PEROXIDATIC ACTIVITY OF CATALASE IN THE SEA URCHIN OVARY

AKIO TOMINAGA

*Department of Anatomy, Ehime University School of Medicine,
Shigenobu, Ehime 791-02*

Received for publication August 3, 1982

Endogenous catalase activity in the ovary of two sea urchin species, *Hemicentrotus pulcherrimus* and *Anthocidalis crassispina*, was investigated by 3,3'-diaminobenzidine (DAB) method.

Osmicated DAB products were located at the lamellar (in *H. pulcherrimus*) or stellate (in *A. crassispina*) structure of the cortical granule (Cg) in the mature egg and that of the presumptive cortical granule (pCg) in the oocyte.

As for the A-type giant granule (A-g.) in the nurse cell, the same product was also found at the cortical granule-like structure (Cgls), but not at the other part of A-g.. In the present study of sea urchin ovary it is clear that the A-g. is "Heterophagosome", and that the heterophagic substance may be regenerated as a nutrient for the growing oocyte after autolysis of nurse cell.

The endogenous granules which show diaminobenzidine (DAB) reaction have been cytochemically investigated in various tissues and cells. The peroxidatic activity of catalase was demonstrated in cortical granules in mature unfertilized eggs of sea urchin species (11).

The morphology of the sea urchin ovary has been reported (2, 10, 13, 16, 21, 23, 24): in early breeding season the ovary contains oocytes in different growing stages and nurse cells which contain glycogen particles and numerous giant granules (A and B types) in their cytoplasm as nutrient materials. They undergo remarkable seasonal changes in a year (3, 6, 12, 14, 18, 20).

The function of the nurse cells has been considered as both for storage of nutrient material for oocyte growth and phagocytosis, namely they eat relict eggs after spawning. Ultracytochemistry on the oogenesis in sea urchin revealed the fact that there are similar stainabilities for several reagents between oocyte components and nurse cell giant granules (16-18).

The purpose of the present investigation was to elucidate the relationship among three DAB positive sites: a) cortical granules in mature egg, b) presumptive cortical granules in oocyte, c) A-granule in nurse cell, from DAB-catalase activity throughout the seasonal changes of a year.

MATERIALS AND METHODS

Sea urchin species used in the present investigation, *Hemicentrotus pulcherrimus*

and *Anthocidaris crassispina*, collected during the breeding season through non-breeding season in the Matsuyama sea area, Ehime, Japan were used. The breeding seasons of these two sea urchin species are from January to March and from July to August, respectively.

Ovarian lobes removed from the coelom wall were fixed in ice-cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 hr and washed three times and stored overnight in cold 0.1 M cacodylate buffer.

The ultrastructural localization of peroxidatic activity of catalase was investigated using an incubation medium containing 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a substrate originally developed by Graham and Karnovsky (7) which was modified by Novikoff and Goldfischer (16) and Fahimi (4), containing 10 mg DAB in 0.1 M Tris-HCl buffer and 0.002% H_2O_2 , pH 8.5.

Small pieces of ovarian lobes or 40 μm thick gelatin sections (23) were incubated in the above mentioned medium for 30 min–1 hr at room temperature. Control experiments were done as follows: a) without incubation, b) incubation medium containing 3-amino-1,2,4-triazol, as an inhibitor, at the final concentration of 0.02 M, c) incubation medium without H_2O_2 .

After incubation, the pieces or sections of ovarian lobes were quickly rinsed in Tris-HCl buffer and put into cacodylate buffer followed by postfixation in ice-cold 2% OsO_4 in 0.1 M cacodylate buffer, pH 7.4 for 30 min. Dehydration was routinely performed in increasing concentrations of ethanol and embedded in Epon or Spurr's resin. Sections were cut on an LKB-8800 Ultratome with glass or diamond knife. For light microscopy, sections about 0.5–1 μm in thickness, unstained or lightly stained with toluidine blue, were examined.

For electron microscopy, unstained golden sections, thicker than routinely used, about 100 nm in thickness were examined with Hitachi HU-12A type electron microscope under high contrast conditions.

RESULTS

In the early breeding season, oocytes in different developmental stages and nurse cells which contain numerous giant granules (A and B types) in their cytoplasm are found under the ovarian epithelium (Figs. 1, 2). Mature eggs are located in the deeper portion of the ovary (Fig. 1). DAB reaction for the localizations of peroxidatic activity of catalase of sea urchin ovary was investigated both in light and electron microscopic levels.

In the light micrograph (Fig. 1) the reaction products were found as follows: 1) in the cytoplasm of oocytes in vitellogenesis stage, 2) in the cortex of mature eggs, 3) some of the giant granules in the cytoplasm of nurse cells.

