In Vitro Cell. Dev. Biol.—Animal 33:536–545, July–August 1997

1071-2690/97 $05.00+0.00

ISOLATING AND MAINTAINING HIGHLY POLARIZED PRIMARY EPITHELIAL CELLS FROM NORMAL HUMAN DUODENUM FOR GROWTH AS SPHEROID-LIKE VESICLES

HANS-JÜRGEN BOXBERGER, THOMAS F. MEYER,[1] MATTHIAS C. GRAUSAM, KRISTIAN REICH, HORST-DIETER BECKER, AND MICHAEL J. SESSLER

Max-Planck-Institut für Biologie, Abteilung Infektionsbiologie, Spemannstrasse 34, 72076 Tübingen, Germany (H.-J. B., T. F. M.), and Abteilung Allgemeine Chirurgie und Poliklinik, Universität Tübingen, Hoppe-Seyler-Strasse 3, 72076 Tübingen, Germany (M. C. G., K. R., H.-D. B., M. J. S.)

(Received 20 February 1996; accepted 14 October 1996)

SUMMARY

A method is described for the three-dimensional (3-D) *in vitro* culture of nontransformed gastrointestinal epithelial cells from the human duodenal mucosa. Biopsies obtained from human duodenum were finely minced. The tissue fragments were suspended in culture medium supplemented with 5% fetal calf serum and the appropriate antibiotics. The suspended mucosal fragments generated spheroid-like multicellular vesicles consisting of highly prismatic absorptive and goblet cells retaining most of the histological features of the tissue *in vivo*. We performed immunocytochemical studies to determine the origin of the vesicles using monoclonal antibodies against EP4. The histochemistry of the vesicles showed alkaline phosphatase activity. Ultrastructural studies revealed that these cells exhibit characteristics of normal duodenal cells *in vivo*: apical microvilli, glycocalyx, tight junctions and desmosomes, lateral membrane interdigitations, mucous droplets, and a well-developed Golgi apparatus. An overgrowth of the vesicles by fibroblasts was not seen during cultivation. In contrast with the two-dimensional cell cultures grown on artificial supports, the vesicle cells show organization similar to that of natural epithelia. The polarization and cytoarchitecture of normal gastrointestinal epithelial cells cultured as 3-D vesicles are comparable to those known for the native tissue. This study was undertaken to provide a morphological baseline for subsequent infection experiments.

Key words: duodenum; spheroid-like vesicles; polarization; human gastrointestinal epithelium; three-dimensional cell culture; *in vitro* model.

INTRODUCTION

Because the microaerophilic bacterium *Helicobacter pylori*, usually detected in the antrum but also in the duodenal cap (Prewett et al., 1992), is accepted as the main precursor for gastric and duodenal ulceration (Bayerdörffer et al., 1992) increasing interest has focused on the interactions between *H. pylori* and the human gastrointestinal mucosa. To this end, cultures of normal primary epithelial cells are indispensable in studying *H. pylori* colonization in different functional areas of the gastric as well as the duodenal epithelium. Although the small intestinal mucosa represents one of the most rapidly proliferating cell populations in the body (Cairnie et al., 1965; Weiser, 1972), epithelial cells from normal duodenal tissue have proven rather resistant to *in vitro* culture.

Several investigators attempting to develop *in vitro* culture systems have emphasized the difficulties of maintaining the typical differentiated functions of duodenal villous cells. Many studies have been performed with cells of murine origin (Harrison and Webster, 1969; Lichtenberger et al., 1973; Raul et al., 1978; Tsuchiya and Okada, 1982; Négrel et al., 1983; Kondo et al., 1984; Quaroni, 1985; Kedinger et al., 1986; Tait et al., 1992) but only a few with cells of human origin (Moyer, 1983; Gibson-D'Ambrosio et al., 1986). However, most of these *in vitro* studies of small intestinal epithelium have been limited by rapid autolysis, dedifferentiation, fibroblastic overgrowth, and the loss of specific intestinal features (Raul et al., 1978; Négrel et al., 1983; Kondo et al., 1984; Quaroni, 1985; Gibson-D'Ambrosio et al., 1986), although organ culture has been described (Trier, 1976).

Although the potential of epithelial cells to polarize is genetically determined, the expression of polarized organization of organelles, cytoskeleton, and enzyme activity, as well as the asymmetric distribution of membrane components, depends on specific external conditions (Nelson and Veshnock, 1987; Rodriguez-Boulan and Nelson, 1989). Likewise, the formation of cell contact structures and cell–substratum interactions plays a critical role in the development of epithelial cell polarity (Streuli et al., 1991). In other words, the culture conditions determine the degree to which tissue characteristics are expressed in primary epithelial cells grown *in vitro*.

Common methods of cell culturing are limiting because they do not allow the spatial arrangement of intact epithelial linings in live organisms to be reproduced (Rodriguez-Boulan and Nelson, 1989). To circumvent the drawbacks of two-dimensional (2-D) cultures and to maintain the original epithelial structure and morphology, we established a multicellular vesicle culture system for normal epithelial cells from the human duodenal mucosa (HDEC) that avoids contact

[1]To whom correspondence should be addressed.

between the cells and any artificial supports, as well as enzymatic disaggregation of the epithelial cell sheet. Therefore, this three-dimensional (3-D) *in vitro* model is a considerable improvement over the conventional monolayer techniques. It represents a useful and reproducible tool for a wide range of studies on basic biological mechanisms, including metabolic measurement, lumen formation, transcytosis, and a variety of pathogenic processes such as the colonization and invasion of normal human mucosal cells by microorganisms.

Materials and Methods

Preparation of human duodenal tissue. Sixty patients presenting for routine upper gastrointestinal endoscopic assessment were selected as donors. The study was approved by the hospital ethics committee. All patients gave their informed consent to the biopsies. Subjects with known malignancy were excluded. Biopsies obtained for this study revealed no alterations in duodenal morphometry or histology. From these volunteers, biopsy specimens (100 mg wet wt each) were obtained from different sites of the duodenal cap. The tissue samples were collected in wash medium (as will be described) and transported to the cell culture laboratory for further preparation within 2 to 3 h. The biopsies were rinsed several times with fresh wash medium, clipped free of loose adventitial connective tissue, and placed in a plastic Petri dish (Falcon, Becton Dickinson, Lincoln Park, NJ) containing 10 ml of wash medium for further processing.

Cell culture media. The wash medium initially used to kill indigenous bacteria was RPMI (Biochrom, Berlin, Germany) containing 25 m*M* HEPES buffer (GIBCO, Paisley, Scotland), 5% fetal calf serum (FCS; Biochrom), 100 U penicillin (GIBCO) per ml, 100 μg streptomycin (GIBCO) per ml, and 50 μg gentamicin (Sigma, St. Louis, MO) per ml. Two to 4 μg Fungizone (GIBCO) per ml was added to prevent yeast contamination. Alternatively, we used a mixture of DME (Biochrom) and Ham's F12 (Vitromex, Vilshofen, Germany) medium (1:1) supplemented with the same ingredients just mentioned. The same mixture without antibiotics was used as growth medium. To dissolve the newly produced mucus, the growth medium was supplemented with 5 to 50 μg mucomyst (*N*-acetyl L-cysteine; Fluka, Buchs, Switzerland) per ml (Buset et al., 1986).

Culture of normal human duodenal epithelial cells (HDEC). HDEC were grown both as 2-D cell layers and as 3-D vesicle cultures. Small intestinal scrapings for conventional cell culture were prepared according to the method described by Kondo and colleagues (1984). Briefly, using sterile techniques, we dissected the biopsies into small fragments which were rinsed again with fresh wash medium. Usually, the fragments of one biopsy were introduced into a small, 3.5-mm-diameter culture dish (Falcon) containing 1 ml of wash medium. The cultures were grown in a humidified incubator at 37° C containing 5% CO_2 for 12 h to allow initial attachment.

