Aquaculture 278 (2008) 14–21

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Development of two cell lines from *Epinephelus coioides* brain tissue for characterization of betanodavirus and megalocytivirus infectivity and propagation

C.M. Wen [a,*], C.W. Lee [a], C.S. Wang [a], Y.H. Cheng [b], H.Y. Huang [c]

[a] *Department of Life Sciences, National University of Kaohsiung, Kaohsiung, 811, Taiwan, ROC*
[b] *Department of Animal Science, National I-Lan University, I-Lan, 260, Taiwan, ROC*
[c] *Institute of Biotechnology, National University of Kaohsiung, Kaohsiung, 811, Taiwan, ROC*

ARTICLE INFO

Article history:
Received 10 February 2008
Received in revised form 5 March 2008
Accepted 10 March 2008

Keywords:
Astroglia
Piscine cell line
Grouper
Iridovirus
Mx protein
Nervous necrosis virus

ABSTRACT

Betanodaviruses and megalocytiviruses are the causative agents of viral nervous necrosis and iridoviral disease, respectively, among marine species farmed in Taiwan. Because there are few cell lines susceptible to these viruses, we wished to identify additional lines with which to study viral pathology and epidemiology. Thus, we established two clonal cell lines designated GBC1 and GBC4 from the brain of an orange-spotted grouper, *Epinephelus coioides*. Both cell lines grew well in Leibovitz's L-15 medium supplemented with 5% (GBC1) or 10% (GBC4) fetal bovine serum at temperatures between 20 °C and 35 °C. Cytokeratin immunofluorescence staining revealed that both cell lines were of epithelial origin. GBC4 cells expressed glial fibril acidic protein suggesting, that they are astroglial lineage cells. The modal diploid chromosome number was 44 and 48 for GBC1 and GBC4, respectively. GBC1 cells were highly susceptible to grouper nerve necrosis virus (GNNV) and yielded titers of 10^{10} $TCID_{50}$ ml^{-1} but were non-susceptible to the giant seaperch iridovirus (GSIV). By contrast, GBC4 cells were susceptible to GSIV with titers approaching 10^{9} $TCID_{50}$ ml^{-1} whereas GNNV infection only yielded titers of 10^{6} $TCID_{50}$ ml^{-1}. GNNV propagated in GBC1 across a wide range of temperatures (15–37 °C) whereas in GBC4, GSIV propagated over 15–30 °C. Induction of the Mx protein upon GNNV infection occurred in GBC4 but not in GBC1, suggesting that the Mx protein inhibits virus production. Our results suggest that these two cell lines provide a valuable tool for the isolation and investigation of betanodavirus and megalocytivirus.

1. Introduction

Piscine nervous necrosis viruses (NNVs) and iridoviruses cause severe epizootic outbreaks resulting in mass mortality and large economic losses in farmed marine fishes in Taiwan as well as other Southeast Asian countries. Piscine NNVs are icosahedral, non-enveloped, single-stranded RNA viruses 25–30 nm in diameter and are classified in the genus *Betanodavirus* of Nodaviridae (ICTVdB Management, 2006b; http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/). They are the causative agents of viral nervous necrosis (VNN) disease in marine species in many countries (Renault et al., 1991; Mori et al., 1992; Chua et al., 1994; Chi et al., 1997; Grotmol et al., 1997; Bovo et al., 1999; Cutrín et al., 2007). The diseased fish commonly display vacuolating encephalopathy and retinopathy that frequently leads to death, especially at the larval and juvenile stages. More than 30 susceptible fish host species have been reported from more than ten families (Munday et al., 2002). All the NNVs can be classified into one of the four following genotypes based on partial sequences of the coat protein genes: SJNNV (striped jack NNV), RGNNV (red spotted grouper NNV), TPNNV (tiger puffer NNV), and BFNNV (barfin flounder NNV) (Nishizawa et al., 1997). The NNVs discovered in Taiwan, including grouper NNV (GNNV) and yellow grouper NNV (YGNNV), have the RGNNV genotype (Lai et al., 2001; Chi et al., 2003).

