Primary Culture of Mesodermal and Endodermal Cells of the Starfish Embryo

Authors: Kaneko, Hiroyuki, Kawahara, Yukio, and Dan-Sohkawa, Marina

Source: Zoological Science, 12(5) : 551-558

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.551

ZOOLOGICAL SCIENCE 12: 551–558 (1995)

Primary Culture of Mesodermal and Endodermal Cells of the Starfish Embryo

HIROYUKI KANEKO, YUKIO KAWAHARA and MARINA DAN-SOHKAWA

Department of Biology, Faculty of Science, Osaka City University, Sugimoto-cho, Sumiyoshi-ku, Osaka 558, Japan

ABSTRACT—A method is described for culturing embryonic cells of the starfish *Asterias amurensis*. A network composed purely of mesenchyme cells can be obtained by treating dissociated cells of embryos at the mesenchyme migration stage with 0.6 M glycine in half strength Ca^{2+}, Mg^{2+} free Jamarin (artificial sea water) for 24 hr and subsequently culturing them for 24 hr. When the treatment period is shortened to 12 hr, aggregates of epithelial cells come to coexist with the network. After 3 days of culture, these aggregates form monolayer sheets constituted of four types of cells which can be identified morphologically and by 8-anilino-1-naphthalenesulfonic acid (1, 8-ANS) stainability. These cell types correspond to cells composing the esophagus, the stomach, the intestine and the coelomic pouch. No ectodermal cells were found in the sheet. These results suggest that there is an order of resistibility to 0.6 M glycine treatment among cells of the three germ layers, i. e. mesoderm, endoderm and ectoderm in descending order.

INTRODUCTION

Primary culture of cells is a valid method for studying differentiation and functions of an embryonic cell by reducing its complex *in vivo* environment to a simpler *in vitro* one. In contrast to the success in culturing vertebrate cells, efforts to culture invertebrate cells have not been as rewarding. In line with such efforts, we recently placed in culture an archenteron complex of the starfish embryo consisting of the archenteron, the extracellular matrix (ECM) surrounding it, and the mesenchyme cells scattered in the ECM (cf. Fig. 3f) [14]. In this "organ" culture, the mesenchyme cells formed a dynamic network on the dish surface which was found to be acellular in nature [14]. In order to analyze further the nature and the molecular mechanism of the functions of the mesenchyme cells, we decided to establish a method for culturing a pure population of this cell type.

In the sea urchin, Okazaki [18] showed that the micromeres isolated from the 16-cell stage embryo differentiate *in vitro* into primary mesenchyme cells. These cells, in turn, form spicules bearing the shape characteristic to the species from which they are derived. Various methods for isolating and culturing the primary mesenchyme cells of the sea urchin have since been proposed by investigators [9, 12, 15, 20]. One of these methods depends on the binding property of the primary mesenchyme cells to wheat germ agglutinin (WGA) [9]. When cells dissociated from intact embryos were inoculated onto the surface of a WGA-coated dish, only the primary mesenchyme cells became attached. Application of this method to starfish embryonic cells was unsuccessful (unpublished data). Coating of the dish surface with various kinds of ECM proteins, such as collagen, fibronectin, laminin and uncoated surface also proved unsuccessful, probably owing to a strong aggregating tendency of the cells (unpublished data). This tendency is considered to arise from the strong reconstructing ability of the starfish embryonic cells including both epithelial and mesenchymal types [5, 6, 8]. In order to prevent the cells from aggregating, we searched for a condition to keep them in a dissociated state for a prolonged period of time.

In this study, cells dissociated from starfish embryos at the mesenchyme migration stage were treated with a glycine solution for 24 hr, after which they were transferred to a culture medium. After 24 hr in culture, all epithelial cells died but the mesenchyme cells survived. When the treatment period was shortened to 12 hr, small surviving aggregates were found spread out in a monolayer over the dish surface by the third day of culture. These cells were identified as being endodermal in nature by comparison with the cells constituting intact archenterons kept in culture for 24 hr.

Accepted June 9, 1995
Received March 20, 1995

MATERIALS AND METHODS

Embryos

Mature eggs of the starfish *Asterias amurensis* were obtained after Kanatani [13], by treating freshly isolated ovaries with 10^{-6} M 1-methyladenine (1-MA) in sea water for 15 min. Eggs were inseminated within about 1 hr after the beginning of this treatment.

Spermatozoa were obtained by making several cuts in a testis on a dry Petri dish. They were kept in a refrigerator and used within 2 days.

Fertilized eggs were allowed to develop in artificial sea water (Jamarin U; Jamarin Laboratory, Osaka) at 18°C. The nomenclature of the developmental stages of the starfish was described previously [7]. The embryos were anesthesized with a small amount of 4% paraformaldehyde [14] and photographed under a light microscope (Olympus BH).

Solutions

Millipore-filtered sea water (MSW): natural or artificial sea

water, Jamarin U, which is run through a Millipore filter (pore size 0.45 μm or 0.22 μm), then combined with penicillin G potassium (50 units / ml) (Meiji Seika Kaisha, Ltd., Tokyo) and streptomycin sulfate (50 μg / ml) (Meiji Seika Kaisha, Ltd., Tokyo).

