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Primary cell culture from embryos of the Japanese scallop Mizuchopecten yessoensis (Bivalvia)

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Abstract

Primary cell cultures obtained from embryos of Mizuchopecten yessoensis (Bivalvia) survived for four months. Although the number of cells progressively decreased during the cultivation, mitotic cells were observed both at the first stages and at the end. A possibility of growing marine invertebrates cells in long term primary culture is discussed.

Introduction

The problem of initiation of a cell line from marine invertebrates has a three-decade history (Flandre, 1971; Maramorosch, 1976). All efforts to decide the problem have not met with success, although cultures of vialble molluscan cells have been maintained for different time intervals (Tripp, 1963; Perkins and Menzel, 1964; Li *et al.*, 1966). Absence of continuous cell lines from marine molluscs, and of reliable methods for maintaining cells *in vitro* for a long time, makes it difficult to study marine molluscan diseases, nutrition and biochemistry.

Brewster and Nicholson (1979) report that among different tissues tested from marine molluscs larval ones are the most promising for establishment of continuous cell lines. They obtained the first primary cell culture from marine mollusc embryos. However, no mitotic cells were observed, although these authors describe rapid increase of cell numbers in such cultures in a relatively short time (1–7 d). Their experiments encountered considerable problems with contamination of embryos with protozoa, bacteria and fungi, which caused quick loss (less than 2 weeks) of most cultures.

This paper reports a possibility of long-term (up to 4 months) cultivation of marine invertebrate embryonic cells in primary culture. No visual increase in the cell number have been observed, but mitotic cells were observed.

Materials and methods

For experiments, the scallops (Mizuchopecten yessoensis) were collected in Vostok-Bay of the Japan Sea in May 1988 and May 1989.

Scallop larvae culture

Embryonic material for primary cell cultures was

obtained after artificial egg fertilization of the scallop. The shell surfaces were disinfected with 70% ethanol, and the animals (males and females separately) were placed in 14–15°C ultraviolet-sterilized sea water. The molluscan spawning was induced by injecting 0.5–1.0 ml of 10^{-3} serotonin creatinsulfate into gonad or adductor muscle (Varaksin and Naidenko, 1989). A sperm suspension from a few males was added to eggs (2–3 spermatozoa per one egg to avoid polyspermy) under microscopic control. The fertilized eggs were washed to remove spermatozoa and cultivated in closed tanks with sterile sea water, which was stirred and constantly aerated with sterile air. Twenty hours after fertilization, larvae of the swimming-blastula stage were transferred into fresh sea water. Developed trochophore larvae were collected on a fine 30 μm mesh nylon screen, rinsed in sterile artificial Ca^{+2}-and Mg^{+2}-free sea water and concentrated to a small volume (0.5–1 ml) by centrifugation (1000 × g for 10 min, 5°C).

Cell culture

Larvae were treated in 0.125% collagenase (product of the Pacific Ocean Institute of Bioorganic Chemistry) and dissociated completely for a period of 1.0–1.5 h at 10–12°C. The resulting cell suspension, which consisted of all types of larval cells, was washed twice in sea water with penicillin G (500 U/ml) and gentamicin (40 μg/ml) by centrifugation (1000 × g for 5 min), and the pellet was resuspended in growth medium. The medium was standard Leibovitz medium L-15 (Flow), containing high concentration of amino acids as well as hemolymph of marine molluscs (Awapara, 1962), with the following modifications: medium osmolarity as equilibrated to that of scallop hemolymph (1100 mOsmol) with NaCl (18.05 g/l), KCl (0.29 g/l), $CaCl_2 2H_2O$ (1.205 g/l), $MgCl_2 6H_2O$ (5.481 g/l), $MgSO_4 7H_2O$ (4.28 g/l). Other ingredients added were taurine (25 mg/l), glucosamine (50 mg/l), glutamine (100 mg/l), embryo bovine extract (2%) and gentamicin (40 mg/l). Taurine is a specific amino acid of scallop hemolymph (Lange, 1963; Smith and Pierce, 1987) which is important for osmotic regulation. The pH was then adjusted to 7.6–7.8.

Cell suspension (0.5–1.0 × 10^6 cells/ml) was dispensed into culture vessels and Leigton tubes. Two different kinds of culture vessels were used: collagen-coated vessels and non-treated ones. The vessels were incubated with collagen solution (1 mg/ml, Serva) during 3–4 h at 15–20°C and then washed with sea water three times. All vessels and tubes with cell suspension were incubated at 15°C. The medium was changed after an initial 24-h period of growth by centrifugation (1000 × g, 5 min) and the pellet was resuspended in fresh medium.

