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Development and characterization of a new tropical marine fish cell line from grouper, *Epinephelus coioides* susceptible to iridovirus and nodavirus

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Abstract

The development and characterization of a new tropical marine fish cell line (GS), derived from the spleen of orange spotted grouper, *Epinephelus coioides* is described. The GS cells grow well in Leibovitz's L-15 medium supplemented with 10% foetal bovine serum, and have been subcultured more than 200 times. The optimal growth temperature was 27 °C. The GS cell culture consisted of mostly fibroblastic cells. The modal diploid chromosome number was 48. GS cell cultures showed advanced cytopathic effects after infection with a pathogenic grouper iridovirus (Singapore grouper iridovirus, SGIV) or with a grouper nodavirus (*Epinephelus tauvina* nervous necrosis virus, ETNNV). Analysis by transmission electron microscopy showed a large number of SGIV and ETNNV particles in the cytoplasm of virus-infected cells, respectively, indicative of high sensitivity to these two viruses. Immunofluorescence microscopy showed that iridovirus-infected GS cells reacted strongly with monoclonal antibody against the grouper iridovirus. It is suggested that the GS cell line has good potential as a diagnostic tool for isolation and propagation of iridovirus and nodavirus. When the GS cells were transfected with pEGFP vector DNA, significant fluorescent signals were observed suggesting that the GS cell line can be used as a useful tool for transgenic and genetic manipulation studies.
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Keywords: Tropical marine fish cell line; GS; Grouper; *Epinephelus coioides*; Iridovirus; Nodavirus

1. Introduction

Grouper, *Epinephelus* spp., is one of the most important fish genera maricultured in China including south part of mainland, Hong Kong and Taiwan, Singapore and other Southeast Asian countries. In recent years, with the rapid development of intensive aquaculture industry, infectious viral diseases have severely affected many highly valued fish species, including grouper, causing heavy economic losses. Iridovirus and nervous necrosis virus (nodavirus), the two newly emerging viral pathogens have been isolated and identified as the most important pathogens infecting grouper in the last decade. Iridoviruses are large double-stranded DNA viruses, icosahedral, 120–300 nm in diameter and contain a spherical deoxyribonucleoprotein core surrounded by a lipid membrane containing protein subunits. Histopathological signs in iridovirus-infected fish may include enlargement of cells and necrosis of the renal and splenic hematopoietic tissues. The mortality rates due to these iridovirus infections range from 30% (adult fish) to 100% (fry). Iridoviruses have been isolated and identified from at least 20 fish species worldwide (Hyatt et al., 2000). Outbreaks of iridoviral diseases in grouper have been reported in Singapore (Chua et al., 1994; Qin et al., 2003), Thailand (Danayadol et al., 1996) and Taiwan (Chou et al., 1998). Fish nodaviruses

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are icosahedral, naked nonenveloped single-stranded RNA viruses in size of 25–30 nm. They caused lesions in fish brain and retina, and the disease caused by nodavirus is also known as viral nervous necrosis (VNN). VNN causes mass mortality, often up to 100%, of affected populations in fry and fingerling stages. Outbreaks of VNN have been reported in grouper in many countries (Chi et al., 1999; Chua et al., 1995; Danayadol et al., 1995; Hegde et al., 2002; Mori et al., 1992).

The establishment of healthy and sensitive fish cell lines is essential for isolation, identification and characterization of infectious viruses from fish. To date, more than 150 fish cell lines have been developed for virus isolation and propagation (Fryer and Lannan, 1994). However, they were mostly derived from fresh water fish and not sensitive to the newly emerging marine fish viruses. Relatively, few fish cell lines susceptible to viruses were derived from tropical marine fish. Chong et al. (1987) developed a cell line from Asian seabass fry, *Lates calcarifer* (SB), and Chew-Lim et al. (1994) established a tropical fish cell line from grouper, *Epinephelus tauvina* (GP). However, GP cells were only sensitive to iridovirus, SB cells only to nodavirus, and the SB cell line was chronically infected with an unidentified virus. Chang et al. (2001) developed a new virus-free cell line from Asian seabass fry (SF) sensitive to several marine fish viruses including iridovirus and nodavirus. Four grouper cell lines were reported from different tissues of eye, fin, heart and swim bladder in *Epinephelus awoara* (Temminck & Schlegel) (Lai et al., 2003). Fish spleen is an important organ for virus infection, particularly for grouper iridovirus infection (Huang et al., 2004). The objective of this study was to establish a new cell line from the spleen of orange spotted grouper, *Epinephelus coioides* (GS cells). Susceptibility of the new GS cell cultures to iridovirus and nodavirus was analyzed. Efficiency of transfection and expression of foreign DNA were also investigated in the new cell line.

