Establishment and characterization of normal and initiated hamster tracheal epithelial cell system

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Abstract. The development and characterization of normal hamster tracheal epithelial (HTE) cell system and its initiated subline is described in the present study. Normal HTE cells grew in a monolayer, had a stable diploid karyotype, were anchorage dependent and non-tumorigenic. The presence of desmosomal attachments and keratin filaments confirmed the epithelial nature of these cells. An initiated subline DTC8 was isolated after treatment of HTE cells with a sub-optimal dose of 9,10-dimethyl-1,2-benz[*a*]anthracene (DMBA). These DTC8 cells grew in a monolayer, had a higher growth rate and saturation density, were weakly anchorage independent and non-tumorigenic. Treatment of DTC8 cells with 100 ng 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), resulted in transformation of these cells which then showed anchorage independent growth on semisolid agar and formed tumours in 85% animals. As DTC8 cells showed heterogeneity in chromosome number, they were further cloned by the limiting dilution method using gamma-irradiated hamster embryonic fibroblasts as a feeder layer. The clone H7I, isolated among these clones had all the properties of initiated cells.

The process of carcinogenesis proceeds through multiple discernible stages which consist of initiation, promotion and progression (Yuspa & Poirier 1988). It is now understood that transition between these stages is driven by different biochemical mechanisms and genetic elements (Weinberg 1989). The nature of epigenetic processes and further genetic changes during the promotional phases of two stage transformation experiments subsequent to the initiation process are little understood. One approach for gaining further insight into these processes is to define specific experimental cells or tissue systems which are relevant to human cancer. The cell culture systems provide a quantitative model to study chemically induced neoplastic transformation and may be useful in identifying potential promoting agents. The murine C3H/10T1/2 cell line was the first cell model used for such analysis (Mondal, Brankow & Heidelberger 1976). Tsuchiya & Umeda (1997) have recently shown the usefulness of the method for optimizing transformation in the BALB/c 3T3 cell transformation assay, where the cells were initiated with *N*-methyl-*N'*-nitro-nitrosoguanidine (MNNG) and then promoted with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), resulting in transformation of these cells. However, the development of an epithelial cell system for such studies is important because the majority of human cancers are epidermoid carcinomas. A number of epithelial cell lines from animal and human tissues have been developed during the

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last few years for such studies using diverse strategies to prevent epithelial differentiation *in vitro* (Thomassen, Saffiotti & Kaighn 1986, Masui 1992).

The hamster tracheal epithelial cell system provides a unique model to study environmental and chemical carcinogenesis due to its low rate of spontaneous transformation and sensitivity to transformation *in vitro* by chemical carcinogens (Walker & Nettesheim 1989). So far, few studies have demonstrated the ability to culture initiated nontumorigenic cells *in vitro* which can be transformed after the treatment of cells with tumour promoters (Tsuchiya & Umeda 1997). Hennings *et al.* (1992) developed an initiated mouse keratinocyte cell line for studying tumour promotion *in vitro*. Nakazawa, Aguelon & Yamasaki (1992) identified and characterized a specific oncogene mutation as a carcinogen-induced initiation event using a BALB/c 3T3 cell transformation model for initiation. However, a clonal epithelial cell line of initiated cells has not been identified and characterized to study the multistage process of carcinogenesis.

In this paper, we describe the development and characterization of a normal hamster tracheal epithelial (HTE) cell system. We have further isolated and cloned an initiated subline from HTE cells by treatment of these cells with a sub-optimal dose of 9,10-dimethyl-1,2-benz[*a*]anthracene (DMBA).

MATERIALS AND METHODS

Chemicals were purchased from following sources: human transferin, insulin (bovine pancreas), bovine serum albumin, hydrocortisone, epidermal growth factor (EGF-tissue culture grade), HRPO labelled mouse anti-rabbit antibody, colchicine, DMBA, TPA, ampholines (pH 5–8) were purchased from Sigma Chemical Co., MO; 9,6-diamidino-2-phenyl indole (DAPI) was a generous gift from Professor W. Gohde, University of Munster, Germany; bactotrypsin (1:250) was from Difco Laboratories, MI; and Ham's F12 medium was purchased from Hi-media, Mumbai, India.

