THE EXPRESSION OF DIFFERENTIATION BY
CHICK EMBRYO THYROID IN CELL CULTURE

II. Modification of Phenotype in
Monolayer Culture by Different Media

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ABSTRACT

Previous results with thyroid secretory cells in monolayer culture seem contradictory with respect to phenotypic stability of this cell type. On the one hand, in "minimal" medium the cells lose structural and functional specializations which can be returned only by three-dimensional growth in organ culture upon addition of fibroblasts derived from the thyroid capsule. On the other hand, in "rich" medium used for cloning, cytoarchitecture and function remain unaltered in either mass or clonal cultures. The apparent discrepancy has been resolved by plating cell suspensions in both media and changing to the alternate medium once the cells have become established. It has been shown that a number of characteristics, including hormone levels, are reversed each time such a change in medium is made. These modulations are discussed in terms of the normal variations in structure and function of the gland in vivo.

INTRODUCTION

An increasing number of normal, differentiated cell types have been shown to exhibit phenotypic stability during two-dimensional growth under clonal conditions. The rapid loss of differentiated features that commonly occurs during monolayer culture of cartilage (Holtzer, Abbott, Lash, and Holtzer, 1960; Stockdale, Abbott, Holtzer, and Holtzer, 1963), retinal pigment cells (Whittaker, 1963), and liver (Sato, Zaroff, and Mills, 1960; Fogel, 1968) has been avoided by single-cell plating and clonal growth of the same cell types (Coom, 1966; Cahn and Cahn, 1966; Coon, 1968). Thyroid cells behave similarly, and furthermore, even retain a number of differentiative characteristics when cultured as monolayers (Spooner, 1968; 1970). This stability in monolayer culture is in sharp contrast to the functional and fine structural "dedifferentiation" that was observed in earlier studies with monolayers of the same cell type (Hilfer, 1962; Hilfer, Hilfer, and Iszard, 1967).

In the present investigation, we have compared the properties of thyroid cells, derived from common primary suspensions, during monolayer culture in both the nutrient medium used by Hilfer (1962) and that used by Spooner (1970), in an attempt to resolve the apparent contradiction on the question of thyroid stability. A sensitive assay procedure has demonstrated that cells growing in both culture media contain detectable amounts of thyroid hormones, although those growing in the former medium always contain
TABLE I

Comparison of Properties of Embryonic Chick Thyroid Cells Growing as Monolayers in Two Different Culture Media

| Property                  | F12S10       | TyHS         |
|---------------------------|--------------|--------------|
| Attachment time           | 30 min       | 12 hr        |
| Mitotic activity          | High         | Undetectable |
| Culture pattern           | Dense epithelium | Irregular patches |
| Nucleolar appearance      | Phase opaque | Phase pale   |
| Endoplasmic reticulum     | Extensive    | Sparse       |
| Detectable hormone        | High         | Low          |

Bars on all figures represent 1 μ.

**FIGURE 1** Thyroid epithelial cells after 5 days of monolayer culture in F12S10. The culture is an exceedingly dense epithelium. Mitotic figures are scattered throughout the culture (arrows). Living, phase. × 150.

**FIGURE 2** Thyroid epithelial cells after 5 days of monolayer culture in TyHS. This culture was initiated with the same primary cell suspension used for the culture shown in Fig. 1. The culture is composed of small patches of cells, and the cells have assumed a fibroblast-like morphology. Note the accumulation of cytoplasmic granules around the nuclei (arrows) and the paleness of the nuclei. Living, phase. × 150.

lower levels. Furthermore, we have found that it is possible to reversibly modify a number of cell properties by simple manipulation of the culture medium.

**MATERIALS AND METHODS**

**Dissociation and Cell Culture**

Thyroid glands from 16-day Rhode Island Red chick embryos were used as the cell source. Only the left gland was used since it is larger and more advanced developmentally at this age (Hilfer, 1962).

