Polarity and differentiation of human rectal adenocarcinoma cells in suspension and collagen gel cultures

SUSAN C. KIRKLAND
CRC Cell Proliferation Unit, Department of Histopathology, Royal Postgraduate Medical School, Hammersmith Hospital, DuCane Road, London, W12 OHS

Summary
HRA-19al.l. cells, derived from a primary human rectal adenocarcinoma, form polarized monolayers when grown on tissue-culture plastic. The apical membrane of the cells is in contact with the culture medium while the basolateral surface is attached to the plastic substratum. Cells cultured on non-tissue-culture plastic form floating colonies. Cells within these colonies are orientated so that their apical membrane is in contact with the culture medium while the basolateral membrane faces the centre of the colony. When these colonies are embedded in collagen gel the cells organize to form glandular structures similar to those observed in xenografts of this cell line. In addition a reversal in the orientation of cell polarity is observed with the basolateral membrane now in contact with the collagen gel, while the apical membrane faces lumina within the colony. This interaction is specific to collagen gel, as in a control experiment where colonies are embedded in agarose gel, neither a reversal in polarity nor the formation of glandular structures is observed. These results demonstrate an induction of glandular organization in human rectal adenocarcinoma cells by collagen gel. In addition an increased absorptive cell differentiation and reversal in cell polarity is observed in response to collagen gel.

Key words: differentiation, colorectal, epithelium, cell culture.

Introduction
HRA-19al.l. rectal adenocarcinoma cells retain many of the differentiated features characteristic of normal colorectal epithelium. The majority of cells have the morphology of poorly differentiated absorptive epithelial cells. They form polarized monolayers on tissue-culture plastic with sparse microvilli located exclusively on the apical membrane (Kirkland, 1986). When established as xenografts in nude mice the cells organize into glandular structures, the absorptive cells in these structures are more differentiated than their in vitro counterparts and mucous and endocrine cells are now observed (Kirkland, 1988). This suggests that HRA-19al.l. cells are responsive to factors present in the in vitro situation, which control their differentiation. However the nature of these differentiation-inducing factors is not understood. To identify some of the conditions affecting differentiation in these cells, culture conditions have been modified in an attempt to induce the differentiated conditions observed in vivo.

HRA-19al.l. cells cultured under different conditions have been examined for the presence of absorptive, mucous and endocrine differentiation. In addition the orientation of cell polarity has been investigated. The ability of epithelia to perform specialized functions such as absorption or secretion is dependent upon the establishment and maintenance of such cell polarity. In intestinal epithelium, ultrastructural studies show the apical membrane to have a brush border, whereas the basolateral membrane is devoid of microvilli. Tight junctions at the apical surface separate the two membrane domains, which have characteristic sets of proteins and lipids (Fujita et al. 1973; Kawai et al. 1974). Although the morphological and biochemical aspects of cell polarity have been well documented, little is known about the mechanisms involved in the generation and maintenance of cell polarity, particularly in gastrointestinal epithelium.

Collagen substrates have been shown to enhance differentiation and proliferation of many different cell types in vitro. Gels of collagen have been shown to influence the differentiated characteristics of several epithelial cell types in vitro. Increased differentiation and proliferation have been observed in mammary epithelial cells (Emmerman & Pitelka, 1977; Yang et al. 1980) and keratinocytes (Bell et al. 1983) when they are grown on collagen gels. In addition corneal epithelial cells have been shown to have increased responses to growth factors when grown on collagen gels (Gospodarowicz & Tauber, 1980).
In this study, the influence of suspension and subsequent collagen gel culture on cell polarity and other differentiated features of human intestinal epithelial cells has been investigated.

Materials and methods

**HRA-19 cell line**

The HRA-19 cell line was established from a primary adenocarcinoma of the rectum (Kirkland & Bailey, 1986). Characteristics of the cell line have been described previously (Kirkland, 1986). Experiments in this study have been performed with a cloned clone of the cell line designated HRA-19a1.1. (Kirkland, 1986).

**Suspension cultures**

Confluent HRA-19a1.1. cells (passage 27) were removed from culture flasks with 0.05% trypsin (three times crystallized and dialysed; Worthington Biochemicals, Lorne Laboratories, Bury St Edmunds, Suffolk) in Versene (Glasgow formula). The cells were washed with culture medium then plated into 9-cm bacteriological Petri dishes (Sterilin, Hounslow, Middlesex). The cells were unable to attach to these plastic dishes and formed multicellular aggregates, which floated in the culture medium. The culture medium used throughout these studies was Dulbecco's Eagles medium (Gibco, Paisley, Scotland) with glucose (4500mg l⁻¹). The medium was supplemented with 10% foetal calf serum (Gibco), kanamycin (100µg ml⁻¹) (Bristol Laboratories, Langley, Slough) and 110mg l⁻¹ sodium pyruvate (BDH Chemicals, Poole, Dorset). Cultures were fed three times a week. The colonies from these suspension cultures were used to initiate collagen gel and agarose cultures.

