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Characterization of a new fibroblast cell line from a tail fin of red sea bream, *Pagrus major*, and phylogenetic relationships of a recent RSIV isolate in Japan

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Abstract

Red sea bream iridovirus (RSIV) is a causative agent of red sea bream iridoviral disease (RSIVD) in marine fish species in Japan. Fibroblast cells were developed from a tail fin of red sea bream, *Pagrus major*, and then underwent single cell cloning. The successful cloned cells were named CRF-1 cells. Most CRF-1 cells had a normal diploid karyotype with $2n = 48$ by chromosomal analysis. RSIV-infected CRF-1 cells showed typical morphological changes that were associated with apoptosis by EGFP-annexin V staining. The serial viral passages were successful in CRF-1 cells but failed in BF-2 cells as judged by MTT assay. The expression of three genes obviously decreased in BF-2 cells compared with CRF-1 cells and finally was below detectable level. Because the expression of 591R gene showed the fastest decrease among three transcripts, the suppression of IE transcript may be responsible for the restricted replication in BF-2 cells. MCP and ATPase phylogenetic trees showed that RSIV strain U-1 belongs to a distinct group from RSIV strain ehime-1. Therefore, possibly recent epizootics of RSIVD in Japan do not originate directly from RSIV strain ehime-1. Taken together, this study confirmed that RSIV strain U-1 is more closely related to Korean RSIV isolates.

Keywords: Red sea bream; Fibroblast cell; Red sea bream iridovirus; Susceptibility; IE transcript; Phylogenetic relationship

1. Introduction

Red sea bream iridoviral disease (RSIVD) was first reported in cultured red sea bream, *Pagrus major*, in an outbreak on Shikoku Island, Japan, in 1990 (Inouye et al., 1992). Red sea bream iridovirus (RSIV), the causative agent of RSIVD, is an icosahedral DNA virus about 220 nm in diameter. RSIV is sensitive to chloroform and ether treatments, and unstable to heat and acid pH, and its replication is inhibited by 5-iodo-2-deoxyuridine (Nakajima and Sorimachi, 1994). Diseased fish become lethargic and have severe anemia, petechia of the gill and enlargement of the spleen. RSIV-infected cells staining deeply with Giemsa were a characteristic microscopic lesion in the spleen, heart, kidney, liver and gill (Inouye et al., 1992). RSIVD has been documented in over 30 cultured marine species in Japan (Kawakami and Nakajima, 2002; Nakajima and Maeno, 1998).

Piscine iridoviruses are classified into either genus *Lymphocystivirus* or *Ranavirus*. Although RSIV genomic DNA has a size and G + C content near to those of genus *Ranavirus* (Kurita et al., 2002), RSIV isolates from cultured marine fish species in Japan have common antigenic properties which were distinguishable from piscine iridoviruses of genera *Lymphocystivirus* and *Ranavirus* by serological analysis (Nakajima et al., 1998). The presence of other piscine iridoviruses that do not belong to either genus has been also reported from China, Southeast Asian countries, Taiwan, Korea and Australia (Chen et al., 2003; Do et al., 2004; Go et al., 2006; He et al., 2001; Sudthongkong et al., 2002; Wang et al., 2003). Recently, these iridoviruses including RSIV have been defined as new genus *Megalocytivirus* in the family *Iridoviridae* as the third piscine iridovirus by the International Committee on Taxonomy of Viruses (ICTV) (Williams et al., 2005).

Grunt fin (GF) cells are susceptible to RSIV infection and used for formalin-inactivated vaccine development (Nakajima et al., 1997, 1999, 2002). Although fish cells are generally cultured in a growth medium supplemented with fetal bovine serum

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(FBS), GF cells require uniquely human serum in the medium (Clem et al., 1961). Because human serum is difficult to obtain, there is a need to develop cell lines that can be cultured conventionally with FBS and that are susceptible to RSIV infection. As cell lines of red sea bream, fibroblast cells have been reported to be established from kidney and tail fin tissues (Yu et al., 1995, Tong et al., 1998). Although our laboratory previously established two fibroblast cell lines from the same tissues in red sea bream, both cells were refractory to RSIV infection (unpublished data). In this study, fibroblast cells were developed from a tail fin of red sea bream once again and further underwent single cell cloning. To confirm viral susceptibility of the successful cloned cells, RSIV isolate in Kochi Prefecture, Japan, in 2001 was used and then sequenced for phylogenetic analysis.

2. Materials and methods

2.1. Virus

RSIV strain U-1 was previously isolated from RSIV-infected red sea bream in Kochi Prefecture, Shikoku Island, Japan, in 2001, propagated in GF cells and stored −80 °C until use (Imajoh et al., 2004).

