Proliferation and differentiation of fetal rat intestinal epithelial cells in primary serum-free culture

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Summary

It has been a subject of controversy whether fibroblastic cells are necessary for the proliferation of intestinal epithelial cells in primary culture. To answer this question, we have developed a serum-free primary culture system which allows reproducible and quantitative assays of proliferation and differentiation of fetal rat intestinal epithelial cells in the absence of fibroblastic cells. Pure intestinal epithelial tissues were obtained from 16.5-day fetal rats without contamination of mesenchymal cells, and were successfully cultured on a collagen gel in a medium consisting of Ham’s F12, bovine serum albumin, epidermal growth factor (EGF), insulin, cholera toxin, transferrin and hydrocortisone. The epithelial nature of the cultured cells was confirmed by the presence of cytokeratin in the cells. Under optimal culture conditions, intestinal epithelial cells readily attached to the substratum in a day, and proliferated rapidly in vitro, increasing their number about 10 times in the first 5 days. EGF, insulin, cholera toxin, transferrin and hydrocortisone synergistically induced the epithelial proliferation, and lack of any one of them resulted in a significant reduction of the proliferation. In contrast, fetal bovine or horse serums, which have been widely used to supplement culture media, severely inhibited the epithelial proliferation.

Histological examination showed that the epithelial cells formed simple cuboidal epithelia with basally-located nuclei when cultured on collagen gels. The intestinal epithelial nature of the cells was affirmed by the presence of villin on their luminal surface. Ultrastructurally, cells were connected by tight junctions and desmosomes at the subluminal region, and microvilli were projecting on the luminal surface, indicating that the cells in primary culture retained some characteristics of absorptive epithelial cells. Biochemical analysis showed that the cells expressed weak aminopeptidase activity while alkaline phosphatase or α-glucosidase activities could not be detected, suggesting that they could not differentiate fully under these culture conditions. We concluded that the fetal intestinal epithelial cells can proliferate in the absence of heterologous cells, but that mesenchymal cells may be necessary for their full functional differentiation.

Key words: small intestine, epithelial cells, primary culture, serum-free culture, villin.

Introduction

Small intestinal epithelium is one of the most actively renewing tissues in the body. Stem cells proliferate in the middle crypt region, and most cells differentiate into absorptive and goblet cells as they migrate to the tip of the villus, while some cells differentiate into Paneth, endocrine and other minor cell types (Trier and Madara, 1981). In the course of differentiation, the absorptive cells of the villus develop a number of enzymes such as maltase (α-glucosidase), aminopeptidase and alkaline phosphatase, which are important for digestive and absorptive functions. These features make the intestinal epithelial cells an excellent model for studying the relationship between cell proliferation, differentiation and morphogenesis. However the control mechanism of their proliferation and differentiation has not been fully understood.

The importance of epithelial-mesenchymal tissue interaction in regulating the proliferation and differentiation of intestinal epithelial cells has been repeatedly reported. We have shown that fetal mouse mesenchymes affect intestinal epithelial differentiation in organ culture (Fukamachi and Takayama, 1980). Also, Kedinger et al. (1986, 1987a) have reported that fetal rat gut mesenchymes or fibroblastic support are important for the morphogenesis and cytodifferentiation of intestinal endodermal cells in culture, and suggested that some hormones act on epithelial cells via mesenchymal cells. It is possible that mesenchymes affect epithelial cells via (a) diffusion of soluble factors, (b) contact with extracellular matrix components and (c) direct cell-cell contact. But it remains to be solved by which mechanism mesenchymes affect intestinal epithelial proliferation and differentiation.

There are many intestinal epithelial cell lines derived from colonic adenocarcinomas (for a review, see Rutzky and Moyer, 1990), some of which respond to combined mesenchymal tissues to exhibit morphological and functional differentiation in vitro (Fukamachi et al., 1987; Rich-
man and Bodmer, 1988; Fukamachi and Kim, 1989). Also, several epithelial cell lines have been established from fetal or neonatal rat small intestine (Quaroni et al., 1979; Blay and Brown, 1984; Négre et al., 1983). But these cells have undergone changes and selection to facilitate long-term proliferation in vitro. For example, Evans et al. (1992) have recently reported that intestinal epithelial cells in primary culture are far more sensitive than permanent cell lines to the quality of culture medium. Thus, established intestinal epithelial cell lines cannot be regarded as an adequate model for studying the mechanism of epithelial-mesenchymal interaction, and it is absolutely necessary to establish a primary culture system where intestinal epithelial cells proliferate and differentiate in the absence of mesenchymal cells in order to examine and identify growth factors and extracellular matrix components which regulate intestinal epithelial proliferation and differentiation.

