STUDY ON THE ULTRASTRUCTURE AND FUNCTION OF THE BOVINE THYROID GLAND IN TISSUE CULTURE

TOMOHIRO SHIN

3rd Department of Internal Medicine, Nagoya University School of Medicine

(Director: Prof. Kozo Yamada)

The trypsinized bovine thyroid cells are cultured in a monolayer. Morphologically, their origin can not be identified under conventional light microscope.

Under electron microscope, the cultured cells exhibit striking changes—loss of intracellular granules, granular and tubular deformation of endoplasmic reticulum as well as hypertrophy of mitochondria. The cells bounded by a common terminal bar are apparently considered as follicular epithelium and their endoplasmic reticulum shows tubular pattern.

As to a metabolic study, cultured cells incorporate \({\text{I}}^{131}\) into iodinated protein until 14th day, but thereafter they do not.

Recently, dispersed cells obtained from human\(^{14}\) and sheep\(^{15}\) thyroid glands by trypsinization have been cultured in a monolayer. In 1959, Pulver-taft \textit{et al.}\(^{16}\) and in 1960, Irvine \textit{et al.}\(^{17}\) reported that the sera of patients with Hashimoto's disease had cytotoxic effects on the trypsinized cells of human thyroid gland.

However, trouble is how to recognize the origin of cultured cells, because they might have little or no resemblance to parents under conventional light microscope. Therefore, the present work is aimed at the observation of the ultrastructure of cultured cells from bovine thyroid gland under electron microscope. The function of these cells is also studied by means of \(\text{I}^{131}\) radioautogram.

MATERIALS AND METHODS

Bovine thyroids were obtained from the local abattoir. They were wrapped in polyethylene, and kept on cracked ice until used. The glands were put in 70\% ethanol, and then washed several times in the phosphate-buffer solution. The sterilized glands freed of fat as well as connective tissue were cut into small pieces with scissors. Approximately 30 g of the small pieces was placed in the phosphate-buffer solution (pH 8.0) containing crystalline trypsin (Difco 1 : 250) in a concentration of 0.25\%. The contents were swirled with magnetic
stirrer at low speed for 30 min. After the first supernatant was discarded, the precipitate was resuspended with 80 ml of phosphate-buffer solution containing trypsin and again swirled in the same way for 15 min. The turbid supernatant was spun down at 1500 r.p.m. for 3 min. The precipitate was suspended in tissue culture medium, which was made up of 80% Earle's solution and 20% bovine serum. The Earle's solution used was composed of the following materials —0.4% lactalbumin hydrolysate, 0.45% glucose, 100 units of penicillin G per 1 ml of saline, 1 mg streptomycin sulphate per 1 ml, and 0.02 mg phenol red per 1 ml. The medium was adjusted to pH 7.8 with 5% NaHCO3.

Five ml of the cell-suspended medium was placed in the sterile square bottle with volume of 50 ml and incubated at 37°C.

For observations by optical microscope, glass cover slips were put at the bottoms of the square bottles. The incubation was terminated at intervals from 1 to 40 days for microscopic examination. The medium was decanted and then the cover slips to which cultured cells adhered were removed. The cells were fixed with ether-ethanol (1 : 1), followed by staining with hematoxylin-eosin.

In order to ascertain that cultured cells were capable of binding iodine into organic forms, carrier-free radioactive sodium iodide (Na131I) was added to the incubated medium at 1, 3, 4, 7, 14, and 30th day, respectively, so as to yield the radioactivity of approximately 10 μc per ml. After the cells were incubated further for 3 to 18 hrs, the medium was discarded and then the cells were washed with physiological solution containing potassium iodide in a concentration of 1 mg per 1 ml. The cells collected were homogenized in the Krebs-Ringer bicarbonate buffered solution (pH 7.4) containing methylthiourearacil. The homogenate was hydrolyzed with pancreatin at 37°C, for 8 hrs after addition of toluene (pH 8.0). Aliquots were chromatographed on filter paper in collidine saturated with water in an atmosphere of NH3. Autoradiographs were prepared by using X-ray films. The radioactive spots were identified with those of pure reference compounds.

In preparation for electron microscopy, the cultured medium was decanted from each bottle and the cells were fixed with Palade's buffered osmium tetroxide plus 0.25 M sucrose. The specimens were embedded in methacrylate. Sections were made with a LKB 3314 Sjöstrand Ultramicrotome and stained with uranil acetate or lead nitrate. Electron micrographs were taken with Hitachi Type HA-11-A electron microscope.

