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Development of a Cell Culture System From Gill Explants of the Grouper, *Epinephelus malabaricus* (Bloch and Shneider)

K. S. SOBHANA*, K. C. GEORGE, G. VENKAT RAVI, GIJO ITTOOP and R. PAULRAJ

Marine Biotechnology Division, Central Marine Fisheries Research Institute,
P. B. No. 1603, Kochi, India – 682018.

Abstract

A cell culture system was developed successfully from gill explants of the Malabar grouper, *Epinephelus malabaricus*. Gill tissue samples aseptically excised from healthy juveniles of *E. malabaricus* were explanted in Leibovitz' L-15 medium supplemented with 0.07 M NaCl and 20% fetal bovine serum (FBS). A mixture of different types of cells emerged from the explants, and these cells were observed to spread and attach to the culture flasks from the second day onwards. Confluent monolayers comprising epithelioid as well as fibroblast-like cells were formed within ten days. The cells were found to grow well at 28 $\pm$ 2°C. The cell monolayers were subcultured by trypsinization and seeded into new flasks, which produced confluent monolayers comprising predominantly epithelioid-like cells in subsequent passages.

Introduction

In vitro cell culture systems are necessary for the isolation and characterization of viruses, the development of diagnostic reagents, the testing of therapeutics, and the production of materials for immunological and vaccination studies. Tissue culture and the development of cell lines from fish are of priority interest for pathogen detection and for studies in toxicology, carcinogenesis, cellular physiology, and genetic regulation and expression.

Groupers are important fish group widely used for mariculture in many countries in the Asian region. In recent years, with the rapid development of intensive aquaculture industry, infectious viral diseases have severely affected many high-valued fish species, including grouper, causing heavy economic losses. Iridovirus and nervous necrosis virus (fish nodavirus) are the two newly emerging viral pathogens that have been isolated and identified as the most important pathogens infecting grouper in the last decade. Outbreaks of iridoviral and nodaviral diseases in grouper have been reported in many countries (Hegde et al. 2002; Qin et al. 2003).

The establishment of healthy and sensitive fish cell lines is essential for isolation,

*Corresponding Author. Tel: +91484 2394867
Email : sobhana_pradeep@yahoo.co.in

identification, and characterization of infectious viruses from fish. More than 150 fish cell lines have been developed for virus isolation and propagation (Fryer & Lannan 1994). However, most of these cell lines are derived from freshwater or anadromous fish species and are not sensitive to the newly emerging marine fish viruses. The limited number of reports on viruses from marine fish compared with those from freshwater fish is due to the shortage of fish cell lines derived from marine fish. The study on marine fish cell lines has developed rapidly in recent years and at least 17 cell lines from tissues of commercially important marine fish have been described since 1980 (Fernandez et al. 1993a).

In India, successful marine fish cell culture systems have been developed only from the Asian sea bass, *Lates calcarifer* (Sahul Hameed et al. 2006; Lakra et al. 2006; Parameswaran et al. 2006a, b). Because cell cultures derived from the same species or a species closely related to that in which the disease occurs would be the most sensitive for virus isolation, cell lines derived from local species should be given high priority. The host and tissue specificity of virus underlines the need for developing cell lines from different species in different regions (Cheng et al. 1993).

In this context, development of grouper cell lines, anticipating problems such as viral disease outbreaks is very important and in the present study, an attempt was made to develop a cell culture system from gill explants of the Malabar grouper, *Epinephelus malabaricus.*

Materials and Methods

Preparation of fish and tissue collection

For initiating primary culture from gill, healthy juveniles of the grouper, *E. malabaricus* (average weight 62 $\pm$ 5 g) collected from the coastal waters of Cochin were used. Fishes were acclimatized in circular fiberglass tanks (having *in situ* biological filtration system holding 300 l of well-aerated and dechlorinated sea water of 30–32% salinity) for a period of about two weeks on a diet of marine shrimp/fish meat. Fishes were subsequently transferred to rectangular perspex tanks (90 cm x 60 cm x 45 cm) holding 50 l of well-aerated and dechlorinated seawater of 30% salinity in the Fish Pathology Laboratory of the Central Marine Fisheries Research Institute.

