

## Effects of Exogastrula-Inducing Peptides on Cell Proliferation in Embryos of the Sea Urchin *Anthocardis crassispina*

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**ABSTRACT**—The effects were examined of exogastrula-inducing peptides (EGIPs), found in embryos of the sea urchin *Anthocardis crassispina* and known to be homologous to EGF, on cell proliferation in restricted areas of embryos at the early stages of development. Blastomeres of embryos were mechanically separated into macromeres and mesomeres at the 16-cell stage. Although the number of isolated blastomeres increased linearly in the culture system *in vitro*, EGIPs inhibited or retarded the division of macromeres during the period between 16 hr and 20 hr after fertilization, which corresponded to the times of gastrulation in normal embryos, but they did not affect the division of mesomeres. These differences in the proliferation of blastomeres were confirmed immunohistochemically with a BrdU-specific antibody on whole embryos. Furthermore, the distribution of the cytoskeleton in embryos during early development was examined with a tubulin-specific antibody for microtubules and with NBD-phalloidin for microfilaments. We found local differences in the distribution of cytoskeletal elements of a normal embryo, as well as differences between normal and EGIPs-treated embryos. These results suggest that EGIPs inhibit or retard the division of the progeny of macromeres, namely, the blastomeres of the vegetal-plate area, via a disturbance in the development of the cytoskeleton at the early gastrula stage, leading to exogastrulation.

### INTRODUCTION

Exogastrula-inducing peptides (EGIPs) have been found in embryos of the sea urchin *Anthocardis crassispina* as intrinsic factors that affect morphogenesis of the embryos. EGIPs induce the extrusion of the archenteron, which is very different from the invagination of normal embryos and exogastrulation results when EGIPs are present outside the embryos [7, 13]. Four EGIPs have been purified from mesenchyme blastulae of the sea urchin and named peptides A, B, C and D [13]. The complete amino acid sequences of these peptides and the positions of the disulfide bonds in EGIP-D have been determined [13–16]. It

appears that EGIPs are homologous to epidermal growth factor (EGF) [1], as indicated by the similarities in terms of amino acid sequence and positions of disulfide bonds between EGIPs and EGF. Therefore, it seems possible that EGIPs may regulate the proliferation of cells in sea urchin embryos. Stephen *et al.* [12], using aphidicolin, an inhibitor of DNA synthesis and cell division, found that the proliferation of cells from the vegetal-plate stage onward is not a prerequisite for gastrulation of embryos of the sea urchin, *Lytechinus pictus*. However, inhibition of cell division before the vegetal-plate stage fails to induce gastrulation. Nislow and Morrill [10] reported that localized proliferation of cells occurs in the vegetal-plate region prior to invagination of embryos of the sea urchin, *Lytechinus variegatus*. These reports suggest the importance of the proliferation of cells in the vegetal-plate region before the vegetal-plate

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stage in *L. pictus* and before or early gastrulation in *L. variegatus* for gastrulation of embryos.

In the present study, we examined the effects of EGIPs on the proliferation of cells in whole embryos and of separated mesomeres and macromeres by counting nuclei that stained with Hoechst dye. We pulse-labeled EGIPs-treated embryos and normal embryos with 5-bromodeoxyuridine (BrdU) and stained cells in S phase using a BrdU-specific antibody to compare regional differences in cell division between normal and EGIPs-treated embryos. Furthermore, we also examined the effects of EGIPs on microtubules and microfilaments since the cytoskeleton plays a key role in cell division.

Our results suggest the importance of the proliferation of the macromeres or blastomeres in the vegetal hemisphere for normal gastrulation since EGIPs suppressed the division of macromeres and of cells in the vegetal hemisphere of intact embryos, with resultant exogastrulation.

## MATERIALS AND METHODS

### *Separation and culture of blastomeres*

Gametes of *Anthocidaris crassispinata* and *Pseudocentrotus depressus* were obtained by injection of 0.5 M KCl into the coelom. Blastomeres were isolated by the method of Livingston and Wilt [9]. In order to facilitate later separation of individual blastomeres, fertilization membranes were removed mechanically by passing the newly fertilized eggs through a small-bore pipette [6]. After washing with normal seawater (SW), the membrane-free embryos were allowed to settle. They were then resuspended in  $\text{Ca}^{2+}$ -free SW and cultured with gentle stirring in  $\text{Ca}^{2+}$ -free SW. At the 16-cell stage under the  $\text{Ca}^{2+}$ -free conditions, the embryos dissociated spontaneously into two different types of blastomere pair: mesomere/mesomere pairs and macromere/micromere pairs. These dissociated blastomeres were placed in  $\text{Ca}^{2+}$ -free SW in a dish coated with 1.2% agarose. Single macromeres were separated from micromeres by passing macromere/micromere pairs several times through a micropipette. The macromeres and the mesomere pairs were separately