After the attachment phase, the wash medium was removed and replaced by 1.25 ml of growth medium. During the 2- to 3-d period permitted for adhesion, the cultures were handled very gently to prevent detachment of the mucosal fragments. The medium was changed three times per week. Every third day we removed the newly produced mucus by gently rinsing the adhered explants with warm phosphate-buffered saline (PBS). Cells obtained by this method were shown by microscopical techniques to be epithelial and were not contaminated with fibroblasts. We have not attempted to subculture these cells.

As an alternative to culture on artificial substrata, HDEC were grown on bovine lens capsules. These natural laminae densae were isolated by a modification of the technique of Starkey and coworkers (1984), washed with sterile Ca^{2+}- and Mg^{2+}-free PBS, and mounted under sterile conditions in teflon Combi-ring dishes (Renner, Dannstadt, Germany) to create a basement membrane chamber (Boxberger et al., 1989).

The duodenal epithelial vesicles were prepared and maintained as described (Moyer, 1983) with the following modifications: the mucosa was separated from the underlying submucosa by our gently scraping off coherent cell clusters with a blunt scalpel. The isolated cell clumps were rinsed twice in fresh wash medium and transferred to small petri dishes (Falcon), each containing 3 ml of wash medium. The vesicles were routinely examined by phase-contrast microscopy to assess morphology. After 24 to 48 h they were collected with a sterile Pasteur pipette and cultured separately in Millicell inserts with a pore size of 3 μm (Millipore, Bedford, MA) which were placed in six-well tissue culture plates (Nunc, Roskilde, Denmark).

The impermeability of the vesicles was tested with trypan blue (Merck, Darmstadt, Germany) as a tracer according to the method of Herzog and Miller (1981). The epithelial nature of the vesicle was confirmed both by electron microscopy and histochemical procedures.

Immunodetection of laminin and collagen type IV. The presence of a basal lamina was assessed by immunofluorescent staining. HDEC vesicles were embedded in 1% agarose (Seakem, FMC, Rockland, ME) and fixed in 4% formalin (Merck). The samples were then dehydrated with isopropanol (Merck) for 20 min at 60° C, embedded in paraffin (Merck) for 20 min at 80° C, sectioned at a thickness of 3 to 4 μm with an HM 400 microtome (Microm, Heidelberg, Germany), and mounted on glass slides. Sections were then cleared in xylene (Merck) for 15 min and hydrated to water in an ethanol series (100%, 96%, 80%, 70%). After being rinsed in Tris-buffered balanced salt solution (TBS) at pH 7.6, the sections were incubated in mouse-derived primary antibodies to laminin (1:30; Dako, Hamburg, Germany) or to collagen type IV (1:40; Dianova, Hamburg, Germany) for 1 h. This incubation was followed, after a TBS rinse, with exposure to rabbit anti-mouse IgG (Dako) for 30 min. After a further rinse in TBS, the Streptavidin/Biotin Complex (Dako) was added. The chromagen (100 mg diaminobenzidine tetrachloride diluted in 200 ml of TBS with 100 μl 30% hydrogen peroxide) was applied to the sections for 30 min and again rinsed with TBS. Controls were labeled with a second antibody only. Finally, the sections were stained with hematoxylin (as will be described) before being mounted with Eukitt (Kindler, Freiburg, Germany) on glass coverslips.

The sections were examined at 450 to 490 nm with a Nikon Microphot-Fx microscope (Nippon Kokagu K.K., Tokyo, Japan) equipped with PSM-1 and HB 10101 high pressure mercury lamps (Nikon). Photographs were taken with an FX-35a camera (Nikon) on Kodak TMax 400 or Ektachrome 160 T film.

Hematoxylin/eosin (HE) staining. The specimens were embedded in paraffin as just described and thick sections were stained with HE before being observed with a light microscope: after hydration to water (described earlier), the sections were stained with hematoxylin (Merck) and rinsed with hot tap water (5 min) and cold distilled water. After being counterstained with eosin (Sigma) for 5 min, the sections were rinsed once again with distilled water, dehydrated both with ethanol (70%, 96%, 100%) and xylene (5 min), and finally mounted as described earlier.

Viability assay. To assess the viability of the HDEC in 3-D culture, the vesicles were treated with the Eukolight Viability/Cytotoxicity Kit (Molecular Probes, Inc., Eugene, OR). After being rinsed with PBS, the vesicles were incubated in 5 μ*M* calcein AM and 5 μ*M* ethidium homodimer-1 for 2 h at 37° C in the dark. The cells were examined at 450 to 490 nm with the Nikon microscope mentioned earlier. Photographs were taken on Kodak Elite 400 film.

Proof of alkaline phosphatase (AP). AP activity was localized as follows: the HDEC vesicles were embedded in paraffin (Merck) and sectioned as described. The sections were stained both with Neufuchsin (Fluka) and hematoxylin (Merck).

Alternatively, 5-μm-thick cryosections were treated with Alkaline Phosphate Substrate Kit-Vector® Blue according to the protocol of Vector Laboratories, Peterborough, England. Incubation time was 2 h at pH 8.2.

Identification of epithelial antigens. To determine the expression of the epithelial cell antigen EP4 on HDEC grown as 3-D cultures, multicellular vesicles were digested with 0.25% trypsin (Merck) for 10 min at room temperature. Single cell suspensions were washed with ice-cold PBS and incubated with mouse anti-EP4 (1:20; Dako, Hamburg, Germany) or isotype-matched control for 30 min at 4° C, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (1:200; Dako). This monoclonal antibody, Ber-EP4, stains a partially formol-resistant epitope on the protein moiety of two glycopolypeptides expressed on the cell surface as well as in the cytoplasm of a wide range of human epithelial cells (Latza et al., 1990). Cells were analyzed with a FACSort (Becton Dickinson, Mountain View, CA) equipped with Cellquest Software. In each individual analysis, 20,000 cells derived from four to six vesicles were counted.

Light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). LM was performed with a Zeiss Axiovert 10 photomicroscope equipped with phase contrast (Carl Zeiss, Oberkochen, Germany). Specimens for SEM and TEM were prepared as previously described (Boxberger et al., 1989).

Results

Primary cultures were made with mucosa from normal human duodenal cap obtained from 60 donors. The use of high antibiotic levels at the beginning of the cultures reduced the possibility of infection by microorganisms and prevented subsequent contamination. Some primary cultures were lost due to overgrowth of bacteria and/or fungi, but most of the conventional cell cultures could be maintained for more than 30 d until cell degeneration became evident.

2-D culture of HDEC on different supports. In the case of the explant cultures, the mucosal cells were grown on plastic supports and bovine lens capsule. The viable cells migrated out of the periphery of the explants after 5 to 12 d following attachment to the plastic. The HDEC growing out from explants were irregular in shape and showed nuclei containing one to three nucleoli (Fig. 1 *A*). The culture of HDEC on artificial supports led to single clusters of flattened cells. After 15 to 20 d, they displayed a loose-packed pattern that could be taken as indicative of an epithelial layer (Fig. 1 *A*). Confluent monolayers which covered the entire surface of the supports were not found.

The 2-D cultures grown on artificial substrates could be maintained for more than 30 d without signs of deterioration. Fibroblasts appeared, if at all, only after 20 d of cultivation. If present, the fibroblasts grew from the explants after the epithelial cells. The presence of contaminating fibroblasts was indicated by the appearance of clusters containing spindle-shaped cells. As the culture time progressed, the fibroblastic cell clusters increased while the epithelial areas became more restricted. In general, fibroblasts did not produce significant populations of contaminating cells under these conditions.