Since the first attempt of Capdeville et al. (1967), many researchers have tried to culture intestinal epithelial cells (for a review, see Keding et al., 1987b). But in most primary culture systems so far reported, epithelial cells were derived from cellular outgrowths of intact intestinal fragments which were allowed to attach to the bottom of culture dishes, and usually many fibroblastic cells as well as epithelial cells proliferated in primary culture. Thus, these culture systems are not adequate for our study because it is difficult to determine whether growth factors and/or extracellular matrix components affect epithelial cells directly or act on contaminated fibroblastic cells which in turn affect epithelial cells. Keding et al. (1987a) used a primary culture system where pure intestinal epithelial tissues obtained from 14- to 15-day fetal rats were cultured on dishes coated with different extracellular matrix components. But their work was focused on the need of fibroblastic cells for in vitro differentiation of intestinal epithelial cells, and they did not quantitatively examine epithelial proliferation in the absence of mesenchymal cells.

We have developed a primary culture system where the proliferation and differentiation of pure intestinal epithelial tissues from 16-day fetal rats can be quantitatively examined. Our procedures allow reproducible proliferation of intestinal epithelial cells in the absence of mesenchymal cells, and would be useful for further analysis of epithelial-mesenchymal interaction.

Materials and methods

Preparation of tissue fragments

Fischer 344/Du rats (Charles River Japan) were mated during the night, and copulation was checked by the presence of spermatozoa in vaginal smears on the following morning. The conceptus was considered 0.5 days old at noon of this day. Animals were killed by cervical dislocation, and duodenal tissues were dissected out from 16.5-day fetuses. They were treated with 0.1% collagenase (CLS I; Worthington) in Hanks’ balanced salt solution (HBSS) at 37°C for 70 min, and then epithelial tissues were separated from mesenchymes by the aid of forceps under a dissecting microscope. The epithelial tissues were further treated with 0.75% collagenase and 0.75% hyaluronidase (HSE; Worthington) in HBSS at 37°C for 50 min to obtain smaller tissue fragments, by repeated pipetting with a Pasteur pipette.

Primary culture

Collagen gels were reconstituted from collagen solution extracted from rat tails according to the method of Imagawa et al. (1984), and were used as substrata. The epithelial cells were cultured in Ham’s F12 medium (Gibco) supplemented with 1 mg/ml of bovine serum albumin (fraction V; Sigma) (basal medium). Various concentrations of epidermal growth factor (EGF; Collaborative Res.), insulin (Sigma), cholera toxin (List Biol. Lab.), transferrin (Sigma) and hydrocortisone (Sigma) were also added to the basal medium to induce epithelial proliferation. No antibiotics were used in this experiment. To examine the effect of sera, fetal bovine or horse sera were obtained from Filitron (Altona, Victoria, Australia), Whittaker M. A. Bioproducts (Walkersville, MD, USA) or Handai Biken (Osaka, Japan), and were added to the culture medium at concentrations of 2 to 20%.

Intestinal epithelial cells from a fetus suspended in 1 ml of culture medium were seeded into each well of 24-well multiwell plates precoated with collagen gels, and incubated in a humidified atmosphere of 5% CO2 in air at 37°C.

Immunofluorescence microscopy with anti-cytokeratin antibody

To confirm the epithelial nature of the cultured cells, some cells were cultured on collagen gels which had been formed on sterilized cover glasses, fixed with cold 95% ethanol, and dried to attach the cells to the cover glass using collagen gels as an adhesive. Before staining, the cells were treated with prechilled methanol and washed with PBS. They were then treated with PKK-1 mouse anti-cytokeratin antibody (Labsystems, Finland) followed by fluorescein-conjugated sheep anti-mouse antibody (Cappel), and observed with an Olympus fluorescent microscope equipped with an epi-illumination excitation system. Normal mouse serum instead of PKK-1 antibody was used for the controls.

