Selective inhibition of gastrulation in the starfish embryo by albuside B, an inosine analogue

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Abstract External application of 0.2–100 μg/ml albuside B inhibits gastrulation of the starfish (*Asterina pectinifera*) embryo. Treated embryos retain the late blastula morphology with the vegetal plate. However, the vegetal plate is unreactive to soybean agglutinin, a probe for observing the progenitor cells of the archenteron (mesendoderm) in a normal embryo. The effective period of the treatment is limited from 4 to 6 h after fertilization, a period immediately before the onset of blastulation. RNA synthesis is unaffected during the period of sensitivity. The selectivity of the inhibition shows that albuside B may be a useful tool for studying the mechanisms of mesendoderm differentiation.

Key words: Gastrula; Mesendoderm; Albuside B; Soybean agglutinin; *Asterina pectinifera*

1. Introduction

Gastrulation is one of the fundamental events in embryogenesis [1]. The starfish embryo is suitable material for studying the molecular mechanisms of gastrulation, because the geometry of gastrulation is simple; the endoderm and the mesoderm form in sequence starting with a simple hollow blastula. In the starfish embryo, the early cleavage stage (1- to 128-cell stages) is followed by blastulation in which loosely arranged embryonic cells enwrapped in the fertiliization envelope pack themselves into a sheet and further into a hollow blastula [2]. After the blastula hatches from the fertilization envelope, the stage is set for the coordinated movements of gastrulation the process which transforms the simple hollow ball of epithelial cells into a multilayered structure with a mesendodermal archenteron produced by tucking cells from the exterior into the interior [3]. Meanwhile, cells escape from the tip of the archenteron and move into the blastocoel to form motile mesenchyme cells.

There are few, if any, specific inhibitors of gastrulation. The present study was initiated in an effort to detect and characterize a selective inhibitor of gastrulation of the starfish (*Asterina pectinifera*) embryo as a first step towards understanding the molecular basis of cellular differentiation accompanying gastrulation in the starfish embryo. We have found that fluorescein-isothiocyanate (FITC)-conjugated soybean agglutinin (SBA) specifically stains the progenitor cells of the mesendoderm just prior to gastrulation. The finding that albuside B (4-oxo-7-β-D-ribofuranosyl)-3*H*-furo[3,2-*d*]pyrimidine; Fig. 1a), an inosine analogue [4], prevents both mesendoderm differentiation and gastrular morphogenesis indicates that these two developmental events are closely related.

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2. Materials and methods

2.1. Preparation of albuside B

Albuside B was converted from albuside (4-amino-7-(β-D-ribofuranosyl)-furo[3,2-*d*]pyrimidine; Fig. 1b) obtained from a culture broth conditioned by the actinomycete *Sterptomyces albus* [4]. Albuside (10 mg) was incubated at 25°C for 2 h in 1.2 ml of 5 mM potassium phosphate buffer (pH 7.4) containing 0.2 mg of 200 units/mg calf intestinal adenosine deaminase (EC 3.5.4.4, Boehringer Mannheim). The reaction mixture was subjected to high-performance liquid chromatography (HPLC) on an ODS-5 column (YMC, Kyoto) using 20% (v/v) methanol as an eluent. The fraction which showed uv maxima at 219 nm and 259 nm (water, pH 7.0) eluting at the retention time of 0.76 relative to that of albuside was collected. Evaporation of the solvents gave a residue (9.0 mg), which was recrystallized from ethanol to afford pure albuside B as colorless needles (melting point, 115°C) [4].

