Effects of Okadaic Acid on Embryonic Development of the Starfish Asterina pectinifera

Susumu Ikegami, Noboru Kajiyma, Yoshihiro Ozaki, Yasunori Ooe, Takahiko Shimizu, Toshihiko Kasahara, Daisuke Uemura,* and Masaki Shiota**

Department of Applied Biochemistry, Hiroshima University, 4-4, Kagamiyama 1-chome, Higashi-hiroshima-shi, Hiroshima 724, Japan
*Faculty of Liberal Arts, Shizuoka University, Ohyaa, Shizuoka 422, Japan
**Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

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The effects of okadaic acid, an inhibitor of protein phosphatases types 1 and 2A, from the sponge Halichondria okadai Kadota, on the embryonic development of the starfish Asterina pectinifera, were investigated. When cultured in okadaic acid from fertilization, the embryo divided synchronously without any abnormalities such as lysis, swelling or morphological changes different from control embryos up to the 32-cell stage. However, okadaic acid prevented development before the onset of blastulation. Cytological examination showed that chromosomes condensed but did not align in a plane in the mitotic apparatus in each of the blastomeres of the treated embryo at the sixth cleavage, suggesting that this was the root cause of the arrest of further development.

There are many marine natural products that inhibit cell division of eukaryotes. However, most of them have a broad spectrum of activity, most often accompanied by non-specific toxicity toward mammalian cells. It was therefore desired to adopt an assay method which is specific or selective to a particular activity as well as simple. In recent years, we have devised an assay system using starfish embryos to discover specific antimitotic agents from microbial metabolites and marine animals. 1) This assay method can detect DNA synthesis inhibitors, RNA synthesis inhibitors, and agents affecting other functions of chromatin than replication and transcription.

In fertilized eggs of the starfish Asterina pectinifera, cell division proceeds rapidly and nearly synchronously without growth for a total of eight to nine cleavages. 2) Completion of these rapid cleavage periods is followed by the immediate activation of a new developmental program, blastulation. Blastulation involves active morphological movement of embryonic cells, during which cells acquire the highly cooperative nature of epithelial cells and become packed into a sheet, which forms a sphere. 3)

In this paper we describe the results of screening for substances capable of arresting starfish embryonic development at the preblastulation stage. It was found that an extract of the sponge Halichondria okadai Kadota, contained a substance with such activity, which was identified as okadaic acid. 4) The mode of action of okadaic acid on the starfish embryonic development was also examined in relation to the function of chromatine in the embryonic cell.

Materials and Methods

Materials. Okadaic acid and norhalichondrin A were prepared as described. 5) 1-Methyldenediene, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), histone H1 (type III-S), and streptomycin sulfate were purchased from Sigma Chemicals. Penicillin G was from P. L. Laboratories. The sea water of a normal composition (NSW) and calcium-free sea water (CaFSW) were obtained from Jamarin Laboratories. Phosphate-buffered saline without divalent cations (PBS) was purchased from Nissui Pharmaceutical Co. Bisbenzimide (Hoechst-33342) was obtained from Calbiochem-Behring. Gamma-[32P]ATP (3,000 Ci/mmole) was from NEN. Sephadex LH-20 and G-10 were from Pharmacia, and an ODS R-5 column for high performance liquid chromatography (HPLC) was from YMC. Plates for thin layer chromatography (TLC) were Kieselgel 60F254 and RP2 F254 from Merck. Specimens of sponges were collected off Ooshima Island in Yokosuka Prefecture and off Shionomisaki in Wajima Prefecture in July, 1986, off Kuchinoerabu Island and Kuchinoshima Island in Kagoshima Prefecture on July, 1987, off Okinoshima Island in Koki Prefecture in December in 1987, off Mitsukai, Kamagi and Hibi Island in Ehime Prefecture on July, 1988, and off Okinoshima Island and Yokosuka Island in Koki Prefecture on July, 1988. The specimens were kept frozen at –20°C until use. Specimens of A. pectinifera were collected along coasts of various parts of the Japanese Islands during their breeding seasons and kept in running NSW at 15°C in laboratory aquaria.

Bioassay. Pieces of ovarian fragments were dissected from female A. pectinifera, washed with NSW, and then treated with 1-methyldenediene at a final concentration of 150 μmol/l. The released mature oocytes were washed twice with NSW and inseminated 40–60 min after the start of 1-methyldenediene treatment. The inhibitory activity of the sponge extract and various fractions purified from it was measured by adding a small number of fertilized eggs of A. pectinifera to serially diluted sample solutions and observing the cytological changes in the embryos cultured at 20°C as described. 6) One unit of activity was defined as the maximum volume (in ml) of a solution which showed sufficient effectiveness in arresting development at the preblastulation stage.