Cell number determination

The number of cells was measured by a modification of the tetrazolium assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) as a substrate (MTT assay; Alley et al., 1988). MTT working solution was made by mixing equal volumes of MTT stock solution (5 mg MTT/ml Ca2+- and Mg2+-free phosphate buffered saline) and Ham’s F12. At the termination of culture, 200 µl of prewarmed MTT working solution was added to each well, resulting in 500 µg MTT/1.2 ml medium per well. After incubation for 4 h at 37°C, MTT-containing media were removed, and replaced with 1 ml of dimethyl sulfoxide (Dojin Chem., Japan) to dissolve formazan precipitates. The amount of formazan was spectrophotometrically estimated by measuring absorbance at 540 nm, and was used to determine cell numbers in a well. To reduce variation between experiments, cell numbers were expressed as a percentage by regarding the cell number in optimal culture conditions as 100%.

Immunohistochemistry with anti-villin antibody

Cells cultured on collagen gels were fixed with cold 95% ethanol, embedded in paraffin and sectioned serially according to the method of Sainte-Marie (1962). The sections were treated with mouse anti-villin antibody (Chemicon, USA) followed by peroxidase-conjugated sheep anti-mouse antibody (Cappel). After the peroxidase activity had been visualized using diaminobenzidine tetrahydrochloride (Sigma) as substrate, sections were counterstained with 1% methyl green in 0.1 M Veronal buffer (pH 4.0). Normal mouse serum instead of anti-villin antibody was used as a control.
Transmission electron microscopy
Some cells were fixed with 2.5% glutaraldehyde (Sigma) in 0.1% cacodylate buffer (pH 7.4) for 2 h at 4°C, postfixed with 1% osmium tetroxide in the buffer for 2 h at 4°C, dehydrated through a graded series of ethanol and n-butyl glycidyl ether and embedded in Embedding Resin (Taab). Ultrathin sections were cut, stained with uranyl acetate and lead citrate and observed with a JEOL 100CX electron microscope.

Enzyme assay
Cultured intestinal epithelial cells were harvested by dissolving collagen gels with 0.1% collagenase for 1 h at 37°C, washed with HBSS, homogenized in iced distilled water, and stored frozen at −20°C. Alkaline phosphatase, aminopeptidase and α-glucosidase activities in total tissue homogenates were determined using p-nitrophenylphosphate, L-alanine-p-nitroanilide and p-nitrophenyl-α-D-glucopyranoside, respectively, as substrates. One unit of activity equals 1 μmole of product formed per min at 37°C. Protein was determined according to the method of Bradford (1976) using γ-globulin as standard.

Results

Observations with a phase contrast microscope
When intestinal tissue fragments were treated with collagenase, epithelial and mesenchymal tissues could be easily separated. As shown in Fig. 1, pure epithelial tissues with little contamination by mesenchymal cells could be easily obtained, and were used as materials in these experiments. Attempts were made to cultivate single epithelial cells which had been disaggregated by enzyme treatment before plating, but so far they have been unsuccessful. Successful proliferation of the epithelial cells could be obtained only when epithelial tissue fragments were used for culture (data not shown). When tissue fragments were seeded on collagen gels on day 0, most of them attached to the substratum in a day, and the epithelial cells proliferated rapidly in the first few days under optimal culture conditions (see below). These epithelial cells attached closely to each other, and exhibited typical epithelial morphology with distinct margination of cell sheets (Fig. 2). Preliminary experiments showed that collagen gels were necessary for the optimal proliferation of intestinal epithelial cells (data not shown). On days 3 to 5 in culture, some cells in the center of a cell sheet piled up and ceased to proliferate, while other cells at the periphery of a cell sheet continued to proliferate. Most epithelial cells usually degenerated in 4 weeks. Attempts have been made to subcultivate intestinal epithelial cells, but so far they have been unsuccessful.

The cultured cells expressed cytokeratin
The epithelial nature of the cultured cells was examined by staining cells with anti-cytokeratin antibody. As shown in Fig. 3, more than 99% of cultured cells expressed cytokeratin on days 1 to 3 in culture, suggesting that most cells were epithelial in nature. Cells at the periphery of a cell sheet were more strongly stained with anti-cytokeratin antibody than those in the center of a cell sheet. On days 4 to 8, some cells in the center of a large cell sheet failed to express the antigen while cells at the periphery always expressed it.

Proliferation of intestinal epithelial cells
The proliferation of intestinal epithelial cells in culture was quantitatively measured by MTT assay. There was a good correlation between the cell number in a well and the amount of formazan in the range of 10^3 to 5 × 10^4 cells (Fig. 4 shows the correlation up to 10^4 cells). Using this assay system, changes in intestinal epithelial cell number could easily and accurately be determined.

