Fine Structural Localization of Endogeneous Peroxidase in the Endostyle of Ascidians, *Ciona intestinalis*. A Part of Phylogenetic Studies of the Thyroid Gland*

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Summary. The fine structural localization of peroxidase activity in the endostyle of *Ciona intestinalis* was studied by cytochemistry at electron microscopic level. Only zone 7 cells in this organ are positive for this enzyme activity. The reaction product is recognized in the external surface of the apical plasma membrane, and in the cisternae of the rough endoplasmic reticulum, of the nuclear envelope, and of the Golgi lamellae, and small vesicles of the Golgi region and of the apical cytoplasm. By electron microscopic autoradiography of ^{125}I , the main site of iodination of thyroglobulin-like protein is considered to be the apical plasma membrane region of the zone 7 cells. The possibility of iodination of protein taking place within the endostylar lumen near the zone 7 cell cannot be ruled out. The relationship between the fine-structural localization of peroxidase activity and the site of iodination of protein is discussed.

It is well known that peroxidase is an important enzyme for iodination of thyroglobulin in the thyroid gland. Though several papers dealing with the localization of this enzyme in the thyroid have been published, many problems remain to be solved as to the relationship between the activity of this enzyme and iodine metabolism in the thyroid-homologous organ of lower vertebrates or protochordates. As BARRINGTON (1964) pointed out, ascidians and amphioxus are important animals for the study of the evolution of the thyroid. These kinds of animals have a hollow organ in the floor of the pharynx, the endostyle, which has been regarded to be homologous to the thyroid of vertebrates (MÜLLER, 1871; DOHRN, 1886; BARGMANN, 1939).

In the present paper, the fine-structural localization of this enzyme in the endostyle of *Ciona intestinalis* is shown to discuss the relationship between iodine metabolism and enzyme activity of this organ. The result might serve for better understanding of the evolution of the thyroid gland.

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MATERIALS AND METHODS

Thirty specimens of *Ciona intestinalis* used in this study were obtained in November at Hiroshima Prefecture in Japan.

The endostyles of five animals were fixed in 3% glutaraldehyde solution buffered at pH 7.4 with 0.2 M Millonig's phosphate or with sea water for 1 hr. After washing in the phosphate buffer or sea water, they were post-fixed in a 1% osmium tetroxide solution buffered with phosphate or with sea water for 2 hrs. After dehydration through graded concentrations of alcohol, the specimens were embedded in Epon epoxy resin. Sections were cut with a Porter-Blum microtome and stained with Millonig's lead acetate.

Five specimens were used for ^{125}I autoradiography. The animals immersed in 5 liters of sea water containing 5 mCi of ^{125}I were sacrificed after 30 min, 1, 4 and 6 hrs. The endostyles were fixed, dehydrated and embedded in Epon epoxy resin by the same method mentioned above. Sections cut on a Porter-Blum microtome, picked up on uncoated grids and stained with Millonig's lead acetate, were coated with Ilford L4 emulsion by the wire loop method. Following exposure for 4 weeks at 4°C, the autoradiograms were developed in Microdol X for 5 min at 18°C and fixed with 20% sodium thiosulfate for 1 min. The grids were then stained with Millonig's lead solution.

Twenty animals were used for peroxidase cytochemistry. Their endostyles were fixed with a Karnovsky's fixative for 30 min and rinsed by sea water for 12 hrs. Fifteen endostyles were used for histochemistry of electron microscopic level. The materials were incubated at 37°C for 2 hrs in a medium containing 5 mg 3, 3'-diaminobenzidine tetrahydrochloride (Sigma), 2.5 ml of 0.1% H_2O_2 , and 2.5 ml of 0.1 M Tris-HCl buffer at pH 8.0. After incubation, the materials were postfixed with 1% OsO_4 , dehydrated with alcohol and embedded in Epon epoxy resin. Sections cut on a Porter-Blum ultramicrotome were stained with Millonig's lead acetate.

All the electron microscopic sections were examined in a Hitachi HU 11D type electron microscope.

For light microscopy, the fixed and rinsed endostyles of five animals were immersed in benzene for 1 min and incubated in a medium containing 45 mg of benzidine, 9 ml of sea water, 1 ml of saturated NH_4Cl , and 1 drop of 3% H_2O_2 for 5-10 min at room temperature.