In the electron micrograph (Fig. 2), an oocyte in vitellogenesis stage and nurse cells containing numerous giant granules (A and B types) in their cytoplasm are seen under the germinal epithelium and muscle layer. DAB positive granules—presumptive cortical granule (Figs. 3, 4)—are scattered in the cytoplasm of the oocyte (arrowheads). DAB reaction is also positive in some of the A-granules, however, they are not necessarily crowded in the nurse cell cytoplasm close to the oocyte. Yolk granules in the cytoplasm of the oocyte show no reaction. The reaction products are not found at the other region of the ovary including germinal epithelium

and muscle layer (Figs. 2, 3).

Presumptive cortical granules in the cytoplasm of the oocyte in vitellogenesis stage show DAB reaction (Fig. 3). The lamellar or stellate structures of the presumptive cortical granule show intensive DAB reaction, while mitochondria induce weak reaction (arrows). The other components of the oocyte show no reaction (Fig. 4).

Anderson (1) suggested that the presumptive cortical granules were formed from Golgi complex in the vitellogenesis stage, however, in the present result the reaction products are not found at organelles as Golgi region, cisternae of endoplasmic reticulum and in the germinal vesicle. No indication suggesting the synthesis, transfer and intake of this enzyme was cytochemically observed.

In the deeper portion of the sea urchin ovary in breeding season, mature eggs are stored. Cortical granules are located in the egg cortex beneath the plasma membrane of the mature egg. The lamellar structure of the cortical granules show intensive DAB-reaction (Figs. 5, 6) as previously reported by the present author (11). The mitochondria show reaction as weak as in oocyte (Fig. 5). The other components of the mature egg show no reaction. Dense lipid droplets are seen in the egg plasm of the mature egg (Fig. 5).

In the cytoplasm of the nurse cells, different kinds of giant granules are found (Figs. 2, 7, cf. 16, 24). The nurse cells contain nutrient materials, which are taken into the growing oocyte during oogenesis. Giant granules of A-type (A-granule) are large, 10–15 μm in diameter, and often contain many small granules (about 1 μm in diameter) of different density. Some small granules show such stellate or concentric lamellar structure as the cortical granules in the same species (Fig. 8, cf. 16).

FIG. 1. Light micrograph. An Epon section of sea urchin ovary in early breeding season. *Anthocidalis crassispina*. Under the germinal epithelium (Ep), an oocyte (Oc), nurse cells (Nrc) containing numerous giant granules (A and B types) and mature eggs (E) are shown. DAB reaction, nostaining. Gv: germinal vesicle. $\times 440$

FIGS. 2–8. Electron micrographs. DAB reaction.

FIG. 2. Survey picture of the ovary. *Anthocidalis crassispina*. Nurse cells contain numerous giant granules. In the cytoplasm of an oocyte DAB positive granules (arrowheads) are shown. In other region of the ovary no reaction product is found. Electron dense granules in the nurse cell cytoplasm are lipid droplets (arrows). M1: muscle layer. $\times 1,400$

FIGS. 3 and 4. An oocyte in vitellogenesis stage. *Anthocidalis crassispina*.

FIG. 3. Presumptive cortical granules (pCg) in the cytoplasm of an oocyte show DAB reaction. B: nurse cell giant granule of B-type, G: Golgi region. $\times 5,000$

FIG. 4. A part of an oocyte. Lamellar or stellate structures of the presumptive cortical granules show intensive DAB reaction, while mitochondria induce weak reaction (arrows). ER: endoplasmic reticulum, Y: yolk granule. $\times 15,000$

FIGS. 5 and 6. Mature eggs found in the deeper portion of the ovary. *Hemicentrotus pulcherrimus*.

FIG. 5. Cortical granules found in the egg cortex show intensive DAB reaction. $\times 1,800$

FIG. 6. Egg cortices of mature eggs. The lamellar structure of the cortical granules show intensive DAB reaction, while mitochondria induce weak reaction (arrows). $\times 10,000$

FIGS. 7 and 8. Nurse cell cytoplasm. Different types of giant granules (A and B) are shown. *Hemicentrotus pulcherrimus*.

FIG. 7. Some of the A-granules contain DAB positive small granules. $\times 2,200$

FIG. 8. Two A-granules in the nurse cell cytoplasm are shown. A-granules contain small granules which show such concentric lamellar structure as the cortical granules. The substance in the lamellar structure shows intensive DAB reaction. Arrows show mitochondria (weak reaction). $\times 3,900$

FIG. 9. Oocyte cytoplasm after incubation with triazol as an inhibitor. The DAB reaction at the presumptive cortical granule was inhibited (arrowheads), while mitochondria show weak reaction by cytochrome oxidase (arrows). *Anthodialis crassispina*. $\times 15,000$

The substance in the lamellar structure shows intensive DAB reaction. Even in A-granules, DAB reaction is not found when they contain small granules without lamellar structure (Upper left of Fig. 7). Mitochondria also show weak reaction (Fig. 8, arrows), however, other components show no reaction. Dense oil droplets are seen also in the cytoplasm of the nurse cells (Fig. 7).

