Journal of Marine Bioscience and Biotechnology 2007, p. 127-132 Vol. 2, No. 3

Review

Innate Immune Response of NNV Infection in Fish and Its Disease Prevention

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Abstract The innate immune response which is seen as the initial defense mechanism induced upon foreign invasion has been well documented in higher vertebrates. This has also been observed in fish infected with NNV. However, the fish immune system based on fully established genome project has not been fully elucidated. Therefore, in this review, we hope to correlate NNV infection in fish that has devastated the aquaculture industry, to its host immune system. Further, we discuss the potential preventive measures in overcoming the widespread of this neurodisease.

Key words : Betanodavirus, animal model, innate immunity

Introduction

Nervous necrosis virus (NNV) belongs to the beta subgroup of the virus family Nodaviridae. The genome consists of two single stranded positive-senses RNA molecules. RNA1 encodes RNA dependent RNA polymerase (3.1 kb), while RNA2 encodes capsid protein (1.4 kb) [27]. Another subgenome, RNA3 consists of an overlap in RNA1, and encodes a novel anti-siRNA function protein B2 [32]. Due to its compact genomic composition, nodavirus has become one of the most important models for studying RNA virus replication. Nervous necrosis virus (NNV) is an RNA virus that lethally infects a myriad of fish species worldwide resulting in severe economic losses in the aquaculture industry [33]. The geographical distribution of NNV infection includes the tropic and cold water regions. Based on genomic phylogenic analysis of its viral capsid protein, there exist four major types of betanodavirus; SJNNV, RGNNV, TPNNV and BFNNV [4] and although there are more than ten fish species reported to be infected with betanodavirus in Taiwan, all of the isolated strains belong to the RGNNV group and they share more than 90% similarity in the capsid protein nucleotide sequence [10].

NNV causes mass mortality in piscine at larva and juvenile stages. The mortality of NNV-infected fish at the larva stage could be as high as 80-100%, and most of the survivors would develop persistent infection. The syndrome in NNV is viral nervous necrosis (VNN), also called Viral Encepathy and Retinopathy disease (VER disease) and the fish infected with NNV usually appear to have dark body color, abnormal swimming, and lack of appetite [24]. In histopathical observation, it is found that NNV would lead to cell necrosis and induce tissue vacuolation [22,23,26]. The mortality could be observed within short period after onset of symptoms in the larval stage. At present, no commercial therapeutic measure has been described successfully. In this review, we will focus on the recent betanodavirus research development especially in host innate immune response and seek new strategies to curb disease outbreak.

Innate immunity of fish

Innate immune system is thought to be the first line of defense against pathogens including virus and bacteria. It has been reported that bony fish through evolutionary conservation share similar immune system organization with other vertebrates [28]. In general,

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there exist some defense mechanisms in fish which have already been reported. Firstly, the activation of antimicrobial components in fish skin mucus such as defensin and cathelicidins which have been found in halibut and flounder mucus [12], liver [49] and gills [8] as part of the innate immune response. Indeed, Bergsson et al. [5] observed that components of Atlantic cod mucus were also active against both Gram-positive and Gram-negative bacteria. Secondly, cytokines have been defined in the fugu (*Takifugu ruburipes*) and zebrafish (*Danio rerio*) genomic projects, which contributed to the increase of fish expressed sequence tag entries in the GenBank. To date, several cytokine homologues have been cloned in fish species including tumour necrosis factor-α (TNFα) and TNFβ [38,39], interleukin-1β (IL-1β) [13], IL-2 [6], IL-4 [25], IL-6 [7], IL-10 [20], IL-11 [45], IL-12 [48], IL-15 [4], IL-18 [50], IL-21 [6], IL-22, IL-26 [6], IFN-γ [6,51] and several chemokines such as IL-8 or CXCL8, gIP-10, CK-1 and CK-2. Thirdly, the complement system of teleost fish, like that of higher vertebrates, can be activated through the three pathways of complement; the classical pathway is triggered by binding of antibody to the cell surface but can also be activated by acute phase proteins such as ligand-bound C-reactive protein or directly by viruses, bacteria and virus-infected cells [19]; the alternative pathway is independent of antibody and activated directly by foreign microorganisms; the lectin pathway is elicited by binding of a protein complex consisting of mannose-binding lectins to mannans on bacterial cell surfaces [27,28]. The complement system is composed of numerous proteins and all pathways generate factor C3, which has been described and isolated from teleost species [29]. (4) Fourthly, all Non-specific cytotoxic cells (NCCs) in teleosts have been shown to express on their surface the NCC receptor protein, NCCRP-1, by which a cytotoxic cell contacts to its target to induce the lytic cycle against the target [21]. NCC and phagocyte responses are followed by secretion of a number of proinflammatory molecules that have pleiotropic effects. Lastly, presence of Interferons (IFNs), whichare secreted proteins that induce vertebrate cells into a state of antiviral activity by transcriptional regulation of several hundred IFN-stimulated genes (ISGs) [37].