Dissociation medium (DM) (pH 6.6): 1.2 M glycine in distilled water supplemented with 1% (v/v) MSW and 6% newborn bovine serum (NBS; M. A. Bioproducts, Maryland).

Treatment medium (TM) (pH 6.7): 0.6 M glycine in half strength Ca^{2+}, Mg^{2+} free Jamarin U.

Culture medium (CM) (pH 8.1): MSW containing 4% (v/v) NBS.

Dissociation of embryos and further treatment of dissociated cells

Embryos were dissociated as described elsewhere [5, 6]. The dissociated cells were treated as follows if not otherwise mentioned. Packed embryos (0.2 ml) at the mesenchyme migration stage (about 28–30 hr after fertilization) were collected using a hand centrifuge and washed three times with 10 ml of MSW. Embryos were suspended in 4 ml of DM and left standing for 5 min, after which they were submitted to 10 strokes of pipetting by a Pasteur pipette. The obtained suspension of the dissociated cells was hand-centrifuged to separate undissociated bits of embryos from single cells. The supernatant containing the dissociated single cells was set aside in a separate centrifuge tube while the pellet was dissociated again in the same manner. The cell suspension obtained from the series of the dissociation procedure (a total of 8 ml) was centrifuged at $250\times g$ (1100 rpm, Hitachi 05PR-2) for 5 min, which yielded 6.0 to 8.0×10^7 cells. The pellet was resuspended in 15 ml of TM, transferred to a Petri dish (6 cm in diameter) and incubated at 15°C for various intervals between 12 to 48 hr. After incubation, surviving cells were collected by centrifugation at $250\times g$ (1100 rpm) for 5 min and were cultured for 24 hr at 18°C in a smaller Petri dish (4 cm in diameter) containing 4 ml of CM (post-treatment culture). Photographs of the cells in each process were taken with a phase-contrast microscope (Nikon TMD).

Cell counting

The population of the isolated mesenchyme cells was determined as follows. After washing the culture with MSW, the cells were detached from the dish surface by incubation with 0.1% (w/v) trypsin (from: porcine pancreas; Sigma) in MSW for 10 min at room temparature. Aliquots of the detached cell suspension were counted with a haemocytometer under the phase-contrast microscope.

Isolation of the archenteron complex

Archenteron complexes (see Introduction) were isolated as described previously [14]. In brief, 0.4 ml of the embryos collected at the mouth formation stage was washed three times with 10 ml of MSW each time. The embryos were suspended in 10 ml of the DM and left standing for 5 min. Next, the DM was exchanged for 4 ml of fresh DM, in which embryos were submitted to about 10 gentle strokes of pipetting with a broad-mouthed pipette (Komagome's pipette; tip inside diameter 1.5–2 mm) to remove the ectoderm. Isolated archenterons, still enwrapped in a large portion of the extracellular matrix, were collected by hand centrifugation, washed two to three times with MSW, inoculated in a Petri dish containing CM and cultured at 18°C for 24 hr. Photographs were taken with the phase-contrast microscope and a light microscope equipped with a fluorescence apparatus (Olympus BH-RFL).

Staining of stomach and intestinal cells in culture with 1, 8-ANS

A fluorescent dye, 8-anilino-1-naphthalenesulfonic acid (1, 8-ANS; Nakarai Chemicals, Kyoto), which is known to stain the stomach cells and part of the intestinal cells at the bipinnaria stage but not to stain any cells of the archenteron at the mesenchyme migration stage [5], was dissolved in ethanol at 10^{-2} M and diluted 100-fold with MSW. Cells cultured on a cover slip, lying at the bottom of the culture dish, were treated with this solution for 20 min at room temperature. The cover slip was set on a slide glass. The cells were observed and photographed under the fluorescence microscope.

RESULTS

Behavior and fate of the dissociated cells during glycine treatment through post-treatment culture

As shown in Figure 1a, most of the cells dissociated from embryos at the mesenchyme migration stage (cf. Fig. 3a) were seen adhering to the surface of the dish in TM at 2 hr after inoculation. They also adhered to each other, but did not aggregate. A small portion of the cells remained floating with their cilia beating vigorously. A large portion of the cells underwent lyses during the 24 hr of treatment (Fig. 1b). When the cells which have survived the treatment period were cultured in the CM, another considerable portion died after 24 hr (Fig. 1c). A typical network of the mesenchyme cells in culture was found on the surface of the dish underneath the cell debris (Fig. 1d). This network consisted of mononucleated and multinucleated fragments connected to each other by fine cell processes. These fragments were spontaneously separating into two and/or fusing with neighboring fragments as reported previously [14]. The number of fragments in a dish averaged 6.28×10^5 fragments (n=4, standard deviation ±0.78). These cells could be maintained stably in culture for about 4 days, after which they gradually disappeared. Dividing cells were not found.

