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Primary Culture of Black-Spotted Grouper Retinal Pigment Epithelium

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The retinal pigment epithelium of black-spotted grouper (*Epinephelus amblycephalus* BLEEKER) was isolated and established in Leibovitz's L15 medium supplemented with 0.3% sodium chloride at 31℃. We found that the success of the cell culture was correlated with the age of the black-spotted grouper.

Mosaic, dendritic, and nerve-like cell patterns were observed to evolve from the retinal pigment epithelium. The binucleated dendritic and nerve-like cells were absent after the third subculture. There was a general decrease in the amount of pigment in the compact mosaic of hexagonal to oval mononucleated cells as tissue culture progressed. Electron microscopy showed that the seventh subcultured cells retained their melansome granules but most of the granules were not pigmented.

The histology of retina was also studied by cross sectional examination.

INTRODUCTION

The retinal histology of striped bass and channel catfish have been throughly studied (Groman, 1982; Grizzle and Rogers 1976). The gangalial components of the retina form several distinct sublayers. Each sublayer can be separated (Shiosaka *et al.,* 1983) and cultivated *in vitro* for a wide range of experiments on retina (Mack and Fernald, 1991, Vecino *et al.,* 1993; Bok *et al.,* 1993). of particular interest to us was the retinal pigment epithelium (RPE) of black-spotted grouper (*Epinephelus amblycephalus* BLEEKER).

RPE cells are responsible for normal photoreceptor function by supplying nutrients to the retina and preserving ionic homeostasis in the subretinal space (Straub *et al.,* 1993). Its pigment formation is inhibited by 5-bromo-2'-deoxyruidine (Raul *et al.,* 1979). The majority of the RPE cells of primates are binucleate (Mark and Ephrain, 1967). The monkey RPE cells are found to synthesize insulin-like growth

factor binding proteins (Waldbilling *et al.*, 1992).

A strain of RPE cells was successfully cultured for three months by Fischer in 1992. Later, attempts were made to culture RPE cells of human, monkey, rat, rabbit, chicken and fish *in vitro* for comparative studiew on phenomena pertaining to RPE cell morphology, function, and differentiation (Hans, 1881; Jean *et al.*, 1973; Mark and ephrain, 1967; Ross, 1977; Raul *et al.*, 1979; Straub *et al.*, 1993; Townes *et al.*, 1988; Whittaker, 1967). Melanin granules in cultured RPE decrease with the age of the culture, become less pigmented, but retain some characteristics of melanin-producing cells (Albert *et al.*, 1972; Jean, 1973; Ross, 1977; Hans, 1981).

In the present study, we observed the retinal histology of black-spotted grouper (*Epinephelus amblycephalus* BLEEKER) and the phenomean of primary culture derived from the RPE.

MATERIALS AND METHODS

Separation of retinal sublayer

The preparation of RPE was performed as described by Albert *et al.*, (1972). The eyes of black-spotted groupers (*Epinephelus amblycephalus* BLEEKER) were ennclated under sterile conditions and immersed in Phosphate-buffered saline modified by supplemented sodium chloride (3 g/L) (PBS3). A small window was cut in the sclera of each eye and the lenses (with retina and choroid) were carefully excised and removed. The lens of each eye were removed and the neural retina was detached and discarded to expose the RPE.

Histological examination

The retina was fixed in a 10% formalin/PBS3 solution, dehydrated in graded ethanol solution, and embedded in paraplast using routine histological techniques. 5-um sections were stained with Price's Giemsa stains.

Primary culture and subculture

The isolated RPE was minced in PBS3 into 1-mm sections and supplemented with 250 units/ml penicillin, 250 ug/ml streptomycin and 10 ug/ml fungizone. A 1% trypsin in PBS3/EDTA was allowed to act on the RPE for 20 min at 25 ℃ . After centrifugation, segments and cells were transferred into T-25 flasks (NUNC, Denmark), cultivated at 31℃ in Leibovits's L15 medium supplemented with 20% fetal calf serum (RCS) (GIBKO, New Zealand LTD), 100 units/ml penicillin, 100 ug/ml streptomycin, 2.5 ug/ml fungizone and three grams sodium chloride per liter. Colonies were formed between one and two weeks, then were trypsinized, but were still cultured in the old T-25 flask. The cells were incubated at 31℃ and subcultured to confluency as described previously (Ku and Chen, 1992).