Development of hamster tracheal epithelial cell line

Primary cultures of hamster tracheal epithelial (HTE) cells were established by the method of Pai, Steel & Nettesheim (1983) with some modifications. Four- to six-week-old Syrian Golden hamsters (*Mesocricetus auratus*) were used for this study. Their tracheas were removed under aseptic conditions. The tracheal rings were cut and seeded in 35 mm petri dishes (Nunc Delta, Roskilde, Denmark) which had their bottom surfaces scored with multiple intersecting grooves to aid attachment of tracheal rings. Approximately 0.1 ml of growth medium, consisting of 1:1 mixture of Dulbecco's modified minimal essential medium (DMEM) and Ham's F12 medium, supplemented with transferrin (20 µg/ml), insulin (10 µg/ml), bovine serum albumin (BSA) (100 µg/ml), hydrocortisone (0.4 µg/ml), sodium selenite (5 ng/ml), epidermal growth factor (EGF) (5 ng/ml), penicillin (200 IU/ml), streptomycin (250 µg/ml) and 10% goat serum, was initially added on explants which were then covered with a glass coverslips. The dishes were incubated at 37 °C in 5.0% CO₂ for 24 h to allow the explants to adhere to the substratum. After 24 h, 3 ml of additional growth medium was added to each petri dish and cultures were incubated further. The growth medium was changed twice a week.

The cultures were observed every alternate day for the outgrowth of tracheal epithelial cells. The tracheal epithelial cells migrated out of the explant on the sixth day. After 6 days the outgrowth of these cells increased and covered a larger area. The outgrowth of the cells was faster between 6 to 15 days and declined thereafter. To obtain healthy epithelial cells,

which can multiply and be passaged after transfer, 15–20-day-old cultures were selected for the study. Use of a specific growth medium for epithelial cells suppressed growth of fibroblasts and these were removed by partial trypsinization with 0.25% trypsin in phosphate buffered saline (PBS). Fresh growth medium was added and the cells were allowed to grow for few more days. At this point, many patches of epithelial cells were seen.

These primary epithelial cells were then passaged by treatment with 0.25% Eagle's low calcium medium for 24 h and trypsinized further with 0.25% EDTA trypsin for 15–20 min. The cells were gently mixed and centrifuged at 1000 rpm for 5 min. The pellet was re-suspended in growth medium and the cells were plated at a density of 1×10^5 cells/35 mm Nunc dish. Within 24 h after plating the cells adhered to the petridishes, they became confluent within a week. These cells were then passaged by direct trypsinization with 0.25% PBS–trypsin for 5–10 min. After the fourth passage, epithelial cell colonies were isolated by using stainless steel cylinders to get epithelial cell clones and then further grown in bottles. The epithelial nature of these cells was confirmed by light and electron microscopic observations (Figure 1, 2 and 3). After the 10th passage the cells were designated as a HTE cell line and characterized further.

Treatment of HTE cells with DMBA

A sub-optimal dose of DMBA was used for the initiation of normal HTE cells *in vitro*. One μg of stock DMBA solution was prepared in 1.0% dimethyl sulfoxide (DMSO) and further diluted 1:10 with growth medium to get the final concentration of 0.1 μg DMBA/ml. HTE cells (1×10^3 cells/dish) were treated with 0.1 μg DMBA/ml with parallel solvent control (0.1% DMSO) for 24 h. Cells were then washed with PBS and incubated at 37° in a CO₂ incubator. More than 50% of the cells died after DMBA treatment. DMBA resistant cells formed colonies around 15–20 days. Since transformed cells grow well in the absence of pyruvate (Wasilenko & Marchok 1984, 1986), they were further selected in pyruvate depleted medium containing 5% goat serum for 2 more weeks. Out of several surviving colonies, nine colonies were isolated by the cylinder method, subcultured in petri dishes and grown further. They were designated as DMBA treated cells 1–9 [DTC1–DTC9]. Clone DTC7, DTC8 and DTC9 were tested for *in vivo* and *in vitro* transformation assays and characterized further.