The fishes were starved for two days prior to killing for dissecting out the tissues and were maintained overnight in sterile, aerated seawater containing 1000 IU ml^{-1} penicillin and 1000 µg ml^{-1} streptomycin. Before killing, the fishes were tranquilized by plunging in iced water for 5 min, disinfected by immersing in sodium hypochlorite (500 ppm available chlorine) for 5 min, washed in sterile seawater, and swabbed with 70% ethyl alcohol. The gill tissue was aseptically excised and collected in sterile petridishes holding Leibovitz' L-15 (GIBCO) medium (serum free) containing 500 IU ml^{-1} penicillin and 500 µg ml^{-1} streptomycin. Tissue pieces were minced into small fragments using a sterile surgical scalpel and again washed in serum-free medium containing 500 IU ml^{-1} penicillin and 500 µg ml^{-1} streptomycin.

Explantation

The tissue pieces were resuspended in 2 ml of growth medium containing 20% fetal bovine serum, (FBS) (PAN Biotech, Germany), 200 IU mL^{-1} penicillin, 200 µg ml^{-1} streptomycin, and 0.25 µg mL^{-1} amphotercin B and were subsequently transferred to 25 cm^2 tissue culture flasks and distributed uniformly, and the flasks were incubated at 28 ± 2°C for 4-5 h. The medium was replaced with L-15 medium (pH 7.2 ± 0.2) containing 20% FBS, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 0.125 µg mL^{-1} amphotercin B and incubated at 28 ± 2°C. The tissue explants were observed for growth and formation of monolayer of cells using an inverted microscope (Nikon TS 100).

Subculture and maintenance

Once confluent monolayers were formed in primary culture, cells were dislodged from the flask surface by treatment with 0.25% trypsin (0.25% trypsin and 0.2% EDTA in PBS). Two milliliters of fresh growth medium (L-15 containing 20% FBS, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 0.125 µg mL^{-1} amphotercin B) was then added to neutralize the action of trypsin. The detached cells were then split into two portions, transferred to new tissue culture flasks, and incubated at 28 ± 2°C.

Results and Discussion

Explants of gill tissue readily got attached to the culture flask on incubation. Primary cultures initiated from gill explants showed promising results. Emergence of different types of cells from the attached gill explants was observed within a day (Fig. 1). Cells were observed to spread and attach to the culture flask from the second day onwards (Fig. 2). Growth of the cells was very fast, and the cells formed a confluent monolayer comprising epithelioid as well as fibroblast-like cells within ten days (Fig. 3).

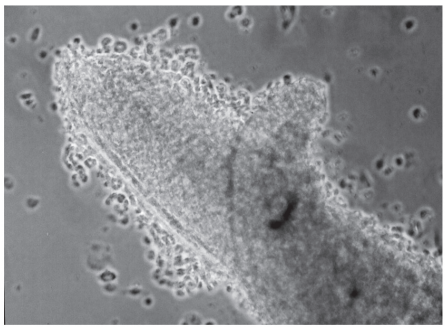

Figure 1. Cells emerging from the gill explants of *E. malabaricus* (X 100)

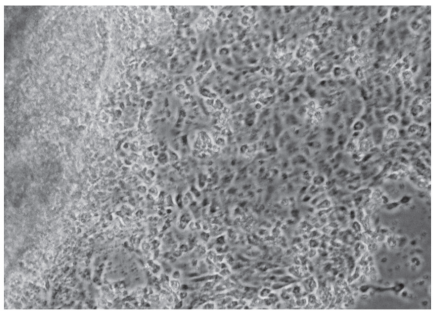

Figure 2. Spreading and attaching cells from the gill explant of *E. malabaricus* on day 2 post-explantation (X 100).

Trypsinization to detach the monolayer yielded individual cells along with cell clumps. The subcultured cells attached well to the flask surface and grew well (Fig. 4). The cell monolayers formed in the subcultured flasks (first passage) were successfully

harvested for passage by trypsinization, which produced confluent monolayers comprising predominantly epithelioid-like cells in subsequent subcultures (Fig. 5 and 6). The cell culture system developed has been successfully subcultured up to sixteenth passage level.

