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Cloning of *crystallin* from orange-spotted grouper and characterization of its activity as potential protective agent

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ABSTRACT

Oxidative stress associated with nodavirus infection is poorly understood, especially pertaining to infection-mediated brain injury. Indirect evidence indicates that infection increases cellular abundance of reactive oxygen species (ROS) with consequent increase in cellular dityrosine production. The detection of dityrosine in nodavirus-infected grouper was demonstrated using immunohistochemical (IHC) staining. Proteomic analyses with eye tissues of healthy grouper revealed more abundant expression of crystallin protein in the eye than in various tissues, which was confirmed by real-time polymerase chain reaction and IHC analyses. Grouper crystallin belongs to a small heat shock protein family with chaperone-like function that prevents heat-induced and oxidative stress-induced protein aggregation. Recombinant crystallin induced nitric oxide (NO) production in RAW 264.7 cells after treatment. The results provide new insight into the pathogenesis of nodavirus and demonstrate an experimental rationale for antioxidant therapy research.

1. Introduction

Betanodavirus is neuropathogenic and inflicts conspicuous damage that is characterized by vacuolation and degeneration of neurons throughout the central nervous system [1,2]. Piscine nodavirus, a member of the *Betanodavirdae* family, is the causative agent of viral nervous necrosis or fish encephalitis that produces high mortalities in hatchery-reared larvae and juveniles of marine fishes in Taiwan, Japan, Australia, and Europe [3]. This virus is unenveloped, possesses a 25–30 nm diameter icosahedral capsid, and contains a genome composed of bipartite, single-stranded, and positive-sense RNA molecules [4]. Considerable progress in the understanding of the *betanodavirdae* pathogenesis has been made. The activation of the host immune response and direct invasion of cells are believed to contribute to this pathogenesis [5,6], which includes induction of inflammatory cells and the host's immune response. For example, ubiquitin conjugating enzyme 7 interacting protein, which functions in apoptosis, and interferon induced with helicase C domain protein1, which contributes to apoptosis and mediates type I interferon production, are differentially expressed in infected and control cells [7].

However, the mechanisms underlying *betanodavirdae* pathogenesis are still not completely clear. When groupers are infected by nodavirus, the virus accumulates in the brain and eyes, and also undergoes replication at these two sites [8]. These two sites are, therefore, very important in the study of virus–host interactions. In addition, fish are under a lot of stress after viral infection: therefore, the immune responses that occur at these sites suffer from the same problems described previously, which are those of antibody production by adaptive immunity, antibody–antigen binding affinity, and immunological memory. However, at the eye, the antibody titer is low. Consequently, virus-induced stress is most important in the eye. If the eye is used to study stresses produced by innate immunity, interference by antibodies can at least be excluded. Also, this allows for examination of whether a secondary antibody production response and subsequent immunological memory are present during stress responses. Therefore, the eye is a very important tissue in the study of nodavirus-induced stress.

The rapid development of proteomic techniques has revolutionized the ability to study protein interactions and cellular changes on a global scale, revealing previously unknown and unanticipated associations. Interestingly, crystallins that are involved in the regulation of cellular redox status are themselves regulated, indicating that nodavirus infection may induce oxidative stress. To clarify this, the present study evaluated the generation of reactive oxygen species (ROS) in nodavirus-infected cells. In addition, in agreement with

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disease's occurrence, immunochemical staining detected protein dityrosine in atherosclerotic lesion of apo-E-deficient mice using a novel monoclonal antibody [9]. As ROS are known to alter proteins [10], the formation of dityrosine [11], which is an indicator of intracellular ROS [12], was used to determine whether nodavirus could alter proteins through ROS activity.

Epidemiological studies have revealed that nodavirus infection is a complicated condition and the pathogenesis of the infection is still not fully defined [13]. Although some nodavirus markers such as coat protein, coat protein antibody, and fish immunoglobulin have been identified and used in diagnosing and monitoring the progress of disease, no single serological test on juvenile groupers can unequivocally diagnose the infection. For example, the detection of nodavirus coat protein is a hallmark for viral nervous necrosis, but the absence of detectable coat protein cannot exclude nodavirus infection. Definitive diagnosis of nodavirus infected viral nervous necrosis still relies on a combination of serological, biochemical, and histological examinations. Using proteomic profiling analysis, the present study aimed to identify biomarkers useful in the diagnosis of viral carrier states in grouper, and how the nodavirus evades host defenses. Detailed analysis of these proteins may reveal valuable information for the diagnosis of nodavirus infected viral nervous necrosis.

One of the identified proteins, crystallin, whose expression was significantly higher in the eye than in other tissues [14], was confirmed by real-time polymerase chain reaction (PCR) and IHC analyses. Crystallin belongs to a small heat shock protein family with chaperone functions that prevent heat-induced and oxidative stress-induced aggregation proteins [15]. In an inflammation-activated mouse model, crystallin pretreatment reduced tumor necrosis factor-α (TNF-α) and nitric oxide (NO) production in lipopolysaccharide (LPS)-activated astrocytes [16]. This suggested the ability to prevent the inflammation-triggered neurotoxicity by crystallin. Recently, as a class of heat shock protein, crystallin exhibits protective function in LPS-induced proinflammation release and therapeutic role in neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease and multiple sclerosis [17–19]. The role of crystallin *in vitro* in relation to the function of macrophage activation during nodavirus-infected grouper is not clear.

