Characterization of Prostatic Epithelial Cell Lines Derived from Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) Model

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Abstract

To develop a syngeneic transplantable system to study immunotherapeutic approaches for the treatment of prostate cancer, three cell lines were established from a heterogeneous 32 week tumor of the transgenic adenocarcinoma mouse prostate (TRAMP) model. TRAMP is a transgenic line of C57BL/6 mice harboring a construct comprised of the minimal -426/+28 rat probasin promoter driving prostate-specific epithelial expression of the SV40 large T antigen. TRAMP males develop histological prostatic intraepithelial neoplasia by 8-12 weeks of age that progress to adenocarcinoma with distant metastases by 24-30 weeks of age. The three cell lines (TRAMP-C1, TRAMP-C2, and TRAMP-C3) express cytokeratin, E-cadherin, and androgen receptor by immunohistochemical analysis and do not appear to have a mutated p53. Although TRAMP-C1 and TRAMP-C2 are tumorigenic when grafted into syngeneic C57BL/6 hosts, TRAMP-C3 grows readily in vivo but does not form tumors. The T antigen oncoprotein is not expressed by the cell lines in vitro or in vivo. The rationale for establishing multiple cell lines was to isolate cells representing various stages of cellular transformation and progression to androgen independent metastatic disease that could be manipulated in vitro and, in combination with the TRAMP model, provide a system to investigate therapeutic interventions, such as immunotherapy prior to clinical trials.

Introduction

Prostate cancer is a major health problem in men in the United States. It is the most commonly diagnosed cancer in men in the United States, with an estimated 334,500 new cases to be diagnosed in 1997 (1). Detection of prostate cancer has greatly improved in recent years due in part to improved public awareness and early detection combining prostate-specific antigen assay, digital rectal exams, and ultrasound-guided transrectal needle biopsies. However, despite the improved detection rate, the death rate due to prostate cancer remains second only to lung cancer, with an estimated 41,800 prostate cancer deaths for 1997 (1). Treatment options available for prostate cancer include: watchful waiting, radical prostatectomy, external beam irradiation, prostate brachotherapy, and hormonal ablation therapy. Although prostate cancer in its early stages is amenable to standard treatments, prognosis for late-stage metastatic prostate cancer is poor.

Despite the magnitude of the problem, rapid progress in prostate disease research has been impaired by the lack of adequate animal models that reproduce the spectrum of benign, latent, aggressive, and metastatic forms of the human disease. Human prostate cancer cell lines (2, 3) and xenographs (4) have been successfully used to study metastatic forms of the human disease. Human prostate cancer cell lines and transplantable tumors in syngeneic Copenhagen rats (7). Here we report the establishment and characterization of tumorigenic cell lines from a C57BL/6 TRAMP mouse that can be grafted into either immune-competent syngeneic C57BL/6 mice or TRAMP mice.

Materials and Methods

Establishment of Cell Lines and Culture Conditions. Tissue used to establish the cell lines was obtained from a 32-week-old prostatic adenocarcinoma from a C57BL/6 male TRAMP mouse. Cultures were initiated by mechanical disruption of the tissue followed by enzymatic digestion with Dispase (1:10 dilution in media; Collaborative Biomedical Products, Bedford, MA) for 1 h at 37°C. Cell suspensions were plated on six-well culture plates and allowed to attach and grow out in DMEM high glucose w/L-glutamine and without diospyruvate media (DMEM-HG; Life Technologies, Inc., Grand Island, NY) supplemented with 5% Nu-serum IV (Collaborative Biomedical Products), 5% fetal bovine serum (HyClone, Logan, UT), 5 μg/ml insulin (Sigma Chemical Co., St. Louis, MO), 25 units/ml penicillin-streptomycin (Life Technologies) and 10^-8 m dihydrotestosterone (Sigma). The culture medium was changed every 2-3 days. During early passage, the cells were differentially trypsinized to enrich for the more adherent epithelial cells. Cells were split when they reached confluency by rinsing in Ca++/Mg++-free HBSS (Life Technologies) for 2 min followed by treatment with 0.25% trypsin, 1 mM EDTA in Ca++-, Mg++-free HBSS (Life Technologies) until the cells released from the plate. Multiple lines were established based on different growth rates and cell morphology. LNCaP and PC-3 cells were obtained from the cell culture core at Baylor College of Medicine and cultured in the above media.

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3 The abbreviations used are: TRAMP, transgenic adenocarcinoma mouse prostate; Tag, T antigen; RT-PCR, reverse transcription-PCR; AR, androgen receptor.
Doubling Time. Doubling times of the cell lines in vitro were determined by plating the cells in six-well plates at 20,000 cells/well and counting triplicate wells every other day for 21 days. Doubling times were calculated from the log phase of the growth curves. TRAMP-C1 and TRAMP-C2 in vitro doubling times were calculated from tumor base area measurements for three experiments, each containing five animals. Five million cells were injected s.c. into the hindquarter of a C57BL/6 male mouse. The two-dimensional tumor base area was measured with calipers every other day, starting on day 7 for 50 days. Doubling times were calculated from the log phase of the growth curves.