The proliferation of intestinal epithelial cells under optimal culture conditions is shown in Fig. 5. They proliferated most rapidly from days 1 to 2, and relatively slowly from days 2 to 5. In total, the cell number increased about 10 times in the first 5 days in culture. In the following experiments, the cell number on day 4 was used to examine the effect of growth factors on the proliferation of intestinal epithelial cells.

Growth factors synergistically induced intestinal epithelial proliferation
The effect of growth factors on the proliferation of intestinal epithelial cells was quantitatively examined, and results are summarized in Fig. 6. The cells proliferated most extensively when the basal medium was supplemented with 20 ng/ml of EGF, 30 μg/ml of insulin, 200 ng/ml of cholera toxin, 100 μg/ml of transferrin and 2 μg/ml of hydrocortisone (complete medium). As shown in Fig. 7, none of the factors supported the epithelial growth fully when only a single factor was added to the basal medium. Rather, these factors synergistically induced epithelial proliferation. This is clearly shown by Fig. 8, where lack of any one of 5 factors severely inhibited the epithelial proliferation, showing that all 5 factors are necessary for the proliferation of intestinal epithelial cells in primary culture. The effect of other growth factors was also examined, but intestinal epithelial proliferation was not stimulated when sodium selenite (100 to 1 μg/ml), ethanolamine (10^{-3} to 10^{-5} M), phosphoethanolamine (10^{-3} to 10^{-5} M), phorbol 12-myristate 13-acetate (10^{-6} to 10^{-9} M), or estradiol 17-β (10^{-7} to 10^{-9} M) were added to the complete medium (data not shown).

Fig. 1. Phase contrast micrograph of duodenal epithelial tissues just after separation from mesenchymal tissues. Note that epithelial tubes with few attaching mesenchymal cells can be obtained by our method. Bar, 1 mm.
Fig. 2. Phase contrast micrographs of fetal rat intestinal epithelial cells on days (A) 1, (B) 3, (C) 5 and (D) 8, cultured under optimal conditions, showing sequential changes of the same tissue fragment in culture. Note that cells at the periphery of the cell sheet always closely attach to each other while cells in the center of the cell sheet pile up. Only a limited area is just in focus on days 5 and 8 because cells pull the collagen gel to make the substratum uneven. Bar, 100 μm.
Primary culture of intestinal epithelium

Serum inhibited the epithelial proliferation

In previous studies, serums have always been used to supplement the culture medium in order to sustain intestinal epithelial proliferation. In the present culture conditions, however, fetal bovine or horse serums at concentrations of 2 to 20% severely inhibited intestinal epithelial proliferation (Fig. 9), although they supported the proliferation of fibroblastic cells and colonic adenocarcinoma cells (data not shown). Also, bovine pituitary extract, which has been widely used to induce proliferation of several kinds of epithelial cells including keratinocytes in serum-free conditions, inhibited intestinal epithelial proliferation (data not shown).

The structure of the epithelial cells

Intestinal epithelial cells formed simple cuboidal epithelia with basally-located nuclei when cultured on collagen gels. Immunohistochemical analysis showed that villin was localized on their luminal surface (Fig. 10). Ultrastructurally, the epithelial cells closely attached to each other, and many long microvilli were projecting on the luminal surface, while their basal surface was relatively smooth with a few cytoplasmic projections (Fig. 11A). They were connected by tight junctions and desmosomes at the subluminal region (Fig. 11B). They were relatively homogeneous, and cells with mucous or serous granules were not found. Thus, the intestinal epithelial cells in primary culture retained some characteristics of absorptive epithelial cells.

Enzyme assay

Duodenal tissues of 16.5-day fetuses expressed a weak aminopeptidase activity (14 mU/mg protein). A very weak aminopeptidase activity (1 to 3 mU/mg protein) was detected in intestinal epithelial cells on day 4 in culture, but the activity could not be found on days 6 or 8. Alkaline phosphatase or α-glucosidase activities could not be detected at all.

