Primary Induction of Horseshoe Crab Embryos

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Introduction

The mechanism of induction by the primary organizer is one of the most exciting problems to be analysed in developmental biology, but the studies on the primary organizer in animals except Chordata are extremely few.

The eggs of horseshoe crabs show surface cleavage as well as those of the other arthropods, and the blastopore appears at the early gastrula stage. Surface cells or blastoderm cells migrate toward the blastopore and enter from there, and a cell-mass is formed under the blastopore. Our morphological observations and previous experimental studies suggest that the germ disc is formed around the cell-mass, and the cell-mass is lately situated at the posterior end of the ventral plate (the embryonic area) and succesively forms segment primordia (Sekiguchi, 1973; Itow, 1984, 1985, 1986).

When the cell-mass of the embryo at early gastrula stage is electrically cauterized, the treated embryo can not develop. When the cell-mass is divided into two or three pieces by the electrocauterization or the cell dissociation, the treated embryos develop into double or triple embryos (Sekiguchi, 1966; Itow and Sekiguchi, 1979). Those experiments suggest that the cell-mass plays the central role of embryogenesis, and it is similar to the mesodermal teloblasts of Annelida and Crustacea. We call it the center cells. But we have not known whether it is the primary organizer or not.

For the perfect understanding of the role of the center cells, we tried the graft experiment. Up to the present the graft of embryonic cells has not succeeded in horseshoe crabs except the preliminary experiments of authors (Itow and Mochizuki, 1988).

The graft of center cells between different species will exactly tell us whether the center cells has the ability of primary induction or not. Besides, we must examine whether the egg extract induces secondary embryos or not, and we must find the substance of induction.

Materials and Methods

Adult horseshoe crabs, *Tachypleus tridentatus*, were collected in the north area of Kyushu, Japan. *Tachypleus (Carcinosporpius) rotundicauda* and *Tachypleus gigas* were collected in the Gulf of Siam in Thailand and sent to us. *Limulus* polyphemus were sent from Woods Hole Marine Laboratory, Massachusetts, the Duke University Marine Laboratory, North Carolina, and the Gulf Specimen Company, Florida. All were transferred to Shizuoka University, where the present study was conducted.

For grafting, a small hole (diameter about 0.05-0.075mm) was opened in the chorion of host horseshoe crab embryos at the early gastrula stage (Stage 7). The position of the hole was at the opposite side of the center cells. The enter cells and other tissues were cut from the donor embryos, and then grafted under the surface cells (extodermal cells) of host embryos through the hole. The treated embryos were cultured in a small dish in about 10ml of sea water containing antibiotics such as 0.5μug/ml streptomycin and 0.5unit/ml peniciline.

Normal and treated embryos were vitally stained with 1/20,000-1/400,000 neutral red and then observed under a stereomicroscope. Secondary embryos were observed at Stages 20, 21 (hatching stage) and the stages after hatching. Whenever a treated embryo bore an extra ventral plate (embryonic area) where there were often extra appendages, we considered it to be a secondary embryo.

The proteins of secondary embryos were analyzed by SDS electrophoresis in order to determine whether they had originated from the donor or the host.

The center cells of 25-50 embryos were homogenized in 0.5ml distilled water or sea water. The concentration of homogenates of the center cells from embryos at Stage 7 was about 0.5-5.0mg (dry weight) /ml.

Absorption granules (Collagen, Spongel, Yamanouchi Co., Tokyo) were added to the resulting homogenates, and the solutions were again homogenized. The solutions including the granules were then injected into the opposite sides of center cells of host embryos at early gastrula stage. The solutions were injected under the surface cells through the small hole, using a glass capillary. About $5\times10^{-8}-2\times10^{-6}$ml of solution as injected into each embryo. In other cases, the punctured eggs were immersed in sea water after adding the solution including the homogenate of center cells, and then their chorions were slightly squeezed. In this way, the solution was able to enter the eggs. For host embryos, we used *Tachypleus rotundicauda* and *Limulus polyphemus*.

Besides, the crude egg extract was separated with the electrophoresis. Native condition of acrylamide gel was used for this purpose. The gel including each substance was divided and was homogenized in the culture medium. In the next day, the homogenate was filtered with millipore filter for the removal of polyacrylamide gel. In other case, the homogenates were centrifuged and the supernatants were used for the injection experiments. The absorption granules were added into the obtained solutions. The granules and solutions were injected into embryos at early gastrula stage.

Some substances which have been already known were also injected into the other embryos at the same stage.

Results and Discussion

1. Intraspecific grafts

Intraspecific grafts of the center cells have been performed in the American horseshoe crab, *Limulus polyphemus* and in three Eurasian species, *Tachypleus rotundicauda, T. gigas* and *T. tridentatus*. Secondary embryos were formed at the high rate (about 20–30%) in the eggs grafted with the center cells of *L. polyphemus* and *T. rotundicauda* (Table 1). In *T. gigas* and *T. tridentatus* , which have larger eggs, most of treated embryos died and the secondary embryos were hardly obtained. The rate of existence of embryos in *Limulus* which have the smallest eggs among all horseshoe crabs was the highest, and the related rate of cells against yolks in the eggs of *Limulus* is the highest. The development of the embryos was also the fastest among all horseshoe crabs, and is completed at about a half periods of that of Eurasian ones.

The success of the intraspecific grafts indicates that the center cells play a central role for the embryogenesis. Then we must examine that the secondary embryos are formed after the growth of the center cells or the host cells, because the center cells are the primary organizer or not.

Table 1 The results of grafts.