2.2. Primary cell culture and subculture

Red sea bream juveniles were obtained from the Ohita Marine Research Center of Nippon Suisan Kaisha Ltd., maintained in a 100 l tank with flow-through water and fed a commercial diet. A red sea bream weighting 14.2 g was disinfected with 70% ethanol. The tail fin was immediately removed and washed three times with phosphate-buffered saline (PBS) supplemented with antibiotics (penicillin, 100 U ml^{-1}; streptomycin, 100 μg ml^{-1}; neomycin, 200 μg ml^{-1}). The washed fin was minced with scissors, digested with 0.15% trypsin solution for 5 min at room temperature and then filtered through a 100 μm nylon mesh. After centrifugation at 350 × *g* for 15 min at 4 °C, the filtered cell pellet was resuspended in Dulbecco's modified Eagle's medium nutrient mixture F12 HAM growth medium (Sigma) supplemented with 20% FBS and 100 μg ml^{-1} of kanamycin. The cell suspension was transferred into a 60 mm^2 tissue culture dish and incubated at 24 °C in an ambient air incubator. The growth medium was changed every 2 days. When the primary cells reached confluence, the cells were washed twice with PBS and trypsinized with commercial trypsin–EDTA solution (Cosmo Bio). Detached cells were resuspended in the growth medium at a split ratio of 1:2 and incubated at 24 °C. After 10 passages, the concentration of FBS in the growth medium decreased to 10% from 20%.

2.3. Single cell cloning

Subcultured cells at passage number 20 were used for single cell cloning. The cells were seeded onto a 96-well tissue culture plate by limiting dilution method and incubated for 7 days at 24 °C. Single colonies in each well were selected, transferred to a 24-well tissue culture plate and cultured at 24 °C. Confluent cells were transferred to a six-well tissue culture plate and finally a single cell was expanded in a 25 cm^2 tissue culture flask. The successful cloned cells were named CRF-1 cells. CRF-1 cells were cultured in the growth medium at a split ratio of 1:3 until 50 passages for the experiments.

2.4. Chromosomal analysis

CRF-1 cells were incubated in a 25 cm^2 tissue culture flask until 70–80% confluence at 24 °C. Colchicine solution (Nacalai Tesque) was added to the cells at a final concentration of 0.2 μg ml^{-1} and then the cells were incubated overnight at 24 °C. After gentle pipetting, detached cells were collected by centrifugation at 200 × *g* for 5 min at 4 °C, treated with a hypotonic solution of 0.65% KCl for 20 min and fixed in 1:3 of acetic acid:methanol for 5 min at room temperature. The fixed cells were collected by centrifugation at 200 × *g* for 5 min at 4 °C and resuspended in fixative solution. The cell suspension was dropped onto a clean slide glass. After air-dry, 5% Giemsa solution was added for 20 min at room temperature to stain chromosomes. One hundred cells at metaphase were counted under a light microscope.

2.5. Viral sensitivity and cytopathic effect

Confluent CRF-1 cells in a 60 mm^2 tissue culture dish were infected with the virus at a multiplicity of infection (MOI) of 10 and then incubated at 24 °C. The development of cytopathic effect (CPE) was observed daily under an inverted light microscope.

2.6. Annexin V and PI staining

Confluent CRF-1 cells in two-well chamber slides were infected with the virus at an MOI of 10 and then incubated at 24 °C. An Annexin V-EGFP Apoptosis Detection Kit (MBL) was used according to the manufacturer's instruction to detect phosphatidylserine translocation in cell membrane at early apoptosis. Apoptotic cells were observed under a Zeiss LSM 510 laser scanning confocal microscope. Fluorescent images were processed by using the Adobe Photoshop 6.0 software.

2.6.1. Viral passage

Bluegill fry-2 (BF-2) cells were used for comparison with CRF-1 cells. Confluent CRF-1 and BF-2 cells in 25 cm^2 tissue culture flasks were infected with the virus at an MOI of 10 and incubated at 24 °C. At 4 days after viral infection, the supernatants for MTT assay and the pellets for quantitative competitive RT-PCR (QC-RT-PCR) analysis were fractionated by centrifugation at 600 × *g* for 10 min at 4 °C. Some of the supernatants were used for the next infection and the same method was repeated until 10 viral passages to examine the change in viral infectivity by serial viral passages.

2.6.2. MTT assay

CRF-1 and BF-2 cells were seeded onto 96-well tissue culture plates and incubated overnight at 24 °C. Viral infection