As described above, DAB reaction found in the sea urchin ovary are lamellar structure of cortical granules in mature egg, that of in presumptive cortical granules in oocyte and cortical granule-like (lamellar) structure in A-granules of nurse cells. These reactions disappear when 3-amino-1,2,4-triazol is used as an inhibitor, however, the reaction in mitochondria has not been inhibited (Fig. 9).

DISCUSSION

Annual change of the sea urchin ovary has been reported by many investigators (3, 6, 12, 14, 18, 20), however, little is known regarding the rise and fall of the nurse cells and the formation and breakdown of the giant granules. At the early breeding season the sea urchin ovary is filled by various developing oocytes, nurse cells with giant granules and mature eggs under the germinal epithelium and muscle layer. After the breeding season, most parts of the ovary are replaced by the elongated nurse cells. The previous reports stated that nurse cells increase in number and size after the breeding season, decrease as oogenesis proceeds and mostly disappear at the end

of the breeding season. According to the recent investigation in our laboratory, it is suggested that nurse cells are lined like the form of "the pseudostratified epithelium" (13, 21). The nurse cells are large in size and complicated in form. They are shown in a section as if they decrease in number when they become small.

Concerning the function of the nurse cells, two kinds of views are reported: a) nurse cell might phagocitize relict ova, b) they store nutrients and supply them to the oocyte. Therefore, the recent investigators have supported the concept as "nutritive phagocyte" for nurse cells because of their dual function. The observations that nurse cells phagocitize relict ova are only made at light microscopic level.

Electron microscopic observations have been reported on the nurse cells and oocytes (2, 14, 16–21, 23, 24). It is considered that glycogen particles and giant granules in the nurse cell cytoplasm are for storage of nutrient materials for oocyte growth. The giant granules in nurse cells were classified by previous authors. Verhey and Moyer (24) described them as 4 types of "yolk like inclusions" in *Arbaica punctulata* and Takashima (16) classified them into 3 types, A, B, and C-granules (cf. review article, Takashima, 18) in Japanese sea urchin species. Among the giant granules, A-granule, 10–15 μ m in diameter, contains small granules showing a stellate or concentric lamellar structure as the cortical granules in the same species.

Ultracytochemistry on the sea urchin ovary revealed that the cortical granules, presumptive cortical granules and lamellar structure in A-granules show the same stainability for phosphotungstic acid (PTA) in several conditions, periodic acid (PA)-silver methenamine *etc.* (17, 18). From this view point, Takashima (18) suggested that materials in the cortical granules are stored in A-granules. Nutrient intake of oocyte from the nurse cell is observed as pinocytosis at the oocyte surface (2, 23). The material intake by pinosomes contributed to yolk formation (17). Anderson (1) suggested that the cortical granules should be formed from Golgi apparatus in oocyte in vitellogenesis stage, however, the source of the materials in them was not clear.

As previously reported, the author detected the DAB-catalase reaction at the cortical granules in mature eggs (11). In the present report, the author examined the relationship among three DAB positive sites in the ovary; cortical granules, giant granules (A-type) in the nurse cells and presumptive cortical granules in the oocyte, in order to elucidate the turnover of the materials in the cortical granules to use DAB-catalase reaction as a marker.

Foerder and Shapiro (5) and Hall (8) have demonstrated the presense of peroxidatic enzyme in cortical granules and called "ovoperoxidase" and "cortical granule peroxidase", respectively. The author supposes that the reaction products in this investigation are due to catalase activity, since the incubation medium used was alkaline solution (at pH 8.5) and reaction was completely inhibited by 3-amino-1, 2,4 triazol. It is difficult to distinguish catalase from peroxidase only by the use of cytochemical DAB methods (9).

The author regards A-granules as "heterophagosomes". In the late breeding season, relict ova and undergrown oocytes were phagocitized by nurse cells and digested as "heterophagosomes", and become A-granules in nurse cell cytoplasm (19). Acid phosphatase (ACPase) activity was shown in these heterophagosomes (unpublished results). In the present investigation the author discussed the annual changes of the sea urchin ovary and keen attention was paid to DAB positive sites.

The relationship among three DAB positive sites, such as cortical granules, presumptive cortical granule and lamellar structure in A-granules was discussed, however, the changing mechanism of these substances is still unclear. It is considered that these substances are dissolved in the molecule state and taken into the oocyte.

ACKNOWLEDGEMENT

I would like to thank Professor Yoichiro Takashima, Department of Anatomy, Ehime University School of Medicine, for his helpful suggestions and encouragement. I would also like to thank Professor Kei-Ichi Hirai, Department of Anatomy, Kanazawa Medical University, for his advice in the field of cytochemistry.