Discussion

In the present study, we established a primary serum-free
culture system where pure intestinal epithelial cells proliferated and differentiated in the absence of mesenchymal cells. Immunohistochemical analysis using anti-cytokeratin antibody showed that more than 99% of cultured cells expressed cytokeratin antigen. It has been well established that cytokeratins are present only in epithelial cells (Sun et al., 1979; Osborn et al., 1982). Thus, we concluded that our cells were epithelial in nature, and that fibroblastic cells were not contaminating the culture. Using this system, we quantitatively examined the effect of growth factors on the proliferation of intestinal epithelial cells in vitro, and found that growth factors synergistically induced their proliferation whereas sera inhibited it.

In previous studies, intestinal epithelial growth was almost always obtained from cultures consisting of epithelial and non-epithelial cell populations. Quaroni and May (1980) reported that suspensions of isolated intestinal cells underwent rapid degeneration in vitro and were not a good source of cells for culture, and that epithelial cell lines could be established only when epithelial cells were contaminated with fibroblastic cells in primary culture. Thus Evans et al. (1992) have suggested that the presence of heterogeneous

![Fig. 6. Effect of various concentrations of growth factors on the proliferation of intestinal epithelial cells in primary culture. The concentration of a factor in the complete medium was changed, while the concentration of other factors was kept constant (EGF = 20 ng/ml, insulin = 30 µg/ml, cholera toxin = 200 ng/ml, transferrin = 100 µg/ml, hydrocortisone = 2 µg/ml). Each bar shows the average of 3 independent experiments.](image)

![Fig. 7. Effect of various combinations of growth factors on the proliferation of intestinal epithelial cells in primary culture. Cells were cultured in media supplemented with various combinations of EGF (20 ng/ml), insulin (30 µg/ml), cholera toxin (CT: 200 ng/ml), transferrin (TF: 100 µg/ml), and hydrocortisone (HC: 2 µg/ml). Average of 2 independent experiments is shown.](image)

![Fig. 8. Proliferation of fetal rat intestinal epithelial cells in complete medium (complete: EGF = 20 ng/ml, insulin = 30 µg/ml, cholera toxin = 200 ng/ml, transferrin = 100 µg/ml, hydrocortisone = 2 µg/ml) or in complete medium with individual deletion of EGF (−EGF), insulin (−insulin), cholera toxin (−CT), transferrin (−TF) or hydrocortisone (−HC). Mean ± s.d. of 6 independent experiments.](image)
Primary culture of intestinal epithelium may be important for intestinal epithelial proliferation, since only in these mixed cultures have growing colonies of epithelium been observed. This is in striking contrast with culture systems for keratinocytes, mammary epithelial cells, hepatocytes, etc., where pure epithelial cells can proliferate in the absence of fibroblastic cells in primary culture. Our present work demonstrates that pure intestinal epithelial cells can proliferate in the absence of mesenchymal cells, and suggests that proliferation of intestinal epithelial cells is controlled by a mechanism common to other types of epithelial cells.

In the present experiment, we have developed a serum-free culture system to induce proliferation of intestinal epithelial cells in primary culture. This is the first report showing that normal intestinal epithelial cells proliferate in a serum-free condition in the absence of mesenchymal cells. There is evidence from in vivo studies that supports the concept of a feedback regulation of crypt cell proliferation by the differentiated villous cells (Rijke et al., 1976). Consistent with this, May et al. (1981) reported that soluble fractions obtained from villous cell homogenates inhibited growth of an intestinal epithelial cell line. Our serum-free primary culture system would be useful for the analysis and identification of such regulatory factor(s) in intestinal tissues.

In this study, we found that serum inhibited the proliferation of intestinal epithelial cells obtained from 16.5-day rat fetuses in primary culture. In previous studies, fetal bovine serum was almost always used to supplement the culture media in culturing intestinal epithelial cells. We suppose that this is a reason why intestinal epithelial cells would not proliferate rapidly in these studies. It is well known that the serum contains a variety of factors which modify differentiated functions of cultured epithelial cells (Masui et al., 1986). Wu and Smith (1982) reported that serum levels higher than 0.1% inhibited tracheal epithelial cell growth, and that serum masked the growth-stimulating activities of EGF and insulin. It remains to be examined which component(s) of the serum inhibit the proliferation of intestinal epithelial cells. We also found that intestinal epithelial cells proliferated most extensively when 100 µg/ml of transferrin were added into the culture media. This may be above the concentration of transferrin required for its function as an iron carrier. Our transferrin was about 98% pure, but it is possible that contaminant(s) in the trans-

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**Fig. 9.** Serum inhibits the proliferation of fetal rat intestinal epithelial cells in primary culture. Growth of the cells in serum-free complete medium (w/o serum) or in complete medium supplemented with various lots of fetal bovine serum (FBS) or horse serum (HS) at the concentration of 20% (oblique lined bars) or 2% (dotted bars). Average of 2 independent experiments.