**Light microscopy of suspension cultures**

Colonies were washed in phosphate buffered saline (PBS), fixed in buffered formalin and suspended in 1.5% agarose, which was allowed to set for 1 h at 4°C. This gel was then processed in a routine way. Sections were stained with haematoxylin and eosin, Alcian blue (pH 2.5) and the Grimelius silver stain (Grimelius, 1968).

**Collagen gel cultures**

Stock solutions of Type I collagen were prepared from collagen fibres dissected from rat tails. Collagen fibres (1g) were suspended in 0.01 M-acetic acid, and stirred for 3 days at 4°C. The solution was centrifuged at 600g for 2 h to remove insoluble material and the resulting clear solution was freeze dried and stored at −20°C. To prepare collagen gels, collagen was redissolved in 0.01 M-acetic acid (0.3g per 100ml), sterilized by irradiation in a ¹³³Cs source and then polymerized by addition of a mixture of two parts 10× Eagles medium and one part 0.34 M-NaOH until pH 7 was reached. Colonies from suspension cultures were rapidly resuspended in the collagen solution. A 1-ml sample of this solution was then placed into
Fig. 3. Haematoxylin and eosin stained section of organoids at 14 days after plating. ×110.

Fig. 4. Transmission electron micrograph of the periphery of an organoid after 7 days in suspension showing abundant microvilli. ×3950.

Fig. 5. A. Section of an organoid from a suspension culture stained with the monoclonal antibody AUA1. ×435. Staining can be seen on cell membranes within the organoid (arrow) while the periphery of the organoid remains unstained.
B. Section of an organoid from a suspension culture stained with the monoclonal antibody HMFG1. ×435. Staining can be seen on peripheral membranes (arrow) but not on cell membranes within the organoid.

Agarose cultures
A 3% solution of Seaprep agarose (Miles Scientific, Slough) maintained at 37°C was diluted with an equal volume of 2× Dulbecco's Eagles medium with 10% foetal calf serum. Colonies that had been in suspension culture for at least a week were resuspended in agarose solution and plated into plastic rings as described for the collagen solution. The agarose was allowed to gel for 1 h at 4°C, then liberated from the plastic rings.

Immunocytochemistry
Immunocytochemistry was performed on formalin-fixed wax-embedded sections of colonies. Sections were dewaxed, rinsed in PBS and incubated in 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. Sections were washed in PBS, then incubated in 5% normal rabbit serum in 0.1% bovine serum albumin (BSA) in PBS for 10 min at room temperature. Sections were drained and incubated overnight at 4°C in primary antibody diluted in 0.1% BSA. AUA1 and HMFG1 monoclonal antibodies were a kind gift from Dr A. A. Epeneoses (Department of Clinical Oncology, Royal Postgraduate Medical
Fig. 6. Transmission electron micrograph of the periphery of an organoid 7 days after embedding in agarose gel showing abundant microvilli. X8190.

School, Hammersmith Hospital, London), and were both used at 1:10 dilution. Following incubation with primary antibody, sections were washed three times with PBS, and incubated in peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (Dako) diluted 1:100 in 0.1% BSA in PBS for 1 h at room temperature. After washing in PBS, slides were developed in diaminobenzidine (DAB) (25 mg ml⁻¹) in 0.03% hydrogen peroxide in PBS then lightly counterstained with haematoxylin.

Electron microscopy
For electron microscopy, cells were fixed in 3% glutaraldehyde in 0.1 M-phosphate buffer at 4°C for 1 h. The cells were postfixed in osmium tetroxide for 1 h at 4°C, dehydrated and embedded in TAAB resin (TAAB Laboratories, Reading, Berks.). Thin sections were stained with uranyl acetate and lead citrate and viewed with an AE1-801 microscope at an accelerating voltage of 60 kV.

Results

Differentiated features of monolayer cultures
HRA-19a1.1. cells form polarized monolayers on tissue culture plastic. Morphological changes are observed in monolayers of these cells with time after trypsinization. By 2–3 days after plating cells generally have a tightly packed morphology; however, cultures contain increasing numbers of cells with large intercellular spaces with time after trypsinization (Kirkland, 1986) (Fig. 1). These morphological changes are thought to be due to the transport properties of these cells (Kirkland, 1986).

