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Tissue culture of the deep-sea eel *Simenchelys parasiticus* collected at 1,162 m

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Abstract We successfully cultivated fin cells of the deep-sea eel *Simenchelys parasiticus* (collected at 1,162 m) in L-15 medium supplemented with fetal bovine serum (FBS) and additional NaCl. We found that the pectoral fin cells proliferated in L-15 medium enriched with 4 g/l of NaCl salt (pH 7.3) containing 10% FBS at 10 °C and 15 °C. No cells were attached to the plastic culture plates when Dulbecco's modified Eagle's medium (pH 7.8) or 0–2 g/l of NaCl was added to the medium or when incubation was carried out at 4 °C. The majority of the explant outgrowth cells were detached when temperature increased to higher than 15 °C. The rate of proliferation of the fin cells was extremely slow and was dependent on the FBS concentration. Cell growth was enhanced by approximately 2.2-fold, and doubling time decreased from 170 h to 77 h when the FBS concentration was increased from 10% to 20% (v/v). Our established deep-sea eel cells were passaged 16 times over a 1-year period under atmospheric pressure conditions.

Keywords Deep-sea organisms · Primary tissue culture · Proliferation rate · *Simenchelys parasiticus* · Subculture

Introduction

Since the establishment of a variety of mammalian cell lines, great interest has been focused on the molecular and cellular biological properties of mammals. Although increasing attention has been paid recently to deep-sea multicellular organisms, no one has successfully established a cell line from them. Primary tissue culture of deep-sea multicellular organisms would offer unusual opportunities to contribute to a wide range of biotechnological fields. In the present study, we attempted to develop a tissue-culture method under atmospheric pressure conditions for the deep-sea eel *Simenchelys parasiticus* (Masuda et al. 1984) collected at a depth of 1,162 m.

Establishment of tissue cultures derived from deep-sea multicellular organisms has been difficult because of the serious damage they sustain upon decompression and exposure to high temperatures. Therefore, almost all deep-sea multicellular organisms die within half a day. Even if deep-sea creatures can be kept alive for a short period, contamination by microorganisms hinders their primary culture. Nevertheless, we maintained a primary culture of the deep-sea clam *Calyptogena soyoae* collected at depths of 1,180, 1,148, and 1,386 m, although only for a short time (Koyama and Aizawa 2000). Several researchers have attempted primary cell cultures from shallower marine clams, although they also were maintained only for a short period (Li and Stewart 1966; Cecil 1969; Odintsova and Khomenko 1991; Takeuchi et al. 1999). In contrast to marine clams, attempts to culture a variety of shallower marine fish cells have been successful over long periods (Clem et al. 1961; reviewed in Wolf and Quinby 1969).

In our previous study, we developed a novel pressure-retaining suction capture device and a pressure-stat aquarium system, both of which were specifically designed for capturing and maintaining deep-sea organisms (Koyama et al. 2002). After slow decompression using those systems, the deep-sea eel *S. parasiticus* was kept alive under atmospheric pressure for 2–5 days. In this study, the pectoral fin cells of *S. parasiticus* collected at 1,162 m were cultured in a salt-enriched medium containing fetal bovine serum (FBS) under atmospheric pressure.

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Materials and methods

Animals

Eight *Simenchelys parasiticus* specimens (Fig. 1; Masuda et al. 1984) were collected by the submersible *Shinkai 2000* (dive no. 1327, 5 December 2001, 1,162 m, 35°00.08′N, 139°13.47′E) using a pressure-retaining suction capture device and a pressure-stat aquarium system (Koyama et al. 2002). The maximum and minimum lengths of the eels were 34.0 cm and 14.3 cm, respectively. To avoid decompression injury of the captured deep-sea eels, the decompression speed of the pressure-stat system was set at 0.6 MPa/h and the inner pressure in the aquarium was slowly decreased to atmospheric pressure over approximately 15 h. After decompression, the eels were transferred and exposed to 20 mg/l of the abrasion agent Elbaziu (Ueno Fine Chemical Industry, Osaka, Japan) in 40 g/l of artificial seawater (Tetra Marine Salt, Tetra, Tokyo, Japan) at 4 °C for 24 h. Five of the deep-sea eels were kept alive for 2 days and the remaining three eels for 5 days at 4 °C under atmospheric pressure conditions.

