Isolation and characterization of propagable cell lines (HUNC) from the androgen-sensitive Dunning R3327H rat prostatic adenocarcinoma

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The Dunning H rat prostate tumor (R3327H) is a widely used experimental model of human prostatic adenocarcinoma (CaP). The Dunning H tumor has been characterized as androgen-sensitive, androgen-receptor (AR) positive, prostate-specific antigen and prostate acid phosphatase (PAP) positive. To date, the tumor has been maintained by serial passage in vivo because of the lack of an in vitro cell line that retains the characteristics of the in vivo tumor. The objective of the present study was to establish a propagable cell line from R3327H adenocarcinoma that maintained androgen sensitivity and expression of AR, PSA and PAP. Tissue harvested from an in vivo R3327H tumor was dissociated with collagenase and placed into Richter’s improved media (with supplements). A cytokeratin-positive epithelial cell line (HUNC-E) and a vimentin-positive stromal cell line (HUNC-S) were generated from the primary culture, subcultured continuously for >300 days, and passed >50 times. Survival of the HUNC-E cell line in vitro depended on several media supplements, including nicotinamide, insulin, transferrin, selenium and epidermal growth factor (EGF). HUNC-E cells expressed AR and produced PSA and PAP throughout the culture period, as confirmed by immunocytochemistry and Western blot analyses. Addition of 14 nM testosterone (T) or dihydrotestosterone (DHT) to HUNC-E cells, stimulated DNA synthesis as well as anchorage-independent growth and PSA production, which demonstrated the androgen-sensitive nature of the cells in vitro. When HUNC-E and HUNC-S cells were combined in a 3:1 ratio and introduced subcutaneously into syngeneic male hosts, tumors formed in 2/3 animals with an average latency of 7 months. RT-PCR and immunocytochemical characterization of the HUNC cell lines revealed that the cells expressed several growth factors and their cognate receptors, including HGF, TGF-α and the TGF-β's, indicating the establishment of potential autocrine loops in the neoplastic cells. The HUNC-E and HUNC-S CaP cell lines, which retain the characteristics of the epithelial and stromal components of the in vivo R3327H tumor, will allow a more thorough and informative molecular and biological analysis of prostatic adenocarcinoma.

Introduction

The Dunning R3327 rat prostatic adenocarcinoma was originally identified in the dorso-lateral prostate of a 22-month-old male Copenhagen rat (1). The tumor is composed of glands and supporting stroma, and is clearly a heterogeneous mixture of several cell types. Various sublines of this tumor have been described, including a hormone-sensitive subline (R3327H) (2). The tumor has been passaged historically in vivo by serial transplantation of a small piece of tumor tissue. While some researchers have reported maintenance of Dunning R3327 variants in tissue culture, in vitro propagation of the androgen-sensitive Dunning R3327H has been limited (3,4). Although the R3327H tumor model closely mimics the features of human prostatic adenocarcinoma, the lack of a cell line that maintains androgen sensitivity and expression of prostate-specific markers limits the degree to which this tumor can be biologically characterized, manipulated, and applied to the study of prostate cancer. To address this problem, we isolated propagable epithelial (HUNC-E) and stromal (HUNC-S) tumor cell lines from the androgen-sensitive Dunning H Tumor. The HUNC-E cells were immunopositive for cytokeratins, prostate-specific antigen (PSA*), prostatic acid phosphatase (PAP) and androgen receptor (AR), and were androgen-responsive in vitro. The HUNC-S stromal line was immunopositive for AR and vimentin, and negative for cytokeratins. The HUNC-E cells retained the capacity to form tumors in vivo only when combined with HUNC-S cells, demonstrating the importance of the presence of both cell types in tumor development. HUNC-E and HUNC-S provide a powerful model for the study of the development and progression of prostate cancer because: (i) cellular communication mechanisms between neoplastic stromal and epithelial prostate cells can be investigated in vitro using the HUNC-E and HUNC-S cells; and (ii) androgen-regulated events can be addressed in vitro for the first time using the androgen-sensitive HUNC-E cells.