Growth rate study of the cells grown as monolayer cultures in tubes was studied by enumeration of cell nuclei according to Sanford *et al* (1951). The viability of these cells was found to be above 95% by the dye exclusion method using 0.4% erythrocin B. The doubling time was calculated by plotting a graph of time versus number of cells using semilog graph paper.

Anchorage independent growth was studied according to the method of Macpherson (1973). In brief, 1×10^4 cells were suspended in 2 ml of 0.3% agar in standard growth medium. This mixture was then layered on previously solidified 0.6% agar in growth medium in 55 mm petri dish. The dishes were incubated at 37°C in a CO₂ incubator for 15 days. The total number of colonies (minimum of 20 cells/colony) were counted and colony forming efficiency was calculated.

Flow cytometry

Cells grown in monolayer culture in bottles were harvested at different time points after subculture, viz. 0, 24, 48 and 72 h, with 0.25% PBS–trypsin. They were washed with PBS and fixed in 70% alcohol. The fixed cells were treated with 0.5% pepsin for 5 min and stained with 5 $\mu\text{g}/\text{ml}$ 9,6-diamidino-2-phenyl indole (DAPI) in tris-HCl buffer pH 8.0 for 15 min. Flow cytometric analysis was done on PAS II (Partec GmbH, Munster, Germany) flow

cytometer fitted with a HBO-100 mercury lamp as described earlier (Dean 1985). Hamster spleen cells were used as a calibration standard. The instrument was aligned optically to give a coefficient of variation (CV) of 1.1–1.4 with spleen cells. The percentage of cells in G₁, S and G₂ + M was obtained using software provided by the manufacturer.

Chromosomal analysis

Chromosome preparations from monolayer cells were made according to Yunis (1981). Cells grown in bottles were treated with 0.2 µg of colchicine/ml of growth medium for 2–3 h, trypsinized, incubated in 0.075 M KCl for 40 min and fixed in cold acetic acid–methanol (1:3). Air-dried preparations were stained for G-banding (Sun, Chu & Chang 1973). At least 25 metaphases were photographed and karyotyped per sample.

Keratin analysis

Keratins were extracted from culture cells according to the method of Eichner *et al.* (1984). Keratins were resolved by their molecular weights by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS–PAGE), as described by Laemmli (1970) and immunoblotted (Towbin, Staehelin & Gordon 1979) using polyclonal keratin antibody to human keratins developed in our laboratory (Vaidya *et al.* 1989). Keratins were resolved by 2-dimensional (2-D) gel electrophoresis with isoelectric focusing as the first dimension in a gradient of ampholines (pH 5.0–8.0) by the method of O'Farrell (1975).

Electron microscopy

Sub-confluent monolayer cells were fixed and processed for electron microscopy *in situ* by the method of Brinkley, Murphy & Richardson (1967). Silver–grey sections were cut on Sorvall MT-5000 ultramicrotome, stained with uranyl acetate and lead citrate and observed in Zeiss EM-109 electron microscope at an accelerating voltage of 80 kV and photographed on ORWO DP2 35 mm positive film.

Hamster embryo fibroblasts cultures

Embryos were dissected from female on the 12th day of pregnancy. They were washed and minced in PBS. The minced material was again washed with several changes of PBS and trypsinized in 0.25% PBS trypsin for 10 min. The cell suspension was centrifuged at 1000 rpm for 7 min. The pellet was resuspended in DMEM supplemented with 10% goat serum and plated at a density of 1×10^6 cells/bottle for further growth. For preparing a feeder layer for cloning, primary hamster embryo fibroblasts cells were seeded at a density of 2×10^5 cells/well in 96 well plates. After 24 h the cells were irradiated at 40 Gy with ⁶⁰Co γ rays.

RESULTS

Characterization of hamster tracheal epithelial cell line

Hamster tracheal epithelial cell line was developed from the primary culture of hamster trachea as described in Materials and methods. Phase contrast microscopy of HTE cells showed tightly packed epithelial cells with large nuclei, 1–2 nucleoli and cytoplasm containing many granules (Figure 1). There was no significant change seen in morphology even after maintenance for 2 years. Ultrastructural studies of these cells showed cells with distinct nuclei and 1–2 nucleoli. The cells were tightly attached and showed frequent desmosomal

Figure 1. Phase contrast photomicrograph of normal hamster tracheal epithelial cells shows densely packed epithelial cells at passage 10 ($\times 120$)

attachments (Figure 2). The cytoplasm showed distinct bundles of intermediate keratin filaments (TF) (Figure 3), pleomorphic and elongated mitochondria, dilated rough endoplasmic reticulum, lysosomes, many dense granules and plenty of ribosomes. Some cells showed mucous secretory granules in the cytoplasm.

