

## PAPERS

## Tissue culture of epithelium derived from Barrett's oesophagus

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### Abstract

**Barrett's oesophagus is a preneoplastic condition in which the squamous mucosa of the oesophagus is replaced by columnar epithelium. Epithelial cells of Barrett's oesophagus were isolated from resected oesophagus specimens by two methods not previously applied to the culture of Barrett's oesophagus cells. These techniques included trypsinisation of small fragments of mucosa, followed by plating in tissue culture dishes, and a direct tissue explant technique. A modified MCDB-153 growth medium was used. Primary trypsin technique cultures were plated on uncoated plastic, or plastic coated with type I collagen, type IV collagen, or fibronectin. Growth on type IV collagen and fibronectin plates was slower but produced less contamination from fibroblasts. By 20-40 days most cultures formed confluent monolayers made up of cells with epithelial morphology. The cells were cytokeratin positive, vimentin negative, and contained alcian blue positive vacuoles, confirming their epithelial origin and suggesting their derivation from Barrett's oesophagus. Electron microscopy showed tonofilaments, microvilli, and desmosomes. Cells proliferated through up to eight subcultures before growth slowed and cells showed senescent changes. This study shows that epithelial cells from Barrett's oesophagus can be grown by comparatively simple tissue culture techniques. These methods can provide sufficient material for a variety of molecular biology and biochemical studies of epithelial cells from Barrett's oesophagus.**

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In Barrett's oesophagus, the normal stratified squamous epithelium is replaced by columnar epithelium. Because Barrett's mucosa is associated with increased risk of adenocarcinoma, it is a useful model in the study of metaplasia and neoplastic transformation.

One limitation in studying the biology of Barrett's oesophagus is the small amount of tissue obtainable by biopsy and the scarcity of resection specimens from patients who have not received radiation or chemotherapy. A possible

solution to this problem is the use of epithelial cells of Barrett's oesophagus grown in tissue culture. In particular, sufficient quantities of epithelial cells for molecular biology and cytogenetics studies can be obtained by either an enzymatic dissociation or tissue explant technique.

### Methods

#### TISSUE SELECTION

Barrett's mucosa samples were obtained from surgical resection specimens with Barrett's oesophagus, previously confirmed by biopsy. The oesophagectomies were performed for persistent high grade dysplasia or for adenocarcinoma. Tissue for culture was selected away from lesions suspicious for carcinoma, in comparatively flat areas of mucosa, generally in the mid portion of the segment of Barrett's oesophagus of the specimen. Obviously ulcerated areas were avoided. About 1 cm<sup>2</sup> of mucosa was harvested per case. Selection of columnar epithelium with intestinal metaplasia, as shown by well formed goblet cells, was confirmed by histological examination of mucosa immediately adjacent to that harvested for culture. Of the seven specimens with longterm tissue culture growth, adjacent mucosa showed no dysplasia (five cases), low grade dysplasia (one case), or was indeterminate for dysplasia (one case), according to the classification used by Reid *et al.*<sup>1</sup> Specimens were collected aseptically, stored in sterile tissue culture medium at 4°C, and processed within 24 hours. Most specimens were processed on the day of collection.

#### INITIATION OF CULTURES

Epithelial cell cultures were initiated by two techniques: tissue explant,<sup>2</sup> and by trypsinisation of mucosal fragments.<sup>3</sup>

In the explant technique, the mucosa was minced into fragments measuring 1 to 2 mm in greatest dimension. These fragments were then placed in 90 mm Petri dishes and anchored under 22 mm × 22 mm glass coverslips before growth medium was added.