Conventional culture of HDEC was considerably more *in vivo*-like when the cells were grown on lens capsules. Following attachment to the natural substratum, the cells migrated to form colonies which subsequently coalesced. Within a week the epithelial cells formed continuous sheets which persisted as monolayers up to 30 d without degenerating. Due to the lens capsule's transparency, light microscopical inspection was always possible.

Cross sections for TEM showed that the HDEC cultured under 2-D conditions developed some but not all of the characteristics of duodenal epithelium *in vivo*. The cells on artificial substrata all displayed an extremely flattened appearance (3–6 μm) but not the characteristic columnar morphology that is usually observed *in vivo*. TEM analysis revealed that the thickness of cells on bovine lens capsules ranged from 5 to 12 μm. Adjacent cells were joined by tight junctions, desmosomes, and membrane interdigitations. The major part of the cells displayed only a few very short microvilli on the apical site (Fig. 1 *B*). Mucus production could be seen for up to 2 wk.

Culture of HDEC as multicellular, spheroid-like vesicles. Fragments of the duodenal mucosa, when scraped from the underlying submucosa and suspended in culture medium, were reproducibly capable of regenerating complex vesicles consisting of highly prismatic epithelial cells often associated with newly produced mucus. This process was completed in 1 to 2 d, followed by the reconstitution of an epithelial surface of regularly shaped cells (Fig. 1 *C* and *D*). Only a low percentage of the fragments formed either solid cell aggregates or attached to the bottom of the culture vessel creating cell layers.

Three hundred milligrams wet wt duodenal tissue (3 biopsies derived from the same person) yielded approximately 11 ± 2.7 vesicles ranging from 100 to 300 μm in diameter. Most of the vesicles attained stable, regular shape and cellular organization which could be maintained for 9.3 ± 4.2 d in culture. Fragments of a larger original area produced irregularly shaped vesicles up to 600 μm in diameter. The released vesicles were composed of cell types resembling the typical simple epithelium of the small intestinal tract (as will be described). The vesicles were often observed to enclose a blood vessel inside the central lumen (not shown). The total number of cells in a vesicle could not be ascertained.

Analysis of HDEC derived from trypsinized vesicles by flow cytometry revealed two major cell populations in the forward versus the side scatter plot. In five different experiments 28.2 ± 9.4% of cells displayed morphologic features characteristic of lymphocytes whereas 69.8 ± 12.2% of cells resembled intestinal epithelial cells morphologically. Furthermore, 68.7 ± 10.1% of all vesicle-derived cells and 89.8 ± 13.3% of cells in the epithelial cell gate expressed the EP4 antigen which is found on human intestinal epithelial cells (Fig. 2 *A–C*).

Although most of the HDEC vesicles did not attach to the bottom of the cell culture dish, they could be maintained *in vitro* for 17 d (the longest period observed). Vesicles exposed to the Eukolight Viability/Cytotoxicity Kit showed extensive, uniform staining typical of live cells (Fig. 3 *A*). After longer periods signs of degeneration occurred, probably as a result of the accumulation of metabolic waste products in the centers of the vesicles. During cultivation, neither an overgrowth of the external surface of the vesicles by fibroblasts was observed, nor was any sign of vesicle enlargement noticed. Metaphases could not be identified by LM.

Further examination by LM showed that the epithelial cells grown as vesicles were impervious to trypan blue (not shown). Furthermore, the epithelial cells of the vesicles showed expression of AP activity at their apical surfaces. Positive staining for AP activity was observed in sections treated with Neufuchsin (Fig. 3 *B*), as well as in sections stained with Alkaline Phosphate Substrate Kit-Vector® Blue (not shown).

Antibodies against laminin and collagen type IV were used to identify the basal lamina within the vesicles. Positive staining for collagen type IV (Fig. 3 *C*) and laminin (not shown) which appeared only basally was observed. At the TEM level, however, alterations in continuity with frequent breaks of the basal lamina were observed.

As judged by SEM/TEM, the vesicles were mainly composed of a single layer of columnar or cuboidal cells similar to those of native duodenal cells. Cells undergoing cell division could not be detected. Two main types of epithelial cells could be distinguished which exhibited characteristic features, suggesting that they, although not characterized by immunological criteria, are absorptive and goblet cells. The apical membrane of the absorptive cells was lined by typical microvilli (Fig. 1 *D*) characteristic of the so-called striated or brush border, which is associated with a thick glycocalyx. All cells were joined near their apices by well-developed junctional structures. The basolateral membranes of adjacent cells were held in close juxtaposition by several elaborate interdigitations and a series of desmosomes. Sometimes, the innermost cell membrane showed signs of an *in situ*-like organization of the basal lamina, but in some vesicles a continuous basal lamina underlay the basal surface of the cells. A loose layer of mesenchymal cells and small capillaries immediately underneath the basal surface of the epithelial cells, as well as reticular fibrils belonging to the lamina propria, underlay the basal lamina. However, direct contacts between the epithelial cell layer and the mesenchymal cells via cytoplasmic projections were not observed. The central lumens of the vesicles were always filled with