Cytological analysis

Cell viability was microscopically examined after staining with 0.01% trypan blue or acridine orange (10^{-7}M) or simultaneous staining with fluorescein diacetate and ethidium bromide (Jones and Senett, 1985) at different stages of cultivation (0,1,3,7,14,60,120 days). Cells were fixed in suspension or on coverslips in the solution of Carnoy or 2.5% glutaraldehyde in sea water at 10°C. The preparations were stained by hematoxylin-eosin or nuclei dye Hoechst-33258 (0.1 μg/ml).

Incorporation of (H^3)-uridin

25 μl (10 μCi) of (H^3)-urdine diluted in the culture medium, was added to each 1-ml aliquot of cell suspension. After incubation during 10 h at 15°C, the content from wells was deposited on microfilters (pore size 0.2μm). The filters were washed with 10 ml trichloratic acid (10%), followed by ethanol, ether and placed in vials with 5 ml standard toluol scintillant. The radioctivity was measured on liqid scintillation counter SL-30 (Intertechnique).

Results

We reared cultures of the scallop throchophore

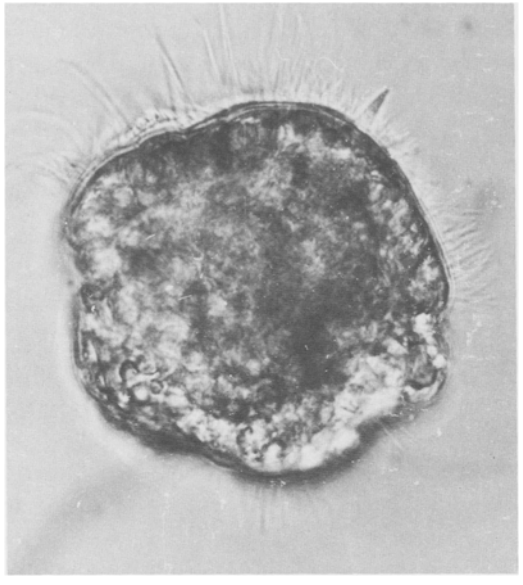

Fig. 1. Trochophore stage of scallop larva culture, × 300.

larvae (Fig. 1) with a concentration of 15–20 larvae/ml. The larvae at this stage were treated by collagenase and 5–6 million cells (per 10000 embryos) were obtained. Cell viability after initiation of culture was 70–80% as estimated by staining with trypan blue.

The cells did not attach to the surface of non-treated vessels but remained suspended and all died after two weeks of cultivation. If the surface of flasks was treated by collagen, a majority of the cells attached and were viable for a long period but did not spread and maintained a spheric form (Fig. 2). They easily detached when the medium was changed but preserved their ability for repeated attachment to collagen-coated vessels. The following cell observations were conducted on the collagen-treated flasks.

After seeding the cells quickly formed aggregates of 10–20 cells (Fig. 3) and within 24 h a majority (nearly 70%) of cells were aggregated. Many clusters of moving ciliated cells could be observed after one week of culture, but no moving cells existed after 2 weeks of cultivation.

Coverslip cultures from Leighton tubes were vitally stained with acridine orange and examined during cultivation. As shown in Fig. 4, the cells had distinctly visible roundish nuclei and uniformly stained cytoplasm. Then coverslip cultures were fixed and stained by Caracci hematoxylin, and mitotic cells were found. The cells in

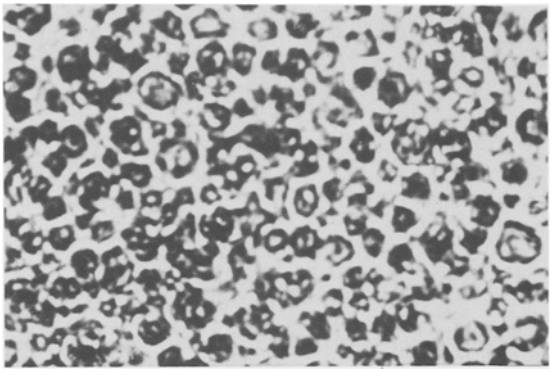

Fig. 2. Embryonic cells in scallop culture with a collagen matrix, × 400.