Materials and methods

Cell line

An explant of the Dunning R3327H tumor was obtained from Dr John Isaacs at Johns Hopkins University (5). The tumor was propagated by implanting a 25-mg trocar piece of tumor subcutaneously into adult male Copenhagen rats obtained from Harland Sprague–Dawley (Indianapolis, IN). Tumors were harvested when they reached 1 cm in diameter. The average length of time for the formation of a 1-cm nodule was 193 ± 12 days (n = 3).

In vivo propagation

Animals were killed by ether overdose and the tumor tissue was excised. One-third of the tumor was formalin-fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin for histological evaluation. The remaining tumor tissue was cut into small pieces (2 mm x 2 mm) and subjected to enzymatic digestion with 0.5 mg/ml collagenase ( Worthington; Freehold, NJ)
and 1 mM EDTA for 4 h at 37°C in a 5% CO₂ incubator. At 30 min intervals, the supernatant (containing dissociated cells) was decanted and centrifuged for 2 min at 800 r.p.m. in a swinging bucket rotor. Cell pellets were resuspended in Richter’s Improved MEM (Irvine Scientific; Santa Ana, CA) containing the following supplements: 10 mM nicotinamide, 20 ng/ml epidermal growth factor (EGF) (Collaborative Biomedical Products; Bedford, MA), 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium (Collaborative Biomedical Products; Bedford, MA), 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone and 2% fetal bovine serum (Gibco BRL; Grand Island, NY) containing the following standard media and confluent cultures were trypsinized with 0.25% trypsin (Gibco BRL; Gaithersburg, MD) and replated at 1:5 dilution.

**Immunocytochemical evaluation**

Cells were harvested by trypsinization, resuspended, and enumerated, and seeded into two- to four-chamber slides (Nunc; Naperville, IL) at 100 000 or 200 000 cells/chamber, respectively. After 24 h, media was aspirated and chambers were rinsed twice in phosphate buffered saline (PBS) to remove unattached cells and traces of media. For evaluation of PAP and PSA, slides were fixed for 20 min in 100% ethanol at room temperature. PAP was identified by incubating the slides with a polyclonal antibody [A0627] DAKO; Carpenteria, CA) diluted 1:250 in PBS at 37°C for 2 h. PSA was detected by probing the slides with a polyclonal antibody (A0627) DAKO; Carpenteria, CA) diluted 1:200 in PBS at 2 h for 37°C. For both PAP and PSA evaluation, the secondary antibody consisted of peroxidase-conjugated goat anti-rabbit IgG [(P0448) DAKO; Carpenteria, CA) diluted 1:200 in PBS. Negative controls for all assays included: no primary, no secondary, no primary or secondary antibody, PBS only, and visualizing utilizing TrueBlue® peroxidase as a chromogen (KPL; Gaithersburg, MD).

**Karyotypic analysis**

Subconfluent, log-phase HUNC-E cells were utilized for karyotype analysis. Cultures were treated with 0.25 µg/ml colcemid for 2 h, then harvested by trypsinization. Dispersed cells were resuspended in hypotonic KCl (0.075 M), incubated at 37°C for 10 min, and pelleted. Cells were fixed in a 3:1 solution of methanol:acetic acid. Chromosome preparations were made according to standard procedures as previously described (6). Chromosomes were stained with Giemsa, and 200 metaphase spreads were evaluated with respect to chromosome morphology and number.

