Establishment and characterisation of two cell lines derived from a primary adenocarcinoma of the duodenum

M Golding, G W H Stamp, T Oates, E-N Lalani

Abstract

**Aims**—To establish two cell lines from a primary duodenal adenocarcinoma; to describe the morphological, growth, ploidy, and immunophenotypic characteristics of these cell lines.

**Methods**—The cell lines, designated DAC/S and DAC/E, were characterised using both in vitro and in vivo cell culture techniques, light and electron microscopy, immunocytochemistry, and FACS analyses.

**Results**—Both cell lines have an epithelial origin, are aneuploid and display characteristics of transformed cells. The cell lines differ from each other in morphology, doubling time and serum requirements. These cell lines are anchorage dependent and do not grow in nude mice.

**Conclusions**—DAC/S and DAC/E cell lines are derived from neoplastic epithelium and could provide in vitro model systems for future investigations of the cell and molecular biology of duodenal neoplasia.


Keywords: duodenum, adenocarcinoma, cell lines, integrins, intermediate filaments.

Development of primary adenomas and carcinomas in the small intestine of humans is a rare event. The low frequency of neoplasia in this part of the gastrointestinal tract has been considered surprising given the high incidence of tumorigenesis in the oesophagus, stomach and large bowel. The scarcity of small bowel cancers has consequently resulted in limited information regarding the clinical, pathological and biological characteristics of such malignancies.

The establishment of continuous cell lines from human cancers has provided the potential to study aspects of the biology of various tumours, and to identify specific genetic alterations present in the neoplastic cells which are not confounded by the stromal elements. However, as many tumours exhibit considerable heterogeneity, it is necessary for studies to be carried out on a number of different cell lines, in order to obtain a panel of data which may illustrate the mechanisms which underlie the disruption in growth control and differentiation which characterise malignant neoplasms. For this reason there is a perpetual requirement for novel cell lines for experimental purposes, in the hope that new data may be generated which contribute further to our understanding of the causes and behaviour of cancers.

Immortalisation, which sometimes occurs during cellular transformation in vitro, is a property which is a prerequisite for the establishment of a continuous cell line from a primary culture. Unfortunately, this only occurs spontaneously in less than 10% of tumour cell cultures. If, however, immortalisation does occur, then this provides an opportunity to establish cell clones from the primary culture for subsequent characterisation. Characterisation typically involves determination of morphology, nutritional requirements, growth rates, differentiation properties, immunophenotype, ploidy, karyotype, and DNA fingerprinting.

In this report we describe the establishment of two continuous cell lines derived from a primary adenocarcinoma of the duodenum.

**Methods**

**INITIATION OF THE PRIMARY CULTURE**

A duodenal tumour was obtained from a 45 year old man at the Hammersmith Hospital, London. A 1 cm³ sample of tumour was taken and placed in sterile, ice cold phosphate buffered saline (PBS; pH 7.2), and thin slices adjacent to the tumour were fixed in 10% formal saline (10% formaldehyde in 0.9% sodium chloride), embedded in paraffin wax and processed routinely for histology. Tissue sections of the sample showed a moderately differentiated duodenal adenocarcinoma invading the intestinal wall and through into the pancreas.

The tumour sample was divided into four portions, each placed into a 10 cm Petri dish (Nunc, Roskilde, Denmark), washed thoroughly in cold (4°C) PBS and finely chopped using crossed scalpels. Five millilitres of warm (37°C) Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS); 10 μg/ml insulin (Sigma, Poole, Dorset, UK); 5 μg/ml hydrocortisone (Sigma); and 0.6 mg/ml glutamine was added to the minced tissue and pipetted vigorously to obtain a fine suspension. The suspension was seeded into 10 Petri dishes (five 1×5 and five 1×10 cm) and incubated at 37°C with 10% CO₂ in air. After 48 hours, the medium was aspirated and all adherent cells were washed thoroughly with cold (4°C) PBS, growth medium (as above) was added and the dishes incubated as normal for seven to 10 days, after which discrete colonies of cells had formed.
CELL CLONING AND PROPAGATION

Each colony of cells exhibiting a distinctive morphology was selected using the cloning ring technique and expanded by seeding initially into 24 well plates and subsequently into six well plates. The resultant cell suspension was then seeded into 25 cm² culture flasks (Nunc) standing vertically. Once confluent, the cells were trypsinised (0-25% trypsin in versene), the flask laid horizontally and incubation was continued until confluent. Using this method, five clones were isolated and subsequently expanded. Only two of the original five clones were viable after one month in culture and these were named according to their morphology, DAC/S (duodenal adenocarcinoma/spindle) and DAC/E (duodenal adenocarcinoma/epithelioid).