In the enzymatic technique, 0.5 to 1 cm<sup>2</sup> fragments of mucosa were harvested, and

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underlying muscle and connective tissue were trimmed away. The squares were floated overnight, mucosal surface down, in a solution of 0.15% trypsin (GIBCO), 10 mM glucose, 3 mM KCl, 130 mM NaCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 30 mM HEPES (Sigma), and 3.3 μM phenol red,<sup>4</sup> at 4°C in room air. The next day the tissue was transferred to a second Petri dish containing a similar solution without trypsin and containing 5 μg/ml soybean trypsin inhibitor (Sigma). The mucosa was removed by scraping with small forceps, and the resulting cell suspension pipetted multiple times to dissociate large cell clumps. The cell suspension was then centrifuged at 250 × *g* for 10 minutes. The cell pellet was resuspended in growth medium and plated in six well multiwell tissue culture plates (Falcon), on uncoated plastic or wells coated with Type I collagen (Sigma), Type IV collagen (Sigma), or human fibronectin (Sigma).

#### GROWTH MEDIUM AND CONDITIONS

Cells were grown in a modified MCDB-153 medium (Sigma) with final concentrations of the following supplements: 5% fetal calf serum (GIBCO), 0.4 μg/ml hydrocortisone (Sigma), 20 ng/ml epidermal growth factor (GIBCO), 10<sup>-10</sup> M cholera toxin (Sigma), 1.8 × 10<sup>-4</sup> M adenine (Sigma), 140 μg/ml bovine pituitary extract (Sigma), 100 units/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), and 0.25 μg/ml amphotericin B (GIBCO). Cells were grown in a humidified atmosphere containing 5% CO<sub>2</sub>, at 37°C.

#### STAINING CHARACTERISATION

After the initial subculture, an aliquot of cells was grown on Lab-Tek eight well chamber glass slides for staining with haematoxylin and eosin, alcian blue (pH 2.5), and antibodies to cytokeratin (AE1/AE3, Boehringer Mannheim), and vimentin (Biomedica Corporation). A standard immunoperoxidase staining procedure using the avidin-biotin-complex system was used for the cytokeratin and vimentin stains.

#### ELECTRON MICROSCOPY

Cells grown on glass slides were fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer for four hours. The cells were washed in 7.5% sucrose buffer in 0.1% sodium cacodylate, postfixed with 1% OsO<sub>4</sub> in collidine, dehydrated in graded ethanols, and embedded on the glass slide in Epon. Ultrathin sections were poststained with uranyl acetate and lead citrate and examined on a Phillips 400 electron microscope.

#### Results

Twelve resection specimens were cultured, typically five Petri dishes per case with the explant technique and four to five multiwell plates with the enzymatic technique. Initial growth of epithelial cells was seen in all 12



Figure 1: Mucosa adjacent to that harvested for tissue culture contained numerous goblet cells, confirming the diagnosis of Barrett's oesophagus. Haematoxylin and eosin, original magnification × 100.

cases. One specimen could not be propagated beyond two subcultures. All cultures from four specimens were lost to contamination soon after initial growth was seen. Seven of 12 specimens were successfully subcultured from six to eight times.

#### METHOD 1

##### *Growth of epithelium from explants of Barrett's oesophagus*

Petri dishes containing 12–16 tissue fragments typically showed epithelial growth at the edges of multiple tissue fragments 3–5 days after incubation with enriched growth medium. The initial growth tended to extend from one edge of the tissue, forming an epithelial monolayer skirt. Growth from a single fragment often became dominant. After one or more fragments had produced sufficient growth (roughly the area of the overlying coverslip), the coverslips and tissue fragments were discarded. Cultures were fed twice weekly by removing 5 ml growth medium and replacing it with fresh medium. Localised collections of cells with a fibroblastic morphology were removed from some cultures by scraping with a cell scraper and rinsing the plate with phosphate buffered saline. Treatment of cultures with 0.02% EDTA in phosphate buffered saline did not succeed in loosening fibroblasts from the culture dish,



Figure 2: Cultured epithelial cells derived from Barrett's oesophagus, at day 20, near confluent growth. These cells were grown by the enzymatic technique, on uncoated plastic wells. Phase contrast micrograph, original magnification × 100.