**Western blot analysis**

For evaluation of PSA, conditioned media was collected from the in vitro cultures of HUNC-E cells at 120 days. Three confluent cultures (150 mm dishes) were washed 10X in PBS to remove traces of media. Cultures were re-fed with 15 ml of non-supplemented MEM + non-essential amino acids (serum-free) and incubated for 48 h at 37°C. The media was decanted and passed through a 4-µm filter to remove debris. Proteins in the conditioned media were concentrated by precipitation with two volumes of 100% acetone for 12 h at ~20°C. The protein pellet was resuspended in 500 µl of lysis buffer (PBS + 1% Triton X-100, 0.5 M EDTA, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin A and 100 µg/ml PMSF). After the conditioned media was collected from the plates, the cells were lysed directly on the plates by the addition of 250 µl lysis buffer. Aliquots (50 µl) of the precipitated proteins from conditioned media and the cell lysate were subjected to SDS-PAGE (8–16% gradient gel) and Western blot analysis by standard methods (7). The blot was incubated for 3 h at room temperature with a polyclonal antibody to PSA (DAKO; Carpinteria, CA) at a concentration of 1.0 µg/ml in PBS, 0.2% Tween-20 and 5% w/v non-fat milk. The secondary antibody was a peroxidase conjugated goat anti-rabbit IgG, and visualization was via enhanced chemiluminescence (Amersham; Arlington Heights, IL). PSA is detected on the blots as a 39-kDa protein. For immunoblottting of androgen receptor (AR), total cell lysates were prepared by lysis of HUNC-E cells in the buffer described above. Cell lysates were prepared after 24-h incubation with or without 14 nM T or DHT. Proteins were separated by SDS-PAGE through a 10% polyacrylamide gel, and transferred to Hybond ECL membrane (Amersham Corp.; Arlington Heights, IL). The blot was incubated with primary polyclonal antibody to AR (sc-815, Santa Cruz; Santa Cruz, CA) for 1.5 h at a concentration of 1 µg/ml in PBS with 5% w/v non-fat powdered milk. Secondary antibody was peroxidase-conjugated goat anti-rabbit IgG (DAKO; Carpinteria, CA), and visualization was by a 10-min exposure to TrueBlue® peroxidase (KPL; Gaithersburg, MD). The AR protein is present on the blots as a 110-kDa protein.

**DNA synthesis detection by BrdU**

Tissue culture dishes (35 mm) were seeded with 100 000 cells in supplemented media (the serum utilized during this assay was charcoal-stripped, low-mitotic index FBS; Sigma; St Louis, MO). T or DHT was added to confluent cultures at a concentration of 14 nM for 12 h in the presence of 10⁻³ M bromodeoxyuridine (BrdU) (Sigma, St Louis, MO). Cells were fixed for 5 min with 100% ethanol at room temperature. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 20 min at room temperature, followed by two rinses with PBS. DNA was denatured by a 10-min incubation with 4 N HCl, followed by two rinses with 0.1 M NH₄Cl. After rinsing with PBS, cells were incubated with anti-BrdU antibodies (clone BU-33, Sigma; St Louis, MO) diluted 1:1000 in PBS + 0.2% Tween for 2 h at room temperature. Plates were washed 2X with PBS + 0.2% Tween, and incubated with biotinylated horse anti-mouse secondary antibody (rat adsorbed) for 1:1000 in PBS, for 30 min at room temperature. After two washes with PBS, cells were incubated with peroxidase/avidin/biotin complex (Vector; Burlingame, CA) and developed utilizing DAB + nickel chloride as a chromogen. Labeling indices were determined as the number of labeled cells/total cells per field. Three fields of 200–400 cells were counted for each dish.

**Anchorage-independent growth assays**

Cells were plated (10 000 cells/plate) in a suspension of 0.5% SeaPlaque agarose (FMC Bioproducts; Rockland, ME) containing all media supplements, with or without 14 nM T or DHT, on top of an idealized layer containing 1% agarose. Plates were overlaid with a thin layer of 1.0% SeaPlaque agarose, and fed weekly with supplemented media ± 14 nM T or DHT. Colonies were counted after 3 weeks, and colony forming efficiencies (CFE) determined.

