified by 1.5% agarose gel electrophoresis and staining with ethidium bromide [27]. The 2946 bp, double-stranded cDNA was purified using the QIAquick™ gel extraction system (Qiagen, Valencia, CA) and subcloned using a pGEMT-easy cloning system (Promega, Madison, WI). The cloned PCR products were sequenced using the dye termination method and an ABI PRISM 477 DNA sequencer (Applied Biosystems, Foster City, CA) and analyzed using the GenBank database BLAST and PROSITE (psort.ims.u-tokyo.ac.jp/) programs.

2.4. Expression of recombinant protein A

E. coli BL21(DE3)/pLys cells were transformed with pET29a, which contained the RNA1 gene inserted at the *Bam*HI and *Eco*RI sites. Cultures (50–200 mL) were incubated overnight with 0.5–2.0 mL of preculture. Optimal expression was obtained by induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when the culture reached an optical density of 0.5 at 600 nm (OD_{600}) [28]. Expression of recombinant protein A was monitored by sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) [29], followed by staining with Coomassie brilliant blue R-250.

For identification of recombinant protein A, expression of an N-terminus His tag using Western blot immunodetection was employed [30]. Blots were incubated with a 1:15000 dilution of a His tag-specific polyclonal antibody (Amersham Biosciences) followed by a 1:7500 dilution of a peroxidase-labeled goat anti-rabbit conjugate (Amersham Biosciences, Piscataway, NJ). Binding was detected by chemiluminescence and captured on Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

For protein purification, cell pellets were resuspended in 4 mL of binding buffer (pH 7.8, 20 mM sodium phosphate and 500 mM NaCl) per 100 mL of cell culture. Lysozyme was added to a final concentration of 1 mg/mL, and cell suspensions were incubated on ice for 30 min followed by incubation on a rocking platform for 10 min at 4 °C. A mixture of Triton X-100, DNase, and RNase was added to each suspension to a final concentration of 1%, 5 μg/mL, and 5 μg/mL, respectively, then incubated for an additional 10 min. The insoluble debris was removed by centrifugation at 3000×*g* (Sorvall SS-34 rotor) for 30 min at 4 °C. Supernatants were applied to $Ni2^{+}$ affinity columns (Qiagen) and washed with 6 column volumes of binding buffer followed by 4 volumes of wash buffer (20 mM sodium phosphate and 500 mM NaCl, pH 6.0). Bound proteins were then eluted with 6 volumes of 10, 50, 100, and 150 mM imidazole elution buffer (20 mM sodium phosphate, 500 mM NaCl, and 10 mM-150 mM imidazole, pH 6.0; 11). Eluted fractions were analyzed for the presence of the polyhistidine-tagged protein by analyzing 20-μL aliquots by 10% SDS-PAGE.

2.5. Preparation of a polyclonal antibody against protein A

A New Zealand rabbit was subcutaneously immunized twice every two weeks with 500 μg/1.2 mL of purified recombinant protein A emulsified in an equal volume of Freund's complete adjuvant and then boosted with 600 μg of antigen emulsified in an incomplete adjuvant at the seventh week. Blood (30 mL) was collected during the eighth week. Collected serum was precipitated with 50%