RESULTS

Observations under the Light Microscope

Dispersed thyroid cells adhered to the glass usually within half an hour or so. The cells adhered to each other, forming an aggregate and then spread to all directions from the original foci. They began to proliferate on the 3rd
or the 4th day. The size of the cells increased at this time. Some became polygonal and others fusiform in shape (Fig. 1). The former proliferated forming islets with a monolayer and the latter multiplied surrounding the islets of the polygonal cells (Fig. 2). The polygonal cells were regarded as epithelial in origin and had round, less stained nuclei than those of another. But, some cells did not fall into either category of them by the conventional microscopic examination.

Mitosis was observed at any time throughout the series of experiment after the 3rd day of cultivation. The phenomenon of “malignant transformation” as described by Pulvertaft et al. (1959) was not observed up to 40th day of tissue culture.

**Metabolic Study**

On the 1st, 3rd, 4th and 7th day of culture, the formation of mono- (or di-) iodotyrosine was detected in the cells. On the 14th day, the cultured cells could incorporate I into iodinated proteins which remained at the origin on chromatograms, but iodinated amino acid was not found. During 30 days of tissue culture, there was no evidence of binding iodine into organic forms. No iodothyronine was detected in the cells at any period of tissue culture.

Chromatographically, radioactive spots of extracts of the medium showed the same distribution as that in the absence of cells. On both preparations of autoradiograph, there were some distinct spots in the area of inorganic iodine, but iodinated organic compound was not recognized. These experiments revealed that there was no measurable release of iodinated thyronines from actively growing cells into the medium. This result agreed with the earlier reports.

**Fine Structure of Follicular Cells**

In intact bovine thyroid, gland cells were seen to bound colloid material with their apical surface and to border on blood capillaries with base. The course of two adjacent plasma membranes was rather straight for a long distance. At intervals, however, it was somewhat intricate and showed irregularities. The follicular cells were hold together with the terminal bar in the neighbourhood of the free cell surface (Fig. 3). The microvilli protruded in colloid material from the apical surface of the cells (Fig. 3). They varied in number and shape from slender finger-like projection to short blunt bulge. At the base of the cell, the plasma membrane was irregularly folded so that the zone of basal cells was divided into a number of compartments of various size and shape (Fig. 5). The basis were separated from perifollicular capillaries by basement membrane (Fig. 5). Among intracellular elements of the follicular cell, mitochondria were outstanding because of their large number and regular structure (Figs. 3, 4, 5). They were scattered throughout the cytoplasm without any regularity. Most of them were rod in shape with various length. The
Golgi apparatus was usually found in the neighbourhood of nucleus, consisting of a few flattened membranes and small clusters of varying size vesicles (Fig. 4). The endoplasmic reticulum was distributed throughout cytoplasm. The cisternae showed the characteristic wide dilatation and contained materials of low electron density (Fig. 4). Ribosomes were found covering the surface of the cisternae membranes.

Also, scattered in the cytoplasm, there were several granules which varied in size, density and internal structure (Fig. 3). Most of them were found in the apical zone of the cells. At least two types of big granules were able to be distinguished. The larger one was of pale staining and spheroidal in shape with smooth membrane. This granule was considered to be the "colloid droplet" under light microscope. Another one was stained densely. It was of regular shape and enclosed with smooth membrane. Some granules contained vacuoles as well as structures of varied density.

In addition to these granules as mentioned above, there were small granules with vesicular structure in every cell. They, varying in number, occurred predominantly in the apical zone—so-called "apical vesicles". Most of them revealed a rounded shape with moderate opacity. They contained a homogeneous material and were enveloped with a distinct surface membrane.

The nucleus was roughly spherical with occasional indentations at the margin and contained osmiophilic particles of fairly constant size (Fig. 4). The particlites were round or irregular in shape. They were densely accumulated just within the nuclear membrane as well as in some interior areas of the nucleus. Throughout the remainder of the nucleus, only a few particles were found, giving rather low opacity.

Fine Structure of Cells in Culture

In some cultured cells microvilli were still present, but they were generally shorter than those of the normal follicular cells (Figs. 9, 10). A few of these cells were joined together with convoluted intercellular connection and terminal bar (Figs. 9, 10). It seemed that the trypsin acted to strip the epithelium from the basement membrane without separating the cells at their terminal bars. The cells were incubated under the condition as mentioned above for two weeks. These cells, therefore, seemed to exhibit still a definite polarity. However, they were denuded of the basement membrane. The irregular folding of the plasma membrane at the base of the cell was not seen, showing smooth surface (Fig. 9). In some cells, many fibrilar structures were recognized at the basal zone (Fig. 9).