transferred to agarose-coated dishes using a micropipette and cultured in Jamalin SW (Jamalin Laboratory, Osaka) at 24°C with or without EGIP-D and a crude fraction of EGIPs that has been prepared by chromatography on DEAE-cellulose and Sephadex G-100 [13] at concentrations equivalent to 5 and 50  $\mu\text{g}/\text{ml}$ , respectively. At these concentrations, EGIP-D and the crude EGIPs were sufficient to induce exogastrulation of 90% of embryos, as described previously [13].

### *Determination of cell number*

To determine the number of cells in embryos and in cultured embryoids, nuclear DNA was stained with Hoechst 33258 (Calbiochem Corp., La Jolla, CA, U.S.A.). Embryos or embryoids were placed in a drop of SW that contained 0.02% Triton X-100 and 2  $\mu\text{g}/\text{ml}$  Hoechst 33258 on a polylysine-coated slide in a moist chamber. Fifteen min later, embryos or embryoids were squashed with a coverglass to facilitate observation of nuclei. The number of cells per embryo or embryoid was estimated by counting nuclei under a fluorescence microscope (EFD2, Nikon, Tokyo).

### *Detection of cells in S phase*

Embryos were pulse-labeled with 5-bromodeoxyuridine (BrdU) (Wako Pure Chemicals, Osaka) which was then detected immunohistochemically by the method of Tanaka [17] with slight modifications. BrdU was added to each suspension of embryos at the mesenchyme-blastula stage, at the early gastrula stage, and at the late gastrula stage at a final concentration of 50  $\mu\text{M}$ . Ten min after the start of incubation with BrdU, the embryos were washed twice with SW to remove the label. Embryos were fixed with 4% formalin in SW at room temperature for 30 min, and then they were placed on polylysine-coated slides. After three washes with phosphate-buffered saline (PBS), pH 8.2, the embryos were transferred in 2 N HCl and incubated for 30 min at 37°C to denature DNA. After three more washes with PBS, 15  $\mu\text{l}$  of a solution of BrdU-specific antibody (Becton-Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) and 35  $\mu\text{l}$  of 0.01% Triton X-100 in PBS were added to embryos, which were then

allowed to stand for 1 hr at 37°C. After three washes of embryos with PBS, 60  $\mu$ l of a solution of fluorescein-conjugated antibodies, raised in goat against mouse IgG (1:50 dilution; Becton-Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.), were added to embryos, which were incubated for another hour at 37°C. After washing with PBS, embryos were observed under a fluorescence microscope.

#### Immunofluorescence staining of microtubules

Embryos were fixed in ice-cold 90% methanol that contained 50 mM EGTA (pH 6.8) for 1 min and then they were placed on polylysine-coated slides. After three washes with PBS, 40  $\mu$ l of a solution of antibody against  $\alpha$ -tubulin (1:500 dilution; Amersham International plc., Little Chalfont, Bucks., U.K.) were added to embryos, which were allowed to stand for 30 min at room temperature. After three washes with PBS, 40  $\mu$ l of a solution of fluorescein isothiocyanate-conjugated (FITC-conjugated) antibodies raised in goat against mouse IgG (1:500 dilution; Cappel, Malvern, PA, U.S.A.) were added to embryos, with further incubation for 30 min at room temperature. After three washes with PBS, embryos were observed under a fluorescence microscope.

For preparation of sections, fixed embryos were embedded in O.C.T. compound (Miles Inc., Elkhart, IN, U.S.A.) and frozen in a deep freezer at -20°C. The embryos were cut at 14  $\mu$ m on a cryostat (Reichert-Jung, Nussloch, F.R.G.) and mounted on polylysine-coated slides. After incubation of slides in 0.5% Triton X-100 for 15 min, immunofluorescence staining was performed as described above.

#### Staining with NBD-phalloidin

Microfilaments were stained by the method of Katow [8] with slight modifications. Embryos were placed on polylysine-coated slides and treated with NBD-phalloidin (Wako Pure Chemicals, Osaka) that has been diluted to 1:15 with permeabilization solution, which was composed of 10 mM EGTA, 0.2 mM dithiothreitol, 0.5% Triton X-100 in 25 mM 2-(N-morpholino)ethanesulfonic acid (Mes; pH 6.7; Dojindo Laboratories, Kumamoto) for 15 min without fixation. After three washes

with PBS, the specimens were observed under a fluorescence microscope.