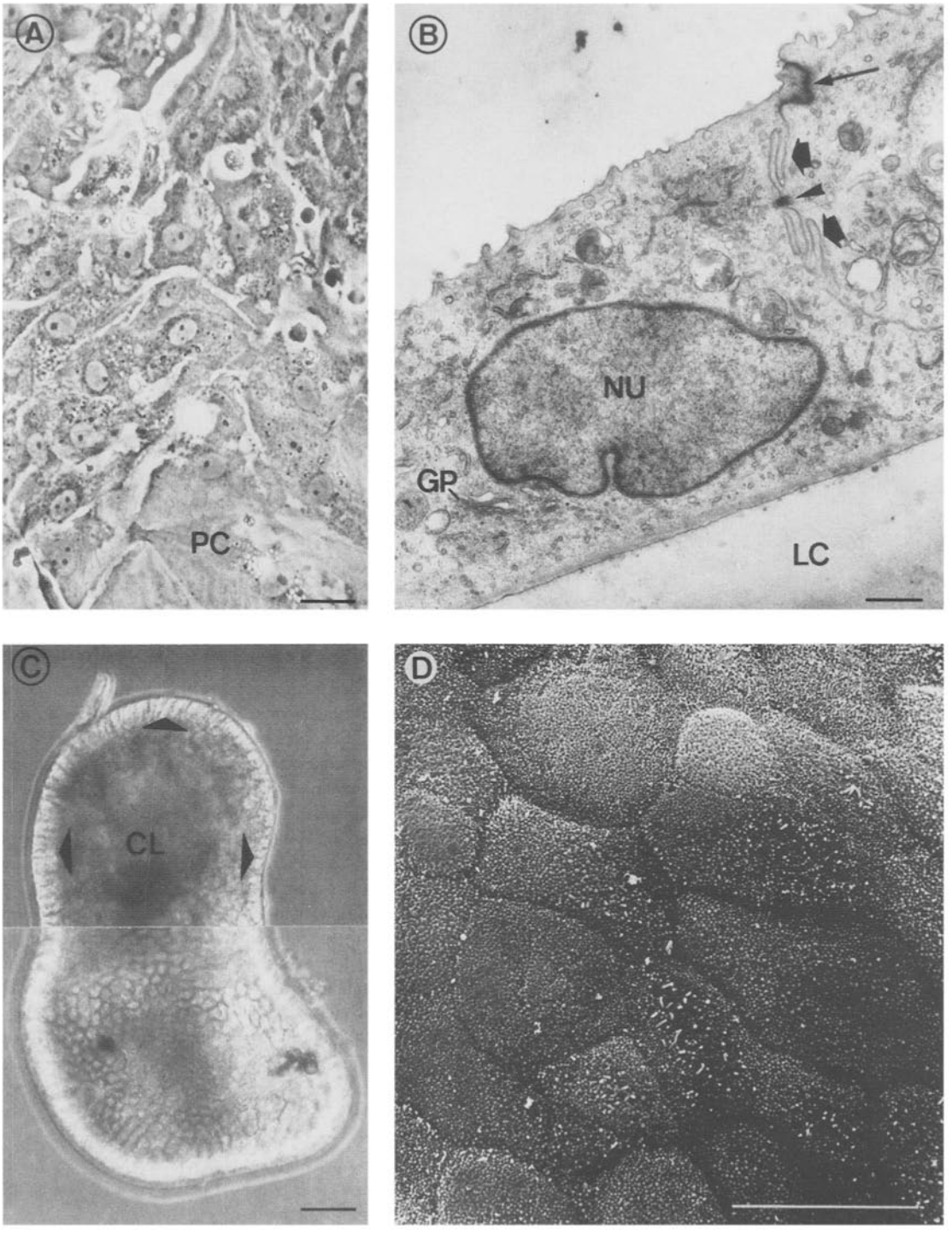

FIG. 1. *A*, LM micrograph of a 10-d-old primary 2-D culture of epithelial cells on a plastic support. The cell sheet is derived from a mucosal explant of a human duodenum. The HDEC contain nuclei with one to three nucleoli in their cytoplasm. Huge pleomorphic cells (*PC*) can be seen scattered in the monolayer. *Bar*: 60 μm. *B*, TEM micrograph illustrating the ultrastructural morphology of a 23-d-old culture of HDEC growing out from a human duodenal explant resting on a lens capsule (*LC*). The cells are polarized with few short and irregular apical microvilli. They are connected near their apices by a junctional complex (*arrow*), a desmosome (*arrowhead*), and by prominent membraneous infoldings (*thick arrows*). The cytoplasm contains Golgi profiles (*GP*), dense granules, and membraneous vesicles. A basal lamina is lacking. *NU* = nucleus. *Bar*: 1.1 μm. *C*, A small fragment of the duodenal mucosa 48 h after mincing. The fragmented mucosa cultured in suspension is forming a spheroid-like vesicle which is floating in the medium. The inside of the central lumen (*CL*) is filled with amorphous material. The upper part of the LM micrograph is depicted as a cross-section showing columnar-shaped epithelial cells (*arrowheads*) which border the vesicle, whereas the lower portion of the micrograph shows the apical "cobblestone" pattern of the sheet of HDEC at the surface of the vesicle (compare with 1 *D*). *Bar*: 20 μm. *D*, Portion of a HDEC multicellular vesicle similar to that depicted in *C*. The SEM micrograph shows the high degree of epithelial organization. The average diameter of the cells is 6 to 8 μm. The apical surface of the cells expresses typical brush border-type microvilli. *Bar*: 15 μm.

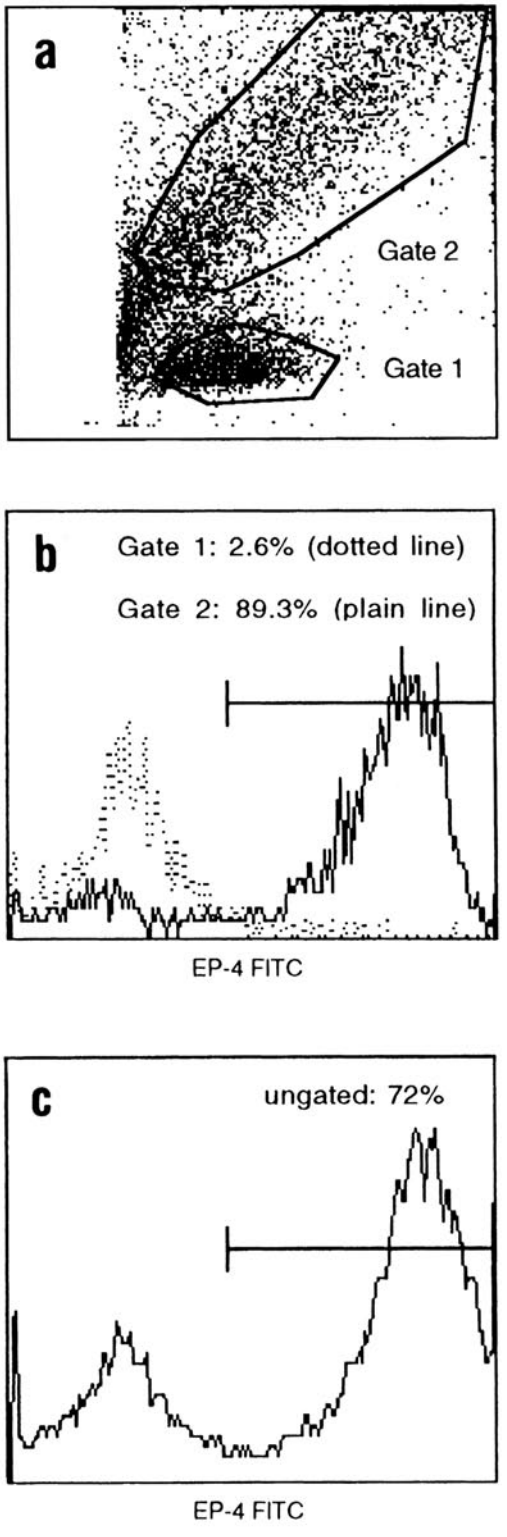

FIG. 2. Typical example for the cellular composition of multicellular vesicles derived from endoscopic biopsies of human duodenal mucosa. Analysis was by flow cytometry after digestion of vesicles with trypsin. *a*, 21.4% of all cells morphologically resemble lymphocytes (*gate 1*) whereas 68.2% morphologically resemble epithelial cells (*gate 2*). *B*, 89.3% of cells within the epithelial cell gate (*gate 2*) are in fact epithelial cells, whereas EP4 is expressed on 2.6% of cells in the lymphocyte gate (*gate 1*). *c*, In contrast, EP4 is expressed on 72% of all vesicle-derived cells.

amorphous material that was composed of cell debris and collagen fibers. Remnants of a lymphatic vessel (lacteal) could be frequently observed within the lumen.

The goblet cells were scattered among the absorptive cells. They contained a well-developed supranuclear Golgi apparatus as well as numerous membrane-bound mucous droplets (Fig. 3 *D*). Each cell contained well-developed intracytoplasmic organelles such as dictyosomes, mitochondria, bundles of filaments, and prominent, basally located nuclei (Fig. 4 *A*).

Our studies show that the HDEC grown as multicell vesicles assumed a nearly column-like shape similar to that known for native cells (Fig. 4 *B*). SEM investigation revealed that the apical diameter of the cells was dramatically reduced compared to that of the flat cells of the conventional cultures. The average size of the tightly packed apical cell membrane was 7 μm in diameter. The morphology of a HDEC vesicle is depicted in Fig. 1 *C*. The shape and polarization of the cells, organelles, and cell contact structures are shown in Fig. 5.

DISCUSSION

In vivo, epithelia are organized as natural barriers which separate compartments of the body from the organism's exterior. Owing to their exposed location, epithelial borders are subject to a range of pathogenic processes such as bacterial infections. Because *Helicobacter pylori* (formerly *Campylobacter pylori*) was isolated from chronically inflamed gastrointestinal mucosa (Marshall et al., 1984), this field has developed with enormous speed. Much evidence has been accumulated showing the pathogenic role of *H. pylori* in active chronic gastritis and duodenal ulcer. However, a number of questions about the histological and topographical features of chronic gastrointestinal ulceration remain unanswered. The majority of studies have been based on histological investigations using biopsy specimens (Bayerdörffer et al., 1992; Prewett et al., 1992; Rugge et al., 1993). *In vitro* culture systems, extensively used for many cell and tissue types other than intestine, would be useful in that respect.