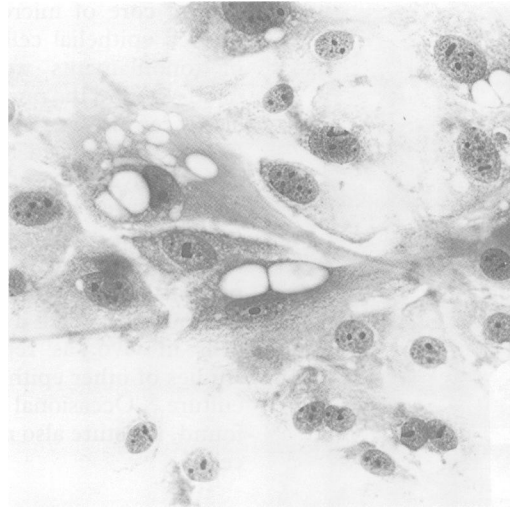


Figure 3: Cultured epithelial cells derived from Barrett's oesophagus, at day 60, after five passages, are irregular in shape, with pale cytoplasmic vacuoles. Haematoxylin and eosin, original magnification  $\times 400$ .

unlike previously described in thymic cell cultures.<sup>2</sup>

After epithelial cells achieved confluent growth over 75% of the dish, the cells were subcultured by incubating with 0.25% trypsin with EDTA (GIBCO), at 37°C for 5–10 minutes. The resulting cell suspension was then transferred to a conical centrifuge tube containing 25 ml growth medium and centrifuged at  $100 \times g$  for 10 minutes. Cells were seeded at  $9 \times 10^5$  cells/well, or 2 to  $3 \times 10^5$  cells/ml. Confluence was reached in three to seven days, and cells could be subcultured up to eight times before cell growth slowed. Cells could be maintained continuously in culture for at least three months. Cells could be retrieved and replated with subsequent growth to confluence after freezing in growth medium containing 10% DMSO and storage in liquid nitrogen. Total cell yield was greater than  $10^9$  cells for subsequent studies.

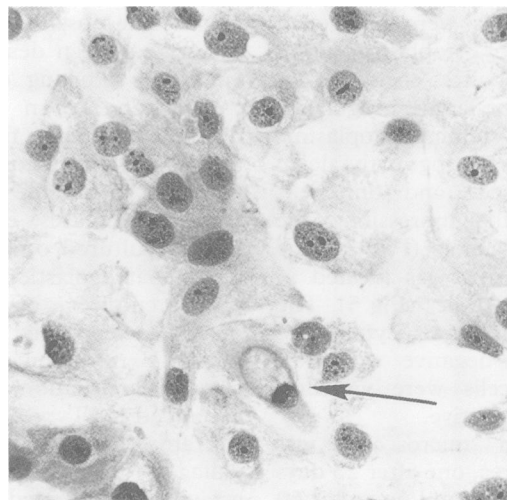


Figure 4: Cells resembling goblet cells (arrow), with compression of the nucleus by a dominant vacuole, could be seen in all cultures. This culture is 23 days old and was initiated by the explant technique. Haematoxylin and eosin, original magnification  $\times 400$ .

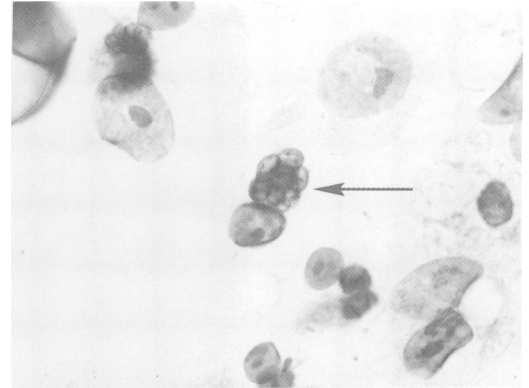


Figure 5: Alcian blue positive material was present in clusters of cytoplasmic vacuoles (arrow). Alcian blue, original magnification  $\times 680$ .