Electron microscopy demonstrates that the cells have morphological features of poorly differentiated absorptive cells. They are polarized in that microvilli are located exclusively on the apical surface and adjacent cells are connected by tight junctions at the apical surface. Microvilli are however sparse and disorganized in appearance (Kirkland, 1986).

Differentiated features in suspension cultures
When HRA-19a1.1. cells were plated onto non-tissue-culture treated plastic they failed to attach but instead organized into multicellular colonies. By 24 h after plating the cells are still rounded and the colonies are without obvious organization (Fig. 2A). However by 48 h, these colonies have smooth outlines and have a more organized structure as observed by phase-contrast microscopy (Fig. 2B). Sections of these organoids after 2 weeks show them to have an organized structure (Fig. 3). These colonies were observed at times up to 2 weeks in suspension culture and remained of a similar morphology except for some increase in size. At 2 weeks the average size of the colonies (measured along the longest axis) was 0.143 ± 0.084 mm (mean ± S.D.) (n = 160). Most organoids were spherical or ovoid in shape but irregular shaped aggregates were occasionally observed (Fig. 3). Electron microscopy, performed on cultures between 7 and 9 days in suspension, shows that the cells are polarized with the apical membrane at the edge of the structure in contact with the culture medium (Fig. 4). The orientation of cell polarity was confirmed by the use of monoclonal antibodies (mAbs) AU1 and HMFG1. AU1 recognizes an antigen expressed by normal colorectal epithelium and located exclusively on the basolateral membrane of these cells (Mooi et al. 1987). Staining of colonies with AU1 showed that staining was restricted to membranes within the organoid, while the periphery remained negative (Fig. 5A), therefore basolateral membranes were located inside the organoids. Conversely, HMFG1, an antibody exclusively staining apical membranes in normal colorectal epithelium, stained only the periphery of the organoids (Fig. 5B). Staining of the organoids with Alcian blue did not demonstrate the presence of mucous cells, but was restricted to a fine line of staining around the periphery of the organoids correlating with the presence of brush border in this region. Endocrine cells were not observed by electron microscopy or after Grimelius silver staining.

Polarity of cells in agarose gel
When floating organoids were embedded in agarose gel their morphology was little changed. After 7 days in agarose the orientation of polarity of cells was investigated. Electron microscopy confirmed that the cells had the same polarity as in the suspension cultures. Apical membranes facing the agarose gel at the edge of the organoid (Fig. 6).

Differentiated features of collagen gel cultures
When floating HRA-19a1.1. colonies were embedded in collagen gel, their morphology changed with a loss of the rounded smooth edges. Debris could be seen surrounding the colonies. The polarity of the cells in these organoids was reversed so that the basolateral membrane was now in contact with the collagen gel (Fig. 7A) and the apical membranes were lining lumina within the

618 S. C. Kirkland
organoids (Fig. 7B,C). Staining with the monoclonal antibodies confirmed this reversal in polarity, AUA1 stained peripheral membranes only (Fig. 8A), whereas HMFG1 stained membranes lining lumina within the organoids (Fig. 8B). This glandular organization is similar to that observed in xenografts of this cell line (Kirkland et al. 1986). Cells within these glandular structures had a more differentiated absorptive cell phenotype (Fig. 7) than when grown on tissue-culture plastic (Kirkland, 1986). Microvilli at the apical cell surface are much more abundant and uniform in size. Tight junctions are again present at the apical surface. Parts of the brush border have an associated glycocalyx, which is not observed in the cells on tissue culture plastic.

Sections of the organoids stained with Alcian blue showed only staining in the lumina without peripheral staining and did not demonstrate the presence of mucous cells. Endocrine cells could not be demonstrated either by electron microscopy or by Grimelius silver staining.

Discussion

Human rectal adenocarcinoma cells, HRA-19al.1.1, form three-dimensional cell colonies (organoids) when cultured in suspension. These organoids are much smaller than previously described for other colorectal carcinoma cell lines, spheroids of 2800 μm have been reported for HT29 cells (Sutherland et al. 1986). The inability for HRA-19al.1.1 cells to form colonies of this size may be related to the differentiated state of these cells. Unlike HT29 cells, the HRA-19 cells polarize within the spheroids and form tight junctions at the periphery which may restrict access of nutrients to the interior. It has previously been shown that the more differentiated cell line

![Fig. 7. Transmission electron micrographs of organoids 7 days after embedding in collagen gel. A. Periphery of an organoid showing a smooth membrane without microvilli. ×6000. B. Cells lining a lumen within an organoid, with numerous microvilli and tight junctions. ×3720. C. Microvilli on cells lining a lumen. ×17 050.](image-url)
Co112 grows more slowly as spheroids than the HT29 cells (Sutherland et al. 1986).