Iridoviruses have been isolated and identified from at least 50 fish species collected in the South China Sea (Wang et al., 2007). The definitive symptom of iridovirus infection is the formation of inclusion body-bearing cells in infected organs. They are icosahedral double-stranded DNA viruses and the virions consist of an envelope, a capsid, and an internal lipid membrane. The capsid is isometric and has a diameter of 125–300 nm and consists of probably 72 capsomers (ICTVdB Management, 2006a; http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/). Piscine iridoviruses are classified into either genus *Lymphocystivirus*, *Ranavirus*, or *Megalocytivirus*. Nakajima and Kurita (2005) further classified megalocytiviruses into three major groups by phylogenic analysis of the major capsid protein (MCP): red sea bream iridoviruses (RSIV), infectious spleen and kidney necrosis iridoviruses (ISKNV), and turbot reddish body iridoviruses (TRBIV). In Taiwan, epizootic iridovirus outbreaks have occurred in several important farmed fish such as grouper (*Epinephelus* spp.), giant

* Corresponding author. Department of Life Sciences, National University of Kaohsiung, No. 700, Kaohsiung University Road, Nan-Tzu District, 81148, Kaohsiung, Taiwan, ROC. Tel.: +886 7 5919231; fax: +886 7 5919404.
E-mail address: wenchiumin@nuk.edu.tw (C.M. Wen).

0044-8486/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2008.03.020

seaperch (*Lates calcarifer*), and red sea bream (*Pagrus major*) resulting in significant economic losses (Chou et al., 1998; Lai et al., 2000; Chao et al., 2002; Wang et al., 2003). Almost all the viral agents belong to the groups of RSIV or ISKNV (unpublished data; personal communication with Dr. Chao Institute for Animal Disease Prevention and Control, Kaohsiung, Taiwan) and only a few are ranaviruses (Murali et al., 2002; Tsai et al., 2005). Although many fish cell lines have been established, relatively few have the ability to propagate betanodaviruses or iridoviruses. The few cell lines (Chew-Lim et al., 1994; Nakajima et al., 1997; Chi et al., 1999a; Chang et al., 2001; Lai et al., 2001, 2003; Qin et al., 2006; Imajoh et al., 2007) capable of viral propagation but produce the viruses slowly and give low viral titers. It thus has become crucial to develop suitable cell lines not only for vaccine development but also for isolation and study of the viruses.

2. Materials and methods

2.1. Fish and primary culture preparation

Young orange-spotted groupers (*Epinephelus coioides*), approximately 10 cm in length, were obtained from a commercial fish farm (Yeong-An, Kaohsiung, Taiwan). The fish were anaesthetized by MS-222 (Sigma) and decapitated aseptically as described previously (Wen et al., 2008). The brain was removed and finely chopped with scissors in phosphate-buffered saline (PBS) (Ca^{2+}- and Mg^{2+}-free). The tissue fragments were then washed several times in an antibiotic solution (PBS containing 500 μg/ml streptomycin and 500 IU/ml penicillin) and were subsequently placed into a 25 cm^2 tissue culture flask (Nunc) containing 2 ml Leibovitz's L-15 growth medium supplemented with 15% fetal bovine serum (FBS) (Gibco) and incubated at 25 °C. Every 4–5 days, half of the growth medium was removed and replaced with fresh medium.

2.2. Subculture and clone isolation

Cells were subcultured when confluent monolayers developed. Cells were washed twice with PBS and were dislodged from the flask surface by treatment with a 0.1% trypsin solution containing 0.2% EDTA and subcultured at a split ratio of 1:2. At the 10th passage, cells were dislodged as before and plated in 3-cm tissue culture dishes at 1000 cells per dish. Clones were allowed to expand for up to 14 days, and single colonies of cells were isolated using cloning rings (Nunc). Cells therein were suspended and plated on 4-well plates, and gradually expanded into 25 cm^2 flasks. Two clones, GBC1 and GBC4, with different phenotypes were selected for further study. FBS in the medium was reduced to 10% after 5 and 60 subcultures for GBC1 and GBC4, respectively. For GBC1, the serum was further decreased to 5% after 100 passages.

2.3. Expression of antigenic markers

Cells grown on coverslips were washed three times with PBS and fixed for 10 min in methanol at room temperature. Mouse anti-human keratin (Clone C-11), mouse anti-porcine glial fibril acidic protein (GFAP) (Clone GA-5), and mouse anti-porcine vimentin (Clone V9) monoclonal antibodies (all from NeoMarkers and diluted 1:200) were used to identify epithelial cells, astroglia, and mesenchymal cells, respectively. For fluorescence immunocytochemistry, FITC-conjugated goat anti-mouse IgG (Cappel; 1:100) was used. Otherwise, the cells were labelled with horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel; 1:10,000) and visualized using TrueBlue peroxidase substrate (KPL). Orcein (KPL) was used as a counter stain. Negative controls (omission of the primary antibody) were included in each experiment.