#### METHOD 2

##### Growth of epithelium after trypsinisation of mucosal fragments

After plating of dissociated epithelial cells in multiwell plates, the plates were left undisturbed for three to four days to permit attachment of cells to the culture dish. Attachment was seen in uncoated wells (Falcon six well plate), wells coated with Type IV collagen, and wells coated with human fibronectin. After three to four days, the unattached cells were rinsed away and the culture refed with fresh growth medium. Time to confluent growth was typically 20 to 35 days. The morphology of the epithelial cells was similar to those cells grown by the explant technique. Cultures contained a variable percentage of cells with fibroblastic morphology. In some cultures the fibroblasts overgrew the epithelial component by the fourth or fifth subculture. Growth on wells coated with Type IV collagen and fibronectin was slower, but some cultures established on these coated plates seemed to contain fewer fibroblasts, although this finding was not seen for all cultures. Cells grown on Type IV collagen or fibronectin could be subcultured fewer times than those grown on uncoated plastic. All cultures grew on uncoated plastic wells, but only 50% of these specimens

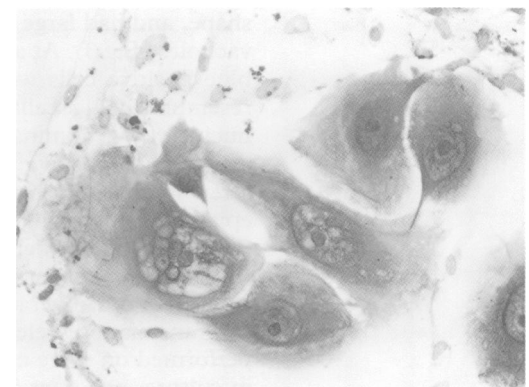


Figure 6: The epithelial cells were cytokeratin positive, while the smaller spindle shaped cells were positive for vimentin but negative for cytokeratin. The cellular enlargement probably represents a senescent change. Cytokeratin, original magnification  $\times 250$ .

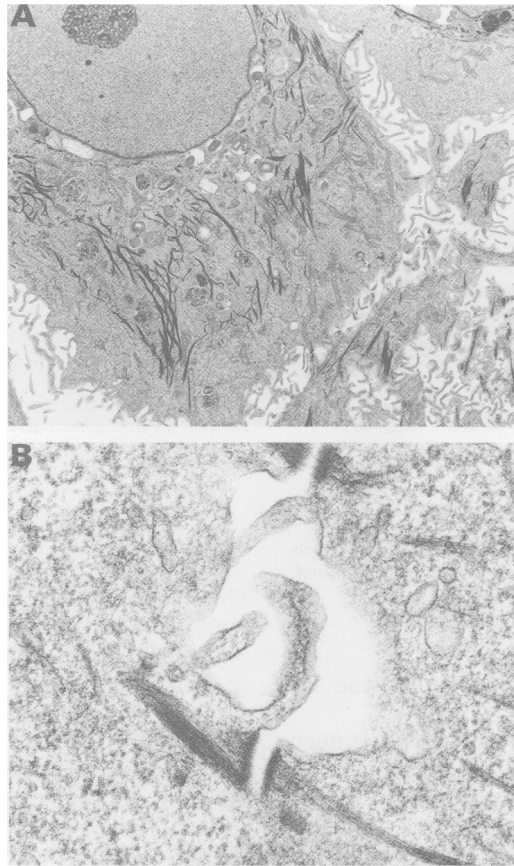


Figure 7: (A) Electron microscopy showed numerous cytoplasmic processes and bundles of tonofilaments in the cytoplasm. Original magnification  $\times 3000$ ; (B) numerous desmosomes were present between epithelial cells. Original magnification  $\times 38\,000$ .

could be cultured on fibronectin or Type IV collagen wells. No epithelial cells could be grown on plastic coated with Type I collagen.