## RESULTS

#### The effects of EGIPs on cell division

The effects of EGIPs on the proliferation of cells at the early stages of embryonic development were examined in embryos reared in SW with or without a crude preparation of EGIPs. Total numbers of cells were counted at various developmental stages by counting nuclei that has been stained with Hoechst dye (Fig. 1). There was no significant difference in terms of numbers of cells in whole embryos between non-treated control and EGIPs-treated embryos during the early development of embryos ( $P > 0.05$ ). This result suggests that EGIPs do not affect the numbers of cells in whole embryos. However, we cannot exclude the possibility that EGIPs might affect the proliferation of certain restricted blastomeres in the embryos.

Isolated blastomeres were cultured separately in

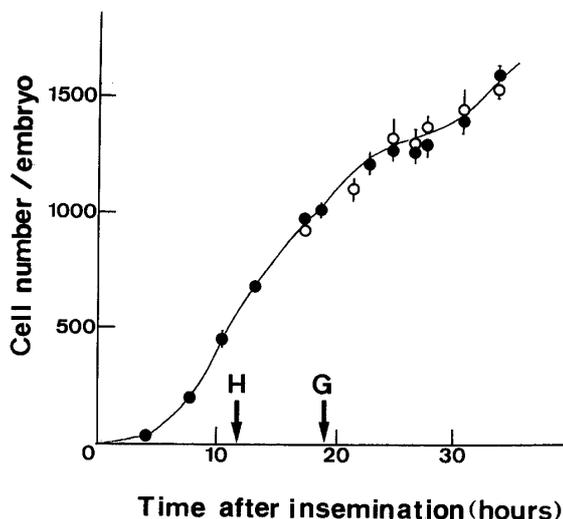


FIG. 1. Effects of EGIPs on the proliferation of cells in whole embryos. *Anthocidaris crassispina* embryos were cultured in the presence (○) or absence (●) of 50  $\mu$ g/ml EGIPs at 20°C, and total cells were counted by counting the number of nuclei that stained with Hoechst 33258 in the developing embryos at various stages. Bars indicate the standard errors ( $n=2-6$ ). H; Hatching; G; the beginning of gastrulation.

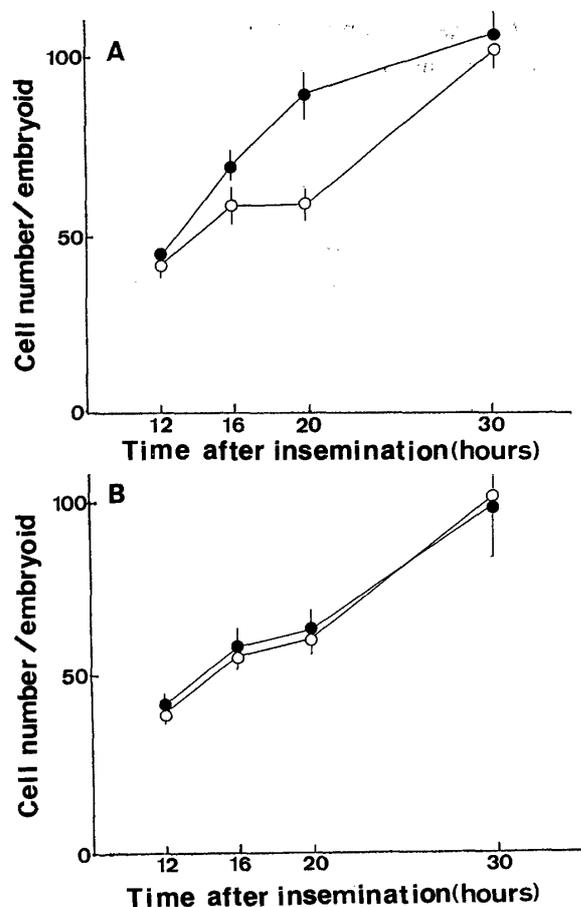


FIG. 2. Effects of EGIPs on the proliferation of isolated blastomeres of *Anthocardaris crassispina* embryos. Isolated macromeres (A) and mesomere pairs (B) were cultured separately in the presence (○) or absence (●) of 50  $\mu\text{g/ml}$  EGIPs at 24°C, and cells were counted as described in the legend to Fig. 1. Bars indicate standard errors ( $n=10-24$ ).

SW with or without EGIPs and the cells were counted by the same method as used for intact embryos (Fig. 2). Cells were counted at the times at which intact normal embryos that has been fertilized at the same time reached the mesenchyme-blastula stage (12 hr), the early gastrula stage (16 hr), the late gastrula stage (20 hr) and the pluteus stage (30 hr).

