Establishment of a gastric epithelial progenitor cell line from a transgenic mouse expressing the simian virus 40 large T antigen gene in the parietal cell lineage

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Received 28 July 2007; revision accepted 3 September 2007

Abstract. *Objective:* In this study the gastric mucosa of transgenic mice expressing the simian virus 40 large T antigen gene in the parietal cell lineage is used to establish and characterize a new epithelial progenitor cell line. In these mice, proliferation and amplification of preparietal cells preclude their maturation into acid-secreting parietal cells leading to achlorohydria, hyperplasia, dysplasia and eventually gastric adenocarcinoma. *Materials and methods:* Enzymatically dispersed gastric epithelial cells were cultured, cloned and screened using immunohistochemical methods, for expression of a variety of biomarkers of differentiated pit, parietal, enteroendocrine and neck/zymogenic cells. *Results:* A biomarker-deficient cell line whose ultrastructural features resembled those of mouse gastric epithelial progenitor cells was established. Treatment with either hydrocortisone or oestrogen significantly enhanced proliferation of these cells, whereas retinoic acid inhibited their growth. No change in differentiation was detected with any of these treatments; however, when these cells were injected subcutaneously into nude mice, they proliferated to form tumours and undergo partial differentiation towards parietal cell lineage. *Conclusion:* This mouse gastric epithelial progenitor cell line could be useful as an *in vitro* model to study growth properties, proliferation and differentiation of a subpopulation of gastric epithelial progenitor cells and also to study gastric carcinogenesis.

INTRODUCTION

Mouse stomach is lined by a variety of mature epithelial cells that produce mucus, pepsinogen, hydrochloric acid, and various other peptides or hormones. These cells are organized to form numerous epithelial pit-gland units. Epithelial progenitors including undifferentiated (stem) and

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poorly differentiated cells are located in the mid-isthmic region of these epithelial units (Karam & Leblond 1992). By using [^3H]-thymidine radioautography combined with electron microscopy, it has been shown that these progenitor cells are responsible for the continuous production of all cell types that populate the pit-gland units (Karam & Leblond 1993a). While little is known about these progenitor cells, it is generally believed that alteration in their proliferation and differentiation programme plays a key role in pathogenesis of diseases such as gastric ulcers and neoplasia (Markert 1968; Pierce 1974; Potter 1978; Till 1982; Del Buono & Wright 1995; Mine *et al.* 1997; Trosko & Tai 2006). In addition, there has been some indication that these progenitor cells express receptors for binding of *Helicobacter pylori*, a major factor involved in the pathogenesis of gastritis, peptic ulcer and even adenocarcinoma (Syder *et al.* 2004; Oh *et al.* 2005). Thus, studies on gastric epithelial progenitors would be very important to provide the basis for understanding pathogenesis of these common gastric diseases and most cancers.

It is difficult to study progenitor cells of the mouse gastric epithelium, as they are found among a heterogeneous population of several differentiating and mature cell types (Karam & Leblond 1992). Because of their small size and relatively small number, the identification of gastric progenitor cells requires use of transmission electron microscopy. Even though Oct-4 has been shown to be a nuclear biomarker for embryonic and various adult stem cells (Tai *et al.* 2005), it has not been yet widely used to characterize subpopulations of gastric epithelial progenitors. Although a primary culture system is a useful tool for studying the features of these progenitor cells (Chen *et al.* 1991; Karam *et al.* 2001), it has restrictions due to their limited lifespan *ex vivo*, and the fact that they cannot readily be subcultured. These hurdles in studying the properties of progenitor cells *in vitro* can be overcome by establishment of immortalized cell lines.