2.2. Cultivation of embryos

Specimens of *A. pectinifera* were collected from coastal waters off Japan during their breeding season and kept in artificial sea water (ASW; Jamarin Laboratory, Osaka) (15°C) in laboratory aquaria. Spawning was induced in vitro by immersion of gonadal fragments in ASW containing 1.0×10^{-6} M 1-methyladenine (Sigma) [5]. Spawned eggs were inseminated during 40–60 min after the start of 1-methyladenine treatment. Fertilized eggs were cultured at 20°C in ASW containing 5 mg/ml streptomycin sulfate and 50 μg/ml penicillin G. To locate FITC-SBA-reactive cells in an embryo, embryos cultivated in the presence or absence of albuside B were fixed for 10 min in cold methanol and washed three times in phosphate-buffered saline (PBS) at room temperature. Specimens were rinsed in PBS and incubated for 15 min in PBS containing 10 μg/ml FITC-SBA (Sigma). Following rinsing in PBS, specimens were observed under UV epifluorescent illumination.

2.3. Incorporation of [^{3}H]uridine into UTP and RNA

Fifty thousand fertilized eggs were incubated beginning 2 h after fertilization in 10 ml of ASW containing 190 kBq [5,6-^{3}H]uridine (2.0 TBq/mmol, Amersham) with or without albuside B. At given times, aliquots were removed and the embryos were washed with cold (4°C) ASW and fixed in cold 5% (w/v) trichloroacetic acid (TCA). Radioactivity in aliquots of acid-soluble fractions was measured as described previously [6]. Radioactivity incorporated into UTP was measured by counting the UTP fraction, which was separated from other acid-soluble fractions by HPLC as described [6].

For extraction of RNA, 5,000 embryos (volume: 30 μl) were collected, fixed in 5% (w/v) TCA and then washed three times with 5% (w/v) TCA. They were suspended in 3 ml of 0.5 N NaOH and digested at 37°C overnight. Then 0.3 ml of 100% (w/v) TCA was added and the acid-soluble radioactivity was counted as described previously [6]. From these data the amounts of UMP incorporated into RNA were calculated.

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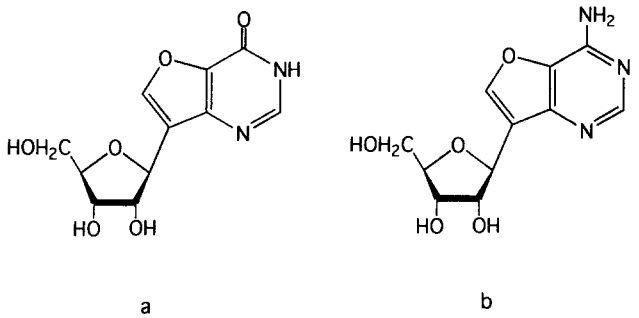

Fig. 1. Structures of albuside B (a) and albuside (b).

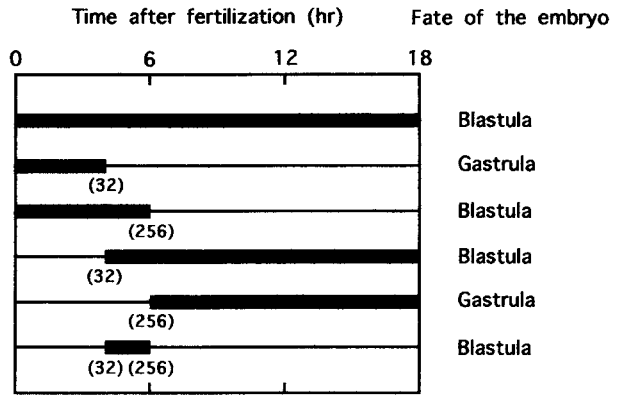

Fig. 3. The fates of *Asterina pectinifera* embryos that were cultured in the presence of 10 μg/ml albuside B for the periods indicated by bold lines and in the absence of albuside B for the periods indicated by thin lines. Numerals in the parentheses indicate the number of cells in each embryo.