Response of the dissociated cells to different treatment conditions

When the treatment period with TM was shortened to 12 hr, aggregates of various sizes coexisted on the substratum with the mesenchyme cells at the end of the post-treatment culture (Fig. 2a). When the period was prolonged to 48 hr, not only did the aggregates disappear, but the mesenchyme cells appeared unhealthy and rounded (Fig. 2b).

When the population of cells to be treated with TM was doubled to 14.3×10^7, large aggregates were found among a dense network of mesenchyme cells at the end of the post-treatment culture (Fig. 2c). On the other hand, when the number of the cells was decreased to 1.3×10^7, the substratum was populated by only a small number of mesenchyme cells. No cell aggregates were found in this case (Fig. 2d).

Behavior of cell aggregates in prolonged culture

The aggregates, such as those shown in Figure 2a and c, spread out on the substratum after about 3 days in culture, forming monolayer sheets of an epithelial nature (Fig. 3e).

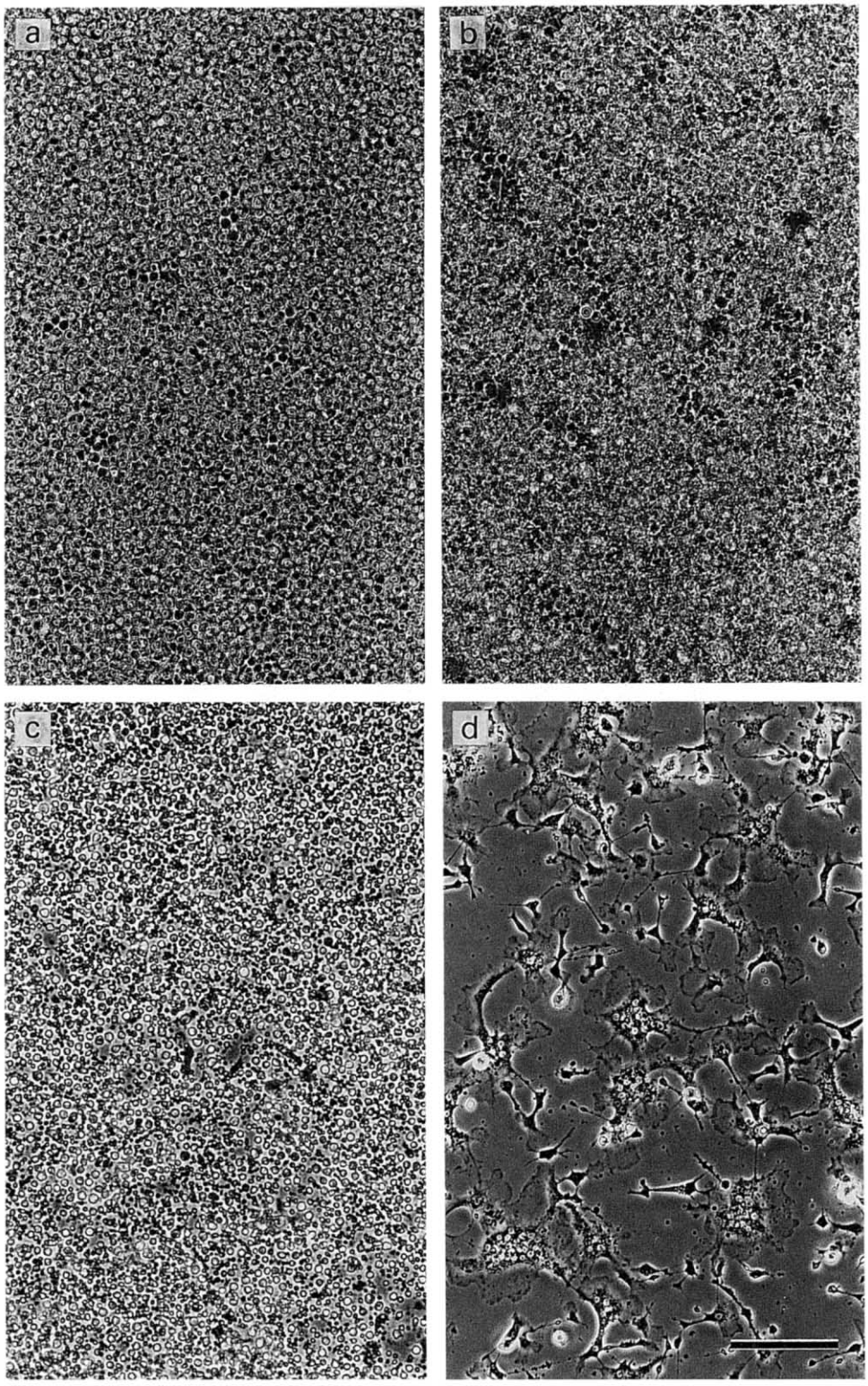

FIG. 1. Behavior and fate of embryonic cells during glycine treatment through post-treatment culture. Phase-contrast micrographs of cells dissociated from embryos at the mesenchyme migration stage and incubated in TM for 2 hr (a) or for 24 hr (b). (c) Living cells in (b) were transferred to CM and cultured for 24 hr. (d) Mesenchyme cells adhering to the substratum with the cell debris of (c) removed. Bar = 100 μm.