**RT-PCR analysis**

Messenger RNA was isolated from HUNC-E and HUNC-S cell lines by cesium chloride gradient followed by oligoDT column separation (Pharmacia Biotech; Piscataway, NJ). Aliquots of 2 µg of polyA mRNA in 4 µl of ultrapure water per sample was reverse transcribed (RT) by incubation with 1 µl Superscriptase II (Gibco BRL; Grand Island, NY) in the presence of 1 µl oligoDT, 2 µl 5X first strand buffer, and 2 µl dNTPs (all from Clontech; Palo Alto, CA). PCR reactions were carried out in EasyStart 50 µl PCR tubes (Molecular Bio-Products; San Diego, CA), using 1/10 (1 µl) of the RT reaction for each primer set and 0.5 µl of AmpliTaq polymerase (Clontech; Palo Alto, CA). All primers were used at a final concentration of 12.5 pm. Primers for c-mer and HGF (8), PSA, PAP and AR (9), TGF-α and EGF (10), IGFI-II, type 1 IGFR, M-6-PIGF-IR (11), 12TGF-B1-3 (13), TGF-B2-14 (14) and c-myc (15) have been described previously, and actin primers were obtained from Research Genetics (Huntville, AL). Primers for rat TGF-B1 were as follows: forward, 5′-TTGCTGCAATCGGATCATG-3′; reverse 5′-CAACACATGTTGACCGAGA-3′. PCR consisted of 35 cycles of: 1 min at 94°C for denaturation, 1 min at an appropriate annealing temperature, and 2 min of extension at 72°C. All PCR reactions were followed with a 10 min extension at 72°C. PCR products were subjected to 1.2–1.5% agarose and visualized with ethidium bromide. A 100 base pair-ladder (Promega; Madison, WI) was used as a molecular size marker in all gels.

**Tumorigenicity assays**

HUNC-E and HUNC-S cells were trypsinized, resuspended and washed twice in cold PBS. The cells were enumerated with a hemacytometer and combined into a single 1:1 (E:S) ratio. Cell suspensions were passed into 10 3-month-old and 12 neonatal (2-day) Copenhagen rats. Rats were monitored weekly for the development of subcutaneous tumors. Tumors were harvested at 0.5–1.0 cm.

**Results**

**Successful in vitro propagation of R3327H cells (HUNC)**

Dunning H adenocarcinoma tumor tissue was obtained from a male Copenhagen rat that bore a 1-cm³ subcutaneous tumor. Tumor latency was 182 days, and histological evaluation revealed that the tumor was composed of moderately differentiated cells organized into glandular structures. The tissue was disaggregated with collagenase and placed in culture with supplemented Richter’s media (see Materials and methods). The cells attached to tissue culture dishes in aggregates, which expanded rapidly during the first 48–96 h of culture. Several cell types were present in the culture, with morphologies ranging from stromal- to epithelial-like. When the culture reached 30 days, several subpopulations were generated by
A novel in vitro model of prostatic adenocarcinoma

Fig. 1. HUNC-E (A) and HUNC-S (B) cells are shown at 60 days in culture. The epithelial cells were compact with well-defined cell margins, while the stromal cells were more elongated with irregular cytoplasmic protrusions.

placing a cloning ring around a single stromal- or epithelial-like colony and transferring the selected cells to a new dish. Multiple subclones were established from the original clones. The in vitro morphology of the cloned HUNC-E and HUNC-S cells after 60 days in culture is shown in Figure 1. While subconfluent cultures of the HUNC-E cells were phenotypically homogeneous, two morphological variants were apparent in confluent cultures, and may be the result of phenotypic plasticity. The stromal HUNC-S cell line was characterized by the development of cytoplasmic protrusions and extensions in culture, giving them a more elongated appearance when compared with the more uniform cobblestone monolayer formed by the HUNC-E cells. To date, the cells have been in culture for >300 days and have been passaged >50 times. Removal of any of the various supplements (defined in Materials and methods) from the media resulted in rapid senescence of the HUNC-E population.

Production of AR, PSA and PAP by H-UNC cells in vitro

The HUNC-E cells were evaluated by immunocytochemistry for production of AR, PAP and PSA at 30–45 day intervals during the culture period. At passage 23 (~160 days), the cells were strongly immunopositive for AR and PAP, while expression of PSA was weaker and decreased with time in culture (Figure 2A–C). However, addition of 14 nM DHT to passage 23 cultures of HUNC-E significantly increased the levels of immunodetectable PSA (Figure 2C and D). Production of the 39-kDa PSA protein was confirmed by Western blot analysis of conditioned media and crude cell lysate from the HUNC-E cells at 120 days in culture (Figure 3A). Expression of the 110-kDa AR protein in the cultured HUNC-E cells was also confirmed by Western blot analysis (Figure 3B).