In mammals, three families of IFNs (type I IFN, type II IFN and IFN-λ) can be distinguished on the basis of gene structure, protein structure and functional properties. Type I IFNs include the classical IFN-alpha and beta, which are induced by viruses in most cells, whereas type II IFN is identical to IFN-gamma and is produced by natural killer cells and T lymphocytes in response to interleukin-12 (IL-12), IL-18, mitogens or antigens. IFNs are a family of structurally related cytokines with a hallmark function of antiviral activity. Despite their distinct activity profiles, IFNs exhibit diverse biological functions as represented by three major biological activities: antiviral activity, anti-tumour activity and immunomodulatory effects. IFNs consist of several types, most intensively studied is IFN-α. Recently, the cytoplasmic RNA helicases retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 have been proposed to bind to viral dsRNA, resulting in activation of the interferon regulatory factor-3 (IRF-3) kinases. The Type I IFN downstream genes include those for the double-stranded RNA (dsRNA)-activated protein kinase (PKR) and the 2-5-oligoadenylate synthetase (2-5-OAS); dsRNA is a molecular motif encountered during viral infection that activates these enzymes. Zebrafish interferon gene (zIFN) has been recently identified as to having anti-virus function and may contribute to both induction and regulation of the innate and adaptive immunity. Downstream interferon activated Mx gene has also been identified in zebrafish, grouper, salmon, trout, and halibut upon infection with aquatic viruses suggesting the importance of the interferon regulatory pathway including RNA-activated protein kinase (PKR) and the 2-5A proteins during viral infection [1,2,9,15,43,47].

Innate immunity and betanodavirus

Betanodavirus is a typical age-dependent virus similar to the neurotropic virus in human such as Semliki Forest virus (SFV), Japanese encephalitis virus (JEV), herpesvirus, and alphavirus. Despite reasons for acute infection in larval stage while persistence in adult stage remains unclear, there is a possibility that innate immune response may play a role in persistent infection.

The first finding of innate immune response in betanodavirus is the interferon related response mainly type I activation [46]. The *in vitro* results indicated that Mx gene expression may play an important role in NNV persistence in Barramundi brain cell (BB cell). Further, the study showed that IFN-like cytokines existed in the culture supernatant of BB cells, and IFN-induced re-

sponse played an important role in protecting the majority of cells from virus lytic infection and regulating NNV persistence in the BB cell line. Another group has utilized Mx gene for a potential drug in gene therapy using Grouper Brain cell (GB cell) [26]. In addition, in our recent studies, had identified NNV's acute and persistence infection being regulated by interferon response *in vivo* as well as *in vitro* [30]. In a published macroarray data of NNV infected sea bass, it had also been shown that an interferon upstream gene, mda5, is upregulated upon infection [14]. These results have concluded that NNV is possibly sensitive to type I interferon related response.