Staining of chromatine. For observations of chromatine, samples of the embryos were checked by fluorescence microscopy after they were fixed and stained with methanol (4°C) containing 10 μg/ml bisbenzimide as described previously. 7)

Cell counts. Embryos were fixed overnight at room temperature in CaFSW containing 3.5% formalin and 0.25% ethylenediaminetetraacetic acid disodium salt. Fixed embryos were stored at 4°C in CaFSW containing 3.5% formalin. A small number of the embryos were placed onto the surface of a glass slide coated with 0.01% poly-l-lysine. The fertilization envelope was removed from each embryo with a fine needle and the cells of each embryo were spread over the surface of the glass. The attached cells on the glass were covered with a drop of a solution containing 0.01 μg/ml DAPI, 0.14 μM NaCl, 0.16 M boric acid, and 0.35 M NaOH and left for 30 min at room temperature. The cells on the glass were washed three times with 1 ml of PBS, air-dried for 10 min, immersed in 10 μl of PBS containing 10% glycerol, and the number of nuclei or
areas of condensed chromatin was counted under a fluorescence microscope.

Histone H1 kinase activities. Histone H1 kinase activities of embryos were measured by the method of Picard et al., with slight modifications. Groups of embryos were rapidly washed (within 10 sec) in an excess of buffer containing 50 mM β-glycerophosphate, 15 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10 mM MgCl2, and 0.7 mM dithiothreitol (DTT) at pH 7.3; then the groups of embryos were taken into a small volume of buffer and frozen in liquid nitrogen. Immediately after thawing, which disrupted the embryos, an identical volume of a mixture containing 100 μM gamma-[32P]ATP (1,500 cpm/μmol), 10 mM MgCl2, and 2 mg/ml histone H1 was added. After a 5-min incubation at 21°C, the reaction was stopped by addition of a mixture containing 30% urea-8% DTT-5% sodium dodecyl sulfate (weight per volume) in 0.5 mM Tris-HCl, pH 6.8, and the proteins were separated by SDS-polyacrylamide gel (PAGE). Protein was measured as described by Lowry et al. using bovine serum albumin as a standard. Finally, the parts of the gels corresponding to H1 histones were cut out and counted by liquid scintillation.

Results

Screening of sponge extracts by the starfish embryo assay

We searched for sponge metabolites capable of arresting the embryonic development of fertilized starfish eggs at the preblastulation stage. Approximately 100 different sponge specimens were collected by SCUBA from several areas along the coasts of the south-western part of the Japanese Islands. One gram each of the sponge specimens was macerated and immersed overnight in 3 ml of methanol. Each extract was diluted 20 times with ASW to make a dilute sample solution. A small number of fertilized starfish eggs were placed in the diluted sample solution immediately after fertilization. Fertilized eggs in 26 of the different diluted sample solutions were unable to reach the blastula stage, while the other diluted sample solutions did not affect embryonic development up to the gastrula stage. When diluted 10-fold, half of the active extracts caused lysis or morphological abnormalities of the fertilized eggs, which were incapable of undergoing the first cell division. Eventually, 5 extracts produced inhibition of embryonic development over a range of 10- to 40-fold dilution at stages shortly before the onset of blastulation, but did not affect early cleavages. One specimen was identified as Halichondria okadai Kadota.

Purification and identification of inhibitors of A. pectinifera development

Several biologically active substances have been isolated from the sponge H. okadai Kadota. To identify the component of the sponge species with such a specific activity against starfish embryos, we purified the active principle from the methanol extract guided by the starfish embryo assay, as outlined in Fig. 1. From 1 kg of the sponge specimen, 30 mg of an oily solid was obtained as an active substance, which was tentatively designated substance A and which inhibited embryonic development specifically at the morula stage at a concentration of 8 μg/ml or greater. The purity of substance A was confirmed by TLC using a Kieselgel 60F254 plate, which was developed with benzene-methanol (3:1, volume by volume). When the plate was irradiated with UV light at 254 nm, substance A made a single spot at Rf 0.36, the value of which was identical to that of authentic okadaic acid. Furthermore, TLC using an RP2 F254 plate, which was developed with acetonitrile-water (1:1, volume by volume), gave a single spot at Rf 0.65 after the plate was sprayed with a 98:2 mixture of 1% p-ansai-1,2-dihydroxy in acetic acid and concentrated sulfuric acid followed by heating at 90°C for 10 min. The spot was pink, as is that of okadaiic acid. The chemical shifts in the 1H NMR spectrum of substance A were identical to those reported for okadaic acid. Furthermore, the inhibition by authentic okadaic acid of the development of A. pectinifera embryos was the same as that of the isolated substance A.