Media

Dulbecco's modified Eagle's medium (DMEM; ICN Biomedicals, Aurora, Ohio, USA) and L-15 medium (ICN Biomedicals) were dissolved in 0, 2, or 4 g/l of NaCl-containing water. Then a 1% (v/v) antibiotic solution, consisting of penicillin (5,000 units/ml), streptomycin (5,000 μg/ml) (Bio Whittaker, Walkersville, Md., USA), and 10% (v/v) FBS (ICN Biomedicals), was added to each medium. DMEM and L-15 medium were adjusted to pH 7.8 and 7.3, respectively.

Primary culture of *S. parasiticus*

To cultivate cells from *S. parasiticus*, the pectoral fin tissue was removed using surgical scissors and transferred to artificial seawater at a temperature of 4 °C containing a mixture of penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), and fungizone (2.5 μg/ml) (Bio Whittaker). After a 15-min exposure, the pectoral fin tissue was rinsed twice with cold (4 °C) artificial seawater containing 1% antibiotics, transferred to a Petri dish, and minced into pieces of approximately 1 mm^3. The minced pectoral fin tissue fragments were transferred into either trypsin/EDTA 0.25 mg/ml (Bio Whittaker) or collagenase-dispase solution consisting of collagenase 0.5 mg/ml (Wako, Tokyo, Japan) and 10% (v/v) dispase (Collaborative Biomedical Products, Bedford, UK) in phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+}. Each transferred pectoral fin preparation was incubated for 20 min at 20 °C. After incubation, the enzyme solutions were removed by centrifugation at approximately 500 *g* for 2 min, and the minced fin tissue was resuspended in 3 ml of medium. The tissue fragments were placed in 25-cm^2 tissue culture flasks (Falcon, Franklin Lakes, N.J., USA) with about 4–6 fragments in each flask. The media in the flasks were removed by aspiration and 1 ml of fresh medium/25 cm^2 growth surface was added. The flasks were tilted gently to spread the pieces evenly over the growth surface and placed in an incubator for 10–20 days at various temperatures. The culture media were changed 1–3 times weekly.

When the explant culture of fin cells ceased to spread, the minced tissues were trypsinized for 3 min at 20 °C, transferred to the collagenase-dispase solution, and further incubated overnight at 4 °C. The disaggregated fin cells were washed by centrifugation at approximately 500 *g* for 2 min and resuspended in L-15 medium containing 4 g/l of NaCl. Following these procedures, the fin cells were cultured at 15 °C.

Doubling time of *S. parasiticus* fin cells

The subcultured fin cells were placed in new culture flasks with trypsinization for 3 min at 20 °C. After overnight incubation at 15 °C, the numbers of fin cells attached to the growth surface were counted in ten random areas (each 1 mm^2). Statistical analysis was performed using Student's *t*-test. The calculations were performed using Microsoft Excel software.

Results

Explant culture of *Simenchelys parasiticus* fin cells

We investigated explant tissue culture techniques because the fin tissue fragments did not disaggregate by overnight enzymatic treatment at 4 °C. Minced pectoral fin tissue fragments (about 1 mm^3) were digested with either trypsin/EDTA or collagenase-dispase for 20 min at 20 °C. The tissue fragments were plated in 25-cm^2 culture flasks in salt-enriched DMEM (pH 7.8) and L-15 medium (pH 7.3) at 4 °C, 10 °C, 5 °C, and 20 °C under atmospheric pressure. Additional sodium chloride (0, 2, and 4 g/l) was added to the medium. The pectoral fin cells showed explant outgrowth from collagenase-dispase-digested fragments in L-15 medium enriched with 4 g/l of NaCl at 10 °C and 15 °C (Fig. 2). No cells attached to the plastic plates in DMEM (pH 7.8) with the addition of 0–2 g/l of NaCl or when incubated at 4 °C. When the temperature was elevated over 15 °C, most of the explant outgrowth cells detached. Moreover, explant culture did not proceed when the enzymatic treatment was omitted, because dead cells at the cutting site were thought to block the explant outgrowth.

