Induction of brain and sensory pigment cells in the ascidian embryo analyzed by experiments with isolated blastomeres

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Summary

Tadpole larvae of ascidians have a brain and two kinds of sensory pigment cells within the brain. These neural tissues are formed through folding of the neural plate and, as in vertebrates, neural induction is involved in the formation of the nervous system. The brain and pigment cells are derived from anterior-animal (a-line) blastomeres, and inductive interaction with anterior-vegetal (A-line) blastomeres is necessary for formation of neural tissues from the a-line cells. In order to investigate the characteristics of this process in detail, blastomeres were isolated from embryos of *Halocynthia roretzi* at the 110-cell (late blastula) stage, and then their developmental capacity was examined. In the first set of experiments, brain- and pigment-lineage cells, isolated prior to neural induction, failed to develop three specific features of epidermis (epithelial morphology, secretion of larval tunic and expression of epidermis-specific antigen). These observations suggested a process different from amphibian neural induction, indicative perhaps of a permissive type of induction in ascidian embryogenesis. Next, in order to identify which type of tissue-precursor cell is the inducer of the sensory pigment cells, various sets of blastomeres were isolated from 110-cell embryos, in which the fates of most of the blastomeres are restricted to a single type of tissue. The results suggested that spinal-cord precursor blastomeres, which are cells of the A-line, are sufficient to induce the a-line cells to form pigment cells. In addition, embryos from which all spinal cord precursors had been ablated, failed to form sensory pigment cells as well as a brain structure. Therefore, it is probable that the inducers of the ascidian sensory pigment cells are the spinal-cord precursor cells.

Key words: neural induction, ascidian, brain, pigment cell, blastomere isolation, permissive induction.

Introduction

The dorsal tubular nervous system of the tadpole larvae of ascidians (Prochordata, Asciidiacea) is formed through folding of the neural plate (Conklin, 1905; Satoh, 1978; Nicol and Meinertzhagen, 1988), which is also the mechanism for formation of the central nervous system in vertebrates. Eventually, a brain with a cavity develops in the head region of the larva (Fig. 1A and B). Two melanin-containing pigment cells lie in the brain. One is a sensory cell for detection of gravity and is referred to as the oolith. The other is a cell in the eye organ and is referred to as the ocellus (Dilly, 1962, 1964). Ascidians are known to exhibit an essentially mosaic type of embryogenesis. However, inductive interactions between cells are involved in the formation of the brain and the two sensory pigment cells (Rose, 1939), as is the case in vertebrates (Spemann and Mangold, 1924).

Previous studies have already revealed the following information about the formation of the brain and the sensory pigment cells. (1) Cell lineage analysis has shown that brain and pigment cells are derived from anterior-animal (a-line) blastomeres. At the 110-cell (late blastula) stage, six bilateral blastomeres are brain-lineage cells. All parts of the brain are derived from these six cells, which, in addition, give rise to the sensory pigment cells and the primordial pharynx (Fig. 1) (Conklin, 1905; Ortolani, 1962; Nishida, 1987). (2) Blastomere ablation and combination experiments have revealed that the inducers of the brain and the pigment cells are the anterior-vegetal (A-line) blastomeres (Rose, 1939; Reverberi and Minganti, 1946a, 1946b; Reverberi et al. 1960; Okado and Takahashi, 1988; Nishida and Satoh, 1989). The A-line blastomeres themselves give rise to mainly spinal cord, notochord and endoderm. 'Spinal cord' is a traditional term. In reality, all cells in the region called the spinal cord, which is derived from the A-line blastomeres, are presumed to be ependimal glial cells and are not neuronal cells in ascidian larvae (Katz, 1983; Nicol and Meinertzhagen, 1988). (3) Embryonic cell dissociation experiments have indicated that induction of the pigment cells is accomplished at around the 180-cell (middle gastrula) stage (Nishida and Satoh, 1989).