MINIMAL GROWTH REQUIREMENT

Experiments were set up as follows: DAC/S and DAC/E cells (1 x 10⁵) were suspended in serum free DMEM medium and seeded into each well of a 24 well plate and incubated for 24 hours. The medium was then aspirated and replaced with one of the following media: DMEM alone; DMEM + 10% FCS; DMEM + 10% FCS and insulin (I), or glutamine (G), or hydrocortisone (H), or all three (I,G,H (all concentrations as above)). The effect on cell growth (quadruplicate samples) was ascertained over 10 days by viewing at daily intervals. As a result of this analysis, DAC/S cells were subsequently grown in DMEM + 10% FCS and DAC/E in DMEM + 10% FCS + I, G and H.

CALCULATION OF DOUBLING TIMES

DAC/E and DAC/S cells (3 x 10⁵) were seeded into each well of a 24 well plate and incubated for 1 ml medium (as above). Cells from three wells were trypsinised at 24 hour intervals, triplicate counts were made and averaged for each cell line at a given time point. This was repeated until the plateau phase was reached, and from the data the lag time, population doubling time, and plateau density were determined.

FACS ANALYSIS FOR PLOIDY STATUS

Cells of both clones were grown until confluent in 75 cm² culture flasks (Falcon, Oxford, UK), trypsinised and a single cell suspension produced by syringing the cells three times through a 25 gauge needle. Cell suspensions containing approximately 10⁶ cells of each clone were centrifuged at 1000 x g, the supernatant fluid aspirated and the cell pellet resuspended in 2 ml cold PBS and gently vortexed to remove any excess medium. The resulting cell suspension was then centrifuged at 1000 x g and the cell pellet was fixed by resuspending in ice cold 70% ethanol with periodic vortexing for 30 minutes. This was followed by washing for five minutes in 100 mM Tris buffer (pH 7-5) and staining with 0-01% propidium iodide. The nuclear DNA content was analysed using a fluorescence activated cell sorter (FACS IV, Becton Dickinson, Gosport, UK). The normal lymphoblastic cell line RPET 001 served as a control.

SOFT AGAR GROWTH ASSAY

Anchorage independent growth was assessed as follows: a single cell suspension of 5 x 10⁴ cells from each clone was seeded in a 0-3% agar layer on top of a 0-5% agar base (both layers contained DMEM medium with 10% FCS) in 5 cm Petri dishes (Nunc). After four days of culture at 37°C in a humid atmosphere of 10% CO₂ in air, the cultures were overlaid with fresh DMEM medium with 10% FCS. These overlays were replaced every three days and after six weeks on this regimen, any colonies of more than 20 cells were counted in duplicate dishes and compared with parallel cultures of the breast cancer cell line T47D, which served as a positive control.

COLLAGEN GEL CULTURES

Cells (5 x 10⁴) of each clone were added to 6 ml collagen Type I (Vitrogen 100), prepared according to manufacturer’s instructions and 1 ml of this mix was placed into each of six pre-warmed (37°C) organ culture dishes (Falcon). The gel was left to set in the incubator for 90 minutes before adding culture medium (as above). Cell growth was analysed daily by phase contrast microscopy. Growth medium was changed every three days, and at weekly intervals for a period of six weeks. One gel of each clone was fixed in formal saline for 24 hours, processed and embedded in paraffin wax for routine histology.

XENOGRADING INTO ATHYMIC NUDE MICE

Cells were grown to confluence in 75 cm² culture flasks, trypsinised as above and re-suspended in DMEM + 15% FCS. Six NIMR Nu/Nu athymic female mice (three for each cell line) were injected subcutaneously in both flanks with 10⁷ cells suspended in 0-5 ml of medium. Progress of the xenografts was monitored by weekly inspection for up to six months.

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DAC/S, DAC/E and T47D (positive control) cells were grown on four well slides (CA Hendley Ltd, Loughton, Essex, UK) until 50% of