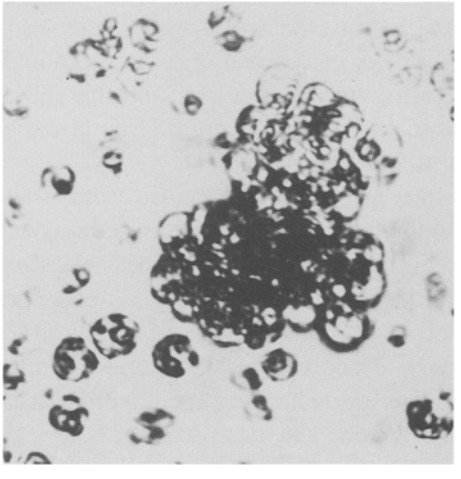

Fig. 3. Aggregated cells in a culture from scallop larva, × 340.

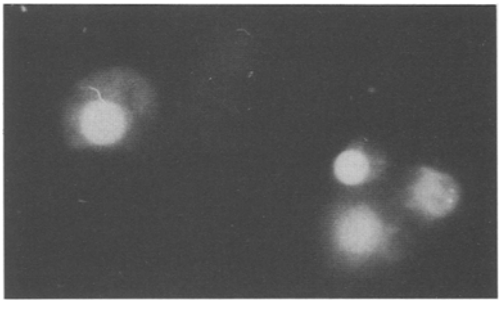

Fig. 4. Cells from an embryonic culture. Acridine Orange staining, × 490.

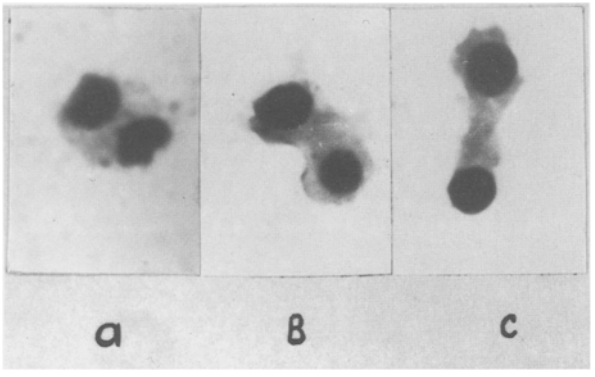

Fig. 5. Mitotic cells in an embryonic culture after 4 months in vitro. Hematoxylin-eosin staining, × 800: a) late anaphase stage; b) and c) telophase stage.

telophase were most clearly visible (Fig. 5). Few (0.2–0.3%) mitotic figures were observed both at the first days of cultivation and at the end of it. Our efforts to accumulate mitotic cells using colcemid (0.1 μg/ml, during 24 h) in the growth medium was not successful.

Primary cell cultures obtained from embryos in this study lasted for four months, although cell degeneration proceeded. This process was displayed in the constant decrease of cell number, perhaps, because of cell destruction, especially in the first few days. However, simultaneous staining with fluorescein diacetate and ethidium bromide showed 60% cell viability at the end of cultivation. Viable cells incorporated 4–6 times more (H^3)-uridine than the control digitonin-killed cells.

Since the number of cultured cells was limited because of the absence of active proliferation and the necessity for periodic staining of fixed cells, after 120 days all the cell population was expended in our experiments. Unfortunately, we failed to observe visual increase in the cell number and therefore could not estimate cell Kinetics.

We also obtained incorporation of (H^3)-uridine in some experiments with tissues of adult scallops. However the viability of these cells fell to 60% during the first days of cultivation and the process of cell degeneration occurred very quickly.

Discussion

Our results confirm the data of other authors about the possibility of long term cultivation of invertebrate cells and present the first example of the long term primary cell culture from scallop larvae. The main difficulties we met during the process of larvae cell line establishment were connected with handling of the molluscan embryos in aseptic conditions in considerable volumes.

We think that the attachment of molluscan embryo cells to the collagen coating helped the culture survive for the long period of time and to yield mitotic cells. The adhesion reactions of molluscan cells must be investigated in further research.

The low mitotic rate (<5 mitotic figures/1000) observed in our investigations was also shown for the white body cells in Octopus vulgaris cultured *in vitro* (Necco and Martin, 1963); for preparations of the intestinal epithelium of the adult scallop M.yessoensis (Uscheva and Leibson, 1988); and even in actively growing tissues such as regenerating mantle epithelium of the oyster Crassostrea virginica (Hillman, 1963). Ellis (Ellis *et al.*, 1985) reported a low mitotic index in cell cultures of oyster heart and mantle (doubling time of population was 10–14 days) and cell degeneration after the third passage.

Our failure to accumulate mitotic cells using colcemid may be explained by the long development cycle of marine invertebrate cells in culture (to 10–14 days) as compared with that for mammals (20–36 h)(Thomas, 1977).