As shown in Figure 2A, macromeres in the absence of EGIPs divided at a constant rate, doubling in number during the period between 12 hr and 20 hr after fertilization, an indication that all macromeres divided once during this period. By contrast, EGIPs-treated macromeres divided normally until 12 hr after fertilization but increases in cell numbers were significantly reduced both at

16 hr and at 20 hr after fertilization ( $P<0.05$ ), an indication that some macromeres did not cleave or that the cleavage of all macromeres was retarded to some extent during this period. However, the numbers of EGIPs-treated macromeres reached normal values by 30 hr after fertilization (Fig. 2A). The period during which EGIPs suppressed the division of macromeres corresponded to the time at which normal embryos began to gastrulate in the absence of exogenous EGIPs and to the time between the inhibition of gastrulation and the induction of delayed exogastrulation when EGIPs were added exogenously to embryos. By contrast, there was no difference in rates of cell proliferation between non-treated and EGIPs-treated mesomere pairs, as shown in Figure 2B. When pure EGIP-D was used in these experiments, the same results as those of the crude EGIPs were also obtained.

These results show that EGIPs inhibit proliferation restrictively, acting only on macromeres, and transiently, at the time that corresponds to the gastrula stage of normal embryos, but they do not affect the proliferation of mesomeres.

#### *The effects of EGIPs on DNA synthesis in embryos*

The results described above suggest that EGIPs affect the proliferation of macromeres *in vitro*. Therefore, it is important to examine whether EGIPs have effects *in vivo* on the division of macromeres in embryos via, for example, DNA synthesis or changes in the cytoskeleton. First, we investigated the effects of EGIPs on DNA synthesis in embryos at various developmental stages by immunostaining with a BrdU-specific antibody.

In normal embryos at the mesenchyme-blastula stage, cells at S phase were distributed uniformly over entire embryos (Fig. 3A). However, at the early gastrula stage, the number of such cells in the vegetal hemisphere ( $81.2 \pm 8.3$ , mean  $\pm$  S.E.,  $n=5$ ) was larger numbers than that in the animal hemisphere ( $47.2 \pm 7.0$ , mean  $\pm$  S.E.,  $n=5$ ) (Fig. 3C). They were distributed uniformly again over the entire embryo at the late gastrula stage (Fig. 3E).

In EGIPs-treated embryos, cells at S phase were observed to be uniformly distributed over all regions of embryos at all stages examined but normal