During the course of purification, we found that a fraction separated from substance A prevented the first division of fertilized A. pectinifera eggs. We isolated this inhibitory substance as outlined in Fig. 1. From 1 kg of the sponge specimen, 0.15 mg of an oily solid was obtained as an active substance, which we tentatively designated substance B. Substance B inhibited the first division of fertilized A. pectinifera eggs at a concentration of 50 μg/ml or greater. The purity of substance B was confirmed by TLC and HPLC. Substance B and authentic norhalichon-
drin A showed the identical $R_f$ value of 0.62 on a Kieselgel 60F plate, which was developed with n-propanol–ammonia water–water (6:3:1, volume by volume), followed by spraying with 2,4-dinitrophenylhydrazine in methanol. Furthermore, the retention volume of substance B was the same as that of the authentic sample of norhalichondrin A on HPLC using an ODS column and 20% aqueous methanol as an eluent. The inhibitory activity of authentic norhalichondrin A on the development of *A. pectinifera* embryos was the same as that of the isolated material. Fragmentation of fertilized eggs that had been cultured in 50 μg/ml norhalichondrin A from fertilization took place when the control embryos reached the 64-cell stage (5 hr after fertilization). This result showed that norhalichondrin A had activities other than interference with the functions of chromatin. Therefore, its effects on starfish development were not investigated further.

### Effects of okadaic acid on the development of *A. pectinifera* embryos

Fertilized eggs of *A. pectinifera* were cultured at 20°C in NSW containing 20 μg/ml okadaic acid. The sixth cell division occurred at 5 hr after fertilization with a 30-min delay compared with normal embryos; development beyond the 128-cell stage was prevented. As shown in Fig. 2, none of the embryonic cells differentiated into epithelial cells. All of the okadaic acid-treated embryos stopped development at this stage; the arrested appearance was always the same. We next examined whether or not applications of higher concentrations of okadaic acid would cause earlier retardation of embryonic development. It was found that a slowing and arrest of embryonic development was observed with the same timing in blastomeres of embryos cultured in a higher concentration (200 μg/ml) of okadaic acid as those cultured in a lower concentration (8 μg/ml) of okadaic acid.

To find out whether the inhibition caused by okadaic acid was reversible, okadaic acid (10 μg/ml) was added to embryo cultures immediately following fertilization and at intervals up to 6 hr; a portion of the culture was washed in NSW and cultured without okadaic acid. The embryos that had been kept in NSW containing okadaic acid for less than 1.5 hr from fertilization developed to the blastula stage after removal of okadaic acid, and they became normal gastrulae. However, washing with ASW of the embryo which had been exposed to okadaic acid for 2 hr or more from fertilization did not aid reversal of the effect of okadaic acid and they stopped development after completion of the seventh cleavage before blastulation. In other experiments, okadaic acid was introduced at later stages of development. When embryos were given okadaic

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**Fig. 2.** Okadaic Acid-induced Blockade of Development of *Asterina pectinifera* Embryos.

(A) and (C), embryos which were cultured in NSW containing 20 μg/ml okadaic acid for 3 and 6 hr, respectively; (B) and (D), embryos which were cultured in the absence of okadaic acid for the same periods as (A) and (C), respectively. The bar indicates 40 μm.

**Fig. 3.** Okadaic Acid-induced Unscheduled Chromosome Condensation in *Asterina pectinifera* Embryos at the 64-Cell Stage.

(A) and (C), embryos which were cultured in NSW containing 20 μg/ml okadaic acid for 4 and 5 hr from fertilization, respectively, and which were fixed and stained in methanol containing 10 μg/ml bisbenzimide; (B) and (D), embryos which were cultured in the absence of okadaic acid from fertilization for 4 and 4.5 hr, respectively, and which were fixed and stained in the same way as for (A) and (D). (A) and (B), embryos at the 32-cell stage; (C) and (D), embryos at the 64-cell stage. They were observed under a fluorescence microscope to see the DNA. The bar indicates 20 μm.
acid treatment starting 4 hr after fertilization, they never blastulated but developed beyond the 32-cell stage. On the other hand, when okadaic acid treatment was started at or after 4.5 hr postfertilization, blastulation invariably occurred but development stopped soon after blastulation. When embryos were given okadaic acid treatment after blastulation (at or after 6 hr postfertilization), they stopped development very soon and eventually died. These results showed that the embryo was sensitive to okadaic acid even before the 32-cell stage but the developmental arrest occurred at later stages.

