LONG-TERM CULTURE OF EPITHELIAL CELLS FROM THE NORMAL RAT COLON

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SUMMARY

Serial passage cultures of colonic epithelial cells from young rats have been maintained for more than 6 months in Eagle’s minimum essential medium buffered with HEPES (25 mM) and supplemented with 2.5% fetal bovine serum, 0.5 µg/ml insulin, 5.0 µg/ml transferrin, and antibiotics. The cells proliferated in this medium with a population doubling time of approximately 53 h. The cells retained differentiated morphology as evidenced by secretory activity and the presence of secretory granules, microvilli, tonofilaments, and desmosomal junctions. Further, cells at the fourth passage had normal karyotypes with 42 chromosomes and exhibited anchorage dependent growth. High concentrations of fetal bovine serum (10 to 15%) exerted toxic effects on the colonic epithelial cell cultures.

Key words: colon; epithelial cultures; normal rat.

INTRODUCTION

An availability of normal epithelial cell cultures, particularly from tissues that are primary targets for carcinogenesis should facilitate studies in the areas of control of cell proliferation and differentiation, aging, and mechanisms of carcinogenesis. Attempts to establish normal epithelial cell lines from several different organs have not been successful because of the contamination of epithelial cultures by fibroblasts and loss of proliferative potential of the epithelial cells. The reduction or elimination of fibroblast contamination by selectively using EDTA-trypsin dissociation procedure (1), supplementing the culture medium with collagenase or cis-4-hydroxy-L-proline (2-4), and culturing of cells in D-valine medium (5,6) have been reported. However, these methods are not successful in the selective removal of fibroblast contamination from the primary cultures of colonic epithelial cells (7). Nevertheless colonic epithelial cells have been separated from fibroblasts and subcultured by the penicylinder method (7). Although epithelial cells in primary and low passage cultures maintained typical epithelial characteristics, their proliferative potential was not sustained. These observations suggested that the culture conditions were not optimal. In this report we will describe the conditions for long-term cultivation of colon epithelial cells in Eagle’s minimum essential medium (MEM) buffered with HEPES and supplemented with a low concentration of fetal bovine serum (FBS), insulin, and transferrin. The monolayer cultures have been serially passaged over a period of 6 months without any adverse effects on morphology or proliferative potential of the cells.

MATERIALS AND METHODS

Preparation of cell cultures. The procedure for dissociation and culture of the epithelial cells was the same as described previously (7) except that the culture medium used in this report was Eagle’s minimum essential medium supplemented with 25 mM HEPES (MEM-HEPES) (GIBCO, Grand Island, NY). HEPES buffer was very effective in stabilizing the pH during subculturing procedures and while observing cells in an inverted phase contrast microscope. Briefly, 2- to 16-d-old suckling rats were sacrificed and their

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colons excised and washed with Ca**+-Mg**+-free Hanks' balanced salt solution (BSS). The colons were opened by making a longitudinal slit to expose the epithelial mucosa that was peeled away gently from the underlying lamina propria. The epithelial sheets were dissociated with 0.1% bovine pancreatic trypsin (GIBCO) in BBS for 30 min at room temperature. The cell suspension was filtered through a layer of 100 μm Nitex (Tetko, Elmford, NY), and the filtrate was combined with equal volumes of culture medium supplemented with 2.5 FBS. The cell suspension was centrifuged at 100 x g for 7 min in a DPR-6000 centrifuge (IEC, Needham Heights, MA). The cell pellet was washed twice and resuspended in the culture medium.

In some experiments, the epithelial mucosas were dissociated with 0.5 mg/ml collagenase (I and III, Sigma, St. Louis, MO) in MEM supplemented with 2.5% FBS for 24 h at 37°C. The cell suspensions were washed and resuspended in the culture medium as described above. Since collagenase dissociation had no beneficial effect over trypsin in reducing fibroblast contamination of primary epithelial cell cultures, trypsin was used routinely as a cell dissociation enzyme.

**Effect of different FBS concentrations on epithelial cell proliferation of "primary cultures."** Because high FBS concentrations have been shown to have growth inhibitory effects on epithelial cell proliferation (8), the effect of different FBS concentrations on colonic epithelial cells was examined. With the autoradiographic method, the proliferative activity of epithelial cells was evaluated by estimating the labeling index (LI) after tritiated thymidine [3H]dThD incorporation. Approximately 4 x 10⁴ cells in 2 ml culture medium were plated in each 35 mm culture dish (Falcon Plastic, Los Angeles, CA) containing a cover slip (7). The culture medium was MEM-HEPES supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml Fungizone, and different concentrations (0, 2.5, 5, 10, and 15%) of FBS. The culture medium was changed twice every week. After 3 and 6 d, cultures were pulse labeled for 2 h with [3H]dThD (1 μCi/ml), with a sp act of 6.7 Ci/mmol. The cover slips were rinsed with BSS, fixed in Carnoy's fixative, and mounted on glass slides (cell side up). Slides were coated with Kodak NTB-2 emulsion diluted 1:1 with triple distilled water, exposed for 4 wk, developed in Kodak D-19, and fixed in Kodak rapid fixer. The autoradiograms were stained with periodic acid Schiff's stain (PAS) and hematoxylin. Cells with six or more grains over the nuclei were considered labeled. The number of labeled epithelial cells was counted in a total of 400 cells from each sample. Three samples were enumerated for each serum concentration, and their mean value was taken.

Because the maximum labeling index (LI) was observed at 2.5% FBS (see Results), all primary cultures are now started at this concentration.

**Passage of primary epithelial colonies.** Primary epithelial colonies consisting of 200 to 1000 cells were passaged by using stainless steel cylinders (6 mm d, Fisher Scientific Co., Pittsburgh, PA). The capped colony was rinsed twice with MEM-HEPES and finally immersed in one drop of 0.1% trypsin solution. After 5 min, the culture medium, supplemented with 2.5% FBS and antibiotics, was added to the cylinder, and the cells were dislodged by repeated pipetting. The cell suspension was withdrawn and added to 10 ml of cold "conditioned" culture medium supplemented with 2.5% FBS, 5.0 μg/ml transferrin, and antibiotics. Conditioned medium is one that has been exposed to epithelial cell cultures for 4 d and mixed in a ratio of 1:1 with fresh medium. Two-milliliter aliquots of the cell suspension were cultured in each 35 mm culture dish. All low passage cultures were maintained in conditioned medium by replacing 1 ml of old medium with 1 ml of fresh medium when the medium was changed.

**Enumeration of cells in epithelial colonies.** Because we were dealing with epithelial colonies consisting of only a small number of cells, at least initially, it was essential to devise a method to enumerate cells without sacrificing the colonies. Such a method enabled us to determine the changes in cell numbers and population doubling times at different times after various treatments.

By using an eye piece graticule (1 cm x 1 cm) and an inverted phase-contrast microscope, the number of cells was counted successively in situ on the same epithelial patch at 100 magnification. In larger epithelial patches, the total area of the colony was successively measured. Then the cells in a 50 to 100 mm² area were counted. Total cell numbers were calculated from the multiple of the number of cells in 1 mm² area and the total square millimeter area of the epithelial patch. The average time required to scan one colony was approximately 1 to 2 min.

Population doublings were determined as \( N = N_0 2^n \), in which \( N_0 \) =