Transmission electron microscope observation

The cells were propagated for two to three days in T-25 flasks until a confluent monolayer was formed. After being rinsed two times in PBS3, the cells were fixed with cold 2.5% glutaraldehyde/PBS3 for 1h, scraped with rub policeman and centrifuged, then postfixed with 1% OsO_4/PBS3 for 30 min. After each fixation the specimens were rinsed two times with PBS for 15 min each. The specimens were dehydrated in a graded series of ethanol concentration, and then embedded in Spurr's resin. Thin sections were cut on a LKB-2088 ultramicrotome and collected on Formvar coated 200 mesh copper grid. The specimens were examined using a Hitachi H-600 transmission electron microscope under 75 KV after staining with uranyl acetate and lead citrate.

RESULTS AND DISCUSSION

This paper reports the observation of the retinal histology of black-spotted grouper (*Epinephelus amblycephalus* BLEEKER) and the phenomena of primary cultures of the RPE.

Figure 1 shows the retinal cross-section of black-spotted grouper. They were structurally similar to those found in striped bass (Groman, 1982). Its basement layer was composed of pigmented epithelium. Rods, single cones, and double cones were located in the deepest part of the retina next to the pigment cells. Applying the method for the separation of retinal sublayers (shiosaka *et al.,* 1984), we estimated the ratio of rods to cones at approximately 100:1, and the cone to double cone at 1:4.

The osmometry was initially determined on the observation of isolated cones cultured in the Leibovitz's L15 medium supplemented with different sodium chloride concentration. Then black-spotted grouper RPE were isolated and cultured in Leibovitz's L15 medium supplemented with 20% FCS and sodium chloride of three grams per liter at 31℃. Successful primary cultures of RPE were obtained from 85 per cent (six of seven cultures) of young black-spotted grouper (about 3 cm) and designated as BGRPE. However, none of the four cultures were obtained from adult black-spotted grouper (about 35 cm).

This result was similar to the results of Jean *et al.,* (1973) and Pfeffer (1991) who showed that the success of mammalian RPE cell cultures were correlated with the age of the donor. Significant migration of explants of RPE were observed one week after the cultures were established (Fig.2A). Early migratory cells were usually heavily pigmented and round. The center of the colony showed numerous heavily pigmented and round. The center of the colony showed numerous heavily pigmented granules, whereas more recently divided cells at the periphery of the colony had fewer pigment granules and were less pigmented. These cell characteristics were very similar to those reported by Jean *et al.* (1973) and Hans (1981). The epithelial-like sheets of pigmented cells appeared within three to four days after explanation.

As growth continued, a compact mosaic of hexagonal to oval pigmented cells was the most common pattern (Fig.2B). The cells varied in size and in amounts of pigment granules. Occassional binucleated dendritic (Fig.2C) and nerve-like cells (Fig.2D) were seen between the colonies. However, after approximately three passages (four weeks after primary culture was established), these binucleated dendritic and nerve-like cells disappeared.