Biological characterization of the HUNC-E cells

Under normal culture conditions, the HUNC-E cultures had an average population doubling time of 35.2 h. Saturation density was evaluated by allowing cultures of HUNC-E cells to remain at confluence for 14 days with replenishment of the media every 3 days. The saturation density was calculated to be $2.3 \times 10^5$ cells/cm$^2$ (standard error $\pm 7 \times 10^3$, $n = 8$). Anchorage-independent growth capacity (a crude marker of
tumorigenic potential) was evaluated using soft agar suspension assays. The HUNC-E cells formed colonies in 0.5% soft agar at an efficiency of 4.8% (±0.6%). Karyotypic analysis of the cells revealed that the cells were derived from rat tissue and were primarily diploid (90% diploid and 10% tetraploid).

Effects of androgen on HUNC-E cells
In vivo, R3327H tumor is sensitive to androgen. Consequently, we evaluated the androgen-responsiveness of the HUNC-E cells in vitro. To study the effects of androgen on DNA synthesis, confluent cultures of HUNC cells were exposed to 14 nM T or DHT for 12 h in the presence of bromodeoxyuridine (BrdU). The proliferative component of the cultures was immunostained utilizing monoclonal anti-BrdU antibodies. The number of BrdU(+) cells was increased by the addition of exogenous T or DHT (Figure 4A–C). The data from three experiments was combined and the labeling indices are shown graphically (Figure 4D). Both T and DHT stimulated confluent cultures of HUNC-E cells to undergo an average 3-fold increase in DNA synthesis when compared with the androgen-free culture. The effects of androgen on DNA synthesis of subconfluent log-phase HUNC-E cells was negligible.

Anchorage-independent growth capacity of the HUNC-E cells was evaluated with or without 14 nM T or DHT. At the end of 3 weeks without androgen, the cells had a CFE of 4.8 ± 0.6%. In the presence of T or DHT, the CFE increased to 13.6 ± 1.2% and 9.3 ± 0.9%, respectively. The HUNC-E cells cultured under normal conditions expressed PSA weakly, and the levels of immunodetectable PSA decreased with time in culture. However, as shown in Figure 2C and D, exposure of the HUNC-E cells to 1514 nM DHT results in a notable increase in immunodetectable PSA (Figure 2C,D).

Characterization of gene expression by reverse transcription-polymerase chain reaction (RT-PCR) and immunocytochemistry
The HUNC-E and HUNC-S cells were analyzed by RT-PCR and immunocytochemistry to evaluate expression of several prostate-associated genes and prostatic growth factors/receptors (Table I). These preliminary results suggested that neither the HUNC-E or the HUNC-S cell lines expressed mRNA for the M-6-P/IGFII Rc, a proposed tumor suppressor gene. As expected, expression of cytokeratins was limited to the HUNC-E cell line. HGF was expressed by both the stromal and epithelial HUNC cells, while c-met (the receptor for HGF) was limited to the HUNC-E cells. Other growth factors and receptors analyzed were expressed by both the stromal and epithelial cell lines.

Tumorigenicity of HUNC-E and HUNC-S cells
Tumorigenic potential of the HUNC cell lines was evaluated, utilizing HUNC-E cells in combination with HUNC-S cells, or HUNC-E cells alone. HUNC-E + HUNC-S cells, in a ~3:1 ratio, formed tumors in 2/3 animals. The morphology of the tumors was reminiscent of the parent H tumor from which the cells were derived, with the formation of some glandular structures along with connective tissue stroma (Figure 5A and B). The cells within the tumor were immunopositive for both PSA and PAP, which confirmed their prostatic origin. When epithelial HUNC-E cells were injected alone into either 3-month-old or neonatal Copenhagen rats, no tumors formed after 14 months incubation: the presence of stromal cells (HUNC-S) was necessary for tumorigenesis.
Discussion

The Dunning R3327H prostatic adenocarcinoma is a widely accepted model of human prostate cancer (15); however, its experimental use has been limited because of the absence of an accompanying in vitro model that closely mimics the tumor in vivo. Although the HUNC-E cells do not require T or DHT for growth, maintenance of the cells in vitro required the presence of several supplements: insulin, transferrin, selenium, nicotinamide and epidermal growth factor (EGF). Removal of the media supplements induced a rapid senescence of the population, indicating that these cofactors were necessary for the survival of these cells in vitro. The presence of EGF appeared to be particularly important in maintaining cell morphology and promoting cell survival, which corroborates previous evidence that EGF is essential for the maintenance of prostatic cells.