When keratocytes from the deep-sea epidermis began to deteriorate and detached from the plastic plates after 7–11 days of culture, the tissue fragments were further treated with enzymatic digestion. Enzymatic

Fig. 1 A deep-sea eel *Simenchelys parasiticus* specimen collected at a depth of 1,162 m. The left side of the pectoral fin was removed with surgical scissors

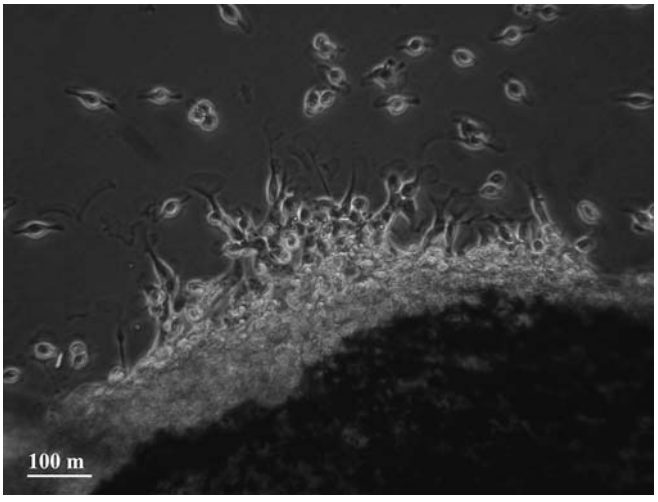

Fig. 2 Explant outgrowth of deep-sea eel pectoral fin cells. Primary explant culture in L-15 medium (pH 7.3) enriched with 4 g/l of NaCl and containing 10% FBS under atmospheric conditions at 15 °C

disaggregation of the tissue fragments succeeded after the keratocytes detached. After the procedure, we obtained homogenous fibroblast-like cells through successful cell passage.

Proliferation of fibroblast-like *S. parasiticus* fin cells

We measured the doubling time of the fibroblast-like fin cells because a minority of the cells divided by mitosis (Fig. 3). Figure 4 shows the rate of cell proliferation. The rate reached statistical significance ($P < 0.05$) at 240 h of cultivation compared with baseline (0 h) (Fig. 4A). Based on the results shown in Fig. 4A, the doubling time of the fibroblast-like fin cells was 170 h. When the FBS concentration increased from 10% to 20% (v/v) in the medium, the cell proliferation rate also increased by approximately 2.2-fold (Fig. 4B). FBS contained growth factor(s) of the fibroblast-like fin cells and therefore enhanced the speed of cell growth with a doubling time of 77 h. Thus, we succeeded in cultivating trypsin-dispersed fin cells of the deep-sea eel *S. parasiticus* in L-15 medium supplemented with FBS and NaCl. Using 20% (v/v) FBS-containing medium, our established deep-sea eel cells were passaged 16 times over a 1-year period.

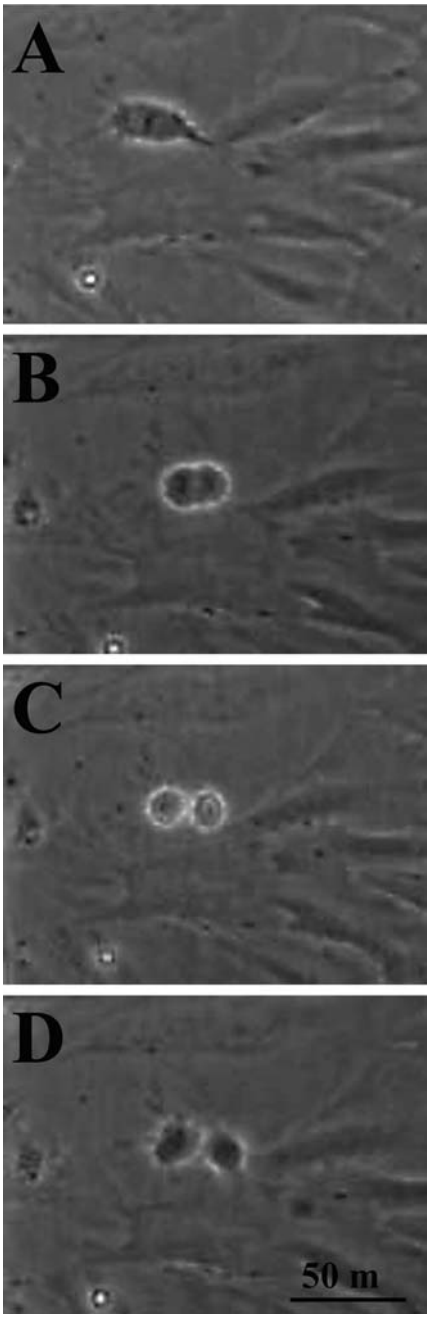

Fig. 3A–D Mitotic fibroblast-like deep-sea eel fin cell. **A** 0 min, **B** 26 min, **C** 38 min, **D** 51 min