3. Results

3.1. Effects of albuside B on starfish embryonic development

Fertilized *A. pectinifera* eggs were placed in ASW with or without 0.2 μg/ml albuside B immediately following fertilization. Cleavages of both albuside B-treated and control embryos progressed normally at almost the same rate up to the blastula stage (6 h after fertilization). After this time, however, the formation of cilia was delayed in the albuside B-treated embryos: rotation of control embryos began at 12 h post-fertilization whereas albuside B-treated embryos started to rotate at 15 h. Control embryos underwent hatching and gastrulation at 14 and 16 h, respectively, while albuside B-treated embryos hatched at 18 h and progression of development was invariably arrested at the late blastula stage showing the appearance of vegetal thickening (Fig. 2).

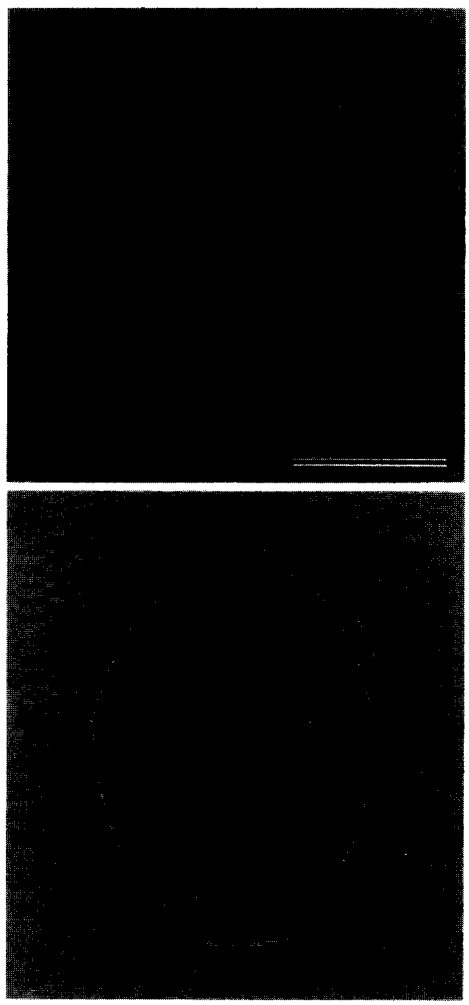

Fig. 2. Albuside B-induced blockage of development of *Asterina pectinifera* embryos at the late blastula stage. Embryos were cultured in the presence (A) or absence (B) of 10 μg/ml albuside B for 24 h after fertilization. Scale bar = 100 μm.

Next, the effect of a much higher concentration of albuside B on embryonic development was examined. It was found that in cultures containing 100 μg/ml albuside B, the arrest was observed at the same stage as in embryos cultured in 0.2 μg/ml albuside B. Therefore, it is unlikely that the rate of albuside B uptake in embryonic cells before the midblastula stage is too low to cause any apparent disturbance of development.

To localize the point in development where albuside B arrests the embryo, albuside B was added to embryonic cultures to give a final drug concentration of 10 μg/ml immediately following fertilization and then, at intervals, an aliquot of the culture was washed in ASW and cultured without albuside B up to 22 h. Embryos given albuside B from the time of fertilization up to 4 h after fertilization (32-cell-stage) and then returned to ASW without albuside B developed normally to the gastrula stage (Fig. 3). However, exposure to albuside B for more than 6 hr from the time of fertilization (256-cell-stage) prevented gastrulation, resulting in the eventual arrest of development at the late blastula stage. In other experiments, albuside B was introduced at later stages of development. When albuside B treatment was started at any time before 4 hr after fertilization (32-cell-stage), the treated embryos never developed beyond the early gastrula stage. On the other hand, addition of albuside B at 6 h after fertilization (256-cell-stage) did not inhibit gastrulation. Furthermore, only 2-h exposure of albuside B starting at 4 h after fertilization was sufficient to arrest the embryos (Fig. 3). Three separate experiments gave the same results, which indicate that the embryo was sensitive to albuside B for only a short period immediately before blastulation and an irreversible change