Investigations using human normal tissues and cells are suggested to provide more relevant information on human pathogenic processes than cells derived from other species (Gibson-D'Ambrosio et al., 1986). To obtain a large number of viable, functionally active cells for primary cultures, human fetal intestine from abortions was used (Moyer, 1983; Gibson- D'Ambrosio et al., 1986). To avoid such ethically questionable methods, we used mucosal biopsies from normal adults. As there is a high risk of damaging the cells during proteolytic disaggregation (Freshney, 1987), we chose the alternative of mechanical techniques. The present study was undertaken to develop an *in vitro* model which could be used to study the interactions of *H. pylori* and other intestinal pathogens with human normal mucosal cells derived from the duodenum.

Conventional 2-D culture of HDEC used here for comparison between monolayers and vesicle cultures is limited by both rapid deterioration of the cells and loss of many typical cytological characteristics of the native mucosa, such as the highly polarized phenotype, the nonrandom arrangement of organelles and cytoskeletal elements, the large number of cell–cell contacts, the expression of correct membrane domains, the specific intestinal enzymatic activity, and the loss of membrane potential. This lack of morphological, physiological, and biochemical features is a commonly lamented disadvantage of the conventional 2-D cell culture on a rigid substratum

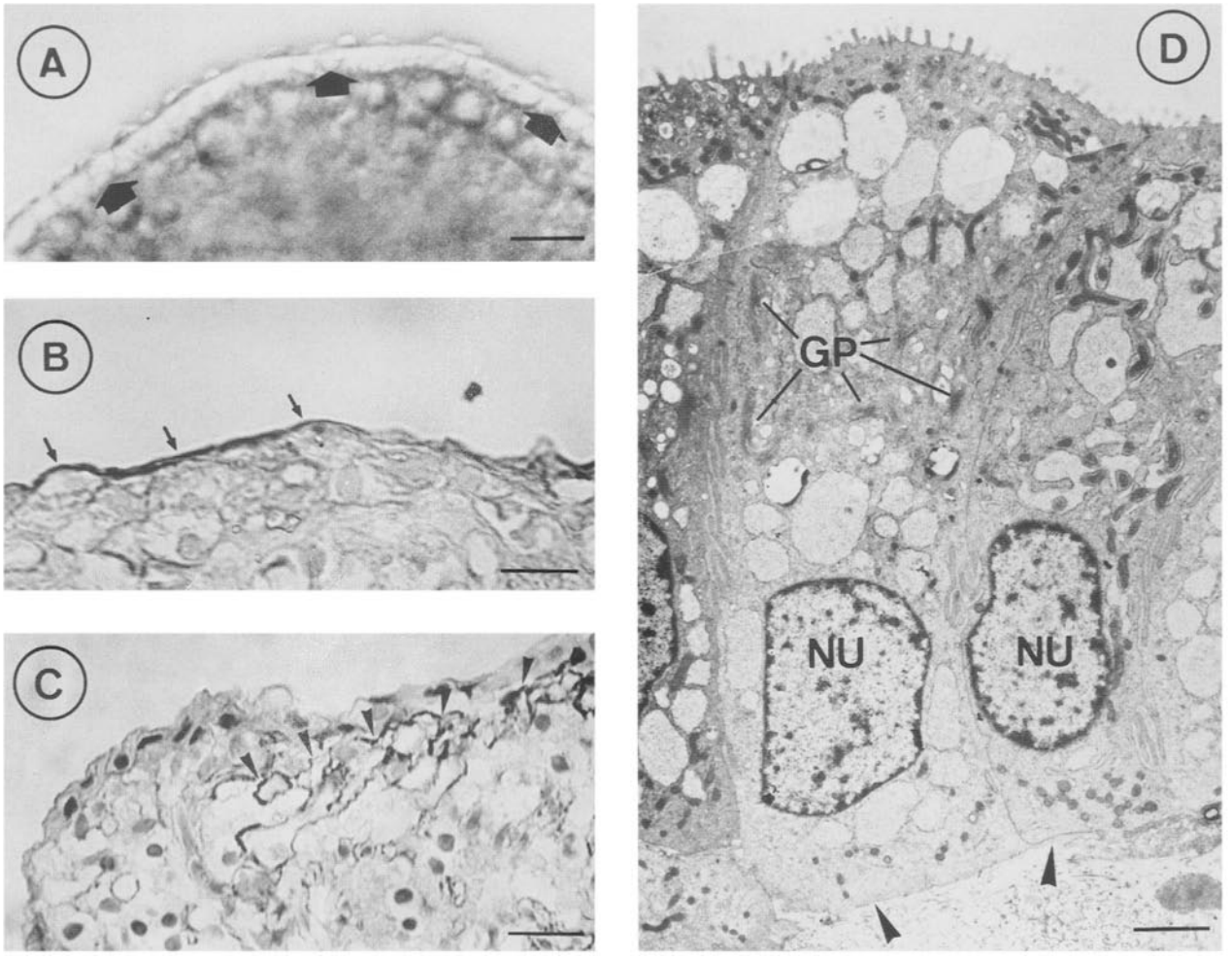

FIG. 3. *A*, HDEC bordering a 3-D vesicle (*arrows*) exposed to the Viability/Cytotoxicity Kit display an extensive, uniform (original green) staining typical for live cells. *Bar*: 60 μm. *B*, LM micrograph of HDEC grown as a 3-D vesicle and processed with Neufuchsin for the detection of alkaline phosphatase (*AP*), observed as a deposit at the apical surface (*arrows*). *Bar*: 20 μm. *C*, Indirect immunofluorescent staining with antibodies specific for basal lamina constituents showed that HDEC vesicles in 3-D culture continued to express collagen type IV. *Note* the presence of dense, dark staining at the basal surface of the epithelial cell layer (*arrowheads*). *Bar*: 40 μm. *D*, Portion of a HDEC multicell vesicle. *Note* the overall columnar appearance of the enterocytes which are connected by tight junctions, desmosomes, and membraneous infoldings (compare with 1 *B*). The apical membrane domains display microvilli and a glycocalyx. The cytoplasm encloses elongated mitochondria, a number of secretory droplets, and extensive supranuclear Golgi profiles (*GP*). The nuclei (*NU*) are situated in the lower part of the cell body. *Arrowheads* indicate a discontinuous basal lamina between the residual collagen fibers filling the central lumen and the basal cell membranes. *Bar*: 2.5 μm.

(Raul et al., 1978; Tsuchiya and Okada, 1982; Kondo et al., 1984; Quaroni, 1985; Minuth et al., 1992). We thus used lens capsule basement membrane as a more flexible support with which cells could interact to enhance their differentiation and polarization.

The basement membrane and some of its individual components are important factors for the expression of the polarized and differentiated phenotype of epithelial cells *in vitro* (McDonald, 1989; Rodriguez-Boulan and Nelson, 1989; Timpl, 1989; Hohn and Denker, 1994). Indeed, the culture of HDEC cells on this material has led to both a more prismatic phenotype and a higher cell density as compared with the cell culture on solid, artificial supports. Our observation was in accordance with previous findings (Boxberger et al., 1995) and similar results obtained by others (Starkey et al., 1984; Mackenzie et al., 1993; Kubat, 1994) which suggest that the basement membrane could affect the degree of morphological and physiological differentiation of epithelial cells, possibly via interaction with transmembrane integrins (Ekblom et al., 1986; Burridge et al., 1988; Watt et al., 1988; Aggeler et al., 1991; Streuli et al., 1991; Schmidt et al., 1993). In the long term, however, even on lens capsules the HDEC could not preserve their polarized state. To approximate the physiology of the *in vivo* situation, we developed the 3-D multicell vesicle culture system for normal primary epithelial cells.