Many other cells appeared to be more round in shape (Fig. 6). The microvilli were absent. The plasma membrane was generally smooth and the cells revealed roughly amoeboid shape as a whole. There was no polarity of the cells.
Concomitant with the changes of the cell border, there occurred tremendous modifications of the cytoplasmic organelles. On the first day of cultivation, some cells contained fine filaments (Fig. 6) which disappeared as tissue culture continued further. Lipid droplets varying in size and quantity were apparently much more in the cells, which still possessed the polarity (Fig. 9).

The mitochondria of cultured cells varied from ovoid to rod in shape as usual, but it was generally smaller than that of intact cells (Figs. 7, 8). The mitochondria contained irregular cristae, some of which were arranged circularly (Fig. 8) and occasionally destructed with swelling (Fig. 7). The content of cristae was relatively scanty (Figs. 9, 10).

The Golgi apparatus was situated usually near the nucleus. They appeared to grow generally well (Fig. 11), however, there were some exhibiting a few aggregates of lamellated saccules, and vesicles adjacent to them (Fig. 12).

On the 1st day of culture, the endoplasmic reticulum already revealed small vesicles irregular in shape (Fig. 6). In some parts of cytoplasm, however, it arranged in tubular pattern (Fig. 7). The large dilated reticulum observed in the normal follicular cell was not found in any cultured cell. In advanced cultures, the most reticulum showed tubular profiles, while some seemed to be arranged parallel to each other (Figs. 10, 12). As mentioned above, striking changes of the endoplasmic reticulum were recognized in the cells cultured for 14 days. It was connected together with a common terminal bar, for which the origin of the cell was regarded as follicular epithelium (Fig. 10).

The intracellular granules of these cultured cells were missing, while in the normal follicular cells they were scattered throughout. A few granules of the cultured cells were occasionally found on the 1st day of cultivation (Figs. 6, 7). The granules themselves were considered to be identical with those of the intact cells, because they had relatively dense, homogeneous content enclosed by a single-layered membrane. They were of round or oval form. After the 3rd day of culture, such granules as mentioned above were not found in the cell. Apical vesicles were hardly recognized even in the cell which still maintained a polarity in vitro (Figs. 9, 10).

The modifications of the nuclei appeared to be less outstanding than those of the cytoplasm, because they concerned only with changes in form and size. The nuclei of the cultured cells were generally round and larger than normal. The nuclei of the cells showing amoeboid form were distorted (Fig. 6).

DISCUSSION

It has been noted in the monolayer culture of bovine thyroid tissue that the epithelial type of cell is predominant and forms sheets, which are surrounded by zones of fibroblastic cells (Fig. 2). The ability for these cells to incorporate iodide into iodotyrosine is gone after 14-day cultivation.
ever, the results of the present investigation do not indicate whether the cells which synthesize iodotyrosine are actively growing in culture or exist quietly. Much less, it can not be ascertained what cells synthesize iodotyrosine.

As mentioned before, the change in intracellular fine structure is particularly remarkable in the endoplasmic reticulum. It has been suggested that rough-surfaced endoplasmic reticulum of the follicular cell is cellular machinery for thyroglobulin synthesis as well as the transporting channel for intracellular products from internal to external milieu. The results from the metabolic study shows that the cultured cells still retain the ability of organic binding for radioactive iodine within two weeks. If granular or tubular reticulum in cultured cells is concerned with protein synthesis as well as transport of cellular products, one must assume that, in the case of tissue culture, the cellular machinery of thyroid gland is functionally a little or not available.

The "colloid droplets" are considered by most investigators to be antecedents of the luminal colloid. Some, like Nadler et al., maintain that they originate from pinocytosis of small portions of colloid by pseudopods from cell apex. The "apical vesicles" of follicular cells have been interpreted to be pinocytotic in character. However, Nadler et al. hold that those vesicles contain the material which is a precursor of colloid. Other cytoplasmic granules may be related to the secretory function of follicular cells but the precise role is not clear. No conclusion is drawn about the significance of these granules and vesicles in the present investigation. A few granules in the cell are found only on the first day of cultivation (Figs. 6, 7), though they disappear after 3 days. Whether the granules and vesicles are secretory or pinocytotic is still a matter of argument. The results mentioned before reveal that cultured cells do not have colloid secreting ability, even though they are capable of synthesizing iodotyrosine during two weeks of cultivation. Indeed, the analytical study of cultured medium fails to show the presence of any organic iodine compound.