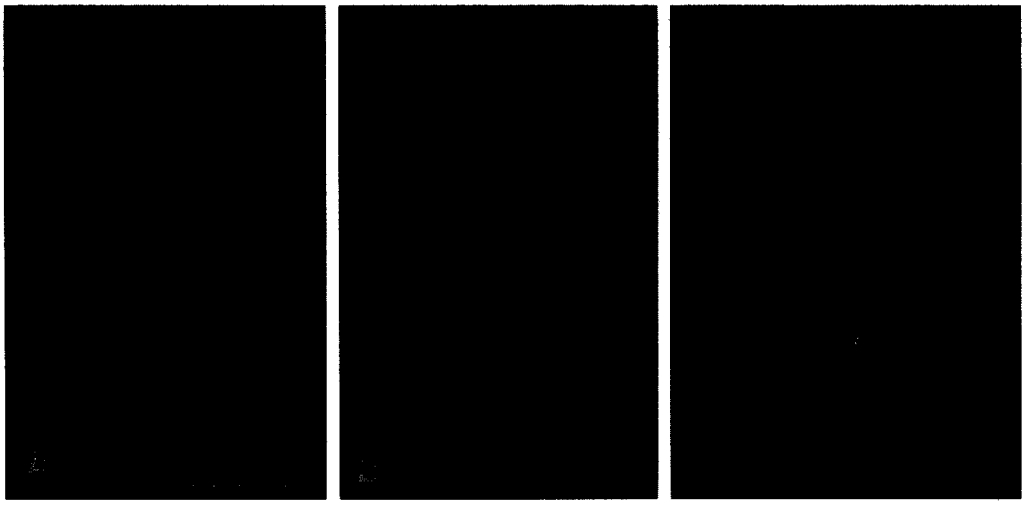

Fig. 4. Albuside B-induced blockade of mesendoderm differentiation of *Asterina pectinifera* embryos at the late blastula stage. Fertilized eggs were cultured in the presence (A) or absence (B, C) of 10 μg/ml albuside B from fertilization. At 16 h (B) or 24 h after fertilization (A,C), the embryos were fixed in methanol and stained in PBS containing FITC-labeled soybean agglutinin and were observed by fluorescence microscopy. Scale bar = 100 μm.

induced by albuside B during this period was sufficient to prevent later developmental arrest.

3.2. Effects of albuside B on mesendoderm differentiation

The above results suggest that the albuside B block point can be localized to mesendoderm differentiation. To address this possibility, we searched for FITC-labeled lectins capable of staining the mesendoderm of the early gastrula without reacting to the ectoderm. It was found that FITC-SBA can be used as a probe of differentiation of the mesendoderm. As shown in Fig. 4C, FITC-SBA stained the archenteron of the control gastrula whereas the ectoderm was unreactive to the lectin molecule. FITC-SBA stained the progenitor cells of the archenteron in the vegetal plate [7] of the control blastula (Fig. 4B) whereas no fluorescence was observed in the vegetal plate of the albuside B (10 μg/ml)-treated embryo at the same stage of development (Fig. 4A). These results show that albuside B not only acts as a selective inhibitor of gastrular morphogenesis but also prevents the formation of the progenitor field for the mesendoderm [7]. To the best of our knowledge, this is the first report on the occurrence of a selective inhibitor of mesendoderm differentiation.

3.3. Effect of albuside B on RNA synthesis of starfish embryos

We examined the effect of albuside B on the rate of RNA synthersis in early embryos. Fifty thousand embryos at the 4-cell stage (2 h after fertilization) were cultured in 1 ml of ASW containing [^{3}H]uridine with or without 10 μg/ml albuside B. Aliquots of the embryo suspension were collected at intervals, and the radioactivity incorporated into the RNA fraction and the specific radioactvities of [^{3}H]UTP in the albuside B-treated embryos were measured. From these data, amounts of UMP incorporated into RNA were calculated. It was found that although the rate of RNA synthesis in the albuside B-treated embryo at 8 h and 10 h after fertilization was 32% and 49% of that of the control embryo, respectively, RNA synthesis during the critical albuside B-sensitive period, from 4 to 6 h after fertilization, was little affected (Fig. 5).