Our studies of the HDEC multicellular vesicles emphasize the histophysiological and morphological similarities of the 3-D cultured epithelial cells to those of the native tissue. The most remarkable features of this method are its high reproducibility and the highly prismatic nature of the mucosal cells in the vesicles. Their columnar shape was in sharp contrast to the flattened morphology observed

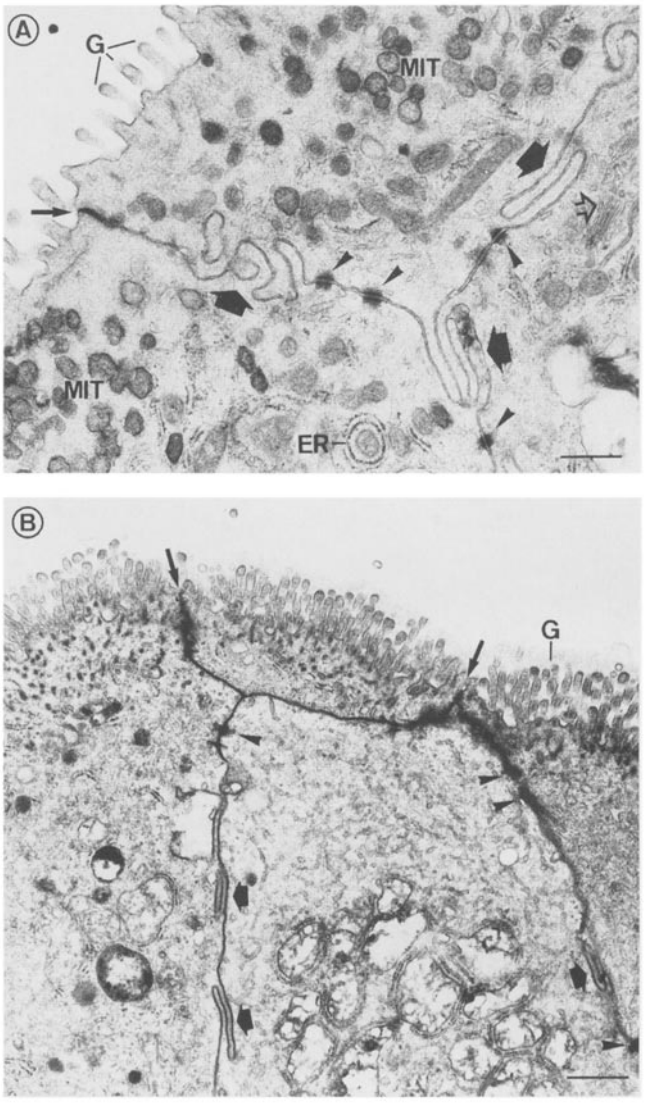

FIG. 4. *A*, Ultrastructure of HDEC grown as 3-D spheroid-like vesicles. A junctional complex at the site of cell–cell contact (*arrow*) and desmosomes connecting the lateral cell membranes (*arrowheads*) are present. The apical poles of the cells demonstrate brush border microvilli associated with a conspicuous fuzz-like coat reminiscent of a glycocalyx (*G*). Membraneous interdigitations (*thick arrows*) are also visible (compare with 3 *D*). Clusters of mitochondria (*MIT*), endoplasmic reticulum (*ER*), and a dictyosome (*open arrows*) inside the cells can be seen. TEM micrograph; *Bar*: 0.6 μm. *B*, TEM micrograph depicting the ultrastructural appearance of the control epithelial cells from human duodenum. This oblique section displays the typical brush border-type microvilli covered by a thick glycocalyx (*G*). Tight junctions (*arrows*), desmosomes (*arrowheads*), and membraneous interdigitations (*thick arrows*) are present. *Bar*: 1.1 μm.

when the cells were cultured on plane substrata. In comparison with the 2-D culture, the multicell vesicles presented also strong evidence of cytological differentiation which was manifested by the lack of cells in metaphase. The reason little proliferation and only differentiated function is obtained is that only the digitiform villi of the tunica mucosa were scraped off. Due to this technique, the isolated vesicles consisted mainly of cut off and resealed villi which were bare of proliferative cells.

In vivo, the tight interaction of the intestinal epithelial cells both facilitates the potential replacement of a wounded epithelium by an intact cell sheet and may serve as a spatial organizer of their highly polarized cytoarchitecture and histophysiological qualities (Nelson and Veshnock, 1986). The remodeling of an intact epithelial cell sheet is thought to be initiated by the release of growth factors which could trigger repair activities such as cell proliferation and locomotion (reviewed in McNeil, 1993). Our data indicate that the HDEC vesicles both retain and restore the original intimate contact that is necessary for the epithelial remodeling of *in vivo*- like structures and functions after mechanical disruption.

The examination of the HDEC vesicles further revealed high cell density and maintenance of mucus production. The appearance of absorptive and goblet cells containing characteristic mucous granules as well as the expression of EP4 and AP activity again reflects both the preserved functional polarity of the multicell vesicles and the different physiological functions of the two epithelial subtypes. The AP activity at the apical surfaces of the vesicles was also a reliable, positive marker in our 3-D cultured cells. Corresponding to our data, Walling et al. (1991) have shown that columnar and polarized murine adenocarcinoma cells (MAC 15j) grown on free-floating collagen gels displayed expression of AP, whereas cells grown on the plastic substratum showed no evidence of AP activity. Furthermore, the morphological features of our 3-D cultured cells, such as the typical brush border-type microvilli covered by a glycocalyx, junctional complexes, membrane infoldings at the lateral cell borders, basal lamina, and the distribution and orientation of intracellular organelles, closely resembles those noted in the native duodenal tissue. In addition, the presence of loose mesenchymal cells, capillaries, and the lacteal inside the central cavity emphasizes the intactness of the complete mucosa composed of the epithelial cell sheet and the underlying lamina propria. These observations are in accordance with the finding of others (Tsuchiya and Okada, 1982; Quaroni, 1985) who observed spherical bodies consisting of a single layer of fetal rat intestinal epithelium in organ culture.

Yet, a few differences still exist between the *in vitro* 3-D organization of the HDEC and the native epithelium. *In vivo*, epithelial cells receive nutrients from the basolateral side facing the blood supply. In a multicellular vesicle, the apical cell poles face the nutritive environment, whereas the basolateral surface has access only to the material that fills the central cavity. Obviously, the lifespan of the 3-D cultured cells is strongly connected with the limited passage of metabolic waste products out of the epithelial vesicles as well as the hindered diffusion of nutrients and growth factors into the vesicles (Mueller-Klieser, 1987; Sutherland, 1988). During cultivation, an overgrowth of the external surface of the HDEC vesicles by fibroblasts was never observed, whereas in a few explant cultures, contaminating fibroblasts could be observed. We suggest that the submucosal tissue including the fibroblasts has been entrapped during resealing of the epithelium and the process of vesicle formation, respectively.

In former studies (Quaroni, 1985; Kedinger et al., 1986; Tait et al., 1992; Mackenzie et al., 1993) it was suggested that the maintenance of interepithelial and epithelial–mesenchymal cell interactions that persist in "organoid" cultures plays an important role in proliferation of gut epithelium. We have made similar observations in cocultures consisting of 3-D duodenal epithelial vesicles and subepithelial fibroblasts. In these cultures we were able to prolong the viability of the epithelial cells up to 23 d (unpublished result).