	Secondary embryos	Developed embryos	Treated embryos
L.p.➡L.p.	18 21.7%	83 51.2%	162
T.r.➡T.r.	3 25.0%	12 7.9%	152
Cells except center cells ➡L.p. & T.r.	0 0.0%	102 31.8%	321
L.p.➡T.r.	3 21.4%	14 11.2%	125
T.r.➡L.p.	4 16.7%	24 17.0%	141

L.p. = *Limulus polyphemus*, T.r. = *Tachypleus rotundicauda*.

2. Interspecific grafts

The center cells were grafted from American horseshoe crab embryos into Eurasian ones. As the results, secondary embryos were formed. The grafting from Asian ones into American ones showed the same results (Table 1). The forms and structures of secondary embryos were same as the host embryos. The protein patterns of secondary embryos were same as the host embryos, too. These results mean that the secondary embryos are formed from the cells of host embryos by the induction of the center cells of donor embryos, and they were not formed by the growth of the grafted center cells.

3. Injection of egg extract and several substances

What facters of the center cells are needed for the induction? For the solution of this question, we tried the grafts of homogenized center cells with the method of microinjection, and found that the homogenizate induced secondary embryos (Table 2).

The crude egg extract was further separated with the electrophoresis. Native condition of acrylamide gel was used for this purpose. We found two bands which induced secondary embryos, and named them S-proteins (Figs. 1 and 2, Table 2). The bands of S-1 and S-2 were common in American and urasian horseshoe crabs.

Table 2 The injection of egg extract.

	Secondary embryos	Developed embryos	Treated embryos
Crude homogenate	23 9.5%	243 42.7%	569
S-1	24 9.2%	262 40.8%	642
S-2	31 11.9%	260 48.2%	539
Control	1 1.6%	64 28.8%	222

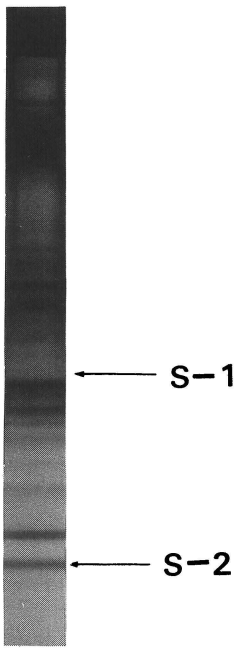

Fig. 1 The pattern of the electrophoresis. The egg extract from embryos at gastrula stage was separated with the electrophoresis under the native condition. S-1 and S-2 were the bands which induced secondary embryos.

We also examined the ability of induction by the already known substances. The following substances did not induce secondary embryos: ascorbic acid (vitamine C), chondroitin sulfate, dibutylic cyclic AMP+theophyline, α-lipoic acid,
thyamine (vitamine B_1) and so on. On the other hand, human fibroblast growth factor (FGF), diacylglycerols, inositole, inositole triphosphate and calcium ionophore induced secondary embryos (Fig. 3, Tables 3 and 4).

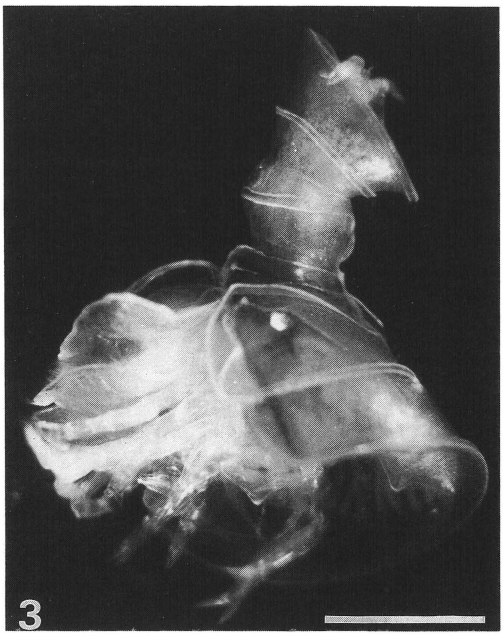

Fig. 2 The secondary embryo (upper side). The embryo was induced after the injection of S-2 protein. Scale=1mm.

Fig. 3 The secondary embryo (upper side). The embryo was induced after the injection of inositol. Scale=1mm.

Table 3 The injection of known substances. These substances did not induce secondary embryos.

	Secondary embryos	Developed embryos	Treated embryos
Ascorbic acid 20-200 mg/ml	0 0.0%	82 69.5%	118
Chondroitin sulfate 10-100 mg/ml	0 0.0%	87 96.7%	90
Dibutyl cyclic AMP 1 mg/ml +	0 0.0%	40 29.6%	135
Theophyline 10^{-2} M			
α-Lipoic acid 5 mg/ml	0 0.0%	61 37.9%	161
Thyamine 5-25 mg/ml	0 0.0%	64 59.8%	107

Table 4 The injection of known substances. These substances induced secondary embryos.

	Secondary embryos	Developed embryos	Treated embryos
Fibroblast growth factor 10 μg/ml	7(6.5%)	107(32.5%)	329
Indole-acetic acid 10-10000 μg/ml	18(5.6%)	320(52.0%)	615
Diacylgrycerols			
sn-DiC8 100-200 μg/ml	4(4.9%)	82	341
rac-DiC8 200 μg/ml	2(4.4%)	45	102
sn-OAG 50-100 μg/ml	4(5.1%)	78	250
rac-OAG 100 μg/ml	14(9.9%)	141	367
Inositol 100mg/ml	6(7.1%)	84(22.3%)	376
Inositol triphosphate 20 μg/ml	3(6.7%)	45(50.6%)	89
Calcium ionophore 10-100 μg/ml	5(23.8%)	21(8.9%)	235

Various possible roles of S-proteins and these substances can be considered as follows; (1) substances inducing the differentiation of center cells, (2) inducers from the center cells, (3) substances which are synthesized in cells accepted the inducer, (4) substances affecting these systems indirectly. The analyse of the induction system is expected in the future.

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