In amphibian embryos, the presumptive neural region, when isolated prior to neural induction, develops into epidermis (Jones and Woodland, 1986).
Fig. 1. (A) Photomicrograph of the head and trunk regions of a larva of *Halocynthia*. (B) Schematic representation of larval tissues and organs. A brain (b) with a cavity is present in the head region. In the brain, two pigment cells, the otolith (ot) and ocellus (oc), are present. Posterior to the brain, the spinal cord (sc) lies dorsally in the trunk region and the tail region, (referred to as the brain stem and spinal cord, respectively, by Nishida (1989) and as the deuterencephalon by Katz (1983)). p, Palp; ph, primordial pharynx; en, endoderm; n, notochord. (C,D) Scanning electron micrographs of the animal and vegetal hemispheres, respectively, of a 110-cell embryo, showing the six bilateral brain-lineage cells (arrowheads). Anterior is at the top of the photomicrograph. The margin of the area isolated in experiments is indicated by black lines. There is no blastocoel in ascidian embryos. (E) Photomicrograph of an isolated mass of cells (3 cells by 5 cells). The midline corresponds to the left side of this isolated mass. (F) Schematic representation of the isolated mass showing the designation of each blastomere. For example, the cell at the top-left corner in this Figure is a8.22. Among five rows, three rows from the top consist of animal-hemisphere (a-line) cells, while two rows from the bottom consist of vegetal-hemisphere (A-line) cells. Double-headed arrows between the second and third rows indicate that those cells are sibling cells. (G) Developmental fate of each cell. Ep, Epidermis; P, palp; B, brain; Ph, primordial pharynx; PC, pigment cell; SC, spinal cord; N, notochord. Each row is named as indicated on the right, according to its fate. (H) Separation of five rows of the isolated mass. Scale bars, 50 μm.

Hence, the induction is of the instructive type (Gurdon, 1987). It is also known that the inducer of neural tissue is chorda-mesoderm. In the present study with ascidian embryos, the following questions were asked. (1) Do the brain- and pigment-lineage cells develop into epidermis, if they are not induced to become neuronal tissues? (2) Which type of tissue-precursor cell is the inducer of the sensory pigment cells? In order to answer these questions, various kinds of blastomere isolation experiment were performed using 110-cell embryos, in
which the fates of most of the blastomeres are restricted to a single type of tissue.

Materials and methods

Embryos

Adults of the ascidian, Halocynthia roretzi (Drasche), were collected in the vicinity of Asamushi Marine Biological Station, Aomori, Japan. They spawned yellowish, translucent eggs, about 280 μm in diameter. Naturally spawned egg were fertilized artificially. Embryos were reared in Millipore-filtered sea water (MFSW) at 13°C, at which temperature embryos hatched about 35 h after fertilization.

Isolation of blastomeres

The 16-cell stage embryos were manually dechorionated with sharpened tungsten needles and reared in 0.9 % agar-coated plastic dishes filled with MFSW that contained 50 μg ml⁻¹ streptomycin sulphate. At the 110-cell stage (9 h after fertilization), identified blastomeres or sets of blastomeres were isolated from embryos with a fine glass needle under a stereomicroscope (Olympus SZH-121). Isolated blastomeres were cultured separately as partial embryos until unoperated controls hatched.

Visualization of the larval tunic

The larval tunic surrounds the larva and forms a transparent fin on the entire larva. Its constituents are secreted by epidermal cells. The transparent larval tunic of partial embryos was rendered clearly visible by dipping partial embryos into sea water that contained sumi ink (Fueki Nori Co., Osaka), which is a suspension of small black particles of carbon. The secreted larval tunic excluded carbon particles and transparent areas were visible around partial embryos.

Immunohistochemistry

A monoclonal antibody, Epi-2, recognizes differentiated epidermal cells (Nishikata et al. 1987). Immunohistochemical staining was performed by the standard method. Partial embryos were stained for indirect immunofluorescence with Epi-2 antibody and rhodamin-conjugated secondary antibody (Nishida, 1990).

Histochemical staining for tyrosinase

Histochemistry for tyrosinase activity was performed with L-dopa as the substrate by the method described previously (Laidlaw, 1932; Nishida and Satoh, 1989).

Results

Isolated brain-lineage cells did not develop into epidermis

The first half of this report deals with the question of whether or not brain-lineage cells develop into epidermis, when they are not induced to form neural tissues.