The cells were seen in telophase on fixed preparations. Cecil (1969) observed different mitotic stages in 14-day-long cell culture from Spisula heart, but no-one else has described different mitotic stages in molluscan cell culture. We suppose that some specific characteristics of mollusc embryo cells may prevent their identification at the first mitotic stages.

The discovery of mitotic cells in embryonic cultures after long time (up to 4 months) cultivation allowed us to claim higher proliferation potential in cultures of embryonic cells compared to cells of adult molluscs. Possibly Hayflick's limit (Hayflick, 1965) for embryonic cells may be several times higher than 3–4 passages, as Ellis (1985) assumed for adult molluscan cells.

However, we observed no growth of cell numbers in primary embryonic cultures. The use of antibiotics and frequent washings of cells with fresh medium although reducing the risk of contamination simultaneously can badly effect the cell's physiological state and, possibly, their ability for proliferation. Since cell degeneration, even in embryonic cultures, predominates over proliferation it is obvious that future research is needed to establish the optimal specific supporting growth factors in the growth medium and conditions of cell state stabilization.

Due to the numerous data on low mitotic activity in tissues of marine invertebrates the observation of Brewster and Nicholson (1979) about a rapid increase in cell number in embryo cell cultures of marine molluscs seems to be at variance. Moreover, they presented no data on proliferation rates and noted the absence of mitotic figures. We believe that their results on rapid increase in cell numbers may be incorrect due to: the difficulties in visual counting of the cell number; the presence of aggregates in the cell culture; and the increase of sizes of these aggregates during cultivation. These authors also observed the presence of large 'balls' of cell attached to the vessel surface, as we did. There were no single cells or cell monolayers in their culture.

Molluscs only spawn once a year and for a short period only which did not allow us to repeat experiments many times. Future research will show if it is possible to obtain cultures of dividing molluscan cells.

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References

Awapara I (1962) Free amino acids in Invertebrates: a comparative study of their distribution and metabolism. Amino Acid Pools. (pp. 158–175). Elsevier, New York.

Brewster F and Nicholson BL (1979) In vitro maintenance of amoebocytes from the American oyster (Crassostrea virginica). J. of the Fisheries Research Board of Canada 36: 441–476.

Cecil IT (1969) Mitoses in cell cultures from cardiac tissue of the surf clam Spisula solidissima. J. Invertebr. Pathol. 14: 407–410.

Ellis LL, Brodey MM and Bishop SH (1985) Preparation of cell culture from Oyster Heart and Mantle. In vitro 3, pt.2: 32A.

Flandre O (1971) The culture of molluscan cells. In: Invertebrate Tissue Culture 1 (pp. 361–383) Acad. Press, New York.

Hillman RE (1963) An observation of the occurence of mitosis in regenerating mantle epithelium of the eastern oyster, Crassostrea virginica. Chesapeake Sci. 4: 172–174.

Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. 37: 614–636.

Jones KU and Senett A (1985) An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. J. Histochemistry and Cytochemistry 33: 77–79.

Lange R (1963) The osmotic function of amino acids and taurine in the mussel Mytilus edulis. Comp. Biochem. Physiol. 10: 133–179.

Li MF, Stewart JE and Drinnan RE (1966) In Vitro Cultivation of cells of the Oyster, Crassostrea virginica. J. of the Fisheries Research Board of Canada. 23: 595–599.

Maramorosch K (1976) Invertebrate tissue culture: Research Applications. Acad.Press. New York.

Necco A and Martin R (1963) Behavier and estimation of the mitotic activity of the white body cells in Octopus vulgaris, cultured in vitro. Exp. Cell Res. 30: 588–623.

Perkins FO and Menzel RW (1964) Mainteance of Oyster cells in vitro. Nature 12: 1106–1107.

Smith LH Jr and Pierce SK (1987) Cell volume regulation by molluscan erytrocytes during hypoosmotic stress: Ca^{+2} effects on ionic and organic osmolyte effluxes. Biol. Bull. 2: 407–474.

Thomas JF (1977) Effects on cell proliferation of metabolites produced by cultured cells. Biochem. Soc. Trans. 5: 1801–1808.

Tripp MR (1963) Cellular responces of molluscs. Ann. N.Y. Acad. Sci. 113: 467–474.

Uscheva LN and Leibson NL (1988) Mitotic cycle of cell of the intestinal epithelium from scallop Mizuchopecten yessoensis. Cytology XXX: 554–558.

Varaksin AA and Naidenko TH (1989) Comparative characteristic of different methods of stimulation of spawning of Japanese scallop. Biologiya morya 2: 66–69.

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