Transgenic mice overexpressing an oncogene placed under the control of regulatory sequences from a cell lineage-specific gene provide powerful models for characterizing molecular mechanisms that underlie initiation and progression of tumourigenesis in defined cellular contexts; this technique is also a powerful system for producing specific cell lines. Human mammary and pancreatic epithelial stem cell lines have been established by using immortalizing SV40 and human papilloma E6/E7 viruses, respectively (Kao *et al.* 1995; Tai *et al.* 2003). In addition, a transgenic mouse model has been developed in which the regulatory elements of the H,K-ATPase β -subunit gene, *Atpb4* (Canfield & Levenson 1991) and an oncogene, the SV40 large T antigen, have been used to induce proliferation of gastric epithelial preparietal cells (Li *et al.* 1995) and thus block their differentiation into acid-secreting parietal cells (Karam *et al.* 1997); in addition, differentiation of preneck cells into zymogenic cells has been blocked at the neck cell stage. Induction of preparietal cell proliferation was associated with a cascade of changes, as summarized in Fig. 1, starting with gastric epithelial hyperplasia, achlorohydria, dysplasia and ending with the gradual development of invasive gastric adenocarcinoma (Syder *et al.* 2004).

We reasoned that these transgenic animals could represent a good source for obtaining a gastric epithelial progenitor cell line. Below, we describe the isolation and culture of such a cell line, as well as characterization of its growth properties, hormone responsiveness and tumourigenicity.

MATERIALS AND METHODS

Transgenic mice

FVB/N strain of mice, bearing the H,K-ATPase β -subunit/SV40 large T antigen fusion gene and generated in the Animal Facility Unit of Washington University School of Medicine

Figure 1. Schematic representation of the normal gastric gland and changes that occur in its isthmal progenitor cells (between the two arrows at the left side) and their descendent cell lineages, during expression of the SV40 large T antigen, in the parietal cell lineage (red). Note amplification of the progenitors of the parietal cell lineage and their transdifferentiation into invasive pre-enteroendocrine cells (yellow). Pit and zymogenic cell lineages appear green and purple, respectively.

(St. Louis, MO, USA) (Li *et al.* 1995), was used in this study. Mice were maintained in a specific pathogen-free state and given a standard irradiated chow diet *ad libitum* in the Animal Resources Center of Kuwait University. Mice were screened utilizing the polymerase chain reaction technique using tail DNA and primers specific for SV40 large T antigen gene as previously described (Li *et al.* 1995). Protocols described below were approved by the Research and Ethics Committees of Kuwait and United Arab Emirates Universities.

Establishment of gastric epithelial progenitor cell line

The stomach of an 18-month-old FVB/N female transgenic mouse was aseptically removed under anaesthesia. The gastric mucosa was scraped, minced and washed several times in sterile phosphate-buffered saline (PBS). Mucosal pieces were then treated with Eagle's basic medium containing collagenase XI (0.75 mg/mL) and were incubated in a shaking water bath, adjusted to 37 °C, for 20 min. Subsequently, disaggregated cells were centrifuged at 900 *g* for 5 min at 4 °C. They were then re-suspended in Iscove's modified Dulbecco's medium containing 5% foetal bovine serum (FBS), streptomycin (50 µg/mL), gentamicin sulfate (100 µg/mL), plated in 25-cm² tissue culture flasks and were incubated at 37 °C in a humidified chamber with 5% CO₂ and 95% O₂.

The cultured cells formed a confluent monolayer after 1 month. At this point, cells were subpassaged, by treating them with 0.25% trypsin-EDTA, and were incubated at 37 °C for ~15 min until they detached from the bottom of the flask. Cells were subsequently harvested, further disrupted by pipetting, and spun at 900 *g* for 5 min. Supernatant containing the trypsin was discarded and the cell pellet was suspended in fresh medium. An aliquot of ~10⁵ cells was seeded into new flasks and multiwell dishes for serial passage, and for other studies. Thereafter, cells were continuously cultured by changing the medium every 3 days, and routinely subcultured by trypsinization after they became confluent.