Experimental design

The brain-lineage cells in the 110-cell embryos comprise six bilateral blastomeres, aligned 3 on each side of the midline, and these cells are bilaterally named a8.19, a8.17, and a8.25 from the midline (Fig. 1C, F and G). The a8.19 cells give rise to the ventral part of the brain and the primordial pharynx. The a8.17 cells give rise to the anterior and posterior parts of the brain. The a8.25 cells give rise to the dorsal and lateral parts of the brain and to two sensory pigment cells (Nishida, 1987). In the 110-cell embryo, the brain-lineage cells, a8.19, a8.17 and a8.25 are sibling cells of palp- and epidermis-lineage cells, namely, a8.20, a8.18 and a8.26, respectively (Fig. 1F and G). The palp is an adhesive organ at the anterior tip of the larva (Fig. 1A and B). The cells of the palp exhibit several features of the epidermis. They secrete larval tunic and express epidermis-specific antigen. Thus, the palp can be thought of as a specific form of epidermis. Therefore, the 110-cell stage is the first stage at which brain lineage and epidermis lineage are separated and segregated into different blastomeres.

Isolation of blastomeres was performed as follows. First, one half of an identified region, consisting of fifteen cells (3 cells by 5 cells), was excised from the 110-cell embryo. Then the animal and vegetal halves were unfolded (Fig. 1C–E). Names and developmental fates of the blastomeres in the isolated region are indicated in Fig. 1F and G. Then each row of three cells was separated from the others (Fig. 1H). For convenience, each row is referred to as the epidermis (Ep) row, the palp (P) row, the brain (B) row, the spinal-cord (SC) row, and the notochord (N) row according to its developmental fate (Fig. 1G). The Ep, P and B rows are cells of the animal hemisphere and are progeny of the a4.1 (anterior-animal) blastomeres of the 8-cell embryos. The SC and N rows are cells of the vegetal hemisphere and are progeny of the A-4.1 (anterior-vegetal) blastomeres of the 8-cell embryos. Isolated rows were cultured separately and allowed to develop as partial embryos until unoperated controls hatched. Then expression of epidermis-specific features was examined. Partial embryos derived from Ep rows (abbreviated as Ep partial embryos hereafter), and P partial embryos were used as positive controls for the expression of epidermis-specific features, and N partial embryos were used as negative controls.

Expression of epidermis-specific features in partial embryos

Differentiation of epidermis in partial embryos was evaluated by expression of three kinds of epidermis-specific features: epithelial morphology of the partial embryos, secretion of larval tunic and expression of the epidermis-specific antigen that is recognized by the Epi-2 monoclonal antibody. The results are shown in Fig. 2 and Table 1.

Most of the Ep and P partial embryos developed into so-called permanent blastulae. They consisted of approximately fifteen simple, columnar epithelial cells with smooth outer surfaces. They often had a cavity at the center. In contrast, the B, SC and N partial embryos developed into irregularly shaped masses of cells. Consistently, most of the Ep and P partial embryos secreted larval tunic and expressed the Epi-2 antigen, while none of the B, SC and N partial embryos did so. These results indicate that brain-lineage cells do not
differentiate into epidermis when they are isolated at the 110-cell stage.

Identification of the inducer of sensory pigment cells
The next part of this report deals with the question of which type of tissue-precursor cell is the inducer of sensory pigment cells.

Experimental design
As in the first experiment, identified regions of 110-cell embryos were excised, but the animal and vegetal halves were not unfolded. The ascidian blastula has no blastocoel. Instead, cells of the animal and vegetal hemispheres only weakly adhere to each other at their base. The various regions isolated in this series of experiments (experiment 1–6) are represented schematically in Fig. 3A. The diagrams show lateral views of the isolated regions. As in the first experiment, each row consisted of three cells, with the one exception that, in experiment 1, the endodermal layer consisted of only two cells, A7.2 and A7.5. Each isolated part includes a single pigment-lineage cell. In some cases both halves of a bilateral region were excised together from embryos. In these cases, each row consisted of six cells. Each isolated part includes two pigment-lineage cells.

The isolated parts of the embryos were cultured until controls hatched. In order to evaluate initially the number of partial embryos that differentiated pigment cells, the number of partial embryos that contained pigment cells with melanin granules was counted. Then the partial embryos that did not contain a melanin

Table 1. Epidermis-specific features in partial embryos expressed as number of embryos that showed epidermal feature divided by the number of embryos examined