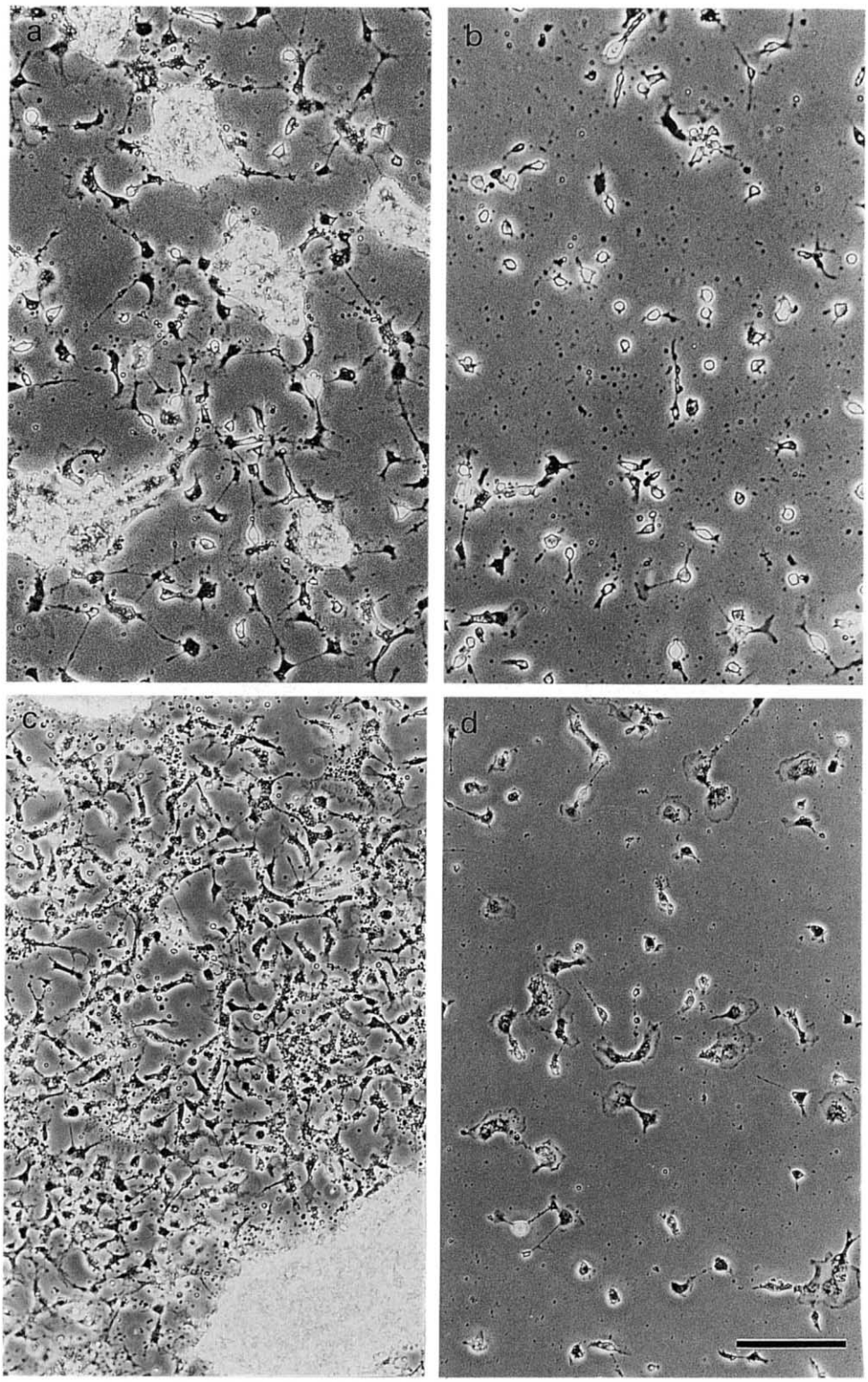

FIG. 2. Response of cells to different conditions of treatment with TM. The duration of the treatment was changed to 12 hr (a) or 48 hr (b), and the number of cells submitted to the treatment was changed to 14.3×10^7 (c) or 1.3×10^7 (d). Treated cells were then cultured in CM for 24 hr. Bar=100 μm.