Light microscopic and histochemical studies

Confluent cells in 25-cm² flasks were routinely examined under a phase contrast inverted microscope and were photographed. Immunofluorescence studies were performed on cells grown on 4- or 8-well chamber slides. Cells were washed three times with PBS, fixed with 4% paraformaldehyde or Bouin's solution and were permeabilized with 0.2% Triton X-100 in PBS for 2 min at room temperature. Cells were probed with a mouse monoclonal antibody specific for cytokeratin 4.63 (Sigma, St. Louis, MO, USA) and a panel of antibodies that we routinely use as biomarkers specific to various cell lineages in the gastric epithelium (Karam *et al.* 2005): mouse monoclonal antibodies specific for H,K-ATPase α -subunit (kindly provided by Dr. Adam Smolka; Smolka & Swiger 1992) and β -subunit (kindly provided by Dr. John Forte; Chow & Forte 1993), and rabbit polyclonal antibodies specific for chromogranin A (DiaSorin, Stillwater, MN, USA), and intrinsic factor (kindly provide by Dr. David Alpers; Wen *et al.* 2000). Antigen-antibody binding sites were visualized by incubation with the appropriate fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated mouse or rabbit immunoglobulin G. TRITC-conjugated *Ulex europaeus* agglutinin type 1 lectin (UEA-1) and FITC-conjugated *Grifforia simplicifolia* II lectin (GSII), known as markers of mucus-secreting pit and neck cells, respectively (Falk *et al.* 1994; Karam *et al.* 2005), were also used. FITC-conjugated phalloidin was used to label actin filaments. Secondary antibodies, lectins and phalloidin were all purchased from Sigma.

Electron microscopic studies

Approximately 1×10^6 cells were plated on 35-mm tissue culture dishes that contained sterile glass coverslips. Cells were grown to near confluence in 5% FBS/Ischove's modified Dulbecco's media, and then were fixed for electron microscopy using mixed aldehyde solution (Karam *et al.* 2001). After post-fixation in reduced osmium, cells were impregnated and then embedded in araldite. Ultra-thin sections were examined using a Philips electron microscope.

Cell population growth and effect of hormones

Confluent cells growing in 80-cm² culture flask at different passages were trypsinized, passed through a 22-gauge needle and were used for studying their replication doubling time and the effect of hormones. After the trypsin was diluted by adding fresh media, cell number was quantified using a haemocytometer. Cells were suspended in the medium at a titre of $\sim 10^4$ /mL, 1 mL aliquots seeded per well in 12-well dishes. To measure cell proliferation, cells were cultured for 5 days, with fresh medium introduced after 3 days. Each day, some wells were trypsinized, and cells counted using a haemocytometer.

For studying hormone responsiveness, cells were plated, as above, in multiwell dishes and after allowing cells to divide for 1 day in complete growth medium containing 5% FBS, the medium was replaced with FBS-free medium containing 0.5 nM, 1 nM or 2 nM of hydrocortisone, oestrogen and retinoic acid. Control cells received FBS-free medium without any hormone. Multiwell dishes were returned to the incubator for 1 day. Cells were subsequently harvested by trypsinization, and quantified using a haemocytometer. This experiment was performed four times. Results are presented as the mean $\pm$ SE of three replicate culture wells, analysis being performed to compare control versus treated cells using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Tumorigenicity study

Mouse gastric epithelial progenitor (mGEP) cells (1×10^6 – 1×10^7) at passage 9 were injected subcutaneously into the flanks of 6–8-week-old Balb/c female athymic nude mice. These mice were observed for growth of xenografts twice a week for 4 months.

RESULTS

The present study describes the establishment of an immortalized gastric epithelial progenitor cell line derived from the gastric mucosa of an 18-month-old female *Atpb4* TAG-transgenic mouse. By this age, the gastric mucosa of the mouse is highly enriched with gastric epithelial progenitor cells. The stomach showed a nodular serosal surface compared to the stomach of its normal littermate mouse of the same sex. When both stomachs were opened along the greater curvature, the normal stomach showed some longitudinal mucosal folds that were absent in the transgenic stomach. The latter exhibited a thick wall and cauliflower-like growths protruding from the mucosal surface.