<table>
<thead>
<tr>
<th>Origin of partial embryos</th>
<th>Ep row</th>
<th>P row</th>
<th>B row</th>
<th>SC row</th>
<th>N row</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Permanent blastula</td>
<td>Permanent blastula</td>
<td>Irregular cell mass</td>
<td>Irregular cell mass</td>
<td>Irregular cell mass</td>
</tr>
<tr>
<td>Secretion of larval tunic</td>
<td>(% (100%))</td>
<td>(% (94%))</td>
<td>(% (0%))</td>
<td>(% (0%))</td>
<td>(% (0%))</td>
</tr>
<tr>
<td>Expression of Epi-2 antigen</td>
<td>(% (97%))</td>
<td>(% (93%))</td>
<td>(% (0%))</td>
<td>(% (0%))</td>
<td>(% (0%))</td>
</tr>
</tbody>
</table>
Fig. 3. (A) Schematic representation of isolated regions from 110-cell embryos in the series of experiments 1–6. Lateral views. Anterior is to the left. Animal pole is uppermost. Each circle with abbreviated nomenclature denotes each row of three cells or row of six bilateral cells. The brain row is indicated with a bold line. The spinal cord row is shaded. The region, isolated in experiment 2, corresponds to that in Fig. 1E. Ep, Epidermis row; P, palp row; B, brain row; SC, spinal cord row; N, notochord row; En, endoderm row. (B) Histogram showing the percentage of the resultant partial embryos that had melanin-containing cells (light shaded) or tyrosinase-containing cells (dark shaded). Numbers shown at the bottom refer to the experiment number and correspond to those in (A). Columns with asterisks show the results obtained with bilaterally isolated parts (6 cells in each row), the columns without asterisks show the results obtained with isolated halves (3 cells in each row). Numbers above each column denote the number of partial embryos examined. A partial embryo derived from the bilateral part (6 cells in each row) often had two pigment cells.

Fig. 4. Partial embryos obtained from the series of experiments 1–6. (A) Experiment 2. The partial embryo has a black melanin-containing pigment cell as well as notochord cells (arrow), which are oval cells with a vacuole. (B) Experiment 3. The partial embryo has a pigment cell but no notochord cell. (C) Experiment 3. A partial embryo subjected to the histochemical reaction for tyrosinase. The embryo has a pigment cell. The embryo swelled somewhat during the histochemical reaction. (D) Experiment 4. The partial embryo has no pigment cell. (E) Experiment 6. A partial embryo, derived from only brain and spinal cord layers, developed a pigment cell. Scale bar, 50 µm.

Granule were subjected to a tyrosinase-specific histochemical reaction, and the number of the partial embryos that produced spots of melanin was counted. Both the former and the latter types of embryo were regarded as embryos in which induction of pigment cell had occurred.

Inducer of sensory pigment cells

Previous studies have revealed that the inducer is the anterior-vegetal (A-line) cells. In the present series of experiments, the SC, N and En rows were cells of the A-line, while the Ep, P and B rows were cells of the a-line (anterior-animal). The pigment-lineage cell (a8.25) is in the B row (Fig. 1G). Results obtained in this study are shown in Fig. 3B and 4. In experiments 1–3, a significant proportion (47–70%) of partial embryos differentiated pigment cells that contained melanin granules or exhibited tyrosinase activity (Fig. 4A–C). In these experiments, isolated embryonic parts commonly contained the SC layer, which was composed of cells of the A-line. In contrast, none of the partial embryos in experiment 4 differentiated pigment cells (Fig. 4D). This clear difference in the results must be attributed to the absence of the SC row in experiment 4. In experiments 5 and 6, the Ep and P rows were removed from the isolated embryonic regions. Although the proportion of pigmented partial embryos was reduced, 14–20% of the isolated regions developed pigment cells (Fig. 4E). As a control experiment, the B row and the SC row were isolated and separated from one another, and none of them developed pigment cells. These results suggest that spinal-cord precursor cells are sufficient for induction of pigment cells.