4. Discussion

Barros et al. [8] introduced actinomycin D into cultures of starfish (*Asterias forbesii*) embryos to analyze whether new RNA transcription is required for gastrulation. Because actinomycin D intercalates into the DNA molecule, it has been widely

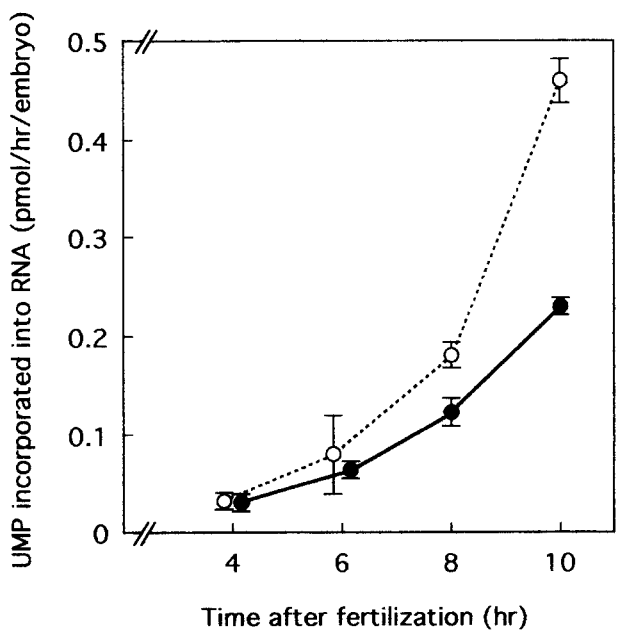

Fig. 5. Amounts of RNA synthesized in *Asterina pectinifera* embryos that were cultured in the presence (●) and absence (○) of 10 μg/ml albuside B. The amount of UMP incorporated into the RNA fraction was determined as described in the text. Vertical bars represent the S.E.M. values (n = 3).

used as an inhibitor of RNA synthesis. They found that a very narrow range of concentartions, 0.5 to 2 μg/ml, suppress gastrulation but that higher concentrations are lethal. The actinomycin D sensitive period was from the time of fertilization to the onset of ciliary movement. Treatments started after the onset of ciliary movement were ineffective in preventing gastrulation. It has been known that DNA replication and DNA strand separation after replication are also affected by actinomycin D [9]. We have shown previously that fertilized *A. pectinifera* eggs cultured in actinomycin D undergo cell division, despite an almost total absence of DNA synthesis from the 32-cell stage up to the 512-cell stage [9]. In the presence of actinomycin D, association of chromosomes with the mitotic apparatus is interrupted. Therefore, chromosomes are not distributed in newly formed blastomeres at each cleavage, resulting in formation of blastomeres that are devoid of nuclei and chromosomes. However, they are still capable of dividing through the formation of spindles and asters. The stage when achromosomal division ceases and embryos begin to die corresponds to the 512-cell stage and the embryos never blastulate. We next examined the effect of other type of RNA synthesis inhibitors on morphogenetic events and RNA synthesis. We found that formycin, an analogue of adenosine, added to a culture of starfish embryos interfered with the formation of UTP and CTP, thereby inhibiting RNA synthesis and halting embryonic development at the early blastula stage. It was notable that the nucleus was present in each blastomere of the development-arrested embryo [6]. Similar results were obtained with albuside [4]. These results strongly suggest that the majority of mRNAs necessary for blastula formation are stored in the egg and that transcripts newly synthesized before and during blastulation are required for development to proceed from the early bastula stage.

