Primary Monolayer Culture of Rat Ependymal Cells: An Ultrastructural Study

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Summary. Dissociated cerebella of newborn rats were cultured in vitro as a monolayer for a long period, and ependymal cell differentiation was investigated by scanning and transmission electron microscopy. Cells with actively beating cilia were recognized on the 3rd day, and gradually increased in number thereafter. At nine months of culturing, a number of areas appeared, some of which consisted of several hundreds of ciliated cells. Cultured ependymal cells showed a striking resemblance in their fine structure to the intact ependyma. They were always found to grow upon the feltwork of astrocytic processes which served as a subependymal structure, as observed in the intact ependyma. It is possible that astrocytes may play some role in ependymal differentiation. Cultured ependymal cells are helpful in understanding the control mechanism for the metachronal cilial movement.

The ependyma is an epithelium which lines the ventricles of the brain and the central canal of the spinal cord, and is characterized by numerous cilia on the luminal surface. Since Purkinje (1836) first observed the motion of the cilia of the ependyma, a number of investigators have, by observing the motion of dark particles or red blood cells put on the ependymal surface of excised brains, recorded the effective, rapid movements of the cilia producing currents of the cerebrospinal fluid in distinct directions (Von Willer and Wigodskaya, 1932; Chu, 1942; Hogue, 1947; Konno and Shiotani, 1956; Hild, 1957; Cathcart and Worthington, 1964). However, no adequate description of the beat pattern and metachronal coordination of fluid-transporting cilia has so far been reported. The effects of various substances, as well as the ion concentrations in the cerebrospinal fluid on the ciliary motion are mostly unknown, and the cell culture method should enable a novel approach to these studies. For such purposes an understanding of the precise ultrastructures of cultured ependymal cells is essential. In the present study, ependymal cells with actively beating cilia were maintained in vitro for a long period, and their fine structure described using scanning and transmission electron microscopy to allow a comparison with that of the intact ependyma, which has been well documented in various species of animals (Fleischhauer, 1975; Tennyson and Pappas, 1962; Brightman and Palay, 1963; Klinkerfuss, 1964; Dohrmann and Bucy, 1970; Yamadori and Yagihashi, 1975; Yamadori, 1978; Hirunagi and Yasuda, 1979).
MATERIALS AND METHODS

Cell culture
Cerebellar tissues including the fourth ventricle were obtained from newborn Sprague-Dawley rats of both sexes. For each series of culture, 5 to 10 animals were used. The whole animal body was twice immersed for a few seconds in 70% ethanol for sterilization, and the cerebellum was carefully removed under a dissecting microscope. The tissues were rinsed several times in Ca, Mg-free Hanks' saline (CMF), and then diced into small pieces with razor blades. The diced tissues were incubated in 0.1% EDTA dissolved in CMF for 20 min at 37°C followed by incubation in 0.04% collagenase (Type I, Sigma) for 50 min at 37°C. The tissues were dissociated by a gentle pipetting and centrifuged at 1,800 g for 5 min. The pellets were washed twice and then resuspended in Eagle's MEM (Nissui, Tokyo) supplemented with 10% fetal calf serum (Gibco). Approximately 5×10⁶ cells, including undissociated small fragments, were inoculated into each dish of 3 cm in diameter (Falcon), which had previously been coated with rat tendon collagen. The medium was changed every 5 days throughout the culturing period. 

Secondary transfer: Some dishes of the primary cultures at various stages of culturing were transferred to the secondary passage to determine whether the cultured ependymal cells were transferable to serial passages. The primary cultures were washed twice with CMF, incubated in 0.1% EDTA for 20 min, and treated either with 0.04% collagenase or 0.2% trypsin (Difco, 1:250) for 30 min at 37°C. The dissociated cells were washed twice with the culture medium, and inoculated into the collagen-coated dish.

Electron microscopy
Cultures were rinsed twice with Hanks' saline, fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 60 min, and postfixed with 1% osmium tetroxide. They were stained in situ with 2% uranyl acetate, and then dehydrated. Ultrathin sections were cut vertically or horizontally to the bottom of the dish. For scanning electron microscopy, cultures were fixed in the same fixative for 2 hr at 4°C. They were postfixed, dehydrated, critical-point dried, and coated with gold-palladium.