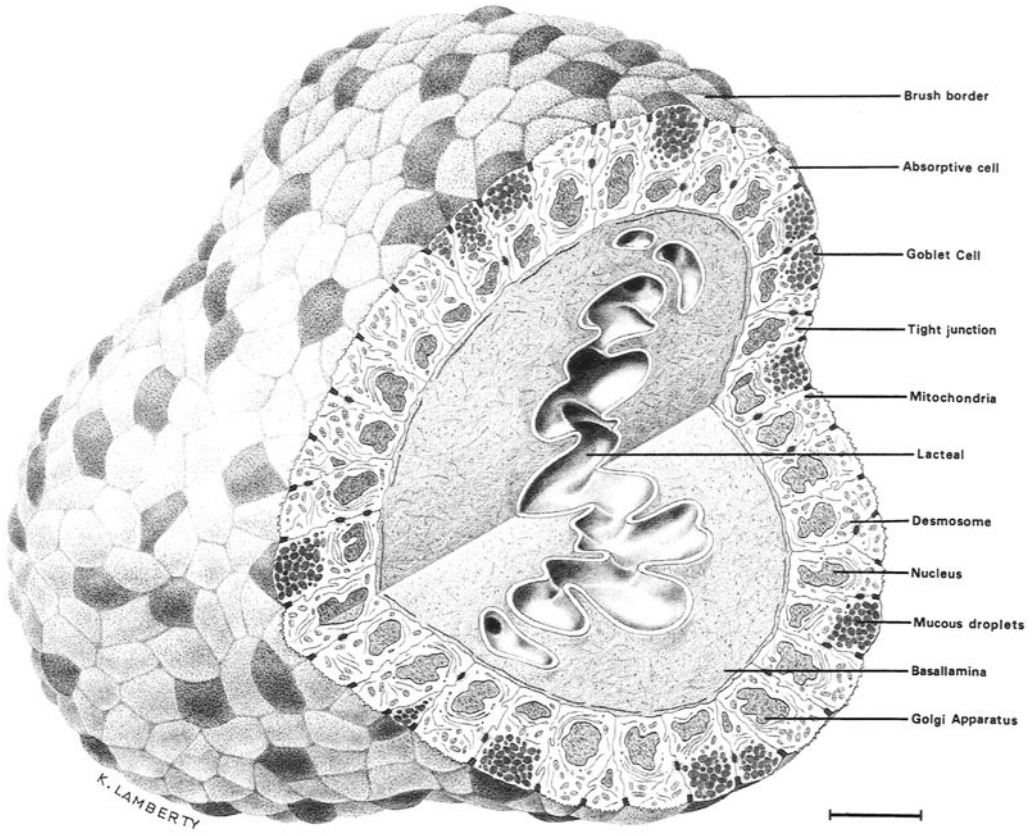

FIG. 5. Schematic drawing of a small HDEC spheroid-like vesicle. This represents a model that is reconstructed from the data obtained by light microscopy, TEM, and SEM. Only the typical features of each cell type are noted. The right portion of the vesicle is depicted as a longitudinal section, whereas the left portion shows the vesicle in a 3-D view. The vesicle is bordered by a sheet of columnar-shaped absorptive enterocytes, with single basophilic goblet cells in between. The apical poles of the cells bear a multitude of microvilli forming the typical brush border. Both cell types are held together by tight junctions, desmosomes, and membraneous interdigitations between the lateral membranes. On their basal sides the epithelial cells are in contact with a basal lamina which is present in fragments only. These fragments are marked as thick lines facing the central lumen. The central lumen (*CL*) is filled with amorphous material which contains collagen fibers, blood capillaries, and the lacteal belonging to the lamina propria that was entrapped during vesicle formation. *Bar:* 25 μm.

Spherical aggregates have been widely used for many years (for further details *see* Mueller-Klieser, 1987; Sutherland, 1988; Knüchel and Sutherland, 1990). Herzog and Miller (1981) and Espanet et al. (1992) reported multicellular follicles from pig thyroid gland cells. It was also reported that fragments of fetal rat intestine evolved into globular structures which were bordered by a single columnar epithelium similar to villous cells (Tsuchiya and Okada, 1982; Quaroni, 1985). To our knowledge, spheroid-like *in vitro* models of HDEC have not been described before. In agreement with other reports (Tsuchiya and Okada, 1982; Moyer, 1983; Négrel et al., 1983; Quaroni, 1985), the use of nondissociated, mechanically removed mucosal fragments as starting material is essential for the successful establishment of HDEC in culture. The treatment of intestinal tissue with disintegrating proteases such as collagenase or trypsin leads to reduced brush border enzyme activity (Kedinger et al., 1986) and is also known to cause shape and polarity changes (Lichtenberger et al., 1973; Raul et al., 1978).

Our study with the HDEC multicell vesicles supports the notion that for an *in vitro* model to reflect the normal histophysiology of an epithelium, preservation of its cellular structure and morphology is essential (Jackson and Newport, 1981). This means the differences in composition between the apical and basolateral cell membrane domains must be maintained. Furthermore, the accurate reorientation of the cytoskeletal elements plays an important role in the polarization of epithelial cells (Nelson and Veshnock, 1986, 1987). Following the concept that a change in cell shape may initiate a corresponding change in cell function, the principal advantage of the 3-D technique appears to be the striking improvement in both cell morphology and polarity over that of conventional cultures. Recent investigations demonstrated that epithelial cell differentiation *in vitro* was greater when the cells were grown in 3-D cultures (Walling et al., 1991; Chevillard et al., 1993; Boxberger and Meyer, 1994; Yamanari et al., 1994; Boxberger et al., 1995).

Our *in vitro* system, which combines the advantages of cell lines and organ cultures, features the use of small vesicles of cultured epithelial cells which appear to possess the original tissue architecture. HDEC vesicles were chosen because they originate from a natural site of *H. pylori* infection. We have developed this model to identify potential virulence factors (adhesins). Besides an *N*-acetylneuraminyllactose-binding hemagglutinine (HpaA) one postulates

further *H. pylori* adhesins which bind specifically to components of the extracellular matrix. Such adhesins could play an important role after the destruction of the epithelium in the late infection phase. Therefore, in our opinion the 3-D vesicle model represents a valuable tool with the potential to give us more insights into the highly complex mechanisms of bacterial–host cell interactions than does the 2-D monolayer cell culture system.

Acknowledgments

We thank Alexandra Ghica for manuscript preparation and critical comments on the manuscript and Dr. Heinz Schwarz (Max- Planck-Institut für Entwicklungsbiologie, Tübingen) for many helpful suggestions. We also appreciate the skillful assistance of Frank Küttner (cell culture), Jürgen Berger (SEM) and Bozena Pichler-Brand (TEM), Klaus Lamberty (art work), and Carmen Müller (photography). This work was supported by the Bundesminister für Forschung und Technologie (01-KI-8920).

References

Aggeler, J.; Ward, J.; Blackie, L. M., et al. Cytodifferentiation of mouse mammary epithelial cells cultured on a reconstituted basement membrane reveals striking similarities to development in vivo. J. Cell Sci. 99:407–417; 1991.

Bayerdörffer, E.; Lehn, N.; Hatz, R., et al. Difference in expression of *Helicobacter pylori* gastritis in antrum and body. Gastroenterology 102:1575–1582; 1992.

Boxberger, H.-J.; Meyer, T. F. A new method for the 3-D in vitro growth of human RT112 bladder carcinoma cells using the alginate culture technique. Biol. Cell 82:109–119; 1994.

Boxberger, H.-J.; Paweletz, N.; Spiess, E., et al. An in vitro model study of BSp 73 rat tumour cell invasion into endothelial monolayer. Anticancer Res. 9:1777–1786; 1989.

Boxberger, H.-J.; Sessler, M. J.; Grund, K.-E., et al. Three-dimensional (3-D) cell culture of epithelial cells from the human colonic mucosa. J. Exp. Clin. Cancer Res. 14 (Suppl. 1):22–23; 1995.

Burridge, K.; Fath, K.; Kelly, T., et al. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. 4:487–525; 1988.

Buset, M.; Lipkin, M.; Winawer, S., et al. Inhibition of human colonic epithelial cell proliferation in vivo and in vitro by calcium. Cancer Res. 46:5426–5430; 1986.