In the present study, we have searched for nucleosides capable of arresting the embryonic development of starfish embryos at the late blastula stage and found that albuside B is one such compound. Our data demonstrate that albuside B does not affect overall inhibition of RNA synthesis during the albuside-B sentitive period. However, this does not exclude the possibility that a small amount of transcription could have been suppressed by albuside B, thereby depriving the embryo of a certain critical amount of the mRNAs that control gastrulation. At present, we have no data indicating that albuside B interferes with the expression of specific morphogenetically significant genes.

In the sea urchin embryo, it has been shown that initial specification of the progenitor field for the archenteron, i.e., the vegetal plate of the blastula stage, occurs during cleavage [7]. There is good evidence that any of the blastomeres of the embryo have the capacity to turn into gut territory founder cells. LiCl treatment causes an isolated animal half of an embryo normally destined to become ectoderm founder cells to differentiate as gut [10]. Treatment of whole embryo with LiCl results in a pattern of differentiation in which an archenteron is exaggerated, and the surface epithelium is reduced. Kominami [11] showed that treatment of *A. pectinifera* embryos with LiCl produced such 'vegetalized' embryos and that the effective peroid of the treatment is limited between 7 to 10 h of development, i.e. just after the albuside B-sensitive period. Treatment with LiCl was incapable of reversing the effect of albuside B, suggesting that the site of action of LiCl differs from that of albuside B (unpublished results).

The present study has revealed that FITC-SBA specifically stains the progenitor cells of the archenteron in the vegetal plate of the late blastula. The nature of the SBA-reactive molecules remains to be determined. In this regard, it is notable that the rate of N-linked glycoprotein synthesis is low in the early stages of sea urchin development and markedly increases at the late blastula-early gastrula stages [12]. In the presence of tunicamycin, an inhibitor of N-linked glycoprotein synthesis, development proceeds normally until the gastrula stage, when the embryo arrested [12]. It was shown that tunicamycin blocks *A. pectinifera* embryogenesis at variious stages of development depending on the concentrations used [13]. It seems possible that tunicamycin at a concentration to block gastrulation may prevent the formation of FITC-SBA-reactive *N*-glycoproteins in the vegetal plate. We wish to understand how the synthesis of the glycoproteins is controlled and whether or not gastrulation would be a downstream event of their biosynthesis. We feel that although difficult, this will be the most productive approach to understand the mode of action of albuside B to block gastrular morphogenesis.

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References

[1] Gilbert, S.F. (1991) Developmental Biology, 3rd Edn., Sinauer, Sunderland.
[2] Dan-Sohkawa, M. and Fujisawa, H. (1980) Dev. Biol. 77, 328–339.
[3] Kuraishi, R. and Osanai, K. (1992) Biol. Bull. 183, 258–268.
[4] Ikegami, S., Isomura, H., Tsuchimori, N., Hamada, K., Kobayashi, H., Kojima, Y., Osano, Y.T., Kumazawa, S. and Matsuzaki, T. (1992) Anal. Sci. 8, 897–898.
[5] Tsuchimori, N., Miyashiro, S., Shibai, H. and Ikegami, S. (1988) Development 103, 345–351.
[6] Isomura, H., Itoh, N. and Ikegami, S. (1989) Biochim. Biophys. Acta 1007, 343–349.
[7] Davidson, E.H. (1993) Development 118, 665–690.
[8] Barros, C., Hand, JR., G.S. and Monroy, A. (1966) Exp. Cell Res. 43, 167–182.
[9] Ikegami, S., Ozaki, Y., Ooe, Y. and Itoh, N. (1991) Dev. Growth Differ. 33, 193–200.
[10] Livingston, B.T. and Wilt, F.H. (1989) Proc. Natl. Acad. Sci. USA 86, 3669–3675.
[11] Kominami, T. (1984) J. Embryol. Exp. Morph. 84, 177–190.
[12] Heifetz, A. and Lennarz, W.J. (1985) J. Biol. Chem. 254, 6119–6127.
[13] Dan-Sohkawa, M., Tamura, G. and Mitsui, H. (1980) Dev. Growth Differ. 22, 495–502.