<table>
<thead>
<tr>
<th>Table 1 Antibodies used for immunocytochemistry</th>
<th>Source</th>
<th>Specificity</th>
<th>Dilution</th>
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<tr>
<td>CAM 5-2</td>
<td>ICRF, London, UK</td>
<td>Cytokeratin</td>
<td>1:2 (sn)</td>
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<td>V9</td>
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<td>Vimentin</td>
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<td>1A4</td>
<td>Sigma, Poole, Dorset, UK</td>
<td>Smooth muscle actin</td>
<td>1:300</td>
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<tr>
<td>PR-3B10</td>
<td>ICRF, London, UK</td>
<td>CEA</td>
<td>Neat (sn)</td>
</tr>
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<td>IG2 integrin</td>
<td>1:50</td>
</tr>
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<td>B de Strooper, Amsterdam, The Netherlands</td>
<td>β1 integrin</td>
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<td>IG2 integrin</td>
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</tr>
<tr>
<td>HEC-1</td>
<td>M Takechi, Kyoto, Japan</td>
<td>E-cadherin</td>
<td>Neat (sn)</td>
</tr>
</tbody>
</table>

sn = hybridoma supernatant.
Establishment and characterisation of cell lines

Figure 1  A, Phase contrast image of DAC/S cells showing fibroblastoid, spindle morphology; B, DAC/E cells displaying a regular polygonal morphology, typical of epithelial cells. Note the nuclear pleomorphism and presence of cytoplasmic vacuoles.

Results

Two clones of cells from the original primary population were propagated.

Electron microscopy

Cells from each clone were grown to confluency on plastic microslips (Thermanox Scientific Ltd, Naperville, Illinois, USA), rinsed with PBS, fixed in 2% gluteraldehyde for one hour, washed with PBS, postfixed in osmium tetroxide for one hour, dehydrated and embedded in TAAB epoxy resin. The cells were then freeze fractured in liquid nitrogen, 50–60 nm sections were cut, stained with uranyl acetate and lead citrate and examined using a Philips CM10 transmission electron microscope.

Confluent. Slides were rinsed well in cold PBS, fixed in a 1:1 mix of methanol/acetone (−20°C) for 10 minutes and air dried. Slides were stained by the standard indirect immunoperoxidase method using mouse antihuman monoclonal primary antibodies (table 1). The cells were incubated with the primary antibody for 40 minutes at room temperature and rinsed, followed by incubation with peroxidase conjugated rabbit anti-mouse immunoglobulins (Dako, High Wycombe, UK) at a 1 in 30 dilution for 40 minutes at room temperature. The slides were rinsed in PBS, developed with 0.25 mg/ml diaminobenzidine (DAB) for three to five minutes, rinsed in distilled water, counterstained with haematoxylin and mounted in aqueous Hydromount. Paraffin wax sections (5 μm) of the original tumour were immunostained using the standard horseradish peroxidase conjugated Streptavidin/biotin complex (Strept ABC) technique.

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Figure 2  A, Electron micrographs of DAC/E cells showing surface microvilli and invagination of nuclei. Note the abundance of euchromatin and irregularly distributed heterochromatin (scale bar = 2 μm); B, DAC/S cells displaying indentigitation of microvilli of adjacent cells. Phagolysosomes and electron dense membrane bound vacuoles similar to zymogen granules are also present. Note the abnormal nucleolus with an electron dense core and the flocculent material within mucus granules (scale bar = 1 μm).

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distributed euchromatin and large nucleoli with an unusual organisation which seemed to contain variable densities of RNA (fig 2A). The abnormal mucus filled vacuoles seen in fig 2B may represent dilated endoplasmic reticulum or an altered mucus content, both of which are characteristic of tumour cells of glandular origin.

GROWTH RATES
Both DAC/S and DAC/E cells attained saturation density at about eight days after seeding, and cell counts were terminated at day 10. From the growth curves (data not shown), the lag time for both cell lines is approximately 24 hours, followed by a log phase of about four days. The calculated cell population doubling times are 24 and 42 hours for DAC/S and DAC/E, respectively.

FACS ANALYSIS
Figures 3A–3C represent typical FACS plots, with relative cell number on the vertical axis and relative fluorescence (DNA content) on the horizontal axis; both are in arbitrary units. Figure 3A shows a typical trace for a normal diploid population with the two peaks representing cells in G1 and G2 phases of the cell cycle, in sharp contrast to fig 3C (representing DAC/S) revealing an ill defined population which is less dramatic for DAC/E (fig 3B). These results indicate that both cell lines are clearly aneuploid, possessing a DNA content of approximately 5N.

GROWTH IN AGAR
After six weeks in culture, neither cell line formed colonies in agar. However, a few cytoplasmic processes were seen emanating from many of the DAC/S cells within the first week in culture, but these regressed after 10 days. In contrast, DAC/E showed no growth in agar and all of the cells had senesced within about three weeks of seeding.

GROWTH IN COLLAGEN GEL
DAC/S cells displayed extensive growth in the gels, extending numerous cytoplasmic processes into the collagen matrix within 12 hours of seeding, although no complex structures developed. In contrast, DAC/E cells did not grow, remaining spherical and showed no obvious interaction with the collagen. Examination of a haematoxylin and eosin stained preparation of DAC/E revealed cells with eosinophilic cytoplasm and pyknotic nuclei indicative of cell death (data not shown).