Light microscopic examination of the gastric mucosa showed massive thickening and formation of multiple epithelial cysts, as previously reported for mice that were one year old (Syder *et al.* 2004). Several sites showed loss of glandular architecture and signs of invasive carcinoma. Submucosal-invading cells were highly proliferative and poorly differentiated, resembling progenitor cells.

Establishment of the new gastric epithelial cell line

When the dispersed gastric mucosal cells were plated in a multiwell culture dish, cell types with two distinct morphologies appeared: large stellate cells and small round or ovoid cells (Figs 2a and 3a). Histochemical studies using phalloidin revealed the epithelial nature of the small cells with peripheral actin distribution and the large stellate cells had mesenchymal features (Fig. 3a).

We observed that the small epithelial cells gradually increased in size, divided and gave rise to small polyhedra-shaped descendants (Fig. 2b–d). Colonies of these cells were trypsinized using a cloning cylinder, passed through a needle, and then were re-cultured as passage 1 (P1). To ensure immortality, cells were cultured, subsequently subcultured and passaged up to P70. When the mGEP cells stored in liquid nitrogen at different passages were thawed and cultured, they grew well and achieved confluence within a few days. Their morphological and population growth properties were subsequently quite stable over serial subpassages.

Characterization of the mGEP cell line

In addition to the peripheral pattern of actin distribution (Fig. 3a,b), the epithelial nature of the cells was confirmed by their expression of cytokeratin (Fig. 3c). Immunohistochemical studies of the cells at P4, with antibodies specific for H,K-ATPase and chromogranin A, revealed very low amounts of these proteins (data not shown). When they reached P10 and on, H,K-ATPase and chromogranin A were down-regulated and became undetectable. Both UEA-1 and GSII lectins, specific for mucus-secreting pit and neck cells, respectively, did not bind to cells at P4 or any of the later passages, indicating the absence of mucous granules characteristic of these lineages (data not shown). In addition, the intrinsic factor specific for chief cells was not detected.

The epithelial nature of the cells was further confirmed by scanning electron microscope that revealed a typical polyhedral appearance with narrow intercellular spaces and multiple contact sites (Fig. 2d). Transmission electron microscopy confirmed that these contact sites represent typical epithelial junctional complexes (Fig. 2e) and could also be gap junctions similar to those previously described by Mine *et al.* (1997). Electron microscopic examination also showed that these cells were characterized by a high nuclear-to-cytoplasmic ratio and to contain few small membrane-bound organelles among many free ribosomes.

Figure 2. Establishment of the mouse gastric epithelial progenitor (mGEP) cell line. Light micrographs showing attached single epithelial-like cell (a), a multinucleate cell (b), small epithelial-like colony (c) and confluent monolayer (d) as they progressively evolve during the first month after dispersion and plating of the transgenic gastric mucosal cells. Scanning electron micrograph showing attached, tightly packed cells (e), transmission electron micrograph of the cells showing distinct epithelial junction at the arrow (f). $\times 400$ (a, b); $\times 200$ (c, d); $\times 800$ (e); $\times 6000$ (f) original magnifications.

Figure 3. Immunohistochemical analysis of the developing mGEP cell line using phalloidin (a), antibodies specific for actin (b) and cytokeratin (c). (a) The two initially attached cell types are stained to reveal actin, but the cell on the left appears typically mesenchymal and the right one appears epithelial-like. (b) Confluent layer of the mGEP cell line stained positive for actin. (c) Monolayer of mGEP cells showing cytoplasmic expression of cytokeratin. $\times 400$ (a); $\times 200$ (b); $\times 300$ (c) original magnifications.