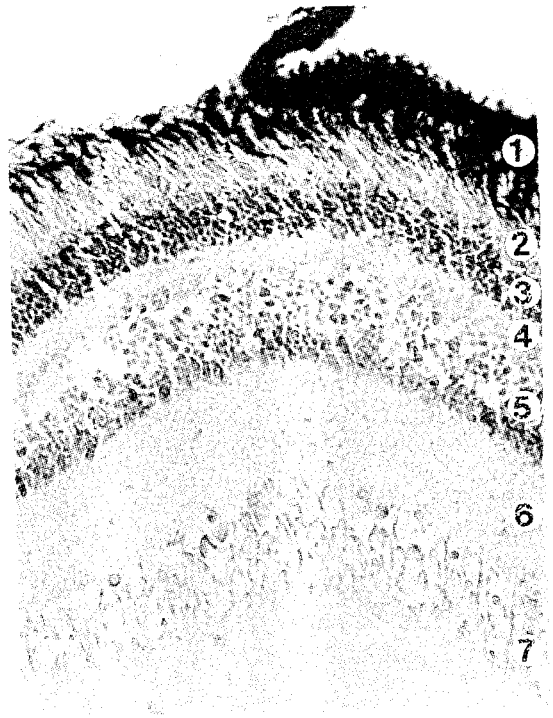

Fig. 1 Retina of black-spotted grouper, 6-month-old fish, (giemsa, formalin), x250. 1. pigment epithelium, 2. layer of rods and cones, 3. outer nuclear layer, 4. outer plexiform layer, 5. inner nuclear layer, 6. inner plexiform layer, 7. ganglion cell layer.

Following trpysinization of the original cultures, the predominant pattern of growth were the cells with small oval nuclei and sparsely pigmented (Fig.3A). The phenotype of these cells was characteristic in papillary (Fig.3B) When observed under scanning electron microscope, the BGRPE cells were flattened with very few bleb on the cell surface (Data not shown). Pigmented granules were almost absent in the fourth passage; and, in subsequent transfers, only an occassional cell contraining pigment granules was seen, as observed by phase inverted microscope. This inability to produce pigment by the RPE does not result from some deficiency in the tissue culture media but is due to decay of tyrosinase or cessation of tyrosinase synthesis (whittaker, 1967).