2. Materials and methods

2.1. Primary cell culture

A healthy orange spotted grouper weighing 20 g, collected from a local fish farm in Guangdong province of PR China was used for primary cell culture. The fish was killed and then swabbed with 70% alcohol. Spleen was removed and washed three times with Leibovitz's L-15 cell culture medium containing high concentration antibiotics (400 IU ml^{-1} penicillin and 400 $\mu g\ ml^{-1}$ streptomycin). After washing, the sample was minced thoroughly with scissors, and transferred to a 60-mm diameter tissue culture dish with 5 ml of 0.25% trypsin solution (Gibco) for 30 min. The trypsinized tissues were passed through a 100 μm steel mesh to give a single cell suspension. The filtered cell suspension was added to 5 ml complete L-15 culture medium containing 20% foetal bovine serum (FBS), 200 IU ml^{-1} penicillin and 200 $\mu g\ ml^{-1}$ streptomycin, and then centrifuged at $200 \times g$ for 10 min. The cell pellet was resuspended in fresh complete L-15 culture medium and seeded into 25 cm^2 tissue culture flasks and incubated at 25 °C.

2.2. Subculture and maintenance

When the primary cell cultures were grown to a complete monolayer, cells were washed with 0.02% EDTA-PBS, and then trypsinized with 0.25% trypsin solution. The subcultures were grown and maintained in fresh L-15 medium with 15% FBS, 200 IU ml^{-1} penicillin and 200 $\mu g\ ml^{-1}$ streptomycin. After 10 subcultures, the concentration of FBS in medium was reduced to 10%, and antibiotics was reduced to the normal concentration of 100 IU ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin.

2.3. Growth study

To determine the optimum cell culture temperature, 10^6 cells ml^{-1} were seeded in 25 cm^2 tissue culture flasks and incubated at 17, 22, 27 or 32 °C, on a daily basis. The cultured cells were treated with 0.25% trypsin such that the cells rounded up allowing the number of cells to be directly counted as cells cm^{-2} from the cell sheet of duplicate culture flasks at each temperature under an inverted light microscope for each of 5 days post-inoculation.

2.4. Chromosome analysis

Chromosome preparations were made from GS cells at subculture 60. One-day-old cell cultures (60% confluent) were treated with 0.2 $\mu g\ ml^{-1}$ colcemid (Sigma, St Louis, MO, USA) for 4 h at 27 °C to arrest cells at metaphase. The cells were harvested by centrifugation at $200 \times g$ for 5 min, and then resuspended in a hypotonic 0.5% KCl solution for 10 min, and then fixed in 3:1 of methanol:acetic acid. Slides were prepared using a conventional drop-splash technique (Freshney, 1994), and stained with 5% Giemsa solution (Sigma). Chromosomes were observed and counted under a light microscope (Leica, Germany).

2.5. Viral susceptibility

A grouper iridovirus (SGIV) (Qin et al., 2003) and a grouper nervous necrosis virus (ETNNV) (Hegde et al., 2002) were used to test viral susceptibility of the GS cell line. Eighty percent confluent monolayers of GS cells were infected with SGIV or ETNNV at a multiplicity of infection (MOI) of approximately 0.1. Cytopathic effects (CPE) caused by the viruses were observed daily using an inverted light microscope. For virus titration, 0.1 ml of 10-fold serial dilutions of virus was inoculated into four wells of subconfluent cells in a 24-well plate (NUNC™

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