More detailed analysis of the cells revealed occasional long microvilli similar to, but thinner than those of mouse parietal cells (Fig. 4a). In some cells, a few small, but distinctive round or ovoid electron-dense granules were observed, similar to those of pre-enteroendocrine cells (Fig. 4b). Cells from all passages examined showed no signs of mucous granule production and no intracellular canaliculi characteristic of parietal cell lineage. Thus, mGEP cells lack signs of differentiated gastric epithelial cell lineages and exhibit several progenitor cell-like features.

Growth properties and modulation by hormones

Mouse gastric epithelial progenitor cells cultured at different passages were found to have a doubling time of ~ 40 h. When they were treated with 0.5 nM hydrocortisone or oestrogen, their proliferation rate significantly increased, whereas 0.5 nM retinoic acid inhibited their replication (Fig. 5); effects of hydrocortisone and oestrogen was dose-dependent (Fig. 5). The hormones had no effect on cell differentiation, as judged by electron microscopy, or by immunohistochemical assays using our panel of lineage-specific antibodies and lectins.

Figure 4. Electron micrographs of the immortalized mouse gastric epithelial progenitor (mGEP) cells showing their ultrastructural features. (a) Note progenitor cell-like features: large nucleus in relation to cytoplasm, much diffuse chromatin, and many free ribosomes. In addition, some microvilli (arrows) cut at different orientations are projecting from the apical surface. (b) Note the few small secretory granules; they appear electron dense and round or ovoid similar to those of pre-enteroendocrine cells. (c) The apical cytoplasm of mGEP cell transplanted into a nude mouse showing numerous tubulovesicular elements characteristic of parietal cell lineage; few scattered electron dense granules are also present. (d) Transplanted mGEP cell showing a small group of electron dense granules characteristic of pre-enteroendocrine cells. $\times 6000$ (a, b, d); $\times 11\,000$ (c) original magnifications.

Figure 5. Effects of hydrocortisone, retinoic acid and oestrogen on the expansion of cells of the mouse gastric epithelial progenitor (mGEP) line. Cells were grown in serum-free medium containing 0.5, 1 or 2 nM of hydrocortisone, oestrogen or retinoic acid. Control cells received serum-free medium with no hormone. After 1 day, cells were trypsinized and quantified using a haemocytometer. Each bar shows data from triplicate wells presented as mean $\pm$ SEM.

Tumorigenicity of the cell line

When the cells from P9 were injected subcutaneously into the flanks of 6–8-week-old athymic nude mice ($n = 3$), tumours developed and masses became visible 3–4 months later. Electron microscopic examination of the tumour cells revealed progenitor-like features as documented in the original, injected cells (Fig. 4c,d). However, a subset of cells in the xenografts exhibited

advanced signs of parietal cell differentiation with prominent long, numerous, microvilli on their apical surfaces, and many small cytoplasmic tubulovesicular structures (Fig. 4c).

DISCUSSION

In *Atpb4*-SV40 TAG-transgenic mice, preparietal cells undergo mitosis and thus their differentiation is blocked. The result is an amplified population of preparietal cells associated with the block in differentiation of both parietal and chief cells (Li *et al.* 1995; Karam *et al.* 1997). With age, preparietal cells increased in number and gastric mucosae of these mice gradually developed severe metaplastic and dysplastic changes ending with invasive carcinoma. Immunohistochemical and electron microscopic analysis revealed the progenitor cell nature of the invasive cancer cells that initiated the development of adenocarcinoma (Syder *et al.* 2004). Thus, this animal model provided new insights into gastric carcinogenesis that fits well with the statement of Potter (1978) 'oncogeny is blocked ontogeny' and the stem cell origin of cancer (Trosko & Tai 2006). This animal model also provides a wonderful source for isolating a huge number of immortalized population of cells that have features of the normally rare gastric epithelial progenitors, that resides in the gastric unit.