Discussion

In the present study, we demonstrated that pectoral fin cells derived from a deep-sea eel proliferated and could be subcultured under atmospheric pressure conditions. The established deep-sea eel cells were passaged 16 times over the course of 1 year. Our primary culture techniques using both enzymatic disaggregation and explant culture could be widely applied in the primary culture of marine vertebrates. Using the same culture technique, we also succeeded in proliferating and freezing stocks of fin cells from the epipelagic eel *Conger myriaster* (data not shown). In a future study, we will attempt to develop freezing preservation methods for deep-sea eel cells.

The primary culture of marine vertebrates has been investigated in animal serum- or sera-containing medium (Clem et al. 1961; reviewed in Wolf and Quimby 1969). However, the culture of marine vertebrate cell cultures has been severely hindered by contamination with microorganisms. To avoid the contaminating

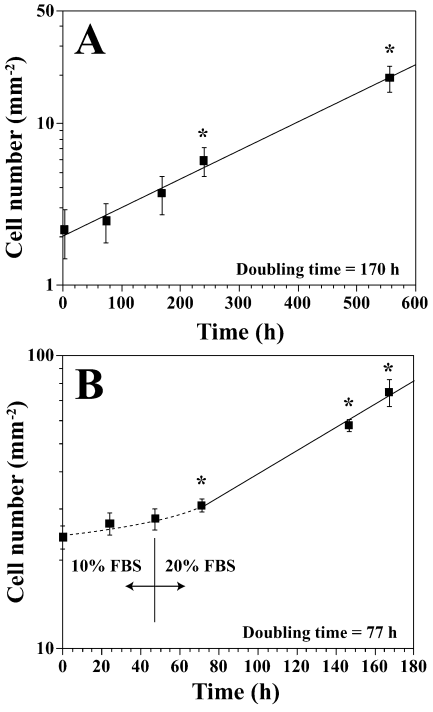

Fig. 4A, B Proliferation of fibroblast-like deep-sea eel fin cells. The number of cells was counted in 10 random 1-mm^2 areas on the growth surface. Values shown are expressed as mean ± SEM ($n = 10$; $*P < 0.05$ compared with time 0 h by Student's t-test). **A** The cells cultured in L-15 medium (pH 7.3) enriched with 4 g/l of NaCl and containing 10% (v/v) FBS under atmospheric conditions at 15 °C. **B** The proliferation rate was dependent on the FBS concentration in the medium

microorganisms, we immersed the deep-sea eels in an abrasion agent (Elbaziu) containing sodium nifurstyrenete for 24 h. When the deep-sea eel cells were not treated with the agent, antibiotic-resistant microorganisms hampered the tissue culture. The fin tissue fragments were disaggregated by enzymatic treatment only after the keratocytes had detached from the plastic plate. The digestion enzymes may penetrate into the tissue fragments when the inner cells are removed due to explant outgrowth.

The doubling time of deep-sea eel cells depended on the FBS concentration in medium and was increased by approximately 2.2-fold when the FBS concentration was increased to 20% (v/v) in the medium; additionally, the cell density increased (Fig. 4). Although the proliferation rate increased with a doubling time of 77 h, the division time (M phase) of the mitotic cells was about only 1 h (Fig. 3). The cells might require higher pressure and/or further additional growth factor(s) for faster growth.

Based on the results of previous studies of deep-sea microorganisms (reviewed in Abe and Horikoshi 2001 and Kato and Bartlett 1997), deep-sea vertebrates should also have piezophilic and piezo-tolerant cell types. Piezophilic cells are defined as those that grow more rapidly under increased pressure than under atmospheric conditions, while piezo-tolerant cells grow at both atmospheric and high pressures. If these cell types occur, they would contain a variety of useful proteins. In future research, we will attempt to determine whether these specific cell types exist.

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