Cairnie, A. B.; Lamerton, L. F.; Steel, G. G. Cell proliferation studies in the intestinal epithelium of the rat. I. Determination of the kinetic parameters. Exp. Cell Res. 39:528–538; 1965.

Chevillard, M.; Hinnrasky, J.; Pierrot, D., et al. Differentiation of human surface upper airway epithelial cells in primary culture on a floating collagen gel. Epith. Cell Biol. 2:17–25; 1993.

Ekblom, P.; Vestweber, D.; Kemler, R. Cell-matrix interactions and cell adhesion during development. Annu. Rev. Cell Biol. 2:27–47; 1986.

Espanet, H.; Alquier, C.; Mauchamp, J. Polarity reversal of inside-out thyroid follicles cultured on the surface of a reconstituted basement membrane matrix. Exp. Cell Res. 200:473–480; 1992.

Freshney, R. I. Culture of animal cells. 2nd rev. ed. New York: Wiley-Liss; 1987.

Gibson-D'Ambrosio, R. E.; Samuel, M.; D'Ambrosio, S. M. A method for isolating large numbers of viable disaggregated cells from various human tissues for cell culture establishment. In Vitro Cell. Dev. Biol. 22:529–534; 1986.

Harrison, D. D.; Webster, H. L. The preparation of isolated intestinal crypt cells. Exp. Cell Res. 55:257–260; 1969.

Herzog, V.; Miller, F. Structural and functional polarity of inside-out follicles prepared from pig thyroid gland. Eur. J. Cell Biol. 24:74–84; 1981.

Hohn, H.-P.; Denker, H.-W. The role of cell shape for differentiation of choriocarcinoma cells on extracellular matrix. Exp. Cell Res. 215:40–50; 1994.

Jackson, C. D.; Newport, G. A simple method for in vitro studies with rodent urinary bladders. In Vitro 17:107–113; 1981.

Kedinger, M.; Simon-Assmann, P. M.; Lacroix, B., et al. Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. Dev. Biol. 113:474–483; 1986.

Knüchel, R.; Sutherland, R. M. Recent developments in research with human tumor spheroids. Cancer J. 3:234–243; 1990.

Kondo, Y.; Rose, L.; Young, G. P., et al. Growth and differentiation of fetal rat small intestinal epithelium in tissue culture. Exp. Cell Res. 153:121–134; 1984.

Kubat, B. The influence of different substrates on tight junction formation and on ADH-response in renal collecting duct cells in primary cultures. Eur. J. Cell Biol. (Suppl. 40, abstract #114); 1994.

Latza, U.; Niedobitek, G.; Schwarting, R., et al. Ber-EP4: new monoclonal antibody which distinguishes epithelia from mesotheia. J. Clin. Pathol. 43:213–219; 1990.

Lichtenberger, L.; Miller, L. R.; Erwin, D. N., et al. Effect of pentagastrin on adult rat duodenal cells in culture. Gastroenterology 65:242–251; 1973.

Mackenzie, I.; Rittman, G.; Bohnert, A., et al. Influence of connective tissues on the in vitro growth and differentiation of murine epidermis. Epith. Cell Biol. 2:107–119; 1993.

Marshall, B. J.; Royce, H.; Annear, D. I., et al. Original isolation of *Campylobacter pyloridis* from human gastric mucosa. Microbios Lett. 25:83–88; 1984.

McDonald, J. A. Matrix regulation of cell shape and gene expression. Curr. Opin. Cell Biol. 1:995–999; 1989.

McNeil, P. L. Cellular and molecular adaptions to injurious mechanical stress. Trends Cell Biol. 3:302–307; 1993.

Minuth, W. W.; Stöckl, G.; Kloth, S., et al. Construction of an apparatus for perfusion cell cultures which enables in vitro experiments under organotypic conditions. Eur. J. Cell Biol. 57:132–137; 1992.

Moyer, M. P. Culture of human gastrointestinal epithelial cells. Proc. Soc. Exp. Biol. Med. 174:12–15; 1983.

Mueller-Klieser, W. Multicellular spheroids. A review on cellular aggregates in cancer research. J. Cancer Res. Clin. Oncol. 113:101–122; 1987.

Négrel, R.; Rampal, P.; Nano, J.-L., et al. Establishment and characterization of an epithelial intestinal cell line from rat fetus. Exp. Cell Res. 143:427–437; 1983.

Nelson, W. J.; Veshnock, P. J. Dynamics of membrane-skeleton (Fodrin) organization during development of polarity in Madin-Darby Canine Kidney epithelial cells. J. Cell Biol. 103:1751–1765; 1986.

Nelson, W. J.; Veshnock, P. J. Modulation of Fodrin (membrane skeleton) stability by cell-cell contact in Madin-Darby Canine Kidney epithelial cells. J. Cell Biol. 104:1527–1537; 1987.

Prewett, E. Y.; Bickley, J.; Owen, R. J., et al. DNA patterns of *Helicobacter pylori* isolated from gastric antrum, body, and duodenum. Gastroenterology 102:829–833; 1992.

Quaroni, A. Development of fetal rat intestine in organ and monolayer culture. J. Cell Biol. 100:1611–1622; 1985.

Raul, F.; Kedinger, M.; Simon, P., et al. Behaviour of isolated rat intestinal cells maintained in suspension or monolayer cultures. Biol. Cell 33:163–168; 1978.

Rodriguez-Boulan, E.; Nelson, W. J. Morphogenesis of the polarized epithelial cell phenotype. Science 245:718–725; 1989.

Rugge, M.; DiMario, F.; Cassaro, M., et al. Pathology of the gastric antrum and body associated with *Helicobacter pylori* infection in non-ulcerous patients: is the bacterium a promoter of intestinal metaplasia? Histopathology 22:9–15; 1993.

Schmidt, J. W.; Piepenhagen, P. A.; Nelson, W. J. Modulation of epithelial morphogenesis and cell fate by cell-to-cell signals and regulated cell adhesion. Semin. Cell Biol. 4:161–173; 1993.

Starkey, J. R.; Hosick, H. L.; Stanford, D. R., et al. Interaction of metastatic tumor cells with bovine lens capsule basement membrane. Cancer Res. 44:1585–1594; 1984.

Streuli, C. H.; Bailey, N.; Bissell, M. J. Control of mammary epithelial differentiation: the separate roles of cell-substratum and cell-cell interaction. J. Cell Biol. 115:1383–1395; 1991.

Sutherland, R. M. Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 240:177–184; 1988.

Tait, I. S.; Flint, N.; Evans, G. S., et al. Progress with small bowel enterocyte culture and transplantation. Transplant. Proc. 24:1061–1064; 1992.

Timpl, R. Structure and biological activity of basement membrane proteins. Eur. J. Biochem. 180:487–502; 1989.

Trier, J. S. Organ culture methods in the study of gastrointestinal-mucosal function and development. N. Engl. J. Med. 295:150–155; 1976.

Tsuchiya, W.; Okada, Y. Membrane potential changes associated with differentiation of enterocytes in the rat intestinal villi in culture. Dev. Biol. 94:284–290; 1982.

Walling, J. M.; Blackmore, M.; Hickman, J. A., et al. Role of the extracellular matrix on the growth and differentiated phenotype of murine colonic adenocarcinoma cells in vitro. Int. J. Cancer 47:776–783; 1991.

Watt, F.; Jordan, P. W.; O'Neill, C. H. Cell shape controls terminal differentiation of human epidermal keratinocytes. Proc. Natl. Acad. Sci. USA 85:5576–5580; 1988.

Weiser, M. M. Concanavalin A agglutination of intestinal cells from human fetus. Science 177:525–526; 1972.

Yamanari, H.; Suganuma, T.; Iwamura, T., et al. Extracellular matrix components regulating glandular differentiation and the formation of basal lamina of a human pancreatic cancer cell line in vitro. Exp. Cell Res. 211:175–182; 1994.