Procedures used for collecting the glands and for cleaning and dissociating the epithelial component have been previously described (Spooner, 1970). However, following the generation of primary cell suspensions with a mixture of trypsin and collagenase, the cells were plated as monolayers without filtration.

Common primary suspensions were plated at $10^6 - 2 \times 10^8$ cells per dish (100 × 20 mm petri style, Falcon Plastics) in 2 parts Tyrode's solution:1 part horse serum medium (TyHS) (Hilfer, 1962) and at 5 × 10^5 cells per dish in the modified Ham's F12:10% fetal calf serum medium (F12S10) (Spooner, 1970). Both media contained penicillin-streptomycin (Baltimore Biological Laboratory), 50 U-50 μg, respectively, per ml, and amphotericin B (Fungizone, Grand Island Biological), 2.5 μg.
Figure 3  Reversibility of properties of thyroid cells growing as monolayers in F12S10 and TyHS. See text for description. Each point represents the average cell number per culture (three cultures per point). Arrows indicate days on which cultures were assayed for hormones. The number in parentheses is the number of samples assayed. (+), hormone level routinely detected in F12S10 cultures. (±), lower but clearly detectable level of hormone.

Microscopy

Living cultures were observed and photographed in an inverted microscope with phase-contrast optics.

Cultures were fixed for electron microscopy with cold, s-collidine-buffered 1.33% osmium tetroxide, pH 8.0, for 30 min. The fixative contained 0.08 M sucrose, and 5.0 mM each of calcium chloride and magnesium chloride (van de Kamp, 1968). Following dehydration, the cells were embedded in Araldite in the culture dish and handled for thin sectioning as described by Spooner (1970). Sections were stained with uranyl acetate in 50% ethanol and with lead citrate (Venable and Coggeshall, 1965). Observations were made with a Zeiss EM-9A electron microscope.

Iodoamino Acid Analysis

Cells were analyzed for thyroxine and triiodothyronine by a sensitive thin-layer chromatographic procedure described in detail elsewhere (Spooner, 1970). Radioiodide was not used because preliminary experiments demonstrated that I² was rapidly bound to the culture medium. Some 60-80% of
RESULTS

Comparison of Cell Properties in Different Media

A comparison of the properties of thyroid cells growing in F12S10 and TyHS is shown in Table I. Cells plated in F12S10 attached and began spreading 30 min after inoculation. Cells plated in TyHS, however, did not attach during the first 2 hr of culture, but were attached and spreading after 12 hr of culture. By the 2nd day of culture, cells in both media had formed sheets of epithelial cells. During the 3rd day, mitotic figures were seen in the F12S10-cultures and were present for the remainder of the culture period. Mitotic figures were never observed in the TyHS-cultures. The rapidly dividing cells in F12S10.
A completely different culture pattern occurred with cells grown in TyHS. The continuous sheet of cells present on day 3 began separating into smaller patches on days 4–5 of culture (Fig. 2). This process continued during the next few days, resulting in the formation of a large number of discrete patches of cells in the culture dish. Individual cells in the patches underwent internal changes during this period. The nucleoli became pale under phase-contrast optics, and dense cytoplasmic granules became clustered around the nucleus (Fig. 2). The cells retained this appearance for the remainder of the culture period. Counts showed no increase in cell number in TyHS medium (Fig. 3).

Effects of Medium Reversal on Cell Properties

If cells grown 6–8 days in TyHS were changed to F12S10, striking changes occurred in the cultures. Mitotic figures began appearing after 2 days in F12S10, and after 6–8 days the cultures were identical with cultures grown continuously in F12S10. The cells had formed a dense epithelium, their nucleoli were no longer pale under phase contrast, and cytoplasmic granules no longer encircled the nucleus. Counts showed that the cell population had increased to the level attained by control F12S10 cultures (Fig. 3). On the other hand, cultures changed from F12S10 to TyHS behaved as though they had been plated in TyHS originally. After 5 days, the dense monolayer had separated into patches of cells. Furthermore, the nucleoli had become pale, and granules had clustered around the nucleus. Mitotic figures were no longer observed, and counts showed a slight decrease in the cell population size (Fig. 3).