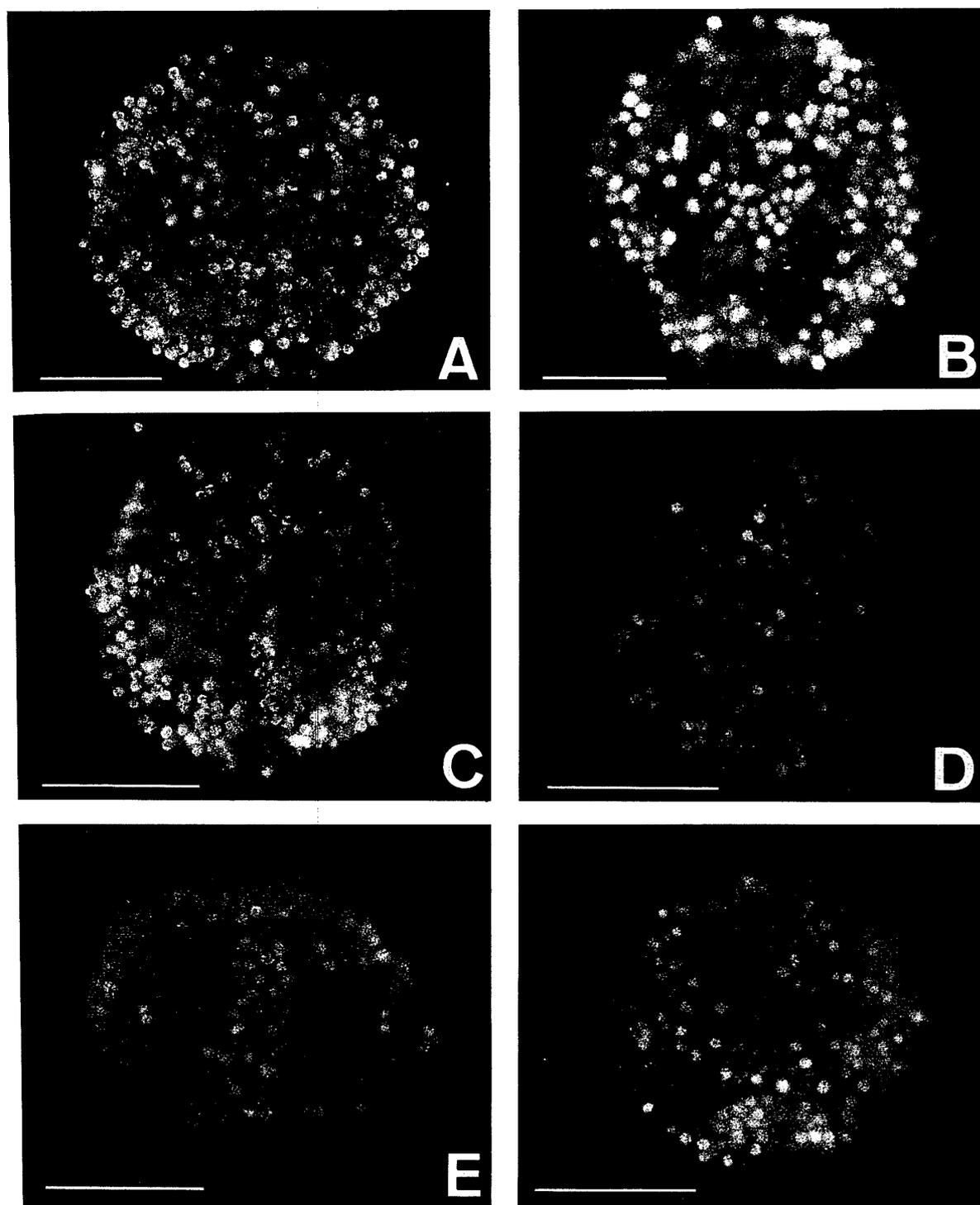


FIG. 3. Effect of EGIPs on DNA synthesis in embryos. Normal embryos (A, C, E) and EGIPs-treated embryos (B, D, F) of *Anthocidaris crassispina* were pulse-labeled with BrdU at various stages of development and immunofluorescence staining was performed 10 min after labeling with BrdU-specific antibody. S phase nuclei appear as white spots in the photographs. A and B, Embryos at the mesenchyme-blastula stage; C and D, embryos at the early gastrula stage; E and F, embryos at the late gastrula stage. Scale bar = 50  $\mu$ m.

invagination was not observed in such embryos (Fig. 3B, D and F). The localized distribution of cells in S phase in the vegetal hemisphere was not

observed in EGIPs-treated embryos at the time at which normal embryos developed to the early gastrula stage (animal hemisphere =  $61.8 \pm 6.6$ ;