2.4. Scanning electron microscopy (SEM)

Cells grown on coverslips were washed three times with PBS, fixed in 2.5% glutaraldehyde (Sigma) and post-fixed in 1% OsO_4 (Sigma) for 30 min. The fixed cells were dehydrated using serial alcohol and critical point drying (Hitachi CP-2). The specimens were coated with a layer of platinum-palladium and observed under a Hitachi S-520 SEM.

2.5. Growth study

To determine the optimum incubation temperature for cell growth, 5×10^5 cells (GBC1 and GBC4 cells at passage 180 and 95, respectively) were seeded in duplicate in 25 cm^2 culture flasks and incubated at 15, 20, 25, 30, or 35 °C. The number of cells from duplicate flasks at each temperature was recorded every day for 5 days. Similarly, the effect of various FBS concentrations (2, 5, 10, or 15%) on cell growth was assessed at 25 °C.

2.6. Chromosome analysis

Chromosome preparations were made from GBC1 and GBC4 cells after 135 and 90 passages, respectively. One-day-old cultures (70–80% confluencey) were treated with 0.1 μg ml^{-1} colcemid (Gibco) for 6 h at 25 °C and dislodged using 0.1% trypsin solution. After centrifugation at 150 ×*g* for 10 min, the cells were suspended in a hypotonic solution (0.04 M KCl) for 30 min at room temperature. After swelling, the cells were fixed with methanol and acetic acid (3:1) fixative. The

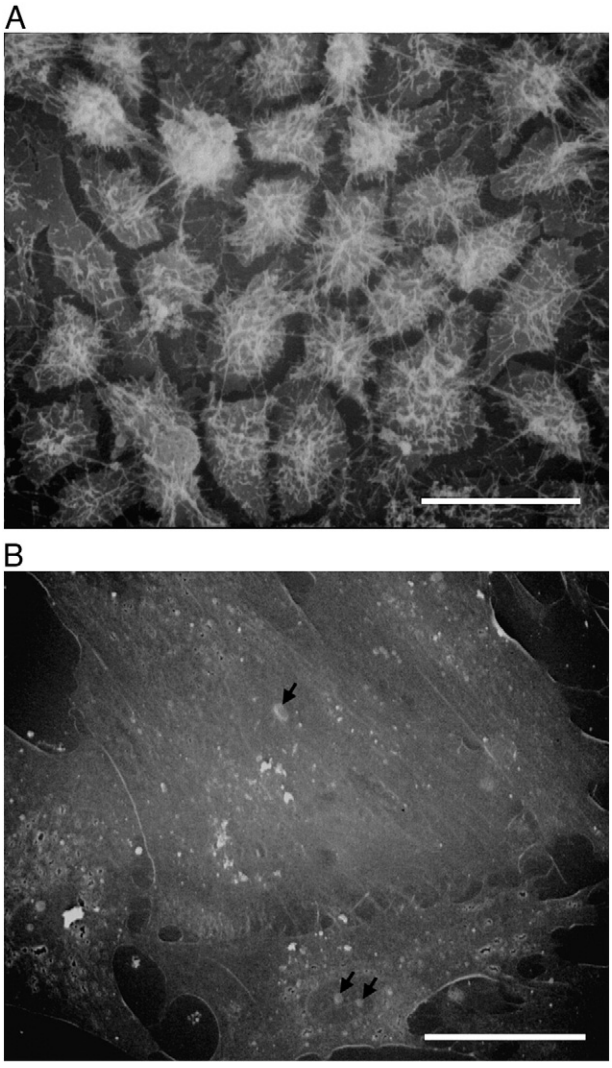

Fig. 1. SEM micrographs of cell lines derived from orange-spotted grouper (*Epinephelus coioides*) brain tissue. (A) GBC1 cells at passage 130. Note depressive extensions and abundant microvilli on the upper surface. Bar = 20 μm. (B) GBC4 cells at passage 85. Note the comprehensive cell spreading. Nuclei and nucleoli (arrows) are also apparent. Bar = 30 μm.