Chromatographic assay for hormones gave results similar to those obtained with cells grown continuously in either of the two media. Thus, cells changed from F12S10 to TyHS contained lower amounts of hormone than cells changed from TyHS to F12S10 (Figs. 3 and 4). This result was the same for 6, 8, and 14 days of culture in the second medium. Furthermore, cells that had been modified by changing the medium would again express their original properties when returned to the original culture medium (Fig. 3).

These results have shown that hormones can be detected in cells grown continuously in TyHS, when assayed by thin-layer chromatography, indicating functional stability under these conditions. Furthermore, it has been shown that...
TyHS and F12S10 have different effects on culture morphology, cell morphology, mitotic activity, and amounts of detectable hormone. However, these changes were not fixed properties of the cells, since they could be reversed by culturing the cells in the other medium.

Effects of Medium Reversal on Cellular Fine Structure

We had previously shown that cells in F12S10 contained large amounts of rough endoplasmic reticulum (Spooner, 1970) and that this organelle was virtually absent in cells in TyHS (Hilfer et al., 1967). The effects discussed above suggested that fine structural investigation would reveal a reversal of the endoplasmic reticulum pattern when the nutrient media were reversed.

Control cultures of cells grown continuously for 8 days in F12S10 contained extensive channels of rough endoplasmic reticulum (Fig. 5). Furthermore, the nucleoli in such cells possessed a prominent granular component (Fig. 6), that may account for the nucleolar density observed with the phase microscope. When sister cultures were grown 4 days in F12S10 and then switched to TyHS for the remainder of the culture period, the cells revealed a strikingly different appearance (Fig. 7): rough endoplasmic reticulum was extremely sparse in the cytoplasm, with only a few small channels being present; and, the granular component was now absent from the nucleolus (Fig. 8).

In the reciprocal situation, cells cultured continuously in TyHS possess little organized endoplasmic reticulum and the nucleolus is agranular (Fig. 9). However, when such cultures are changed to F12S10, large amounts of rough endoplasmic reticulum reappear (Fig. 10). Furthermore, the nucleolus acquires an extremely prominent granular component (Fig. 11). These fine structural results show that the endoplasmic reticulum
and nucleolar morphology of thyroid cell monolayers can be reversibly altered, in the same way that cell morphology, culture pattern, mitotic activity, and hormone levels change as a function of the nutrient supply.

DISCUSSION

The functional stability of thyroid cells from the chick embryo observed in cell culture by Spooner (1970) appeared contradictory to the functional dedifferentiation that occurred during monolayer culture of the same cells in an earlier investigation (Hilfer, 1962). In that study, thyroxine and triiodothyronine could no longer be detected after 5 days of culture. The apparent contradiction has been resolved in the present study by the observation that thyroid hormones could be detected for periods up to 16 days of monolayer culture in cells grown continuously in the TyHS medium used in the earlier study. The greater sensitivity of the current chromatographic assay in comparison with the paper chromatography used in the early study accounts for the failure to detect hormones in TyHS-grown cells.

Nevertheless, there is still a clear and striking difference in the amounts of hormone detectable in cells grown in F12S10 versus TyHS. The latter cells, besides containing less hormone, failed to divide and formed irregular patches in culture, while F12S10-grown cells divided frequently and formed dense sheets of epithelium in culture. Furthermore, TyHS-grown cells had pale nucleoli that lacked a granular component, and accumulated cytoplasmic granules around the nucleus. Nucleoli were phase opaque in F12S10-grown cells and contained a prominent granular component. Granules did not accumulate around the nucleus of such cells. Finally, rough endoplasmic reticulum was sparse in TyHS and extensive in F12S10, thus confirming our earlier independent studies (see Hilfer et al., 1967; Spooner, 1970). None of the features investigated are “fixed” properties of the cells, since hormone levels and morphological properties can be reversed at any time by alterations in the medium.