OBSERVATIONS

The endostyle of *Ciona intestinalis* is a groove in the floor of the gill chamber. The organ is easily distinguished by the naked eye because of its white thread-like structure running longitudinally through the gill region (Fig. 1). By light microscopy 8 zones are distinguished in the transverse section (Fig. 2). Concerning the fine structure of each zone cell, the present authors agree with the description in the previous paper (FUJITA and NANBA, 1971).

When the *Ciona* was immersed in sea water containing ^{125}I , radioisotope was

Fig. 1. Benzidine reaction of an endostyle of *Ciona intestinalis*. Peroxidase activity is shown as two dark lines corresponding to zone 7. $\times 15$

localized on the apical plasma membrane region of zone 7 cells, 1, 4, 6 and 16 hrs after immersion (Fig. 3). Grains were also recognized in the multivesicular bodies and lysosomes after 4, 6 and 16 hrs, especially 16 hrs as FUJITA and NANBA (1971) reported. Similar results were published also by THORPE et al. (1972). No silver grains for ^{125}I were detected on any cells from zone 1 to 6, while zone 8 cells show a few grains on their apical plasma membrane region.

It is clear to the naked eye that a pair of a certain zone is linearly stained blue by benzidine for peroxidase activity (Fig. 1). By light microscopy, it becomes distinct that the zone 7 cells are strongly positive, while the other zone cells are negative to this reaction (Fig. 2). By electron microscopy, the electron-opaque reaction product is localized in the external surface of the apical plasma membrane, cisterna of the nuclear envelope, small flattened cisternae of rough endoplasmic reticulum, and all the cisternae of the Golgi apparatus of the zone 7 cell (Fig. 4). In addition, most small vesicles located near the

Fig. 2. A transverse section of an endostyle of *Ciona intestinalis*. 6, 7, 8 Zones 6, 7 and 8. Peroxidase activity is positive in the zone 7 region between the points marked by arrows. $\times 200$

In addition, most small vesicles located near the

Fig. 3. Parts of zone 7 cells of an endostyle of *Ciona intestinalis*. Four hrs after immersion in sea water containing ^{125}I (1 mCi/l). Silver grains are found on the apical surface region of the cell. $\times 25,000$

Golgi apparatus or at the apical cytoplasm of this type cell are also positive for peroxidase activity (Fig. 5). No reaction products were recognized in any other zone cells of the endostyle by electron microscopic cytochemistry.

DISCUSSION

It has been believed that the endostyle of the ascidian is homologous to the thyroid gland of the higher vertebrate (MÜLLER, 1871; DOHRN, 1886; BARGMANN, 1939). The zone 7 and 8 cells of the endostyle of *Ciona intestinalis* have been considered to be homologous to the thyroid cell (OLSSON, 1963; BARRINGTON and THORPE, 1965b; FUJITA and NANBA, 1971; THORPE et al., 1972). Using light microscopic autoradiography,

Fig. 4. Peroxidase activity in zone 7 cells of an endostyle of *Ciona intestinalis*. Reaction products are localized in the cisternae of rough endoplasmic reticulum, of the Golgi apparatus and of the nuclear envelope, in apical small vesicles, and in the apical cell membrane region. $\times 19,000$

BARRINGTON and THORPE (1965) considered that the zone 7 cell of the endostyle of *Ciona intestinalis* is a specialized region with exceptional capacity for binding iodine and the main site of iodine-binding is intracellular. Recently FUJITA and NANBA (1971) found numerous silver grains by electron microscopic autoradiography in the apical plasma membrane region of zone 7 and 8 cells of the ciona endostyle 1, 4, 6, 16 and 24 hrs and in the multivesicular bodies and lysosomes, 4, 6, 16 and 24 hrs after immersion in sea water containing ^{125}I . In the present study, silver grains for radioactive iodine are

Fig. 5. Peroxidase activity in the zone 7 cell of an endostyle of *Ciona intestinalis*. Reaction products are localized in the Golgi cisternae and apical small vesicles. $\times 28,000$

chiefly flocalized over the apical plasma membrane region of the zone 7 cells of an endostyle of *Ciona intestinalis*, 1, 2, 4, 6 and 16 hrs, and over the multivesicular bodies and lysosomes 4, 6 and 16 hrs after immersion. These facts essentially agree with the results of THORPE et al. (1972).