Fish model for NNV infection and pathogenesis studies

The NNV infection in different fish species has been observed to elicit similar clinical signs. Therefore, it is important to clarify the systematic infection procedure for the system. Some reports had shown that artificial infection of NNV can lead to the same clinical syndrome in turbot and wolf fish. In wolf fish model, the author investigated the pathogenesis of nodavirus infection in spotted wolf fish during the acute stage [23,24]. The authors also investigated survivors of the disease outbreak at 16 wk post-bath-challenge (PBC) for pathological changes and presence of the nodavirus. Another group has attempted to use immune complete Atlantic halibut as a model fish to study the betanodavirus tissue distribution and immune response during NNV infection [22]. In the study, quantitative RT-PCR results revealed that the amount of virus in head samples from the intraperitoneal (i.p.) -challenged group increased throughout the experiment. Further, by using ELISA developed to detect anti-nodavirus activity in plasma, anti-nodavirus antibody response was detected from Day 19 post-challenge in i.p-challenged fish, while no antibody response was detected in the bath-challenged or control fish. Despite efforts to study virus pathogenesis in its natural host, results have not been encouraging due to limitations such as lack of fish bio-resources; including genomic database, mutants and fundamental understanding of fish immune system, that are essential in elucidating the viral-host interaction mechanism. Therefore, many research groups have tried to lobby a suitable model fish species to study important areas like the organ development, host-pathogen interactions, immune system, and signal transduction. Zebrafish and medaka are two of the most important NNV infection model fish species in recent times. Both have near completed genomic database which can be useful tools to facilitate the mechanistic study in virus-host interaction. Medaka was first reported as an infection model in NNV infection [16] whereby results have indicated that, medaka larvae are highly suscetiple to NNV at high dosage and leading to almost 80% mortality. The clinical syndrome and histological analysis on infected Medaka verified NNV's ability to replicate in neuronal cells similar to the natural host. Some fresh water fish species such as rainbowfish was also been examined susceptible to betanodavirus [17]. Additionally, recent reports have proven zebrafish to be also an infection model mimicking natural host infection [30].

Disease prevention

The transfection routes in betanodavirus have been well- defined in stripes jack, both via vertical and horizontal transmission. Prevention of betanodavirus infection and replication is a major concern for the aquaculture industry. The environmental control is considered very important for NNV inhibition especially in hatcheries. Several chemicals have been used to inactivate betanodavirus on contaminated rearing equipment and working staff. Sodium hypochlorite, calcium hypochlorite, benzalkonium chloride, iodine, formalin, cresol, ethanol, methanol, ether and chloroform have all been tested for their efficacy to inactivate SJNNV [3]. Except for formalin, ether and chloroform, all others exhibited inhibitory effects depending on concentration and treatment period. Physical treatments such as heat, pH value, ozone, and UV were also employed for the inactivation of NNV particles. Nonetheless, the most practical treatment for betanodavirus disinfection would be washing of eggs or water supplies and hatchery effluent using ozonated water [18]. Similar observation has also been found in Atlantic halibut whereby the authors suggested washing eggs before hatch out by ozone to remove vertical transmission of betanodavirus possibly present on the eggs surface thus increasing the survival rate of larvae.

The other constructive effort was to develop the virus vaccine, in which, peptide vaccine was first considered as a candidate. By using four synthetic peptides of 15

amino acids (aa) each and heat inactivated *Dicentrarchus labrax* encephalitis virus (DlEV), the results indicated potential protectivity although the mortality rate was not so consistent [11]. The *Escherichia coli* expressed subunit peptide was shown to have significant protection when the challenge was performed at 10 wk post-vaccination [41]. Recombinant Virus-Like Particles (VLPs) were not infectious and did not induce any noticeable side effects, as demonstrated by the insignificant level of mortality recorded in vaccinated fish compared to control. Due to the multivalent structure of the VLPs, which mimics that of native virions, it was likely that the stimulation of the fish immune system closely resembles that induced during a natural infection and thus does not necessitate the use of an adjuvant. Betanodavirus VLPs had also been obtained by using Baculovirus and *E. coli* expression system [27,31]. The *in vivo* examination in sea bass and grouper verified notable protection against betanodavirus infection [29,44]. Another interesting finding was the application of DNA vaccine encoding the Viral Hemorrhagic Septicemia Virus (VHSV) glycoprotein which had induced early protection against the Atlantic halibut nodavirus (AHNV) [40] resulting in lower cumulative mortality in the control group reached 54%. This result demonstrated another potential strategy for NNV prevention in hatcheries by using DNA vaccine.

Acknowledgements

We would like to thank Council of Agriculture of Taiwan (96AS-6.1.6-FA-F1) and National Science Council of Taiwan (NSC 96-2313-B-001-004) for supporting part of this review results. We also thank to Mrs. Siti Khadijah Kasani for editing this review.

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