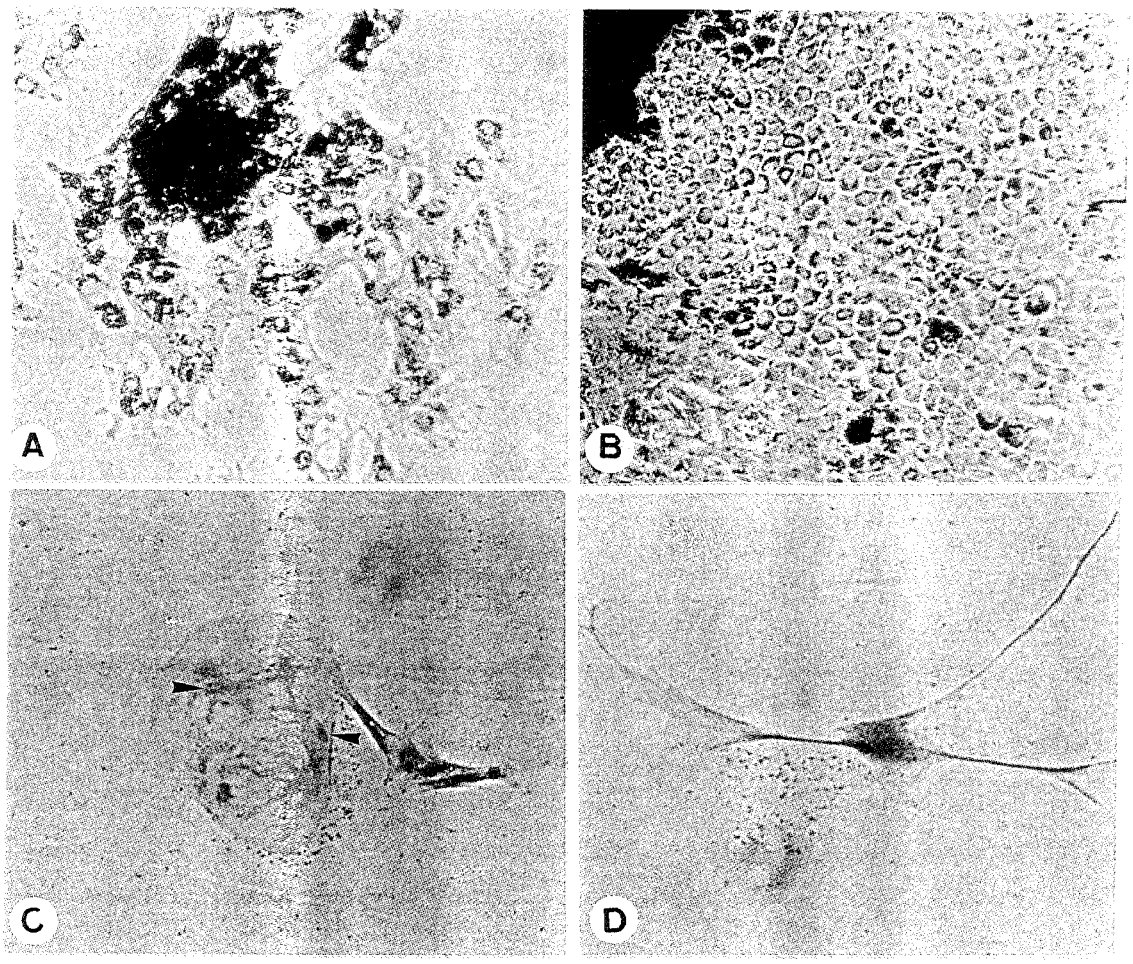

Fig. 2 Cell types in monolayer cell cultures from black-spotted grouper, x100.
A. Cells growing from one week old explant after the culture was started.
B. Two weeks after the culture was started. Cells were generally closed packed and epithelioid in character. Variations in size and degree of pigmentation were present.
C. Dendritic cell with two nuclei (arrows).
D. Nerve-like cell.

Electron microscopic observations showed BGRPE was characteristic in cluster of pigmented (Fig. 4A) and scattered lipid granules (Fig. 4B), and bunch of microfilament (Fig. 4C) in the cytoplasma. In addition, mitochorndria was seldom observed. It was noted that the seventh passage BGRPE cells retained their melansome granules although most of the granules were not pigmented (Fig. 4A).

BGRPE has beeen subcultured ten times within 120 days, but its growth rate decreased gradually after the third passage, and could not grow at 18°C. The cells were susceptible to Abserotype of infectious pancreatic necrosis virus and reovirus, ubt refractory to eel herpesvirus from Formosa (Data not shown). At present, we are trying to maintain the cell line and transform the cell line for use in virological

research. In conclusion, the successful primary culture of RPE cells derived from young black-spotted grouper makes it feasible to provide a ready supply of material for comparative studiew on phenomena pertaining to RPE cell morphology, function, and differentiation.

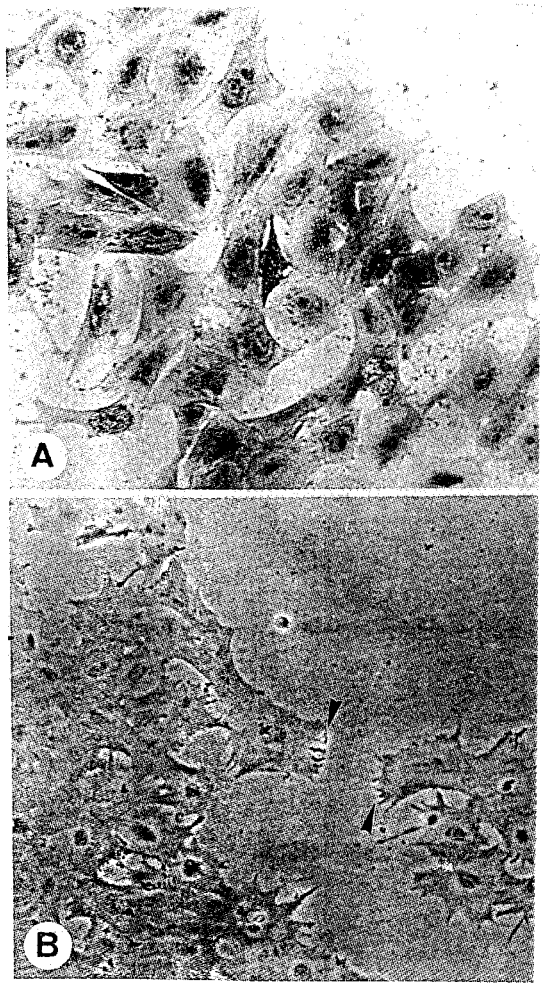

Fig. 3 Monolayer cultures of BGRPE, stained with Giemsa, x 100.
A. Cells with small oval nuclei and sparsely pigmented (the fourth passage).
B. Cells (the sixth passage) were characteristic in papillary (arrow).