To date, the HUNC-E cells in culture have maintained important characteristics associated with the in vivo tumor. The in vivo Dunning R3327H tumor (like human CaP) is positive immunohistochemically for PAP, PSA and AR (17,18). Furthermore, Beckman et al. reported that both acinar epithelial cells and larger epitheloid-type cells in the R3327H in vivo tumor exhibit acid phosphatase enzymatic activity (19). We have demonstrated (by immunohistochemistry and Western blot) that the HUNC-E cells produce PSA in vitro throughout their history in culture. Expression of PAP was confirmed in the HUNC cells by immunohistochemical techniques. Additionally, AR protein was present in the HUNC-E cells, as shown by immunocytochemistry and Western blot analysis. These data provided compelling evidence that the HUNC-E cells retained salient features of the in vivo tumor while being maintained in vitro for almost 1 year.

The R3327H tumor has been characterized as androgen-sensitive. Castration of host animals leads to transient suppression of tumor growth, and a slight reduction in tumor size, followed by acquisition of androgen-independence and tumor progression (2,5,19,20). When comparing the effects of in vivo and in vitro environments on cellular growth and differentiation, conditions of confluence (rather than subconfluence) in vitro best represent the in vivo environment where cells are contacted on most surfaces and can display contact inhibition. Under normal culture conditions, the HUNC-E cells displayed contact inhibition at confluence with a labeling index of only 0.7%. In the presence of testosterone or DHT, the labeling index of confluent cultures was increased ~3-fold. The interaction of androgen with AR stimulated growth of confluent HUNC-E cells, probably by enabling them to overcome contact inhibition. Removal of androgen by castration in the in vitro tumor model may contribute to suppression of tumor growth by restoring the capacity of the tumor tissue to exhibit contact inhibition, and by inducing apoptosis. Consistent with this hypothesis, anchorage-independent growth of HUNC-E cells was induced by androgen, with a 2- to 3-fold increase in colony forming efficiency in the presence of T or DHT. The androgen effects on HUNC-E cells in vitro may occur through mechanisms similar to androgen modulation of tumor growth in vivo, since androgen appeared able to induce growth under conditions that normally inhibit proliferation in both environments.

Importantly, the HUNC-E cells retained the capacity to form tumors in vivo in syngeneic hosts only when combined with HUNC-S cells. This observation indicates that both stromal...
and epithelial cell types are essential in the development of rat prostatic adenocarcinoma, and that these cell lines retain the capacity to form tumors after being cultured in vitro. Our data corroborate previous reports that stromal cells are influential in the development of prostate cancer (21, 22).

RT-PCR and immunocytochemical analyses of the stromal and epithelial HUNC cells revealed several differences between the HUNC-E and HUNC-S cells. Expression of cytokeratin was limited to the HUNC-E cells, while only the HUNC-S cells expressed vimentin. Failure to detect mRNA encoding the M-6-P/IGFII receptor in the HUNC cells may indicate that these cells have lost expression of this proposed tumor suppressor gene. These results were particularly interesting since normal prostate epithelial cells in vitro maintain expression of M-6-P/IGFII R (S.C.Presnell, unpublished observation).

While most of the growth factors and receptors analyzed were detectable in the HUNC-E and HUNC-S cells by RT-PCR, c-met expression was limited to the epithelial cells, which also produce ligand (HGF). While normal prostatic stromal cells often produce growth factors and normal epithelial cells express cognate receptors, neoplastic cells may acquire expression of both the growth factor and receptor, thereby creating autocrine loops that may contribute to autonomous proliferation. Production of HGF, TGF-α, and the TGF-βs along with their cognate receptors by the HUNC-E cells may reflect their transformed nature and tumorigenic capacity. Generation of the propagable receptors by the HUNC-E cells may mimic the androgen-sensitive nature and tumorigenic capacity. Produc-

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