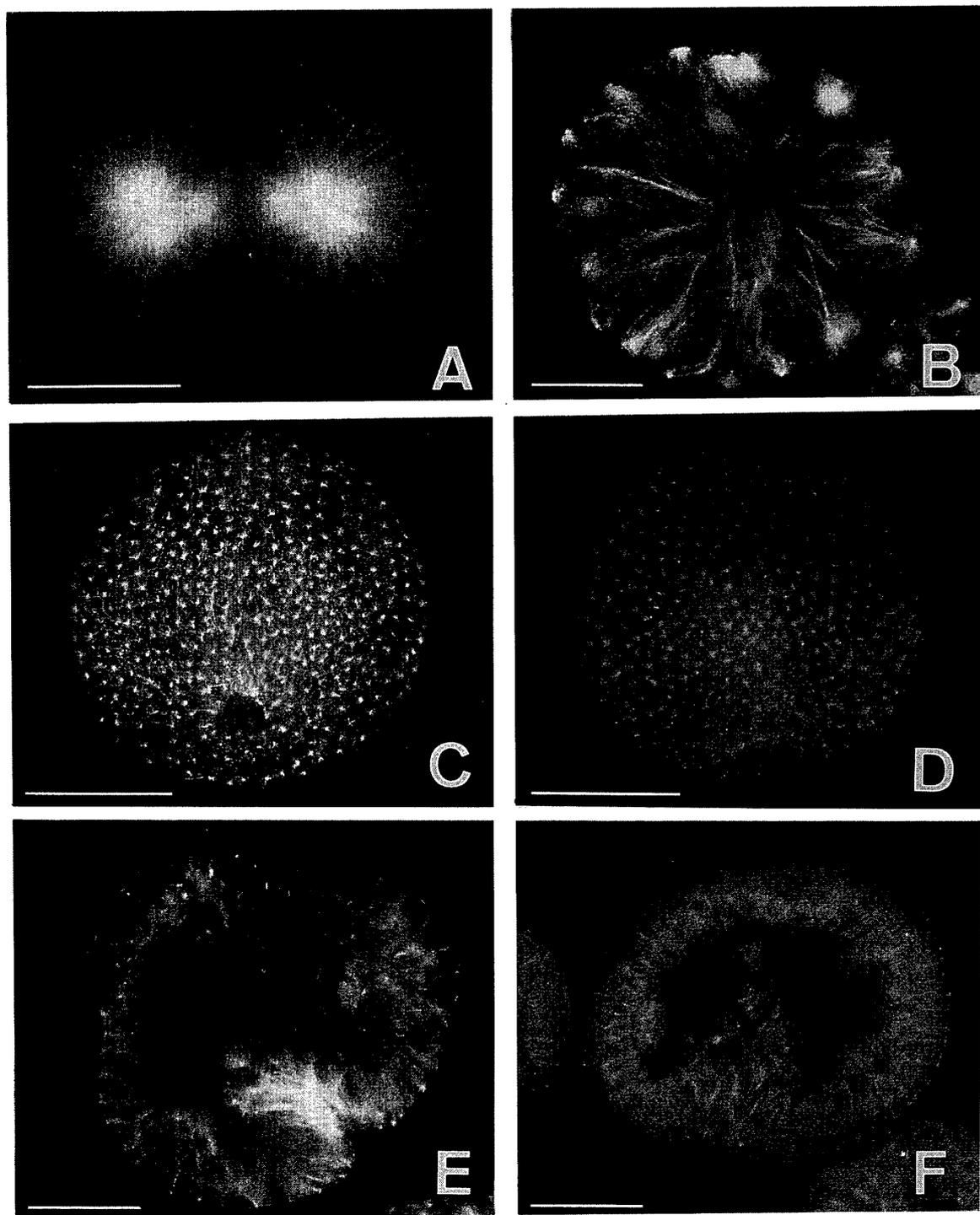


FIG. 4. Immunohistochemical staining of microtubules of embryos of *Anthocidaris crassispina*. Normal embryos at the 2-cell stage were stained with antibody specific for  $\alpha$ -tubulin to confirm its specificity. Specific staining of the mitotic apparatus can be seen in A and B. Normal embryos (C, E) and EGIPs-treated embryos (D, F) at the early gastrula stage were immunostained with the same antibody. C and D, Whole mounts; E and F, sections. Scale bar =  $50 \mu\text{m}$ .

vegetal hemisphere =  $63.8 \pm 7.2$ , mean  $\pm$  S.E.,  $n = 4$ ). These results suggest that local proliferation of cells occurs in the vegetal hemisphere of normal

embryos at the early gastrula stage. EGIPs appear to suppress this proliferation and the formation of the archenteron.