Growth rate studies of HTE cells showed a mean doubling time of 23.6 ± 0.63 h, which remained stable for many passages (Figure 4). HTE cells reached plateau of their growth at a mean cell density of $1.07 \pm 0.19 \times 10^6$ cells/55 mm dish. The plating efficiency of these cells was $16.9 \pm 1.4\%$ which was increased to $25 \pm 2.0\%$ with the addition of 1×10^{-5} M dexamethasone, to the growth medium. However, these cells did not form colonies at low seeding densities, viz. 100, 200 and 400 cells/dish. The plating efficiency was not increased, even after feeding these cells with medium conditioned by HTE cells. All the above assays were repeated at least three times.

Chromosomal analysis showed the normal diploid karyotype in 90% of metaphases studied (Figure 5). The modal chromosome number determined after counting 50 well spread metaphases was 44.

Cell cycle analysis of HTE cells at different time points, viz. 0, 24, 48 and 72 h, is shown in Table 1. At seeding (0 h), the majority of cells ($83.5 \pm 2.12\%$) were in the G_1 phase. Between 24 and 72 h the percentage of cells in G_1 phase declined to $59.5 \pm 0.7\%$ with an increase in the proliferating fraction ($S + G_2 + M$) from 16.5 ± 2.12 to $40.5 \pm 0.7\%$.

To further confirm the epithelial nature of these cells, the cytokeratin expression in

Figure 2. Electron micrograph of hamster tracheal epithelial cells showing the desmosomal attachments (D) ($\times 50\,000$)

HTE cells were studied by SDS PAGE and 2-D gel electrophoresis and immunofluorescence. HTE cells expressed 58, 56, 54, 52, 50, 46, 45 and 40 kDa keratin polypeptides (Figure 6a). The keratin specificity of these peptides was further confirmed by 2-D gel electrophoresis and immunoblotting with a polyclonal antibody raised against human keratins. The amounts of keratins as seen by SDS-PAGE were substantially reduced at later passages. However, qualitative differences in keratin expression at different passages were not investigated.

Characterization and cloning of initiated cell line

Three clones, DTC7, DTC8 and DTC9, were subcultured further and their ability to form colonies on soft agar was studied. Among these, DTC8 cells were weakly anchorage independent and formed $3.43 \pm 0.63\%$ colonies in soft agar (Figure 7b), whereas DTC7, DTC9 and DMSO control cells did not form any colonies in soft agar (Figure 7a). For *in vivo* tumorigenicity assay, 1×10^6 DTC8 and DMSO control cells were injected into hamster cheek pouches. Tumour development was not observed in any of these animals over a period of 6 months, indicating that DTC8 and HTE cells were nontumorigenic. This observation also suggested that DTC8 may have undergone initial transformation changes. Hence, DTC8 and HTE cells were treated with 100 ng TPA/ml of growth medium for 24 h, washed with PBS and allowed to grow further for 5 more days. DTC8 cells grew faster than HTE cells

Figure 3. Electron micrograph of hamster tracheal epithelial cells bundles of keratin filaments (TF) ($\times 50\,000$).

after TPA treatment. Studies on anchorage independent growth showed that DTC8-TPA cells formed significantly higher number of colonies ($12.3 \pm 1.71\%$) in soft agar, as compared to untreated DTC8 cells ($P < 0.001$) (Figure 7c). The control HTE cells did not form colonies in semi solid agar. DTC8-TPA cells and control HTE cells (1×10^6) were injected into the hamster cheek pouch. Seven out of eight hamsters injected with DTC8-TPA developed squamous cell carcinomas within 3 months. Tumours did not develop in animals injected with control cells over a period of 6 months. These cells were selected for further characterization.