As far as examined under electron microscope, definite secretory droplets appear to develope from small vesicular or granular elements of Golgi apparatus in most exocrine as well as many endocrine glands. The functional significance of marked hypertrophy of the Golgi apparatus seen in some cultured cells is open to speculation (Fig. 11).

There is a general agreement about cultured cells in view that after long term they lack their proper physiochemical functions in serially propagated cells derived from specialized tissues of many types. Though the exact evaluation of disappearance of the specialized functions is difficult, they might be due to genetic changes, inadequate nutritional environment, and the lack of cellular organization and interaction. The selective proliferation of non-functioning cells should also be taken into account.

On the other hand, highly specialized cells may have the most fitted
CULTURE OF THYROID

structure for performance of its function in the body. Under electron microscope, intracellular fine structures of the cultured cells show striking changes. These cells are connected together with terminal bar, and thus considered to be follicular epithelium (Fig. 10). The intracellular findings of these cells are also similar to those of cultured cells after longer term (Fig. 12), which can not be distinguished from the undifferentiated fibroblast in fine structure9. It is possible that fibroblastic alteration in the ultrastructure takes place in the follicular cells cultured in vitro. These cells thus result in losing of the ability to synthesize iodothyronine and iodotyrosine due to what is often referred to as dedifferentiation in function.

ACKNOWLEDGEMENT

Grateful acknowledgement is made to Prof. K. Yamada and Dr. S. Ogawa, Department of Internal Medicine, Nagoya University, for their constant interest and guidance in this investigation, and to Dr. S. Kozuka and Dr. H. Watanabe, Department of Pathology, Nagoya University, for their helpful discussion.

REFERENCES


EXPLANATION OF FIGURES

Bm: Basement membrane
Ca: Capillary lumen
Er: Rough-surfaced endoplasmic reticulum
Go!: Golgi apparatus
Is: Intercellular space
M: Mitochondrion
N: Nucleus
C: Colloid
tb: Terminal bar
mvl: Microvilli
av: Apical vesicle
cd: Colloid droplet
gv: Golgi vesicle
G: Intracellular granule
L: Lipid droplet
F: Fibrilar structure
f: Fine filaments

FIG. 1. Histological appearance of cells cultured for 4 days. The polygonal cells proliferate as islets. Hematoxylin- and eosin-stained. ×100.
FIG. 2. Histological appearance of cells cultured for 10 days. The fusiform cells multiply surrounding the sheets of polygonal cells. Hematoxylin- and eosin-stained. ×100.
FIG. 3. Electron micrograph of follicular cells from control bovine. A number of relatively short microvilli are projecting into the colloid. Below them there is a zone containing small vesicles of different contents. A number of droplets varying in size and density are present. ×15000.
FIG. 4. Supranuclear region of a follicular cell in bovine thyroid. The sacs of the Golgi apparatus and numerous Golgi vesicles are present. The endoplasmic reticulum is large and dilated. ×25000.
FIG. 5. The basement of follicular epithelium of bovine thyroid. The plasma membrane is irregularly folded. The basement membrane is consisted of three layers. ×30000.
FIG. 6. Electron micrograph of a cell cultured for one day. The cell is round in form. The intracellular granule is considered to be the same as in the normal follicular cell, because the former has a relatively dense homogeneous content and is enclosed by a single-layered membrane. ×10000.
FIG. 7. Electron micrograph of a cell cultured for one day. The same granule as in Fig. 6 is found. The endoplasmic reticulum appears in the form of small vesicles and tubules. ×10000.
FIG. 8. Electron micrograph of a cell cultured for 3 days. The mitochondria contain irregular cristae. The cristae are arranged circularly and lamellarly in some mitochondria (arrows). ×16000.
FIG. 9. Electron micrograph of a cell cultured for 7 days. Microvilli are still present and the cells are connected together with convoluted intercellular space and terminal bar. The basement membrane is missing and the cell surface is smooth. Many fibrilar structures are recognized at that part. ×12000.
FIG. 10. Electron micrograph of a cell cultured for 14 days. Short microvilli and terminal bar are still recognized. The endoplasmic reticulum shows tubular profiles. ×40000.
FIG. 11. Electron micrograph of a cell cultured for 14 days. The Golgi apparatus appears to grow well and occupies the major part of the cytoplasm. Some mitochondria are swollen with destructed cristae. ×30000.
FIG. 12. Electron micrograph of a cell cultured for 40 days. The endoplasmic reticulum is tubular in form. ×30000.