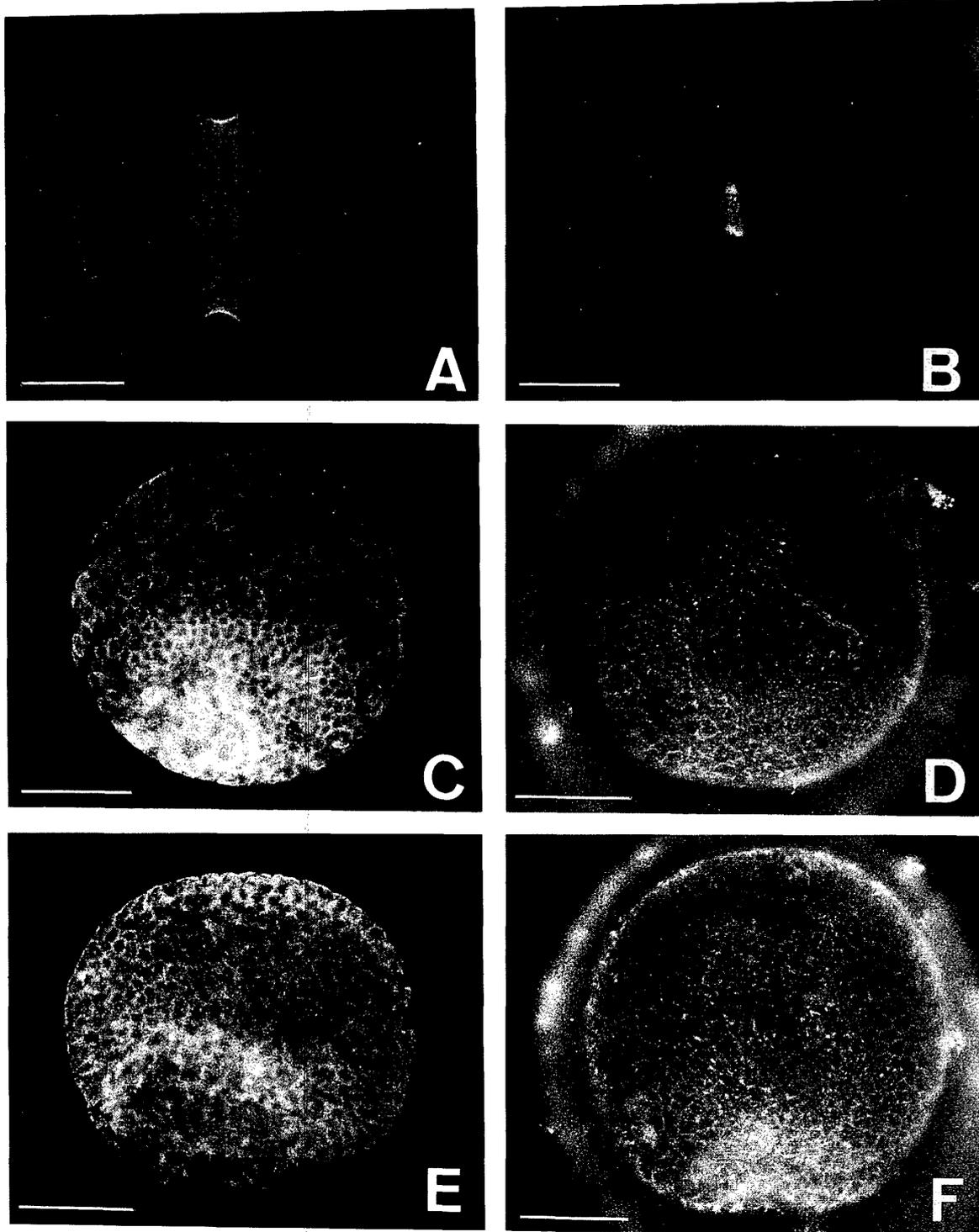


FIG. 5. Staining of microfilaments in embryos of *Pseudocentrotus depressus*. Normal embryos at the cleavage stage were stained with NBD-phalloidin to confirm the specificity of staining. Specific staining of the contractile ring can be seen in A and B. Normal embryos (C, E) and EGIPs-treated embryos (D, F) at the early gastrula stage were stained by the same procedure. Scale bar = 50  $\mu$ m.

The fact that the proliferation of separated macromeres was inhibited by EGIPs in culture *in vitro* reflects the effects of EGIPs on DNA synthesis in embryos since the vegetal hemisphere of embryos is composed of descendants of macromeres.

#### *The effects of EGIPs on the cytoskeleton*

Since cell proliferation and DNA synthesis are affected by EGIPs, the cytoskeleton might also be affected by EGIPs because the cytoskeleton plays a key role in cell division. Therefore, we examined the effects of EGIPs on the behavior of microtubules and microfilaments.

##### a) Microtubules

The distribution of microtubules in normal embryos and EGIPs-treated embryos was examined by immunostaining with an antibody that was specific for  $\alpha$ -tubulin. The staining of sections of normal embryos at the early cleavage stage resulted in specific staining of the mitotic apparatus, confirming the specificity of this antibody for microtubules (Fig. 4A and B).

In whole embryos at the early gastrula stage, the vegetal hemisphere was more strongly stained than the animal hemisphere and the apical side of the invaginating archenteron was more heavily stained in sections of embryos (Fig. 4C and E). EGIPs-treated embryos, examined at the time at which normal embryos developed to the early gastrula stage, were compared with normal embryos. The staining was weak in entire regions of treated embryos and no strong staining of cells in the vegetal hemisphere was apparent in EGIPs-treated embryos (Fig. 4D and F). These results suggest that EGIPs affect microtubules at the early gastrula stage and disturb the normal development of microtubules including spindles in the vegetal hemisphere of the embryos.

##### b) Microfilaments

*Pseudocentrotus depressus* embryos, in which exogastrulation were induced by EGIPs, were used as materials. The effects of EGIPs on the distribution of microfilaments were examined with NBD-phalloidin. When whole mounts of 2-cell embryos were stained with NBD-phalloidin, the contractile ring was stained specifically. This result confirmed the specificity of phalloidin for poly-

merized microfilaments (Fig. 5A and B).