To examine further the role of the spinal cord cells in the induction of pigment cells, ablation of blastomeres was carried out. Since it was difficult to destroy all of the spinal cord precursor blastomeres at the 110-cell stage for technical reasons, ablation was performed at the 64-cell stage. All of the spinal cord precursors, the bilateral A7.4 and A7.8 cell pairs, were destroyed with a sharpened tungsten needle while notochord and endoderm precursors remained intact. None of 51 embryos developed melanin- or tyrosinase-containing cells as well as brain structures (Fig. 5). These results
The results could be an artifact of the isolation and lineage cells developed the features of the epidermis culture methods. However, the isolated epidermis stage failed to develop three features of the epidermis. The 180-cell stage (Nishida and Satoh, 1989). Therefore, been shown that induction is not accomplished before sensory pigment cells, it has respect to induction of sensory pigment cells, it has intrinsic differences from the time when they are generated at the 110-cell stage. These differences may explain why only the six defined cells are invariantly fated to form the brain during normal embryogenesis. Thus, it seems that inductive interactions between cells may be required for the process of differentiation of the neural phenotype, rather than there being a requirement for the choice of a neural pathway among the repertoire of developmental pathways. In this regard, the interactions may be considered to represent permissive induction (Gurdon, 1987). It was shown previously that muscle cells in the tip of the tail of ascidian larvae also require cell interactions to develop into muscle (Nishida, 1990). In this case again, muscle-lineage cells, isolated before the determination of muscle, did not differentiate into epidermis, and the properties of the interactions between cells are suggested to differ from those of mesodermal induction in amphibian embryos. Ascidian embryos essentially show a mosaic mode of development. Permissive induction, which is involved in the formation of the brain and minor (secondary) muscle cells of ascidian larvae, may represent a primitive form of induction which evolved to become the more complex, instructive type of induction seen in vertebrates.

Another explanation of the present results is possible. Interactions that repress epidermis fate and interactions that evoke brain fate may be different processes and only the former interactions may be accomplished before isolation of blastomeres at the 110-cell stage. Such a situation has been suggested to occur in neural induction in Xenopus (Savage and Phillips, 1989). However, if it is the case in ascidians, we must presume that the interactions that repress epidermis fate are very complicated. Since segregation of brain versus epidermis fates of cells has not yet occurred by the 110-cell stage, the interactions should determine that cells of the brain-epidermis lineage produce epidermis and non-epidermis precursor cells together as their progeny. At a later stage only non-epidermis precursor cells may be induced to form brain by interactions that specifically evoke brain fate.

**Inducer of the sensory pigment cells**

Neural differentiation during ascidian embryogenesis can be examined electrically via detection of neural-type action potentials or neuron-specific ion channels (Hirano et al. 1984; Okado and Takahashi, 1988). However, in the present system, no easily detectable marker of differentiation of neural cells is available as yet. Therefore, the latter half of the present study concerned only the induction of sensory pigment cells of the brain.

The results of the isolation and ablation experiments showed clearly that spinal cord precursor cells are
sufficient and necessary for the induction of the pigment cells in Halocynthia. This result seems paradoxical since spinal cord itself is part of the central nervous system. In ascidian larvae, however, all cells in the region called the spinal cord, which is derived from the anterior-vegetal (A-line) blastomeres, are presumed to be endodermal glial cells and are not neuronal cells (Katz, 1983; Nicol and Meinertzhagen, 1988). Although spinal cord is also formed by the folding movements of ectoderm in the tail region, (Satoh, 1978; Nishida, 1986; Nicol and Meinertzhagen, 1988), there is no evidence at present that inductive interactions are involved in the formation of the spinal cord.

Reverberi et al. (1960) obtained totally different results about the inducer of pigment cells using Ascidia aspersa and Phallusia mammillata. The results of their blastomere ablation and transplantation experiments unambiguously indicate that spinal cord precursor blastomeres do not have inductive potency, but notochord and endoderm precursors do. The reasons for the discrepancy between their results and ours is unknown and may be attributable to differences between species. During normal embryogenesis in Halocynthia, in the 110-cell embryo, spinal cord precursor cells make contact laterally with brain-lineage cells, but notochord and endoderm precursors do not make contacts with brain lineage cells (see scanning electron micrograph, Fig. 1G in Nishida, 1986). During the movements associated with gastrulation notochord precursors make contact basally with brain-lineage cells. Since differentiation of pigment cells occurs in cells of the correct lineage, even if morphogenesis and cell movement are continuously inhibited after the cleavage stages by cytochalasin B (Whittaker, 1973; Nishida and Satoh, 1989), gastrulation after the 110-cell stage is not necessary for induction of pigment cells. In such cytochalasin-arrested embryos, the spinal cord precursors retain contact laterally with the pigment-cell precursors. Therefore, it is likely that spinal cord precursors are the natural inducers during normal development.

I thank Dr N. Satoh for his constant encouragement and for his critical reading of the manuscript. Thanks are also due to Dr T. Numakunai at the Asamushi Marine Biological Station for supplying the living materials and to Dr T. Nishikata, Kyoto University for providing the monoclonal antibodies. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (02854095).

References


(Accepted 6 February 1991)