A Novel Human Cell Culture Model for the Study of Familial Prostate Cancer

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Abstract

Research into molecular and genetic mechanisms underlying familial prostate cancer would be greatly advanced by in vitro models of prostate tumor cells representing primary tumors. We have successfully established an immortalized human prostate epithelial cell culture derived from primary tumors of familial prostate cancer patients with telomerase. The actively proliferating early-passaged 957E cells were transduced through infection with a retrovirus expressing the human telomerase catalytic subunit, human telomerase reverse transcriptase (hTERT). A high level of telomerase activity was detected in 957E/hTERT cells, but not in 957E cells. 957E/hTERT cells are currently growing well at passage 40, whereas 957E cells senesced at passage 5. 957E/hTERT cells exhibit epithelial morphology. Expression of an androgen-regulated prostate specific homeobox gene NKX3.1 and an epithelial cell-specific cytoesin 8, but not prostate specific antigen or androgen receptor, was detected in 957E/hTERT cells. Prostatic stem cell antigen and p16 were also expressed in this line. 957E/hTERT cells showed growth inhibition when exposed to retinoic acid and transforming growth factor β1, potent inhibitors of prostate epithelial cell growth. Chromosome analysis showed that the 957E/hTERT cell line (passage 10) was near diploid human male (XY), with most chromosome counts in the 44–46 range. However, there was random loss of chromosomes 8, 13, X, Y, and alteration in chromosome 4q. The late passage 957E/hTERT cell line (passage 32) was karyologically similar to the early passage 957E/hTERT cell line (passage 10) and also had the same alteration of 4q observed in the early passage 957E/hTERT cell line (passage 10) as well as a trisomy of chromosome 20. The well-characterized human cancer lines derived from such patients will be useful for the identification and characterization of prostate cancer susceptibility genes. This is the first documented case of an established human prostate cancer cell line from primary tumor of a familial prostate cancer patient.

Introduction

Prostate cancer is the most common male cancer in the United States, as well as in the Western world, and the second leading cause of male cancer death in the United States (1). The recent progress made in identifying cancer genes and understanding cancer genetics has been impressive. However, our understanding of the molecular and genetic mechanisms underlying prostate carcinogenesis remains limited, particularly when compared with other cancers, e.g., colon, renal, and breast (2). Although mutations have been observed in a wide variety of oncogenes and tumor suppressor genes in prostate cancer, a site-specific model for prostate cancer has not emerged. Indeed, little is known regarding the genes that control prostate cancer susceptibility.

Hereditary factors are estimated to be responsible for about 9% of all cases of prostate cancer in the United States Segregation analysis of familial prostate cancer has supported an autosomal dominant mode of inheritance of prostate cancer susceptibility alleles with some evidence of heterogeneity (3). Genome-wide scan analysis implicated a region of chromosome 1 (1q24–25) as being the most likely region of the genome to contain a major prostate cancer susceptibility gene (4). Subsequent analysis of the linkage data indicates that evidence for linkage to a hereditary prostate cancer locus, termed HPCI, is primarily restricted to families containing five or more men affected with prostate cancer, in which the average age at diagnosis is under 65 years (5). In addition to HPCI, at least four other candidate prostate cancer susceptibility loci have been reported, although the genes at these loci have not been identified (6). More studies are needed to identify these prostate cancer genes.

Elucidation of the molecular and genetic events involved in familial prostate cancer progression remains poorly understood. This is in part attributable to the lack of suitable in vitro models for the study of familial prostate cancer. To study early genetic and molecular lesions of familial prostate cancer, cell lines derived from primary tumors are urgently needed. However, such human prostate cancer cell lines are presently not available. Successful generation of immortal malignant prostate epithelial cell cultures from primary tumor specimens by HPV2 or simian papovavirus (SV40) has been described previously (7–9). These models are of limited value because the immortalized cells frequently contain oncogenic viral DNA and accompany major cytogenic alterations and growth degradation.