**Fig. 10.** Light micrographs of fetal rat intestinal epithelial cells on day 4 in culture stained with (A) antivillin antibody or (B) control serum. Note that villin was localized on the apical surface of the cells. Asterisks indicate collagen gels. Bar, 50 µm.
ferrin preparation promote proliferation of the epithelial cells. It is a problem for the future to examine such possibilities.

The optimal culture condition for a particular type of cells usually differs depending on the stage of development of tissues. It remains to be examined whether neonatal or adult intestinal epithelial cells proliferate under serum-free conditions in the absence of fibroblastic cells, and whether their proliferation is inhibited by the serum. In previous studies using neonatal or adult intestinal tissues, it has been very difficult to obtain pure epithelial cells because fibroblastic cells proliferate rapidly in serum-supplemented media. In the present study, we found that disaggregated mesenchymal cells could not proliferate in serum-free conditions while they did proliferate in serum-supplemented media (data not shown). This indicates that our serum-free culture system may be useful for the elimination of contaminated fibroblastic cells from primary epithelial cell cultures. By using a serum-free condition, pure intestinal epithelial cells may be readily obtained from neonatal or adult animals.

In the present study, we found that cultured intestinal epithelial cells formed simple cuboidal epithelia on collagen gels. Immunohistological studies showed that villin, a Ca\(^{2+}\)-regulated actin binding protein specifically expressed in epithelial cells of some internal organs including intestine (Robine et al., 1985), was localized on the luminal surface of the cultured intestinal epithelial cells. Ultrastructurally, the epithelial cells exhibited polarity with many long microvilli on the luminal surface, and were connected by tight junctions and desmosomes at the subluminal region. Also, we found that the cells expressed very weak aminopeptidase activity, while the activity was less than that expressed in fetal tissues. Thus, we concluded that the intestinal epithelial cells in primary culture retained some characteristics of absorptive epithelial cells. However, we could not find any alkaline phosphatase or α-glucosidase activities in the present study. These results are consistent with the report of Kedinger et al. (1987a) that some intestinal epithelial cells were positively stained with anti-aminopeptidase antibodies, but no cells were stained with anti-maltase, or anti-alkaline phosphatase antibodies when 14- or 15-day fetal rat intestinal epithelial cells were cultured. Our results indicate that intestinal epithelial cells functionally differentiated only a little, even when they exhibited morphologic differentiation in the absence of mesenchymal cells. Kedinger et al. (1987a) reported that fibroblastic support was necessary for the functional differentiation of intestinal epithelial cells. Thus, it is probable that the proliferation and morphological differentiation of intestinal epithelial cells can be induced in the absence of mesenchymal cells, but that interaction between epithelial and mesenchymal cells may be necessary for the functional differentiation of intestinal epithelial cells.

Under present culture conditions, we found that most epithelial cells differentiated into cells with characteristics of absorptive cells, and we could not find any cells with serous or mucous granules. It has been reported that mam-

Fig. 11. Transmission electron micrographs of fetal rat intestinal epithelial cells on day 5 in culture. (A) Epithelial cells exhibit polarity with many long microvilli on the luminal surface, basally located nuclei, and smooth basal surfaces. Bar, 5 µm. (B) Higher magnification of a cell-cell junction facing lumen, showing a tight junction (arrowheads), and desmosomes (arrows). Bar, 0.5 µm.
mary epithelial cells differentiate better when cultured on floating collagen membranes (Emerman et al., 1977) or embedded within collagen gels (Yang et al., 1980). Also we have found that uterine epithelial cells exhibit better differentiation when cultured on basement membrane-like substrata than on collagen gels (Fukamachi and McLachlan, 1991). It is an important subject for the future to find culture conditions which allow full differentiation of intestinal epithelial cells in the absence of mesenchymal cells.

So far, we have not succeeded in subcultivating intestinal epithelial cells. It is also a problem for the future to develop a culture system where normal intestinal epithelial cells can be subcultivated in a serum-free condition. Attempts are now in progress to establish permanent cell lines by transfecting intestinal epithelial cells with SV40 large T antigen genes.

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