RESULTS

Light microscopy
All the cultures consisted of various types of cells on the 3rd day of culturing, these being mainly divided into flat epithelial cells and round cells (Fig. 1A). The rapid motion of cilia was observed on the 3rd day among the flat epithelial cells. Ciliated cell areas were located with a marker pencil on the outer surface of the dish, and observed daily. The number of marked areas increased gradually in the first two months. At this time of culture, the culture consisted of epithelial cell sheets and cell-aggregates (Fig. 1B). Ciliated cells were usually found among the epithelial cells. After two months of culturing, large areas of several hundred ciliated cells developed, in which areas almost all the cells possessed vigorously moving cilia. Some marked areas where the ciliary motion had been observed previously seemed to lose any visible motion and be subsequently replaced by non-ciliated epithelial cells. On the other hand, ciliated
Fig. 1. Phase contrast micrographs of cultured ependymal cells. x 100. A. An epithelial sheet is found on the upper part of the micrograph. A few ependymal cells have appeared on it. Round cells with ramified cellular processes may be presumably granular cells. 12th day. B. Cells in the area demarcated by a dotted line possess actively beating cilia. Arrows indicate large cell-aggregates. 94th day. C. Large ciliated area. Almost all cells in this area possess moving cilia. 300th day.
Fig. 2. Scanning electron micrographs of ependymal cells in 94-day cultures.  
A. The length of cilia and their position on the cell surface vary among cells.  x 930.  
B. Tuft of cilia and numerous microvilli.  x 12,000
cells occasionally appeared where on ciliated cells had previously been observed. After nine months of culturing, the number of ciliated areas ranged from 5 to 20 per dish. The number of ciliated cells in one area varied considerably; some consisted of several hundred cells (Fig. 1C), and others only a few dozen.

The primary cultures were transferred at different culture stages, i.e. one, two, and three months after the first inoculation. In the ninth month of total culturing, only in the cultures which had been transferred during the first month developed ciliated cells.

**Electron microscopy**

The cultures were fixed on the 1st, 3rd, and 9th months and were submitted to electron microscopic observation. The fine structure of the cells was basically the same among the three stages of culturing.

The scanning electron microscopic observation revealed ciliated epithelial cells presenting a polygonal surface-contour of various shapes (Fig. 2A). They showed a tuft of cilia usually at the center of the apical surface, where microvillous protrusions were apparent, especially at the boundaries of adjacent cells (Fig. 2B).

Transmission electron microscope observation revealed that ciliated cells grown in cultures showed a remarkable resemblance to intact ependymal cells. In vertical sections, ciliated cells usually lay directly on astrocytic cells and no basal lamina could be seen between them (Fig. 3). Astrocytic cells, which were discerned by the dense distribution of glial filaments in the cytoplasm, often overlapped each other. A number of microvilli protruded at the apical surface of the cells. They were short and occasionally bifurcated (Fig. 3).

The nuclei—with fine chromatin—usually had simple oval shapes, and occupied a large part of the cell. The nucleolus commonly occurred adjacent to the nuclear envelope (Fig. 4). The cytoplasm contained the usual organelles (Fig. 4, 5): numerous mitochondria and dense bodies or lipofuscin granules of various sizes and appearances (Fig. 4). A few moderately developed Golgi complexes occurred at the apical portion of the cell (Fig. 5A). The rough endoplasmic reticulum also was not well extended. Clusters of free ribosomes, however, were widely distributed (Fig. 5).

The cilia contained nine peripheral pairs of microtubules and one central pair, a pattern common to most cilia (KLINKERFUSS, 1964). A basal foot was attached to each basal body. The fibrillogranular aggregates and ciliary rootlets which are present in the intact ependymal cells of the rat could hardly be found in the present culture. Alternatively, fine filaments of a brush-like arrangement were well developed and attached to the wall of the basal body, extending into the cytoplasm where the filaments were encompassed by a cloud of fine granular material (Fig. 5A). Intermediate filaments and microtubules appeared to show a random orientation. The former were often arranged in fascicles, while the latter usually ran separately (Fig. 5B).