Telomerase is an enzyme responsible for replicating telomeres, and is composed of an RNA subunit containing an integral catalytic subunit, hTERT (10). Telomerase is expressed low in most normal tissues in vivo, but is known to become activated during tumorigenesis, including prostate carcinogenesis (11). Recent findings have directly implicated telomerase in the escape from cellular senescence (10). Indeed, transfection of hTERT into selective human cell types (fibroblasts, retinal pigment epithelial, mammary epithelial, and endothelial cells; Refs. 10, 12–16) can itself induce immortalization. Interestingly, telomerase expression in human somatic cells does not induce any change associated with a transformed phenotype or an altered genetic phenotype (12, 13). Primary cells transduced with the gene that delays replicative senescence will show increased growth potential without converting transformation nor showing karyological artifacts, thus making them ideal in vitro models for the study of prostate carcinogenesis. To our knowledge, no successful establishment of immortal human prostate epithelial cells with telomerase has been reported. Our goal is to generate new, continuously proliferating human prostatic epithelial cell lines from primary prostate tumors of familial prostate cancer patients by using telomerase.

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2 The abbreviations used are: HPV, human papillomavirus; PSA, prostatic-specific antigen; AR, androgen receptor; RA, retinoic acid; TGF, transforming growth factor; TRAP, telomeric repeat amplification protocol; CK, cytokeratin; SKY, spectral karyotyping; FISH, fluorescent in situ hybridization; hsr, homogeneously staining region; PSCA, prostatic stem cell antigen; hTERT, human telomerase reverse transcriptase; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde(3) phosphate dehydrogenase.

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Materials and Methods

Cell culture. The tumor tissue (957E) used for generating the cell line was obtained from radical prostatectomy specimen of a 44-year-old patient with a family history of prostate cancer. The patient had clinical stage B2 adenocarcinoma. The presence of prostatic adenocarcinoma with well-to-moderate differentiation (Gleason 3+3) was confirmed under light microscopy. Fresh prostatectomy specimen was obtained under sterile condition by an experienced pathologist. Tumor tissue on gross inspection was dissected separately for the purpose of generating cell culture. The tumor tissue was chopped into small fragments, 1–2 mm in size, with a sterile blade. The small cell clumps were placed into several type-I collagen-coated dishes (Becton Dickinson, Boston, MA) containing growth medium and were allowed to attach for a week to the bottom surface of the culture dishes. The cells were incubated at 37°C in a humidified air of 5% CO₂ until reaching confluence. Aliquots of the primary cultures were then frozen and stored in liquid nitrogen until the cells were reestablished in secondary culture for additional serial passages. For serial passages, routine trypsinization was used once a week in the collagen-treated culture dishes, and the split ratio of the cells was 1:2. Keratinocyte serum-free medium supplemented with bovine pituitary extract and recombinant epidermal growth factor (Life Technologies, Inc., Gaithersburg, MD) was used for growing and maintaining the cells (17, 18).

Generation of the 957E/hTERT Cell Line. At passage 4, the actively proliferating 957E cells were infected with a recombinant retroviral construct, LXSN-hTERT (Ref. 16; generously provided by Vimla Band, Ph.D., New England Medical Center, Boston, MA) containing the hTERT and a neomycin resistance gene. Briefly, cells were transduced through infection using Polybrene at the concentration of 10 μg/ml and incubated at 37°C 5% CO₂ overnight. The infected cells were washed with PBS, then incubated and subcultured weekly for further serial passages. No G418 selection was necessary because the uninfected 957E cells senesced at passage 5.

Telomerase Assay. Cellular extracts were assayed for telomerase activity with TRAP assay (19). This is a PCR-based assay using a telomerase extension (telomerase substrate oligonucleotide) primer (5’-AATCCGTCCGAGCA-GAGTT-3’) and a downstream (complementary to telomerase repeat) primer (5’-TCCCATCCGCTACCTTAGTAA-3’). This assay amplifies telomeric repeats; repeat length is longest in cells exhibiting telomerase activity and shortest in cells exhibiting little or no telomerase activity. The 267B1 cells were used as positive control for this assay, because they express activated telomerase (20).

RT-PCR Assay. RT-PCR assay was performed as described previously (21). Briefly, total RNAs from culture cells were extracted with RNazol B (TEL-TEST Inc., Freindwood, TX) according to manufacturer’s protocol and quantified with Beckman DU640 (Beckman, Somerset, NJ). One μg of total RNA was reverse transcribed into cDNA with RNA PCR-kit (Perkin-Elmer, Foster, CA) and 1/10 of the reverse-transcribed product from each sample was used for PCR to amplify NKX 3.1, AR, PSA, PSCA, CK 8, and p16, respectively. The expression of CK 8 was used as an internal control for input RNA as well as the marker for epithelial cells, because NKX3.1 expression was restricted to epithelial cells. To verify the validity of CK 8 as the internal control, in parallel we performed CK 8 and the house-keeping gene, GAPDH, in the same cDNA samples. The condition of PCR for individual genes was optimized to amplify amplified product in the linear range of amplification by adjusting amplification cycles for each set of primers. The primer sequences and the expected size of PCR products were same as described previously (21).