Three types of cells were morphologically recognizable in these sheets, namely, large and small cells with clear nuclei (large and small arrows in Fig. 3e, respectively) and still smaller cells, the nuclei of which were slightly out of focus in this figure (arrowhead in Fig. 3e). Judging from the clearness and the size of the nuclei, the former two types were

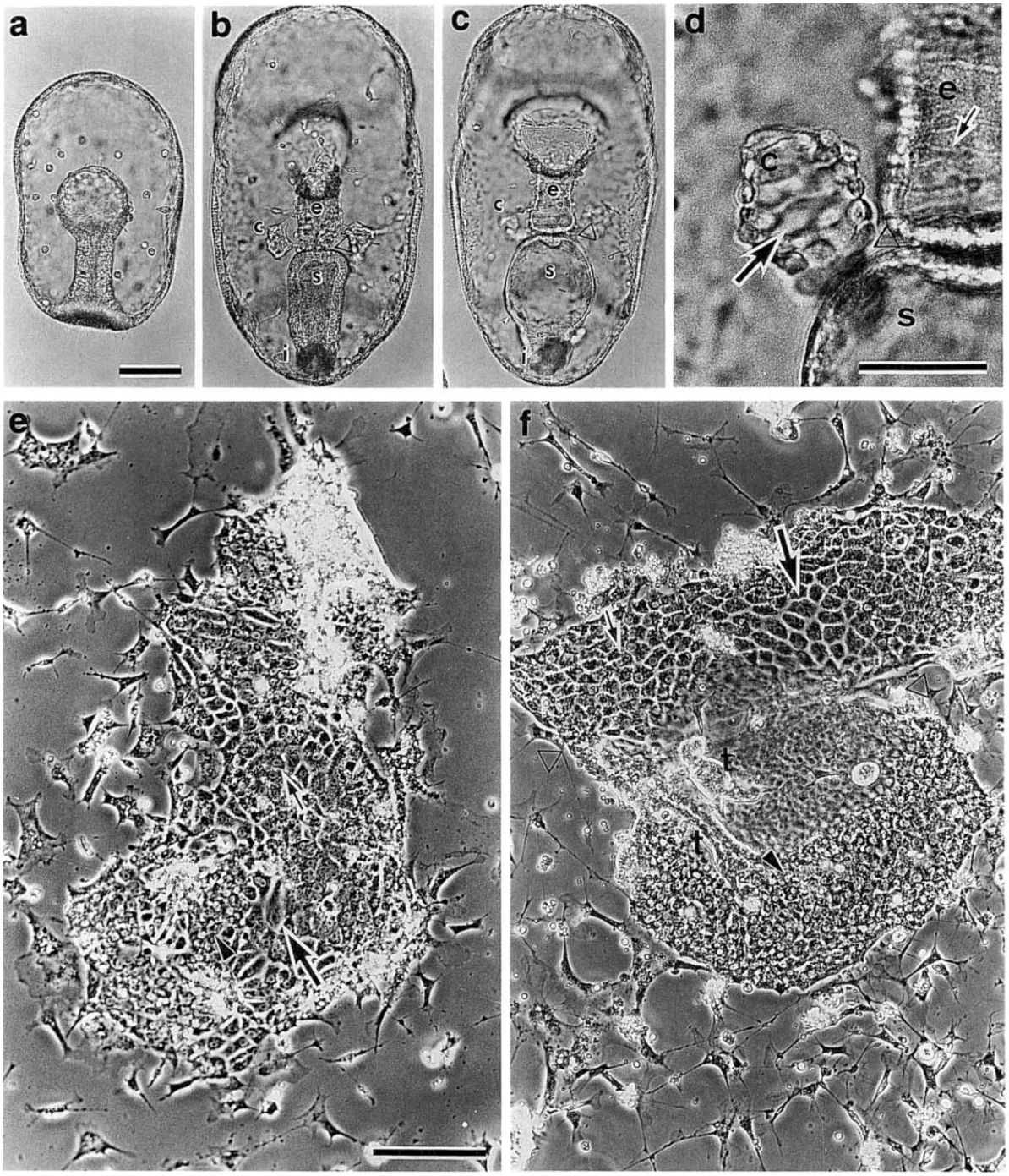

FIG. 3. Comparison of cells constituting the monolayer sheet and the archenteron in culture. (a)-(d): Light micrographs showing different stages of starfish embryos. (a): Mesenchyme migration stage (28–30 hr after fertilization), from which the cells were dissociated. (b): Mouth formation stage (52–54 hr after fertilization, dorsal view), from which archenteron complexes were prepared. (c): Bipinnaria stage (3–5 days after fertilization, ventral view). (d): Coelomic pouch of the embryo in (c) shown at a higher magnification. Note the large and spindle-shaped morphology of the coelomic pouch cells. (e): An aggregate, similar to those shown in Figure 2a and 2c, after 3 days in culture. (f): An archenteron cultured for 24 hr. Mesenchyme cells are scattered around the flattened archenteron. In (b)-(d), c: coelomic pouch, e: esophagus, s: stomach, i: intestine, triangle: sphincter separating the stomach and the esophagus. In (d)-(f), large arrows: large squamous cells, small arrows: small squamous cells, arrowheads: small columnar cells, triangles: sphincter separating the anterior and the posterior halves of the archenteron, t: tubular structure. Bars: (a)-(c)=100 μm, (d)=50 μm, (e) and (f)=100 μm.

considered to be squamous and the latter to be columnar in shape. Each of these cells bore a cilium.