Light and electron microscopic studies did not reveal any gross morphological differences between DTC8 and DTC8-TPA cells. Both these cell lines had a faster growth rate and a higher saturation density than that of HTE cells (Figure 4). DTC8 and DTC8-TPA cells had a mean doubling time of 19.6 ± 1.40 h and 16.9 ± 1.40 h, respectively. The saturation densities of DTC8 and DTC8-TPA cells were of $1.5 \pm 0.29 \times 10^6$ cells and $3.5 \pm 0.41 \times 10^6$ cells/55 mm dish, respectively. Chromosome analysis of DTC8 cells showed heterogeneity in chromosome numbers. DTC8 cells expressed 58, 56, 54, 52, 50, 46, 45 and 40 kDa keratin polypeptides (Figure 6b).

The cell cycle analysis of DTC8 and DTC8-TPA cells at different time points, viz. 0, 24, 48 and 72 h, is shown in Table 1. At seeding (0 h), the majority of DTC8 cells ($90.0 \pm 2.82\%$) were in the G_1 phase. Between 24 and 72 h the percentage of DTC8 cells in G_1 phase declined

Figure 4. Cell growth rate study of normal (HTE ●), initiated (DTC8 ■ and H7I ▼) and TPA promoted (DTC8-TPA ▲ and H7I-TPA ○) cells.

to $61.5 \pm 2.12\%$ with an increase in the proliferating fraction (S + G₂ + M) from 10.0 ± 2.82 to $35.5 \pm 3.53\%$. Whereas, treatment with TPA resulted in a decline of cells in the G₁ phase (56.5 ± 3.93) and an increase in the proliferating fraction (S + G + M) to $43.5 \pm 3.53\%$. As such, DTC8 cells did not show much difference in cell cycle parameters when compared to HTE cells. However, treatment with 100 ng TPA changed the cell cycle parameter of DTC8-TPA cells.

As chromosomal analysis showed heterogeneity in chromosome number of DTC8 cells, they were further subcloned by using the limiting dilution method in 96 well plates and gamma irradiated hamster embryo fibroblasts as a feeder layer. Plates were observed for the growth of colonies every day. Colonies were visualized between 20 and 30 days after seeding. Sixteen clones were picked from 16 different wells and grown individually. Out of 16 clones isolated, three clones were retested for an initiated phenotype by *in vivo* and *in vitro* transformation assays. One of these clones, H71 was found to be an initiated clone, on the basis of criteria described earlier. H71 cells grew as an epithelial monolayer and had a saturation density of 3.5×10^6 cells in a 55-mm dish. The growth rate of these cells was slightly higher than that of HTE cells and had a mean doubling time of 16.6 ± 1.40 h (Figure 4). These cells showed anchorage independent growth on semi solid agar as well as forming tumours in seven out of eight animals (85%) after the treatment with 100 ng of TPA *in vitro*, as shown in Figures 7 and 8. Chromosome analysis of 100 well spread metaphases revealed a modal number of 42. Karyotypic analysis of 18 metaphases showed a random loss of chromosomes. However, loss of chromosome numbers 12 and 17 was a clonal anomaly seen in five different metaphases.

Figure 5. G-banded normal diploid karyotype of HTE cells.

Table 1. Percentages of cells in different phases of the cell cycle in cell lines at different time points

Cell lines	Cell cycle phase	0 h	24 h	48 h	72 h
HTE	G ₁	83.5 ± 2.12	77.0 ± 2.82	59.5 ± 0.70	61.0 ± 1.41
	S + G ₂ + M	16.50 ± 2.12	23.0 ± 2.82	40.5 ± 0.70	39.0 ± 1.41
DTC8	G ₁	90.0 ± 2.82	81.5 ± 0.70	61.5 ± 2.12	64.5 ± 3.53
	S + G ₂ + M	10.0 ± 2.82	18.5 ± 0.70	38.5 ± 2.12	35.5 ± 3.53
DTC8-TPA	G ₁	56.5 ± 3.93	58.0 ± 7.00	44.0 ± 5.65	48.5 ± 6.36
	S + G ₂ + M	43.5 ± 3.53	42.0 ± 7.00	56.0 ± 5.65	51.5 ± 6.36

Mean ± SD.