Growth Factor Response. Both human recombinant transforming factor-β1 (TGF-β1; Boehringer Mannheim, Mannheim, Germany) and RA (Sigma Chemical Co., St. Louis, MO) were tested for their inhibitory effect on the 957E/hTERT cells. Fifty thousand cells per well were plated with keratinocyte serum-free medium. Growth factors were added singly, 24 h after plating, at the concentrations ranging from 1 to 10 ng/ml. The medium was changed every 48 h. Cells were counted after 6 days of treatment.

Cytogenetic Analysis. Chromosome counts, ploid distribution, and Giemsa (G)-banded karyotypes were prepared by standard protocol as described previously (22, 23). SKY was done using the manufacturer’s protocol (24), and the SKY results were compared with G-banded chromosome data. Further analysis was carried out using FISH, which was done on unbanded chromosome preparations with p53 probe in accordance with the recommendations of the manufacturer (VYSIS, Downers Grove, IL).

Results

To determine whether human prostate cancer cells would be immortalized by the expression of hTERT, we introduced a retrovirus construct expressing hTERT into early-passage (passage 4) 957E cells through infection overnight in the presence of Polybrene (10 μg/ml). Noninfected cells could not be propagated serially beyond 5 subcultures. In contrast, the hTERT-infected 957E cells have an apparently unlimited life span and have been successfully subcultivated for more than 40 passages over the course of 1 year with no evidence of decreased proliferation capacity. The 957E cells had the typical epithelial morphology (Fig. 1). The cells grew as adherent cells and were more piled-up on each other in some areas.

To confirm that the immortalized 957E/hTERT cells do contain the transduced hTERT, the TRAP was carried out. A high level of telomerase activity was detected in 957E/hTERT cells (Fig. 2).

RNA samples from 957E/hTERT and 267B1 cells were analyzed to determine the expression of specific markers by RT-PCR. The 267B1 cells were used as a positive control. 957E/hTERT cells expressed an androgen-regulated prostate specific homeobox gene, NKX3.1, an epithelial cell-specific CK 8, and GAPDH, but did not express PSA and AR. PSCA and p16 were also expressed in this cell line (Fig. 3).

The cell line was analyzed for its ability to respond to growth-inhibitory factors known to regulate prostate cell growth. 957E/hTERT cells showed growth inhibition when exposed to TGF-β1 and RA, which are potent inhibitors of prostate epithelial growth (25). RA, at a concentration of 10 ng/ml, showed a dose-dependent inhibition of growth.

Fig. 1. Morphology of the 957E/hTERT cell line. This culture shows typical epithelial morphology with transformed foci (top).
growth at 50% that of control. TGF-β1 significantly inhibited growth only at the concentration of 10 ng/ml.

According to G-banding, the 957E/hTERT cell line (passage 10) was nearly diploid. Random loss of chromosomes 8, 13, X, and Y in the karyotypes was observed (Fig. 4). There was some halo under one of the chromosome 4 but no staining; therefore, we thought it was an artifact.

Further analysis was done by SKY. The SKY results on the 957E/hTERT cells (passage 10) were uniform. All of the metaphases were diploid. The SKY was done on seven karyotypes. It was then noted that the light region under one copy of chromosome 4 was not an additional artifact, but rather an additional piece of chromosome 17 at the q terminal of chromosome 4. The G-banded karyotypes were reevaluated, and this marker was observed in every karyotype. This chromosome is marked by arrows in both the SKY and G-banded karyotypes (Fig. 5). We had limited material for SKY analysis; therefore, there is some speculation that it may be chromosome 10 material rather than chromosome 17 because dye mixtures for chromosomes 10 and 17 are close. The strong opinion that it is chromosome 17 material, we further analyzed this cell line by FISH, using p53 probe (presence on chromosome 17p13.1), and found that p53 was intact at least at the level of analysis (Fig. 6). Further study with comparative genomic hybridization is in progress to determine the origin of this hsr observed with chromosome 4q. The G-banding showed that late-passage 957E/hTERT cell line (passage 32) was hyperdiploid and essentially the same as the early passage 957E/hTERT cell line (passage 10). The same alteration of chromosome 4q observed in the early-passage 957E/hTERT cell line (passage 10) was also observed, as well as a trisomy of chromosome 20.