GROWTH IN NUDE MICE
Six months after inoculation of cells, tissue from the area of injection was taken from two of the animals, which, after staining with haematoxylin and eosin, revealed no tumour growth and only fibrotic scar tissue remaining (data not shown).
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Table 2 summarises the immunocytochemical profiles of both cell lines. Figure 4A shows the moderately well differentiated adenocarcinoma, from which DAC/S and DAC/E were derived, invading deep into the submucosa and muscularis propria of the duodenum.

Both cell lines express cytokeratin and vimentin (figs 4B and 4C, respectively) illustrating the characteristic filamentous nature of these proteins. Figure 4D shows the membrane specific localisation of β1 integrin on the

<table>
<thead>
<tr>
<th>Antigen</th>
<th>T47D</th>
<th>DAC/S</th>
<th>DAC/E</th>
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<tr>
<td>Cytokeratin</td>
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<td>CEA</td>
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<tr>
<td>E-cadherin</td>
<td>+++</td>
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</table>

Staining intensity: − = negative; −/+ =equivocal; + = weak; ++ = moderate; +++ = strong.

Figure 4 A, Haematoxylin and eosin stained section of the well differentiated duodenal adenocarcinoma, here seen invading the intestinal muscularis. DAC/E cells stained for (B) cytokeratin and (C) vimentin. D, T47D cells immunoreactive for the β1 integrin subunit. DAC/E and DAC/S stained for the (E) α2 and (F) β1 subunits. Intense, granular staining of (G) DAC/E and (H) DAC/S with PR 3B10; the majority of cells (>95%) were CEA positive.
epithelial cell line T47D. Interestingly, such membranous expression is not reflected in the DAC/S or DAC/E cells where the integrin subunits α2 and β1 seem to be located predominantly within cytoplasmic granules (figs 4E and 4F). Both cell lines are intensely positive for carcinoembryonic antigen (CEA) (figs 4G and 4H), which is also distinctly located within granules widely distributed throughout the cytoplasm of the majority of the cells.

In summary, we propose that DAC/S are rapidly growing 5N cells with a spindle morphology capable of serum free growth. They seem to be anchorage dependent, are not tumorigenic in nude mice but display extensive, poorly organised growth in Type I collagen. Immunophenotypic analysis suggests an epithelial (cytokeratin positive), dedifferentiated (CEA positive) phenotype. DAC/E cells have similar properties, except that they are epithelioid, are slow growing and senesce when cultured in collagen.

Discussion

Primary adenocarcinoma of the duodenum is a very rare lesion, constituting only about 0.3% of all gastrointestinal cancers, and consequently data concerning the cellular and molecular properties associated with such tumours are very limited. We have established two cell lines, DAC/S and DAC/E, from a primary duodenal adenocarcinoma, which have subsequently been characterised to determine their origin and tumorigenic potential.

DAC/E cells have a regular, polygonal morphology and grow as monolayers with each cell having a well defined boundary with adjacent cells when confluent (fig 1B). This morphology is characteristic of epithelial cells, in sharp contrast to DAC/S which are spindle shaped and form parallel arrays and whorls on confluency (fig 1A).

Ultrastructurally, both cell lines have an unusual complement of morphological characteristics with long, extensive but irregular microvilli a prominent feature. Microvilli are usually uniform in size and regularly arranged, although they may be drastically deformed in some pathological conditions, particularly adenocarcinomas. Cells containing large abnormal mucus vacuoles were observed, suggesting that these cells have a secretory function, as would be expected of cells derived from glandular tissue. The mucus of normal cells is usually of a more electron dense nature from that seen here, and the presence of severely distorted vacuoles is abnormal. The nuclear characteristics of DAC/S and DAC/E cells are also unusual. Nuclei with large, coarse clumps of heterochromatin with invagination of the nucleus (fig 2B) is a common feature of neoplastic cells. Enlarged, distorted nucleoli, characteristic of adenocarcinoma cells, are also evident.

Both cell lines also varied in their growth requirements, which was exemplified by the ability of DAC/S to grow in serum free medium. This indicates that the cells are capable of secreting essential growth factors which could act in an autocrine/paracrine manner, which further reflects the less well differentiated DAC/S phenotype. Such properties indicate a decreased ability to respond to regulatory environmental cues, a common feature of neoplastic cells, and it would be interesting to determine the putative growth factor(s) involved in this autocrine/paracrine mechanism. In contrast, DAC/E required supplementation with FCS, insulin, glutamine and hydrocortisone for optimal growth. This is indicative of a better differentiated phenotype, in that these cells will only respond to appropriate growth stimuli, similar to the situation experienced under normal in vivo conditions.