DISCUSSION

The present study reports the development and characterization of a normal hamster tracheal epithelial cell line and its initiated subline. The normal cell line was developed under the conditions that enhanced growth of epithelial cells in culture (Rheinwald & Green 1977, Bertolero *et al.* 1984). These cells grew as a monolayer with a defined saturation density but had a low plating efficiency which was enhanced by addition of 1×10^{-5} M dexamethasone (Namba & Kimoto 1979). The growth rate of these cells remained constant, even after 65

Figure 6. (A) Keratin profile in HTE cells: (a) molecular weight marker, (b) SDS PAGE, (c) and (d) immunoblots and (e) 2-D gel electrophoresis (numbers indicate molecular weight) (B) Keratin profile in DTC8 cells: (a) molecular weight marker, (b) SDS PAGE, (c) and (d) immunoblots and (e) 2-D gel electrophoresis (numbers indicate molecular weight)

passages and they had a stable diploid karyotype. They have typical features of epithelial cells confirmed by the presence of desmosomal junctions and keratin filaments in all cultured cells.

Cells from several normal tissues of rodent origin can be malignantly transformed *in vitro* by various agents and can also undergo spontaneous neoplastic conversion after variable periods in culture (Sanford & Evan 1982). The hamster tracheal epithelial cell system has been used by several investigators to study chemical and environmental carcinogenesis *in vitro* because of its stable diploid karyotype and susceptibility to environmental chemicals (Nettesheim, Terraghi & Klein Szanto 1982, Nettesheim & Marchok 1983, Jetten *et al.* 1989). Several studies have shown that human cells are rather resistant to *in vitro* transformation by chemical carcinogens (Dipaolo 1983, Barrett & Tennant 1985). Only SV40 induced transformation and spontaneous transformation of human cells have been reported by few investigators (Rhim *et al.* 1985, Reddel *et al.* 1988, 1995, Boukamp *et al.* 1990). In the present study, normal HTE cells were treated with a sub-optimal dose of DMBA and a subline which showed an initiated phenotype was isolated. The initiated DTC8 clone showed fewer colonies on semi-solid agar but did not form tumours when injected into the hamster cheek pouch. However, after treatment with TPA, these cells showed anchorage independent growth and

Figure 7 Colony formation in soft agar (a) HTE cells (b) DTC8 cells and (c) DTC8 TPA cells ($\times 50$)

formed tumours when injected into the hamster cheek pouch. Thus this system can be considered to be an initiated cell system which can be used for the study of the multistage process of carcinogenesis. These cells grow in a monolayer, have slightly changed morphology, increased saturation density and show no changes in cell growth kinetics.

It has been stated that inhibition of keratinization might be an early step in carcinogenesis. The pattern of keratin expression of DTC8 cells was almost identical to that in normal HTE cells and they also maintained these properties at higher passage. Similar findings on a series of chemically and spontaneously transformed human and rat cells have been reported (Nettesheim & Marchok 1983, Breitkreutz *et al.* 1986, Katimura *et al.* 1986, Hennings *et al.* 1992, Nakazawa *et al.* 1992). Thus DTC8 cell line with its initiated phenotype can offer a model to study the regulatory mechanisms in the process of promotion and progression of tracheobronchial epithelial cells.

The significance of chromosomal alterations in different stages in cell transformation is not yet established. The early appearance and stability of the distinct cytogenetic changes in the initiated DTC8 cells strongly indicate that they are essential for the early disturbances in growth resulting in an indefinite life span. The subsequent occurrence of aneuploidy and its correlation with higher growth capacity '*in vitro*' has also been reported in human cell lines (Hennings *et al.* 1990) and in transformed mouse keratinocyte lines (Fusenig *et al.* 1986). Therefore, polyploidization which further contributes to genomic imbalance and altered gene expression may be crucial for the acquisition of autonomous growth in culture but is certainly not sufficient for malignant conversion. Thus, these cell lines should provide a valuable model system to study the mechanisms of carcinogenesis, to study the mode of action of stage I and stage II tumour promoters and may have a potential as a screening system for testing environmental chemicals having tumour promoting activity.

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