#### MORPHOLOGICAL CHARACTERISTICS

Histological examination of tissue adjacent to the samples for culture confirmed the presence of Barrett's oesophagus (Fig 1). Early in culture, the epithelial cells were polygonal, with a moderate amount of cytoplasm (Fig 2). As cell growth slowed after six to eight doublings, the cells became larger, irregular in shape, and had large pale staining cytoplasmic vacuoles (Fig 3). At all stages of growth, alcian blue positive cells were seen, and some cells resembled goblet cells, with compression of the nucleus by a dominant vacuole (Fig 4). The alcian blue positive material was located in a few large cytoplasmic vacuoles (Fig 5). The epithelial appearing cells were cytokeratin positive and vimentin negative (Fig 6). Fibroblastic appearing cells were vimentin positive and cytokeratin negative.

Transmission electron microscopy was performed on three cultures, one after 23 days in culture, one after 55 days, and one after 60 days. In all three cultures, desmosomes, numerous surface cytoplasmic projections, and tonofilaments were found (Fig 7). The cytoplasmic projections generally lacked the

central core of microfilaments found in well formed epithelial cell microvilli. The bundles of tonofilaments were organised in broad bands around the nucleus. Tonofilaments were also seen in association with desmosomes. Other cells showed few tonofilaments, with a comparatively featureless cytoplasm. In the older cultures, many autophagic vacuoles were seen. Some cells, particularly in the youngest culture, contained abundant rough endoplasmic reticulum and Golgi apparatus. Desmosomes had a wider central gap than cells in vivo, as reported in ultrastructural studies of other epithelial cells grown in tissue culture.<sup>5</sup> Occasional convoluted nuclei were found, a feature also reported in other cultured cells.<sup>5</sup>

#### Discussion

The epithelial cells of Barrett's oesophagus can be cultured using an enzymatic dissociation technique or a tissue explant technique, in addition to the non-enzymatic method used by Garewal.<sup>4,6</sup> In our hands, cells could be subcultured through at least eight passages, yielding significant amplification of cell numbers, sufficient for RNA and DNA extraction for molecular biology studies. Although this developmental work has been performed on tissue from oesophagectomy specimens, these techniques, especially the tissue explant method, are applicable to biopsy specimens as well.

Barrett's epithelium will grow on plastic or glass, and will show intestinal differentiation, as evidenced by alcian blue positive mucin. Cells of similar epithelial morphology were obtained with the two techniques. Electron microscopy confirmed epithelial differentiation, showing numerous cell interdigitations, surface cytoplasmic projections, desmosomes, and bundles of tonofilaments.

Only in the last decade or so have techniques for non-malignant epithelial cell culture been developed. General problems in culturing epithelial cells include senescence of the cells lines and overgrowth by fibroblasts. The possible requirement for stromal factors has been dealt with by some investigators by growing epithelial cells, such as thymic epithelium and keratinocytes, on irradiated mouse fibroblast feeder layers. Successful growth on feeder fibroblasts may reflect a requirement for collagen, and in some other cases, epithelial cells are more readily cultured on collagen substrates than on uncoated plastic. It has been shown that some cultures of non-transformed and neoplastic epithelial cell lines may produce Type IV or basement membrane collagen, and differentiation of epidermal cells is enhanced by Type IV collagen. The cells of Barrett's epithelium grown on Type IV collagen grew more slowly than cultures grown on uncoated plastic, and could be subcultured fewer times. The morphological appearance of the cells as viewed with an inverted microscope was similar, but it is possible that the slower growth on Type IV

collagen reflected greater differentiation of the cells.

Quantitative, not qualitative, differences in growth media seem to be important in successful culture of non-malignant human epithelial cell lines.<sup>7</sup> It is unclear if the medium currently used, or the one used by Garewal, fully satisfies the longterm requirements for these cultures, although short term growth is satisfactory. After eight or so subcultures, the epithelial cells became enlarged and irregular in shape and proliferative activity ceased. Electron microscopy showed that cells from older cultures contained many secondary lysosomes, a change associated with aging. No culture has proved to be immortal. The conditions described support short term growth of epithelial cells derived from Barrett's oesophagus, and sufficient quantities of comparatively pure epithelial cells can be obtained for a variety of molecular biology and physiological studies.

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