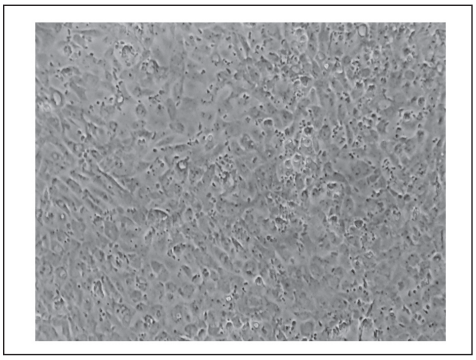

Figure 3. Cell monolayer formed from the gill explant of *E.malabaricus* in primary culture (X 100)

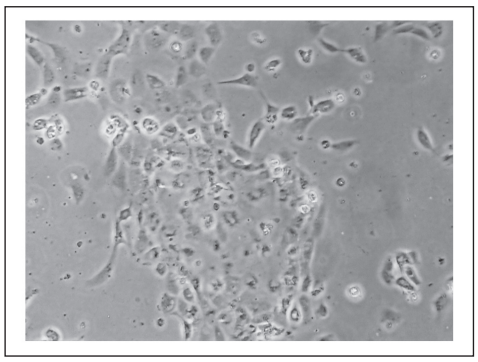

Figure 4. Attachment and formation of monolayer by the subcultured gill cells in the 1st passage (X 100)

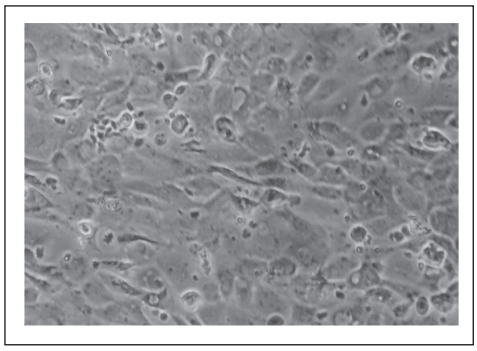

Figure 5. Complete monolayer formed by the subcultured gill cells in the 1st passage (X 100)

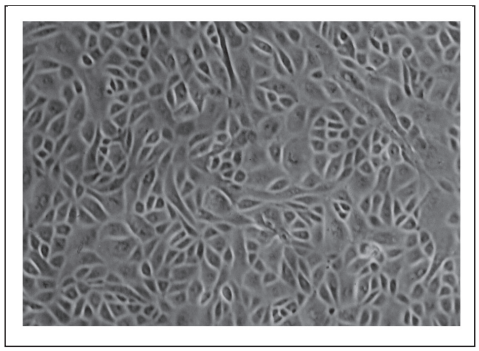

Figure 6. Cell culture system from gill explant of *E. malabaricus* at 6th passage (X 100)

In the present work, primary cell culture was developed from gill of *E. malabaricus* by means of explant technique, which has many advantages over the use of cell suspensions, such as speed, ease, maintenance of cell interactions and the avoidance of enzymatic digestion which can damage the cell surface (Parkinson & Yeudall 1992; Avella et al. 1994). Explants *in vitro* are supposed to be structurally closer to the organ *in vivo* than cultures obtained using cell suspensions.

In the present study, the Leibovitz's L-15 medium supplemented with 20% FBS supported good growth of the cells from gill tissue of *E. malabaricus*. The suitability of L-15 in supporting fish cell lines compared with that of other media has been documented by Fernandez et al. (1993a) when they compared the growth of many fish cell lines in different culture media at different temperature and sodium chloride concentrations.

Leibovitz medium was designed to maintain pH in the physiological range under normal atmosphere without added CO_2. Several researchers have studied the suitability of various mammalian and insect cell culture media and the required supplements for growth of fish cells. Among the various media tested [Leibovitz' L-15, Medium 199 (M-199) and Eagle's minimum essential medium (Eagle's MEM)], L-15 was found to be most suitable for the attachment and proliferation of cells (Lakra et al. 2005). Faster growth and better proliferation was noticed in cells cultured with L-15 medium at pH 7.4. The use of FBS at levels above 10% has been recommended for primary cultures, as well as for the initial passages, whereas an optimal concentration of 5% is enough in later stages (Sahul Hameed et al. 2006; Parameswaran et al. 2006a, b).