On the lateral surfaces of the neighboring cells, apposed membranes formed intercellular junction which consisted of gap junctions (Fig. 6A) and zonulae adherentes (Fig. 6B). The latter appeared to occupy relatively extensive areas of the apposed membranes. Beneath such areas, microfilaments were concentrated and ran in parallel array to the plasma membrane (Fig. 6B).
Fig. 3. Vertical section of ependymal cells in a 90-day culture. They contact each other by zonulae adherentes. Beneath the ependymal cells, multi-layered astrocytes (A) are seen, in which glial filaments are conspicuous. × 9,900
Fig. 4. Horizontal section at a relatively basal level of ependymal cells in a 30-day culture. Cilia and basal bodies are indicated by arrows. *db* Dense bodies. ×3,800
Fig. 5. Legend on the opposite page.
DISCUSSION

In their scanning electron microscope studies of organotypic rat cerebellum cultures, Dalen et al. (1971) and Silberberg (1975) briefly reported the existence of ciliated cells, but no detailed observation has been made thus far on cultured ependymal cells. The present study established the retention of the ciliated cells' properties of differentiation for a long period of culturing. These ciliated cells were identified as ependymal cells by the close similarities in their fine structure to the intact ependymal cell. The surface features of the cultured ependymal cells revealed by scanning electron microscopy resembled those of the intact ependyma (Bruni et al., 1973; Chamberlain, 1973; Peters et al., 1976).

Cultured ependymal cells seemed to grow not directly on the dish, but usually lay on the underlying feltwork of astrocytic processes. No basal lamina was interposed between them, as has been pointed out in the intact ependyma of the cat (Klinkerfuss, 1973).

Fig. 5. Apical portion of ependymal cells in a 90-day (A) and 270-day (B) cultures. A. This cell contains numerous mitochondria and a few Golgi complexes (g). Fine filaments of a brush-like arrangement are encompassed by a cloud of granular materials (arrowheads). db Dense bodies. x 14,000. B. In the cytoplasm thick filaments (f) run as a swirling fascicle. Basal bodies (bb) are sectioned tangentially or transversely. Free ribosomes (r), microtubules (m), and vesicles (v) are observed. x 23,000.

Fig. 6. Junctions between ependymal cells found in a 90-day culture. A. Gap junction. x 64,000. B. Zonulae adherentes. Microfilaments (mf) run in parallel array to the plasma membrane. x 26,000.
1957) and rat (Brightman and Palay, 1963). These facts suggest an essential role for the astrocytes in the differentiation of ependymal cells and in retention of their properties.

However, some differences were noticed in the features of the ependymal cells in situ and in vitro. The cultured ependymal cells were flat rather than cuboidal, and partially overlapped each other. They contained numerous lipofuscin granules which were rather scarce in the intact ependymal cells. Although the cilia of the cultured ependymal cells had a structure common to most cilia, certain appendages, i.e., fibrillo-granular aggregates and ciliary rootlets seemed to be absent. The number of cilia developed on cultured ependymal cell was smaller than that of an intact ependymal cell. The ciliary distribution might have some correlation to the shape of ependymal cells, as Yamadori and Yagihashi (1975) pointed out that the flat ependymal cells in the fourth ventricular floor had sparse distribution of cilia.

It is reported that most, if not all, of the ependyma are formed during the embryonic development (Fujita and Fujita, 1964; Das, 1979). Blakemore and Jolly (1972) suggested that in the dog some of subependymal pale cells undergoing division might replace the cells in the ependyma. In the developmental stages, the number of ependymal cells increases as the volume of the ventricle grows. In the cultures, the number of ciliated areas and the number of ependymal cells in one area increased gradually, and mitotic figures were found occasionally among the ependymal cells. This suggests that the cerebellum of the newborn rat contains cells which can proliferate and differentiate into ependymal cells, although it is uncertain whether ciliated ependymal cells can divide, or cells with undifferentiated properties or astrocytes undergo mitosis to give rise to ependymal cells. This ability of proliferation and differentiation, however, seems to disappear as cultures continue, because the ependymal cells did not differentiate in the secondary cultures transferred from the more than two-month primary cultures.

In the present cultures, the motion of cellular particulates which approached the ependymal cilia indicated that the cilia actually produced a current of the medium (not shown). Judging from the motion of cellular debris, the coordinated metachronal movement of the cilia presumably yielded a constant flow in a direction in some locations, whereas in other areas the cellular debris did not show a constant flow but rather haphazard, meandering traces with several turns. The differences in the fine structure between these areas could not be elucidated in the present study.

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