Modification of phenotypic expression by culture media has also been demonstrated for cartilage (Coon, 1966) and retinal pigment (Cahn and Cahn, 1966) cells in vitro. In those studies, specific cell functions were affected by different fractions of embryo extract. Embryo extract was not responsible for the modifications observed in the present investigation. Instead, it seems highly probable that TyHS represents a condition of nutritional inadequacy. The absence of cell division in TyHS cultures may reflect such an inadequacy.

Despite these considerations, the significant finding is that differentiated thyroid cell function is intrinsically stable during cell culture even under the poor nutrient conditions in TyHS. The techniques used cannot distinguish between low-level hormone synthesis and detection of residual hormone when the cells are maintained in a nondividing condition (i.e., TyHS medium). The quantitative measurements of Tong, Kerkof, and Chaikoff (1962) had previously shown that nondividing monolayers of adult sheep thyroid cells synthesize thyroxine in vitro. Thus, it could well be that low-level synthesis of hormone proceeds in TyHS and that the rate is accelerated when F12S10 is added. Irrespective of the former point, however, is the fact that the cells did remain

FIGURE 8  Nucleolus of cell under the same conditions as Fig. 7. The fibrous core is larger than in F12S10, and the granular component is completely lacking. Note the nuclear pores (arrows). X 17,000.
FIGURE 9  Portions of two cells after 7 days in TyHS. The nucleolus has only a fibrous component, the endoplasmic reticulum exists as a few, narrow channels, and many vesicles with dense homogeneous contents (lipid?) characteristically lie close to the nucleus. X 20,200.

FIGURE 10  Cytoplasm of cell 4 days after being switched to F12S10 after an initial 4 days in TyHS. A pronounced Golgi region (G), canalicate rough endoplasmic reticulum, and large mitochondria fill the cytoplasm. Fibers are visible but are somewhat obscured by the cytoplasm. X 21,200.
FIGURE 11  Nucleolus under the same conditions as Fig. 10. The fibrous core is surrounded by strings of the granular component. \( \times 24,100 \).

stable and did retain the ability to make large quantities of hormone when provided with an appropriate nutrient supply. To be fair, however, it must be emphasized that the stability demonstrated in TyHS is under conditions of low or nondivision. Thus, it is not known if such non- or low expressive cells can transfer the capability to produce larger amounts of differentiated product to progeny cells, as can chondrocytes (Coon, 1966) and pigment cells (Cahn and Cahn, 1966). We do know, however, that the differentiated phenotype is transferred to clonal progeny (Spooner, 1970). In any event, the TyHS-cultured cells certainly do possess sufficient stability of differentiative type to reestablish their secretory phenotype: that is, an extensive rough endoplasmic reticulum reappears after TyHS is replaced by F12S10 (just as occurs in organ culture when thyroid fibroblasts are present, Hilfer et al., 1967). The formation of endoplasmic reticulum in cell culture indicates that the control of cytoplasmic fine structure in thyroid cells is independent of normal tissue level organization, since there are no detectable follicles in our cultures.

Finally, it is worth asking if the ability to modify cellular phenotype is simply an interesting culture phenomenon or whether it can be related to the differentiated state in vivo. Such thyroid properties as follicular cell height and levels of hormone production are known to be modulated by the environment in vivo (via the components of the circulatory system). The environmental (culture medium) modulation of both cell shape and hormone levels demonstrated in vitro reflects this same flexibility. These reversible changes are, therefore, in accord with known thyroid features and reflect a normal characteristic of the differentiated thyroid. Although phenotypically modified, the cells are always recognizable as thyroid by the presence of hormone.

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