In normal embryos at the early gastrula stage, phalloidin bound to the margins of cells, and the fluorescence of NBD was very intense in the vegetal hemisphere (Fig. 5C and E). In EGIPs-treated embryos at the time corresponding to the early gastrula stage of normal embryos, staining was weak over the entire embryo and only limited staining of the vegetal hemisphere was apparent (Fig. 5D and F). This result suggests that EGIPs affect the distribution of microfilaments at the early gastrula stage.

The mitotic apparatus and microfilaments at various stages other than the early gastrula stage appeared not to be affected by EGIPs (data not shown).

## DISCUSSION

The purpose of the present study was to obtain the clues to the mechanism responsible for normal gastrulation, by studying the mechanism of induction of exogastrulation which is evoked when EGIPs are added exogenously to the sea urchin embryos.

In our present study, we examined the effects of EGIPs on the proliferation of cells in whole embryos and of separated blastomeres by counting nuclei stained with Hoechst dye. Furthermore, we also examined regional differences in cell division between normal and EGIPs-treated embryos by pulse-labelling embryos with BrdU and detecting cells in S phase. We found that EGIPs suppress the proliferation of separated macromeres transiently, at the time that corresponds to the early gastrula stage of normal embryos. These results seem to be correspondence with those obtained by labelling experiments that locally enhanced proliferation of cells occurs in the vegetal hemisphere of normal embryos at the early gastrula stage and that EGIPs suppress the localized proliferation of cells at the early gastrula stage.

Macromeres participate in formations of the vegetal plate and a part of ectoderm, and a vegetal half of macromeres contributes to the formation of the vegetal plate. However, in the present study, it is very difficult to distinguish how many and which macromeres participate in formation of the vegetal

plate. The numbers of cells that participate in formation of the vegetal plate occupy about 13% of blastomeres at the 60-cell stage [2, 11]. Furthermore, the vegetal plate cells could occupy only below 10% of total blastomeres following cleavages, because division of the cells that participate in formation of the vegetal plate were suppressed by EGIPs, as shown in Figure 3D. These low percentages of each founder cell might be a reason why the effects of EGIPs on cell proliferation could be detected only in a separated culture of isolated blastomeres.

Nislow and Morrill [10] proposed that two mechanisms are at work during gastrulation: regionalized proliferation of cells occurs during the early stage (before invagination of *Lytechinus variegatus*) of gastrulation and is followed by rearrangement of cells during secondary elongation of archenteron, as demonstrated by Ettensohn [3], Hardin and Cheng [4] and Hardin [5]. This regional cell proliferation seems to occur with different timings depending on species. Stephen *et al.* [12] used aphidicolin to inhibit the cell division before the vegetal plate stage led to the arrest of gastrulation in embryos of *Lytechinus pictus*. In the present study with *A. crassispina*, localized proliferation of cells in vegetal hemisphere was observed at the early gastrula stage and was essential for normal gastrulation because the inhibition of cell division by EGIPs was effective to induce exogastrulation.

The development of EGIPs-treated embryos proceeds in a similar manner to and simultaneously with that of normal embryos as far as the blastula stage. However, EGIPs-treated embryos appear to "hesitate" to invaginate at the stage when normal embryos initiate their invagination, the treated embryos at the late blastula stage swell and the swollen state persists for several hours while control embryos in normal SW almost complete their gastrulation [7]. Then the treated embryos exogastrulate some time after the gastrulation of normal embryos. The delay in the morphogenetic movement of the vegetal-plate in the EGIPs-treated embryos seems to correspond to the period during which EGIPs suppress the proliferation of cells in vegetal hemisphere. These observations suggest that this proliferation of cells

is essential for gastrulation.

This suggestion is supported by our observations of the cytoskeleton from the blastula stage to the early gastrula stage. Immunohistochemical staining of the cytoskeleton indicated that development of microtubules was more active in the vegetal area than in the animal-pole area in normal (control) embryos at the early stage of gastrulation, whereas EGIPs suppressed this enhanced activity of microtubules. The staining with NBD-phalloidin showed a stronger staining at the vegetal hemisphere and suppression with EGIPs. Therefore, it seems to be conceivable that the transient proliferation of cells occurs as a result of active formation of the cytoskeleton in the vegetal hemisphere at the early stage of gastrulation and that exogenous EGIPs inhibit such proliferation of cells by disturbing the development of the cytoskeleton.

These effects of EGIPs must be mediated by receptors in surface membranes since, in an assay *in vitro*, EGIPs from sea urchins did not affect the polymerization of purified tubulin from porcine brain or of purified actin from rabbit striated muscle (unpublished data). Therefore, it is now important to elucidate the localization and stage-specificity of receptors or binding proteins for EGIPs, especially on macromeres, during the development of sea urchin embryos.

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