In this report, the focus was on the well-characterized nodavirus-mediated neuropathogenesis of grouper, aiming to reveal any association between nodavirus infection an oxidative damage to brain area. Nodavirus infection was associated with increased production of ROS. Dityrosine, a useful marker for protein oxidation, was involved in amino acid hydroxylation of brain and eye tissue during nodavirus infection in groupers. Injury mediated by free radicals, particularly by ROS, is an important common pathway of such varied pathological processes as inflammatory damage [20] and neurodegenerative diseases [21]. These previous and present observations indicate that recombinant crystallin is capable of activation of macrophages [22], which is accompanied by production of nitric oxide (NO). A crystallin cDNA from orange-spotted grouper *Epinephelus coioides* was cloned and its expression was characterized. Grouper crystallin possessed chaperone functions that prevented heat-induced and oxidative stress-induced aggregation proteins. Collectively, these observations indicate that crystallin has the potential to act as an anti-inflammatory agent in neuroprotective processes.

2. Materials and methods

2.1. Cell culture and reagents

The grouper cell line GF-1 [23] was grown at 28 °C in Leibovitz's L-15 medium (GibcoBRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS). GF-1 grouper cells, which are susceptible to nodavirus infection and replication, were obtained from the Taiwan Bioresources Collection and Research Center. Transient transfections were performed by introducing 1–2 μg of plasmid encoding grouper crystallin into cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA). After transfection, cells were grown for 24–30 h. Intracellular localization of crystallin proteins was examined using a model IX70 microscope (Olympus, Tokyo, Japan). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, OR, USA) was dissolved in 20% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). An alkaline phosphatase-conjugated substrate Western blotting detection system kit was purchased from Bio-Rad (Hercules, CA, USA). Alkaline phosphatase-conjugated anti-mouse, anti-rabbit, and anti-goat IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:5000 prior to use.

2.2. Measurement of intracellular ROS

The production of intracellular ROS was measured using H_2DCFDA as previously described [24]. H_2DCFDA reacts with ROS to form the highly fluorescent compound dichlorofluorescein. To measure ROS GF-1 cells starved by growth in low-serum Leibovitz's L-15 medium with 1% FBS were treated with nodavirus (10^4 $TCID_{50}$ mL^{-1}) for 24 h followed by 10 μM H_2DCFDA for 20 min. Control cells received DMSO. Cells were collected following exposure to nodavirus or DMSO, and fluorescence was determined with excitation and emission wavelengths of 485 and 520 nm, respectively, using a microplate reader (Thermo Labsystems, Waltham, MA, USA). To determine the production of ROS during nodavirus infection, cells were first incubated with the ROS scavenger, N-acetylcysteine (NAC), for 2 h, followed by nodavirus infection. The ROS level was determined by dividing the absorbance of the infected group by the absorbance of the control group.

2.3. Preparation of dityrosine standard and isocratic reverse-phase high-pressure liquid chromatography (HPLC)

Dityrosine can be formed by a horseradish peroxidase-catalyzed oxidation of tyrosine in the presence of hydrogen peroxide (H_2O_2). Ten milligrams of horseradish peroxidase was dispensed in 500 mL of 5 mM tyrosine prepared in 0.1 M borate buffer, pH 9.1. Then, 142 μL of 30% H_2O_2 was added and mixed by brief swirling. After incubation at room temperature for 60 min, 175 μL of 2-mercaptoethanol was added to the reaction mixture. The resulting solution was immediately frozen in liquid nitrogen and lyophilized. The lyophilized material was dissolved in 250 mL of distilled water and the pH was adjusted to 8.8 with a few drops of 0.01 M NaOH. The resulting solution was loaded onto a DEAE column that has been pre-equilibrated with 20 μM $NaHCO_3$, pH 8.8, and was eluted using 200 μM borate buffer, pH 8.8. The dityrosine-containing solutions were pooled and lyophilized. The dityrosine produced in the mixture was chromatographically purified. To do this, the supernatant was loaded onto a BioGel P-2 column equilibrated with 100 mM NH_4HCO_3. The column was eluted with 100 mM NH_4HCO_3 with a flow rate 40 mL/h. The lyophilized dityrosine was dissolved in 20 mL of 100 mM formic acid and the pH was adjusted to 2.5 by adding concentrated formic acid. The column was eluted with 100 mM formic acid, and the dityrosine-containing solution was lyophilized and stored at −20 °C [10]. An isocratic reverse-phase HPLC system also was used to analyze dityrosine in conjunction with both absorbance detection systems. The 4.6×250 mm^2 reverse-phase column (ODS II Spherisorb; LC-Resources, Deerfield, IL, USA) has an 11%