The population doubling time (PDT) of DAC/S is approximately 24 hours, which is comparable with normal epithelial cell lines. The relatively slower PDT of DAC/E is unremarkable in that tumour cell lines derived from other regions of the gastrointestinal tract have been shown to vary greatly in their growth rates, from 12 to 273 hours.

Ploidy analysis was performed to determine the DNA content of both cell lines. Naturally, all normal cells within the gastrointestinal tract are diploid, but both DAC/S and DAC/E have a predominant ploidy of 5N. These cell lines could be further analysed by karyotyping and genetic fingerprinting. However, 65% of colorectal cancers are aneuploid, with many cell lines derived from such lesions having variable karyotypes.

Collagen gel can provide a suitable matrix for the morphogenesis of primitive epithelial structures such as glands and, indeed, in vitro studies of this nature have been successful in inducing glandular differentiation. DAC/S proliferated and grew rapidly in collagen, yet this growth seemed to occur in a random fashion with no evidence of any attempt at glandular differentiation even when the cells were closely associated in clumps. In contrast, DAC/E cells showed no obvious interaction with the collagen, possibly because of their requirement for a more complex support matrix such as Matrigel, which may be more effective in inducing differentiation.

The assessment of anchorage independent growth in agar showed both DAC/S and DAC/E to be non-viable in such a system. Generally, anchorage independent growth shows a high correlation with tumorigenicity, though only a very small proportion (<1%) of tumour derived cells are clonogenic in semi-solid media. This is because agar seems to enable only the most highly malignant cells to clone.

Both cell lines were non-tumorigenic when xenografted into nude mice, which was disappointing but not surprising considering that the frequency of “takes” (that is, growth of transplanted tumour in the host) is, on average, only 30%. However, there are many examples of cell lines derived from poorly differentiated tumours that readily form colonies in soft agar, but are not tumorigenic in nude mice and the technique and site of xenografting as well as the strain of host animal may have a bearing on the “take” rate. We are currently attempting to define the conditions required to
Establishment and characterisation of cell lines

This is the tumour not the growth in vitro are expression from muscle or originated protein. However, CEA expression is not conclusive evidence of malignancy, as this protein can be expressed, albeit rarely, in non-malignant conditions.

The pattern of integrin expression by cultured cells may be different from that observed in tissues in vivo, which reflects the regulation of integrins by the complex extracellular matrix.

The cells of many tissues express α2β1 and α6β1, which bind collagen and laminin, respectively. Both cell lines were negative for the α6 integrin subunit, but were positive for the β1 and α2 integrin components, yet did not display the membrane specific localisation that is characteristic of integrins (fig 4D). This might be because of mutational loss or abnormal expression of certain integrins which frequently occurs in colorectal adenocarcinomas and associated with more aggressive, poorly differentiated and hence invasive phenotype in melanomas and breast cancer. Indeed, many examples exist where a change in integrin expression is associated with a change in cells from a normal to a malignant phenotype. Moreover, the β1 and α2 integrin subunits are clearly confined to cytoplasmic granules in DAC/S and DAC/E cells, suggesting a defective intracellular receptor translocation mechanism. In addition, neither of the cell lines expressed the cell adhesion molecule E-cadherin, a protein normally associated with all normal epithelia, but which is frequently lost in carcinomas. The absence of E-cadherin immunoreactivity in DAC/E and DAC/S could be a result of the loss of the HECD-1 epitope through mutation or deletion. It may be connected with the inability to form colonies in soft agar because of the lack of intercellular cooperation.

In conclusion, two continuous cell lines have been established from biopsy material obtained from a primary duodenal adenocarcinoma. Both cell lines, namely DAC/S and DAC/E, are immortal, transformed and it is likely that they represent cell lineages which have originated from an epithelial component of the original tumour. Both cell lines have very similar immunophenotypes, but different morphologies and growth properties. The tumorigenic potential of both cell lines has not yet been firmly established, but further experiments are under way to evaluate this.

Virtualy all human cell lines are characterised only after years of intensive work and additional, extensive studies are required to fully characterise these unique cell lines. This may then provide an opportunity to elucidate the molecular mechanisms operating in DAC/S and DAC/E, which will ultimately contribute to our understanding of gastrointestinal carcinogenesis.

This work is supported by the Medical Research Council.

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doi: 10.1136/mp.49.1.M33

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