Identification of the epithelial cells in culture

i) Cells constituting the archenteron in culture

In order to identify the three types of epithelial cells, the

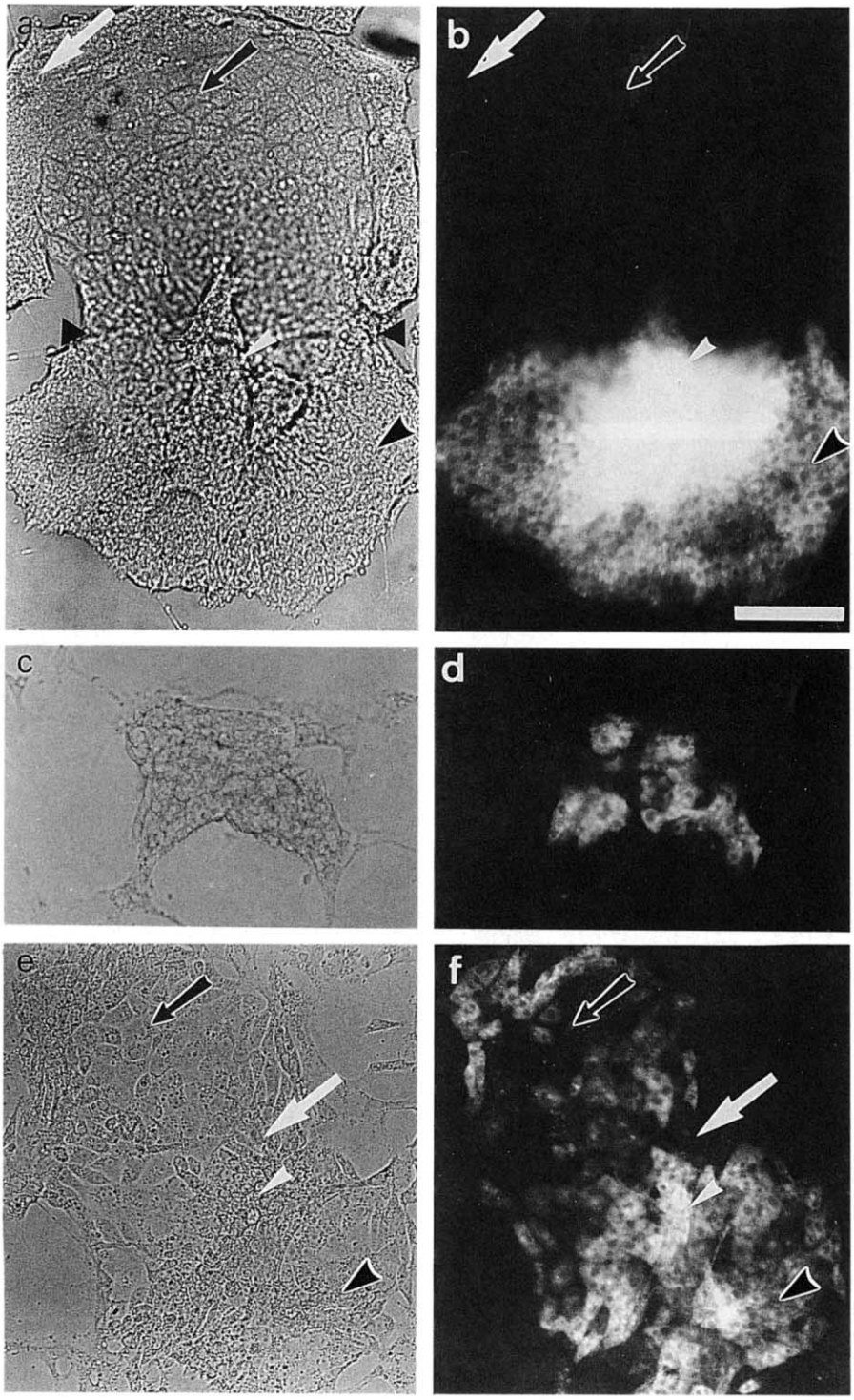

FIG. 4. Comparison of the cells constituting the cell aggregate and the archenteron in culture with reference to 1,8-ANS stainability. (a), (c), (e): Light micrographs, (b), (d), (f): fluorescence micrographs. (a), (b): An archenteron cultured for 24 hr. Cells forming the stomach (white arrowhead) are stained intensively, while those constituting the intestine (black arrowhead) are stained more weakly. Cells of the esophagus (white arrow) and the coelomic pouches (black arrow) are not stained. Triangles: the sphincter separating the esophagus and the stomach. (c), (d): An aggregate cultured for 24 hr after treatment with TM. Some cells are stained. (e), (f): A monolayer sheet 3 days in culture. Four kinds of cells are distinguished, namely, small cuboidal cells stained intensely (white arrowhead), small cuboidal cells stained weakly (black arrowhead), small squamous cells not stained (white arrow) and large unstained squamous cells (black arrow). Bar: 60 μm.