Lakra et al. (2006) observed optimum growth and attachment of sea bass caudal fin cell culture systems using 20% FBS and 1% fish serum. Homologous fish muscle extract and prawn muscle extract have been used for successful development of fish cell culture systems (Kumar et al. 1998; Lakra et al. 2006). In the present study, a successful primary culture was obtained using the tissue culture medium L-15 supplemented with 20% FBS without using fish serum/fish muscle extract. In general, growth and development of cell monolayer from gill tissue explants were good and easy to maintain.

Cells from the gill tissue of *E. malabaricus* grew well in L-15 with additional NaCl (0.07 M NaCl), which is needed for marine fish cells (Clem et al. 1961; Law et al. 1978; Li et al. 1984; Fernandez et al. 1993a). Clem et al. (1961) were the first to establish monolayer cell cultures from marine teleosts and obtained best results in commercial medium modified with 0.07 M NaCl. The JSKG cell line established from gonads of Japanese striped knife jaw, *Oplegnathus fasciatus* and PAS cell line from skin of purplish amberjack, *Seriola dumerili* were initiated at a higher NaCl concentration of 0.206 M but gradually adapted to a low NaCl concentration of 0.116 M after several subcultures (Fernandez et al. 1993 b). However, several authors have reported development of cell lines from marine fish without using increased NaCl concentrations in the cell culture medium (Chong et al. 1990; Chew-Lim et al. 1994; Chang et al. 2001).

Sahul Hameed et al. (2006) reported that for the establishment of SISK cell line from sea bass kidney, additional NaCl was not necessary. Similarly, the SISS cell line developed from spleen of Asian sea bass has good adaptation for growth in Leibovitz's L-15 without special requirements, such as NaCl addition (Parameswaran et al. 2006b).

In the present study, the gill tissue of *E. malabaricus* epithelial cells and fibroblast-like cells coexisted in the primary culture. However, as the culture progressed, epithelioid cells predominated in the subsequent subcultures. Chi et al. (1999) reported presence of both epithelial cells and fibroblast-like cells in primary culture of grouper fin (GF-1) cells. However, they reported that in subsequent subcultures, the fibroblast-like cells proliferated more rapidly than the epithelial cells and ultimately predominated. A similar morphological change has also been observed in orange spotted grouper *E. coicoides*

fin (GF-1) (Chi et al. 1999) and spleen (Qin et al. 2006) cells and in yellow grouper *E. awoara* fin (GF) and heart (GH) cells (Lai et al. 2003).

Many serum factors derived from platelets have a strong mitogenic effect on fibroblasts and also tend to inhibit epithelial proliferation, subsequently causing fibroblasts to overgrow in subcultures (Freshney, 1994). In general, a predomination of fibroblastic cells over epithelioid cells in cell cultures from fish has been reported (Lai et al. 2003). Production and maintenance of epithelioid cell line is reported to be comparatively difficult (Wang et al. 2003). However, in the present study, although epithelioid as well as fibroblast-like cells were present in the primary culture, epithelioid-like cells predominated as the culture progressed. The SF cell line developed from Asian sea bass fry consisted of both epithelial-like and fibroblast-like cells in initial subcultures. However, once the culture progressed, the predominant cell type was epithelial-like cells with small groups of fibroblast-like cells (Chang et al. 2001). Sahul Hameed et al. (2006) also observed both epithelial-like and fibroblast-like cells in initial subcultures and observed presence of only epithelial-type cells after 20 subcultures of the SISK cell line developed from sea bass kidney.

The results of the present study have clearly demonstrated good growth and formation of confluent monolayer of cells from gill tissue explants of *E. malabaricus*, which has been successfully subcultured. Gill tissue appears to be ideal for cell culture as it is easy to collect for use in cell culture. Hence, there is scope and prospect for development of cell line from gill tissue of *E. malabaricus.*

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