Based on this observation, the present authors believe that iodination of thyroglobulin-like protein occurs chiefly in the apical plasma membrane region of the zone 7 cells and probably in the endostylar lumen near the zone 7 cells. The iodinated protein is considered to be reabsorbed into the zone 7 cell and contained in the multivesicular bodies or lysosomes. The endostylar lumen is open to the pharyngeal lumen and the materials in the endostylar lumen could be easily washed away during the fixation, dehydration, and embedding of the tissue. The possibility of the iodination of protein taking place within the endostylar lumen near the zone 7 cells cannot be ruled out but, as FUJITA (1975) pointed out previously. If so, the main site of iodination of thyroglobulin-like protein is the apical plasma membrane region and endostylar lumen. This iodine metabolism pattern is quite similar to that of the thyroid gland in higher vertebrates.

It is well known that peroxidase is the most important enzyme for iodination of protein (SERIF and KIRKWOOD, 1958; ALEXANDER, 1959). Endogeneous peroxidase has been demonstrated histochemically in the rat thyroid follicle cell (DEMPSEY, 1944; DE ROBERTIS and GRASSO, 1946) and in the follicle colloid (DE ROBERTIS and GRASSO, 1946). The fine-structural localization of peroxidase in the thyroid glands of some vertebrates has been shown by STRUM and KARNOVSKY (1970, 1971), NAKAI and FUJITA (1970), SHIN et al. (1970), NANBA (1971), FUJITA (1972) and TICE and WOLLMAN (1974). Most of them reported that the reaction products are recognized in the cisternae of the nuclear envelope, of rough endoplasmic reticulum, of Golgi apparatus, and sub-apical small vesicles, and the external surface of the apical plasma membrane. In the present paper, similar results were obtained in the zone 7 cells of *Ciona intestinalis*. The present authors wish to accept the opinion of STRUM and KARNOVSKY (1970) that the peroxidase protein is synthesized in the rough endoplasmic reticulum and transported to the Golgi apparatus to be condensed, and that the matured peroxidase activity exists chiefly on the external surface of the apical plasma membrane. If so, it is easily understood that iodination of thyroglobulin-like protein takes place over the plasma membrane region of the zone 7 cells and the peroxidase reaction is also positive at the zone 7 cells.

Recently it has been reported in several species of ascidians other than *Ciona intestinalis* that the thyroid-homologous cells may not be restricted to the zone 7 cells, but the whole branchial sac has the ability to bind iodine to protein (SUZUKI and KONDO, 1971), and peroxidase activity has been demonstrated also in the branchial sac of some tunicates, such as *Ascidia zara*, *Styela plicata*, *Pyura michaelsoni*, *Ascidia sydneiensis samea*, *Boltenia isbasii*, and *Pyura mirabilis* (H. KOBAYASHI, personal communication). THORPE and THORNDYKE (1975) and KOBAYASHI (personal communication) also found peroxidase activity in the zone 7 cell of *Ciona intestinalis*. Also in the endostyles of *Molgula manhattensis*, *M. occidentalis*, and *Styela plicata*, DUNN (1975) regarded the zone 7 cells as homologous to the thyroid cells, though he found an accumulation of ^{125}I not only in the zone 7 cells but also in the apical part of the zone 8 and 9 cells. The thyroid homology in ascidians has many problems to be solved in the future. In our previous study (1971), ^{125}I was accumulated over the zone 7 as

well as zone 8 cells in *Ciona intestinalis*. The differences might be caused by unknown animal conditions such as age, season and so on. This is also a problem for the future.

The present paper clearly indicates that only the zone 7 cells are positive for peroxidase reaction and iodination of thyroglobulin-like protein takes place mainly on the apical plasma membrane region of this cell in the endostyle of *Ciona intestinalis*.

ユウレイボヤ (*Ciona intestinalis*) の内柱におけるペルオキシダーゼの局在。 甲状腺の系統発生的研究の一部

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11月に広島県で採取した ユウレイボヤの内柱 (甲状腺と相同器官といわれる) におけるペルオキシダーゼの局在を 電顕レベルの組織化学によって明らかにし、ヨードの有機化の場との関係を論じた。

ユウレイボヤの内柱では 第7型細胞のみに酵素活性が陽性に出る。内柱腔に面する細胞膜の表面、核膜腔、粗面小胞体腔、ゴルジ装置の内腔、ゴルジ装置から細胞表面にかけて存在する小胞などに酵素活性が認められる。 ^{125}I の電顕オートラジオグラフィーの結果、ヨードを有機化する場合は おもに7型細胞の内柱腔に面する細胞膜領域と考えられる。また7型細胞近くの内柱腔内でヨードの有機化が起こる可能性も否定できない。

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