**Discussion**

The present study appears to represent the first documented case of a telomerase-immortalized human prostate cancer cell line established from the primary tumor of a patient with a family history of prostate cancer. The immortalized 957E/hTERT cells show a transformed morphology and are grown in serum-free medium. They expressed CK8, NKX3.1, PSCA, and p16, but did not express AR and PSA. They showed growth inhibition when exposed to RA and TGF-β1. The cell line was near diploid and showed random loss of chromosomes 8, 13, X, and Y. SKY analysis showed some alteration in chromosome 4q.

Some of the same loss of chromosomes 8, 13, X, and Y observed in the 957E/hTERT cell line has been already reported in the literature. It is interesting to note that the loss of chromosomes 8 and 13, specifically observed in this cell line, is the most frequently observed chromosome change in sporadic prostate cancer. In addition, interestingly, the evidence of a prostate cancer susceptibility locus on the X chromosome has been reported. Further SKY analysis shows that there is an addition of either chromosome 10 or 17 material with chromosome 4. Possible evidence of a prostate cancer susceptibility locus on chromosome 4q has been reported. There are reports of loss of heterozygosity for both 10p and 10q in prostate cancer de-
scribed in the literature (28, 29). Alteration of the p53 tumor suppressor gene is associated with a number of cancers, including prostate (30). The p53 gene in this cell line was normal as shown by FISH in our study. A similar result is noted in the Asian population of prostate cancer patients (31). There is a report describing erb-2 gene (17q13) amplification in prostate cancer patients after androgen deprivation therapy (32) that we have not yet examined. Additional evaluations are necessary to ascertain which chromosome genetically affects familial prostate cancer.

We have previously developed reliable methods for generating and characterizing prostate cancer cell lines derived from primary tumors using HPV-16 E6 and E7 genes (8). These models are not ideal, because the immortalizing cells frequently contain viral oncogenic DNA and accompany major cytogenic alterations and growth deregulations. The immortalizing agent may introduce many genetic and epigenetic artifacts into these cells, making it difficult to investigate specific alterations unique to the tumors. In addition, attempts to culture primary tumor cells reportedly produce cultures that invariably exhibit a normal karyotype (33) and lack transformed phenotypes (7, 8). Development of in vitro human cell culture models that mimic human prostate cancer progression would be ideal. HPV infection does not appear to be related to prostate cancer development although

Fig. 5. Spectral karyotype composite of the 957E/hTERT cell line. Top, composite karyotype showing G-banded and pseudocolored chromosomes. Bottom, G-banded preparation of metaphase chromosomes from 957E/hTERT cells (middle), hybridized to SKY paints (left), and after pseudocolor application (right). hsr was observed with chromosome 4 (blue marked by arrow).

Fig. 6. FISH analysis was done on unbanded chromosome preparations with p53 probe. White arrow (left), intact p53.
it is present in many prostate specimens (34–36). The question of whether oncogenic HPV is involved in the pathogenesis of prostate cancer has been a subject of great controversy. As described above, we have succeeded in immortalization by the introduction of telomerase into primary human prostate epithelial cells derived from familial prostate cancer patients. As shown by other human cells (10, 12–16), we anticipated that the hTERT-immortalized cell would maintain the phenotypic and genotypic characteristics of its primary cells. It has been reported that the telomerase expression in human somatic cells did not induce changes associated with transformed phenotype and additional genetic changes (12, 13). Interestingly, it has recently been reported that human endothelial cells also bypassed replicative senescence after introduction of hTERT, that hTERT expression in these life-extended endothelial cells did not affect their differential and functional phenotype, and that they maintained their angiogenic potential in vitro (15). Furthermore, hTERT-expressed endothelial cells had a normal karyotype and did not exhibit a transformed phenotype (15). Conceptually, the successful establishment of spontaneous immortalized human prostate cancer cell lines derived from familial prostate cancer patients would be ideal and a major breakthrough in prostate cancer research. However, the generation of such cell lines is an extremely rare event and, to date, has not been reported.

The well-characterized human cancer cell line derived from primary tumor of familial prostate cancer patients described here will be useful for the identification and characterization of prostate cancer susceptibility genes. The novel in vitro model will also provide the means for testing new modalities for both prevention and progression of prostate cancer, as well as the methods for testing both chemoprevention and chemotherapeutic agents.

References


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