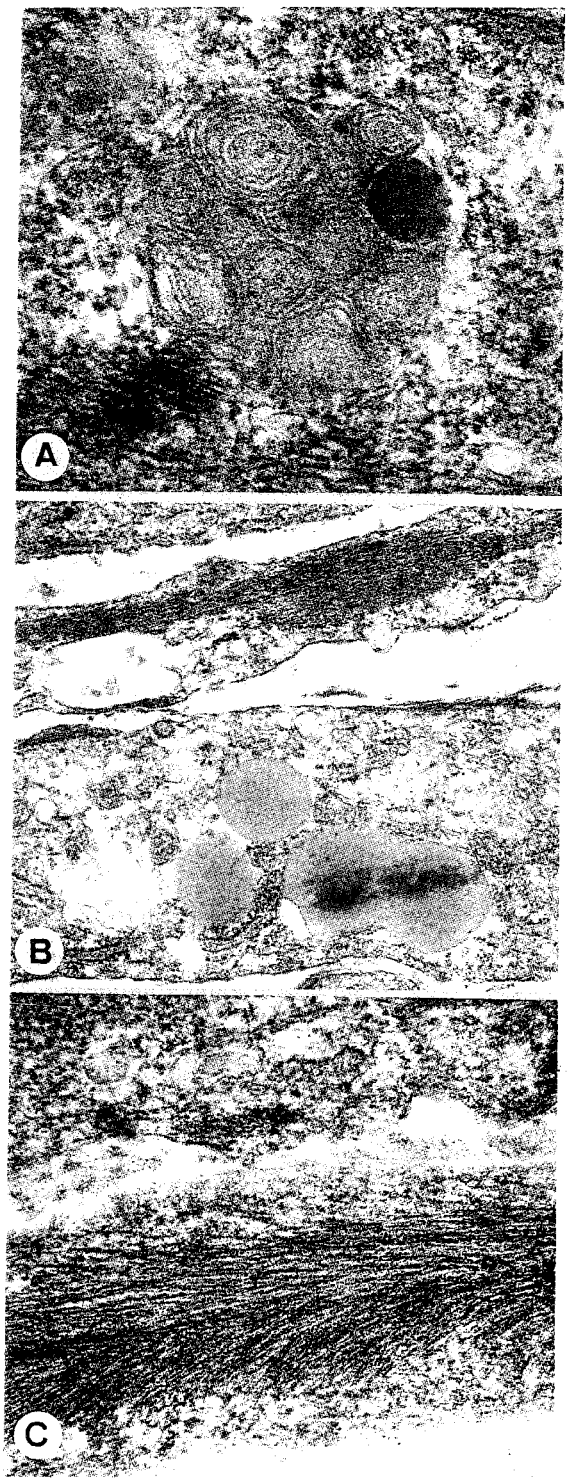

Fig. 4 Transmission electron microscope of the seventh passage BGRPE. (A).The Cells retained melansome granules but most of the granules were lack pigmentation, x 60k. Cells were also characteristic in (B). scattered lipid granules, x 20k, and (C). bunch of microfilament, x 40k.

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鑲點石斑視網膜色素細胞之初級培養

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鑲點石斑(*Epinephelus amblycephalus* BLEEKER) 的視網膜色素細胞可以被分離，並成功的培養在每升添加 3 克氯化鈉的31℃、Leibovitz's L15 培養基中。結果顯示其初級培養的成敗和鑲點石斑的年齡有關。

在其初級培養的細胞中可發現鑲崁狀、灌木狀及神經纖維狀等三種細胞。然而繼代三次後，雙核灌木狀及神經纖維狀細胞會因無法生長而消失。而六角到卵形的單核鑲崁狀細胞則可繼續培養，但其色素顆粒會隨細胞的繼代而減少。電子顯微鏡觀察發現其第七代細胞的色素顆粒仍在，但多數顆粒已不含黑色素。

本文爲便利於視網膜色素細胞層的分離，在進行初級細胞培養前也探討鑲點石斑視網膜的組織結構。