archenteron of embryos at the mouth formation stage (Fig. 3b) was isolated and kept in culture for 24 hr to use the constituent cells as a reference. At this stage, the archenteron is differentiated into three portions, the esophagus, the stomach, and the intestine. The latter two organs, however, are not as clearly separated as the former two, which are separated by a sphincter (triangle in Fig. 3b). We could still distinguish this configuration in a well preserved archenteron *in vitro* (Fig. 3f), the normal counterpart of which corresponds to that shown in Figure 3c. The formerly anterior portion (the upper side of the sphincter: triangles in Fig. 3f) is composed of large, squamous cells (large arrow in Fig. 3f) surrounded by small squamous cells (small arrow in Fig. 3f). These cells correspond to those constituting the esophagus (small arrow in Fig. 3d) and the coelomic pouches (large arrow in Fig. 3d). The formerly posterior portion of the archenteron (below the sphincter in Fig. 3f) is composed of small and cuboidal cells (arrowhead in Fig. 3f). The anterior half of this portion is lifted from the dish surface while the posterior half is attached to it as a flattened sheet. Two tubular structures, which are considered to be remnants of the lumen of the stomach, are suspended in the space under this lifted region. The flattened region corresponds to the intestine and probably the posterior portion of the stomach.

ii) Reaction of cultured cells to 1, 8-ANS staining

When the flattened archenterons were stained with 1, 8-ANS, as shown in Figure 4 a and b, the tubular structure (white arrowheads in Fig. 4a, b) and the lifted region of the stomach were stained most intensely, while the flattened intestine was weakly stained (black arrowheads in Fig. 4a, b). The upper side of the sphincter, constituting of the esophagus (white arrows in Fig. 4a, b) and the coelomic pouch (black arrows in Fig. 4a, b), was not stained. Neither were the ectodermal cells stained, judging from the cases of the prepared archenteron which happen to accompany a considerable portion of the ectoderm, usually at its posterior end (photo not shown). The mesenchyme cells were also unreactive to the dye.

Although there was no cell reactive to 1, 8-ANS in the embryo at the mouth formation stage (cf. Fig. 1a, a' of [5]), a small portion of the cells in the aggregates cultured for 24 hr were stained (Fig. 4c, d: see also Fig. 2a). In the spread aggregates cultured for three days, small cuboidal cells of two different staining intensities were found (white and black arrowheads in Fig. 4e, f). The two types of stained cells formed mixed clusters both after 24 hr and 3 days in culture. The small and large squamous cells did not respond to the dye (white and black arrows in Fig. 4e, f).

While more than 50% of the area of the monolayer sheet is 1,8-ANS positive (Fig. 4f), the proportion of the positive area of the aggregates at 24 hr is much smaller (Fig. 4d). Considering that the aggregates are multilayered, the proportion of stained cells in the aggregates should be much smaller than that of the monolayer sheets.

DISCUSSION

We have presented a method leading to the primary culture of embryonic cells of the starfish *Asterias amurensis*. Cells were dissociated from embryos at the mesenchyme migration stage, treated with TM and subsequently cultured in CM. When the TM treatment was performed for 24 hr, a pure population of mesenchyme cells was obtained after 24 hr of post-treatment culture (Fig. 1d). When the treatment was shortened to 12 hr, cell aggregates were found to coexist with the network of mesenchyme cells (Fig. 2a). These aggregates spread into monolayer sheets within 3 days (Fig. 3e) consisting of cells comparable to those obtained after culturing the intact archenteron for 24 hr (Fig. 3f). These results suggest that there is a difference in resistibility to this treatment among the cells, i.e., the mesenchyme cells, the cells constituting the coelomic pouch and the endoderm, and the ectoderm, in descending order. The mechanism underlying the differential resistibility of the cells belonging to three germ layers is being analyzed.

The pure population of mesenchyme cells obtained in this study behaved in the same manner as that reported previously for the "organ" culture of the archenteron complex [14]. This indicates that the 24-hr treatment with TM does not substantially damage these cells. The mesenchyme cells thus isolated are homologus to the secondary mesenchyme cells of the sea urchin embryos. This notion is based on the following facts. Both of these cell types differentiate at the tip of the archenteron and ingress into the blastocoel. They are reported to take roles in the embryonic morphogenesis such as formations of the mouth and the coelomic pouches both in starfish [1, 3, 4] and sea urchin [10, 11]. They also share a characteristic to form a network *in vivo* [2, 19]. These characteristics and functions commonly assigned to the two cell types contrast sharply with the confined function of spicule formation of the primary mesenchyme cells of the sea urchin. In light of the fact that the secondary mesenchyme cells of the sea urchin neither have been isolated nor cultured, our method offers, for the first time, a useful tool for studying the molecular mechanism underlying the general functions of the echinoderm mesenchyme cells.