In these floating organoids, cells have a similar morphology to those cultured on tissue-culture plastic. No evidence for increased absorptive cell differentiation or differentiation into mucus or endocrine cells was obtained, therefore a three-dimensional cell conformation is not sufficient to affect differentiation significantly.

The cells within the organoids are polarized so that the apical surface contacts the culture medium. Such orientation of polarity in floating cultures has previously been described for porcine kidney epithelial cells (Wohlwend et al. 1985), MDCK cells (Rodriguez-Boulan et al. 1983) and thyroid epithelial cells (Chambard et al. 1981). Cell–cell contact is thought to be important in establishing the orientation of polarity in floating colonies of cells, as single MDCK cells have been shown to lose their polarity in suspension culture (Rodriguez-Boulan et al. 1983). The orientation of cell polarity is reversed in collagen gel cultures so that the basolateral membrane is in contact with the gel at the periphery of the organoid. These results suggest that the cell–cell contact, which is thought to be important in the generation of cell polarity in floating organoids, is overridden by interaction of the apical membrane with the collagen gel. This interaction is specific to a component of the collagen gel as agarose gel does not reverse cell polarity. Collagen gel has been shown to induce a similar inversion in polarity both in thyroid epithelial cells (Chambard et al. 1981) and porcine kidney epithelial cells (Wohlwend et al. 1985). These results suggest an interaction between the apical membrane of the cell and the collagen gel leading to an inversion in polarity. It seems probable that cells have receptors for collagen, as has been shown for laminin (Terranova et al. 1983), which are involved in the maintenance of polarity in vitro.

In addition to the changes in the orientation of cell polarity, cells within collagen gels were found to organize into glandular structures similar to those observed in xenografts of this cell line. The absorptive cells within these structures had a better differentiated phenotype than those on tissue-culture plastic, therefore collagen also induces differentiation in these cells. Collagen gel has previously been shown to induce differentiation in a variety of epithelial cell types including mammary epithelial cells (Emerman & Pitelka, 1977) and keratinocytes (Bell et al. 1983). In addition, isolated human colonic crypts were only able to survive for extended periods in vitro by growing them in collagen gel culture with a feeder layer of bovine aortic endothelial cells (Whitehead et al. 1987).

In gastrointestinal tract virtually nothing is known about the factors that control differentiation and proliferation; however an important role for epithelial–mesenchymal interactions has been demonstrated (Haffen et al. 1987). Association experiments using grafting procedures with dissociated foetal intestines have demonstrated inductive effects of mesenchyme on morphogenesis of endoderm from various levels of the gastrointestinal tract (Haffen et al. 1987). These workers have also provided evidence that epithelial–mesenchymal interactions have a role in controlling cytodifferentiation in the adult intestine. A cell line of undifferentiated crypt cells (IEC-17) when associated with either foetal rat or mouse gut mesenchyme and grafted under the kidney capsule of adult rats or nude mice, formed well-vascularized structures with morphogenesis of crypts and villi and the appearance of the four epithelial cell types found in the small intestine (absorptive, goblet, endocrine and Paneth) (Kedinger et al. 1986). Foetal rat mesenchyme has also been shown to induce gland formation in human
colon carcinoma cells in vitro (Fukamachi et al. 1987). The factors mediating these mesenchymal effects have yet to be identified.

In this study the effects of extracellular matrix on the differentiation of HRA-19al.1. human rectal carcinoma cells in vitro have been studied. A role for extracellular matrix in gland formation and differentiation in human rectal carcinoma cells has been demonstrated. However the components of collagen gel alone cannot substitute for the xenograft conditions, which are able to induce the formation of mucous and endocrine cells by these multi-potential cells. In addition, extracellular matrix has been shown to direct the orientation of cell polarity in gastrointestinal epithelial cells, in a way described for other epithelial cell types. As carcinoma cells were used in this study it is clear that neoplastic gastrointestinal epithelial cells can remain responsive to signals from extracellular matrix that direct the orientation of cell polarity.

I am grateful to Mr R. Janccynski for technical assistance, Mrs Y. Price for histology, and Mrs V. Emons and Mr A. Westmuckett for preparation of specimens for electron microscopy.

References


(Received 21 July 1988–Accepted 23 August 1988)