**Effects of okadaic acid on structural changes of chromatin**

To investigate if chromatin of the blastomeres was affected by okadaic acid, the blastomeres of embryos which had been cultured for 5.5 hr in the presence and absence of 20 µg/ml okadaic acid from fertilization were stained with the DNA-specific dye bisbenzimide and examined under a fluorescence microscope. As shown in Fig. 3, chromosome condensation was observed in the blastomeres of the okadaic acid-treated embryo at the 64-cell stage. Condensed chromosomes were not aligned in a plane due to the absence of a mitotic apparatus and remained dispersed. This abnormal condensation took place during the sixth M phase and chromatins remained condensed during the seventh cell cycle. The embryo divided once and further cleavages were interrupted.

Generally, as cells enter mitosis, histone H1 phosphorylation takes place.\textsuperscript{1,2,3} As cells leave mitosis, the phosphates on H1 are rapidly lost from the histone. To investigate whether histone H1 kinase activities were elevated in blastomeres whose chromosomes began to condense abnormally at the sixth M phase, okadaic acid (20 µg/ml)-treated embryos were disrupted every 10 min starting from the time before entering the sixth cleavage up to the end of the cleavage. As shown in Fig. 4, a cycling of histone H1 kinase activity was observed. The activity reached a maximal value before the cleavage, and it dropped down at the time of cleavage as was observed for the control embryo. The elevation of the enzymic activity in the okadaic acid-treated embryo was less synchronized than that was found in the control embryo.

**Discussion**

A selective assay system using fertilized starfish eggs appears to be a useful probe for detecting natural antimitotic agents that inhibit normal chromatin functions. This has been shown by our isolation of okadaic acid from the sponge *Haliclona okadai* Kadota guided by the starfish embryo assay. Okadaic acid has been obtained by Tachibana et al.\textsuperscript{4} as an inhibitor of the growth of KB cells. It has been demonstrated that okadaic acid is a potent inhibitor of protein phosphatases types 1 and 2A,\textsuperscript{1,4} both of which have been detected in starfish oocytes.\textsuperscript{15} Picard et al.\textsuperscript{9} showed that microinjection of okadaic acid induced germinal vesicle breakdown of starfish (*Marthasterias glacialis* and *Astropecten aranciatus*) oocytes and activation of MPF and histone H1 kinase. They microinjected okadaic acid into a single blastomere at the four-cell stage during early development. The microinjected blastomere was arrested with condensed chromosomes and with microtubules polymerized. In our investigation we found that the treatment of fertilized starfish eggs with okadaic acid did not halt development until the 32-cell stage. A possibility exists that the discrepancy between the result of Picard et al.\textsuperscript{9} and ours was due to the incapability of the early *A. pectinifera* embryo to incorporate sufficient amounts of okadaic acid from outside the embryo. Therefore, we microinjected okadaic acid into the fertilized embryo soon after fertilization to give a final intracellular concentration of 8 µg/ml. It was found that the arrest of the cleavage cycle was observed with the same timing as that found in the embryo that received external application of 8 µg/ml okadaic acid from fertilization (data not shown).

We are now trying to find a clue to the reason why chromatin of *A. pectinifera* embryos before the 32-cell stage of development is immune to the action of okadaic acid.

Entry into and exit from M phase of the cell cycle are controlled by changes in protein phosphorylation-dephosphorylation reactions, which are attributed to fluctuation in the activity of maturation-promoting factor (MPF).\textsuperscript{16,17} MPF was shown to be a heterodimer containing one molecule of cyclin B and one molecule of a homologue of a yeast mitotic kinase encoded by the cdc2 gene.\textsuperscript{18} The kinase phosphorylates histone H1 efficiently.\textsuperscript{19} Our results showed that, although chromosomes were kept anomalously condensed in the presence of okadaic acid, histone H1 kinase activity reached a maximal value just before cleavage, then it dropped down and cleavage took place (Fig. 4). Okadaic acid did not affect the cycling of histone H1 kinase activity during progression of the sixth cell cycle. Since chromosomes were not associated with a mitotic apparatus, the sixth and seventh cleavages were achromosomal (Figs. 2 and 3). It remains to be seen why chromosomes of the blastomeres of the okadaic acid-treated embryo at stages later than the sixth cleavage were kept condensed in spite of the normal cycling of histone H1 kinase activity.

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