Four types of cells having features common with those forming the archenteron were identified in the monolayer sheets, namely, the coelomic pouch cells, the esophageal cells, the stomach cells and the intestinal cells (Fig. 4e, f). This suggests that, at least, the stomach cells and the intestinal cells have differentiated *in vitro* during the process of the present method. This statement is based on the fact that the archenteron of the material embryo consists of only three types of cells. While its bulged tip (Fig. 3a) is occupied by primordia of the coelomic pouches (the upper thin portion) and the esophagus (the lower thick portion) [16, 17], the rest of the archenteron is undifferentiated morphologically or in 1, 8-ANS responsiveness [5]. The apparent increase of the proportion of 1, 8-ANS positive cells among the epithelial cells during the culture period (Fig. 4d, f) also supports the

above statement. These facts indicate that our culture method can also be used for analyzing the mechanism of cell differentiation.

The distribution of 1, 8-ANS positive cells both in the aggregates (Fig. 4d) and in the monolayer sheet (Fig. 4f) shows that cells of a certain type tend to cluster together. Thus, the cells may sort themselves out while differentiating. We do not know, at present, when these cells started to differentiate, i.e., whether in the TM or only after being involved in the aggregate. Further experiments are neccessary to find the answer.

ACKNOWLEDGMENTS

We thank the members of the Ushimado Marine Laboratory of Okayama University, Akkeshi Marine Laboratory of Hokkaido University, Asamushi Marine Biological Station of Tohoku University, and Dr. S. Ikegami and his group of the Faculty of Biological Production, Hiroshima University for kindly providing the starfish and necessary equipment.

REFERENCES

1 Abed M, Crawford, BJ (1986) Ultrastructural aspects of mouth formation in the starfish *Pisaster ochraceus*. J Morph 188: 239–250

2 Chia F-S (1977) Scanning electron microscopic observations of the mesenchyme cells in the larvae of starfish *Pisaster ochraceus*. Acta Zool (Stockh) 58: 45–51

3 Crawford BJ, Abed M (1983) The role of the basal lamina in mouth formation in the embryo of the starfish *Pisaster ochraceus*. J Morph 176: 235–246

4 Crawford BJ, Chia, F-S (1978) Coelomic pouch formation in the starfish *Pisaster ochraceus* (Echinodermata: Asteroidea). J Morph 157, 99–120

5 Dan-Sohkawa M, Kaneko H (1989) Sorting out of presumptive stomach cells of the starfish embryo. Dev Growth Differ 31: 503–508

6 Dan-Sohkawa M, Komiya Y, Kaneko H, Yamanaka H (1986) Morphogenetic capability of epithelial and mesenchymal cells dissociated from swimming starfish embryos. In "Progress in Developmental Biology Part A" Ed by Slavkin HC, Alan R Liss Inc, New York, pp 57–60

7 Dan-Sohkawa M, Tamura G, Mitsui H (1980) Mesenchyme cells in starfish development: Effect of tunicamycin on their differentiation, migration and function. Dev Growth Differ 22: 495–502

8 Dan-Sohkawa M, Yamanaka H, Watanabe K(1986) Reconstruction of bipinnaria larvae from dissociated embryonic cells of the starfish, *Asterina pectinifera*. J Embryol exp Morph 94: 47–60

9 Ettensohn CA, McClay DR (1987) A new method for isolating primary mesenchyme cells of the sea urchin embryo: Panning on wheat germ agglutinin-coated dishes. Exp Cell Res 168: 431–438

10 Gustafson T, Kinnander H (1956) Microaquaria for time-lapse cinematographic studies of morphogenesis in swimming larvae and observations on sea urchin gastrulation. Exp Cell Res 11: 36–51

11 Hardin J (1988) The role of secondary mesenchyme cells during sea urchin gastrulation studied by laser ablation. Development 103: 317–324

12 Harkey MA, Whiteley AH (1980) Isolation, culture, and differentiation of echinoid primary mesenchyme cells. Wilhelm Roux's Archives 189: 111–122

13 Kanatani H (1969) Induction of spawning and oocyte maturation by 1-methyl-adenine in starfishes. Exp Cell Res 57: 333–337

14 Kaneko H, Takaichi S, Yamamoto M, Dan-Sohkawa M (1990) Acellularity of starfish embryonic mesenchyme cells as shown *in vitro*. Development 109: 129–138

15 Katow H (1986) Behavior of sea urchin primary mesenchyme cells in artificial extracellular matrices. Exp Cell Res 162: 401–410

16 Kuraishi R, Osanai K (1992) Cell movements during gastrulation of starfish larvae. Biol Bull 183: 258–268

17 Kuraishi R, Osanai K (1994) Contribution of maternal factors and cellular interaction to determination of archenteron in the starfish embryo. Development 120: 2619–2628

18 Okazaki, K (1975) Spicule formation by isolated micromeres of the sea urchin embryo. Am Zool 15: 567–581

19 Tamboline CR, Burke RD (1992) Secondary mesenchyme of the sea urchin embryo: Ontogeny of blastocoelar cells. J Exp Zool 262: 51–60

20 Venkatasubramanian K, Solursh M (1984) Adhesive and migratory behavior of normal and sulfate-deficient sea urchin cells *in vitro*. Exp Cell Res 154: 421–431