Cell immortalization is highly valuable for studying several biological phenomena, provided that the immortalized cells retain critical biological features characteristic of the parent cells (Vidal *et al.* 1993; Kao *et al.* 1995; Gudjonsson *et al.* 2002; Tai *et al.* 2003). Indeed, the mGEP cell line established in the present study shows several features characteristic of gastric epithelial progenitors (Karam & Leblond 1993a). These features are the large nucleus-to-cytoplasm ratio, the numerous free ribosomes, and the few small cytoplasmic membranous organelles: rough endoplasmic reticulum, Golgi apparatus and mitochondria. In addition, nuclei were characterized by prominent nucleoli and much diffuse chromatin. On the other hand, the cells exhibited no ultrastructural evidence of acid production, pepsinogen secretion or mucous granule formation. In addition, using light microscopic immunohistochemical analysis employing a panel of validated cell-specific lectins and antibodies, we found that none of the differentiated gastric cell markers (H,K-ATPase, intrinsic factor, chromogranin A, and GSII and UEA-I lectins) was detectable in these cells. However, detailed electron microscopic analysis revealed that cytoplasm of mGEP cells carries a few distinctive pre-enteroendocrine cell-like granules similar to those previously described in the mouse stomach (Karam & Leblond 1993b; Syder *et al.* 2004).

The finding that the immortalized mGEP cells established in the present study had characteristic ultrastructural feature of mouse pre-enteroendocrine cells is not surprising. They are derived from the gastric mucosa of an old SV40 transgenic mouse with invasive gastric adenocarcinoma. Electron microscopy and molecular profiling of the invasive gastric cancer cells showed their pre-enteroendocrine cell-like phenotype and demonstrated their origin from the amplified preparietal cells (Syder *et al.* 2004).

An interesting feature of the immortalized mGEP cells was that, when transplanted into subcutaneous tissue of nude mice, they underwent differentiation and showed some features characteristic of the parietal cell lineage: numerous cytoplasmic tubulovesicular elements and long apical microvilli. Thus, this mGEP cell line could serve as a unique *in vitro* model system to study differentiation of gastric progenitor cells. Differentiation of cell lines with progenitor cell-like features has previously been reported in other cell lines such as that derived from basal epithelial cells of the rat prostate (Danielpour 1999; Shim *et al.* 2004). That a population of mGEP cells form a tumour, which gradually shows signs of differentiation, fits well with the

observation that many tumours when probed with antibodies specific for the stem cell marker Oct-4, some cells were labelled and others were negative, indicating their differentiated state (Tai *et al.* 2005; Atlasi *et al.* 2007).

Cultured mGEP cells have a doubling time of around 40 h in a medium containing FBS. To investigate modulation of cell population growth by hormones known to influence cell proliferation of other epithelial cell lines, cells were cultured in the presence of hydrocortisone, oestrogen and retinoic acid. The results showed that while hydrocortisone and oestrogen stimulated cell expansion and proliferation, retinoic acid had an inhibitory effect. By increasing the dose of hydrocortisone, the cell replication rate also increased. These results were revealed by an increased proliferative cell fraction as indicated by using continuous bromodeoxyuridine labelling for 1 day, and immunohistochemistry.

That this cell line was tumourigenic when injected into nude mice indicates that cells of the line were at a malignant stage and thus, could be used to study gastric tumourigenesis both *in vitro* and *in vivo*. This model system could also provide tools for stepwise analysis of mechanisms involved in the control of proliferation and expansion of these cells. Efficacy of an inhibitory agent can first be assayed on this cell line *in vitro*, then the down-regulation of primary tumour growth can be evaluated by treating *in vivo* transplanted nude mice. Because the preperietal-like features of this cell line tended to become prominent when grown in subcutaneous tissue of nude mice, it could provide a good model to dissect the molecular events involved in the differentiation of preperietal cells.

ACKNOWLEDGEMENTS

This study was supported by Funds from Kuwait Foundation for the Advancement of Sciences and Terry Fox Fund for Cancer Research (to S.M.K.). The authors are grateful to Dr. Jeffrey Gordon for providing the transgenic mice used in the study and for the constructive comments on the manuscript.

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