REFERENCES

1. Anderson, E.: Oocyte differentiation in the sea urchin, *Arbacia punctulata*, with particular reference to the origin of cortical granules and their participation in the cortical reaction. *J. Cell Biol.* 37; 514-539, 1968.
2. Bal, A. K.: Ultrastructural changes in the accessory cells and the oocyte surface of the sea urchin *Strongylocentrotus dröbachiensis* during vitellogenesis. *Z. Zellforsch.* 111; 1-14, 1970.
3. Chatlynne, L. G.: A histochemical study of oogenesis in the sea urchin, *Strongylocentrotus purpuratus*. *Biol. Bull.* 136; 167-184, 1969.
4. Fahimi, H. D.: Effect of buffer storage on fine structure and catalase cytochemistry of peroxisomes. *J. Cell Biol.* 63; 675-683, 1974.
5. Foerder, C. A. and Shapiro, B. M.: Release of ovoperoxidase from sea urchin eggs hardens the fertilization membrane with tyrosine crosslinks. *Proc. Natl. Acad. Sci. U.S.A.* 74; 4214-4218, 1978.
6. Fuji, A.: Studies on the biology of the sea urchin. I. Superficial and histological gonadal changes in gametogenic process of two sea urchins, *Strongylocentrotus nudus* and *S. intermedius*. *Bull. Fac. Fish. Hokkaido Univ.* 11; 1-14, 1960.
7. Graham, R. C. and Karnovsky, M. J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14; 291-302, 1966.
8. Hall, H. G.: Hardening of the sea urchin fertilization envelope by peroxidase-catalyzed phenolic coupling of tyrosines. *Cell* 15; 343-355, 1978.
9. Hirai, K.: Diaminobenzidine in cytochemistry. *Acta histochem. cytochem.* 8; 31-40, 1975.
10. Holland, N. D. and Giess, A. C.: An autoradiographic investigation of the gonads of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.* 128; 241-258, 1965.
11. Katsura, S. and Tominaga, A.: Peroxidatic activity of catalase in the cortical granules of sea urchin eggs. *Develop. Biol.* 40; 292-297, 1974.
12. Kobayashi, N. and Konaka, K.: Studies on periodicity in oogenesis of sea urchin. I. Relation between the oogenesis and the appearance and disappearance of nutritive phagocytes detected by some histochemical methods. *Sci. Eng. Rev. Doshisha Univ.* 12; 27-45, 1971.
13. Kume, T., Tominaga, A. and Takashima, Y.: Studies on the nurse cells and oogenesis in the sea urchin ovary. I. Morphology of the nurse cell (Light microscopy of Epon serial section). *Med. J. Osaka Univ.* 28; 173-182, 1978.
14. Masuda, R. and Dan, J. C.: Studies on the annual reproductive cycle of the sea urchin and the acid phosphatase activity of relict ova. *Biol. Bull.* 153; 577-590, 1977.
15. Novikoff, A. B. and Goldfischer, S.: Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. *J. Histochem. Cytochem.* 17; 675-680, 1969.
16. Takashima, Y.: Electron microscopic observations on the nurse cells in sea urchin ovary.

- Med. J. Osaka Univ.* 19; 113–126, 1968.
17. Takashima, Y.: Cytochemical studies on the components of yolk granules and cortical granules in sea urchin eggs. *Med. J. Osaka Univ.* 22; 109–127, 1971.
 18. Takashima, Y.: Ultrastructure of nurse cells and oogenesis in the sea urchin. *Gunma Symposia Endocrinol.* 13; 105–127, 1976.
 19. Takashima, Y., Tominaga, A. and Kume, T.: Formation and behavior of the nurse cell giant granules in the sea urchin ovary. In 9th Int. Cong. Electron Microsc., Toronto, vol. 2, 1978, pp. 554–555.
 20. Tennent, D. H. and Ito, T.: A study of the oogenesis of *Mespilia globulus* (Linne). *J. Morphol.* 69; 347–404, 1941.
 21. Tominaga, A., Nagato, T., Kume, T. and Takashima, Y.: Studies of the nurse cells and oogenesis in the sea urchin ovary. II. Morphology of the nurse cell (TEM and SEM observations). *Med. J. Osaka Univ.* 28; 183–203, 1978.
 22. Tominaga, A. and Takashima, Y.: Gelatin embedding for enzyme ultracytochemistry; Acid phosphatase activity in sea urchin eggs. *J. Electron Microsc.* 30; 339–340, 1981.
 23. Tsukahara, J.: Electron microscopic studies on the behavior of glycogen particles during oogenesis in the sea urchin. *Develop. Growth Differ.* 13; 367–378, 1971.
 24. Verhey, C. A. and Moyer, F. H.: Fine structural changes during sea urchin oogenesis. *J. Exp. Zool.* 164; 195–226, 1967.