Histology and Immunohistochemistry. Cell lines were grown on eight-well chamber slides. Slides were prepared for staining by rinsing three times in HBSS (Life Technologies), fixed for 5 min in zinc-buffered formalin (Medical Chemical Corp., Santa Monica, CA), and rinsed three times in PBS. Slides were stored in 70% ethanol until ready to use. Tumor tissue was fixed overnight in zinc-buffered formalin and paraffin-embedded by standard methods. Sections (4 μm) were cut from paraffin-embedded tissues. Tissues for histological analysis were stained with H&E by standard methods. Immunostaining for cytokeratin was performed using Dako’s (Carpinteria, CA) pan-cytokeratin antibody (Z0022) at 1:1000 dilution with a 5-min protease K treatment for antigen retrieval. AR antibody (PG-21) was a gift from Gail Prins (University of Illinois, Chicago, IL) and used at a dilution of 1:500 with citric acid antigen retrieval essentially as reported previously (11). E-cadherin antibody was obtained from Transduction Laboratories (Lexington, KY) and used at 1:5000 dilution with citric acid antigen retrieval. Tag staining was performed as described previously (9). p53 staining was performed as described previously (12). Staining was visualized using Vectastain Elite ABC immunoperoxidase system (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine peroxidase substrate kit (Vector). Cells were counterstained with either a weak hematoxylin stain or methyl green (Sigma). Primary antibody was replaced with either normal rabbit serum or block for negative controls.

RNA Isolation and Analysis. Total RNA was isolated from cell lines and tumor tissue using Trizol (Life Technologies) according to the manufacturer’s recommendations. The RT-PCR was performed with 1 μg of total RNA essentially as described (13). Ribosomal L-19 was used as an internal control for each reaction. The following primers (5′-3′) were used for PCR amplification: Tag forward, PB + 1/28: cttgctaggttgggctgaatcc; Tag reverse, Bii: agctgatccaggttcagctatcc; mouse AR forward, AR9f: tggctgtctctgcagttcctactacag; mouse AR reverse, AR9r: ggtcctggagttggtgg; mouse probasin forward, mPBf: atcattccttgcacagttgg; mouse probasin reverse, mPBr: acagtctgctgcctgatagca; L-19 forward: ctggaaggtcagaggtgat; and L-19 reverse: gcagagctttctggtcag. PCR products were fractionated on an agarose gel. Tag and probasin PCR products were then transferred to Hybond-N+ membrane (Amersham Corp., Arlington Heights, IL). Membranes were hybridized with a 32P-labeled oligonucleotide (Tag SV 40 rev: ctccutcaagacctactattccagt; mouse AR reverse, AR9r: gguttgggtattagggtttccaaa; mouse probasin reverse, PB + 1/28: cttgtcagtgaggtccagatacctacag; Tag reverse, PB + 1/28: cttgtcagtgaggtccagatacctacag; Tag forward, PB + 1/28: cttgtcagtgaggtccagatacctacag; Tag reverse, PB + 1/28: cttgtcagtgaggtccagatacctacag). Membranes were washed and exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY).

Soft Agar Assay. Soft agar assays were performed to determine anchorage-independent growth of the cell lines as described previously (14). Briefly, the cell lines were plated at 5 × 104 cells/well and grown for 10 days on a layer of 0.6% agar in basic media containing 0.3% agar. The number of colonies was counted (defined as 16 or more cells). PC-3 cells were used as a positive control for the assay.

Tumorigenicity of Cell Lines. Cells were grown to 95% confluency, washed three times with serum-free medium, trypsinized to release cells, washed with 5-fold excess media to neutralize trypsin, and recovered by centrifugation. The number of live cells was determined by trypan blue exclusion and resuspended in serum-free medium at 5 × 107 cells/ml. Male C57BL/6 mice were s.c. injected into the hindquarters with 0.1 ml of cell suspension (5 × 108 cells) using a 21-gauge needle. The injection tract was occluded for 1 min following injection to prevent cell suspension leakage. Animals were assessed for tumors every other day starting 1 week after injection.

Results

We report on the establishment of three cell lines, TRAMP-C1, TRAMP-C2, and TRAMP-C3, that represent various stages in prostate cancer progression. These cell lines were all established from a prostate tumor from a single 32-week-old TRAMP mouse. Briefly, cell lines were established by mincing the tumor, treating with DsRed-1 for 1 h, and plating the cells in tissue culture dishes. During early passage epithelial cells were selected by differential trypsinization. Cells were grown in DMEM-HG media supplemented with 5% NuSerum, 5% FBS, 5 μg/ml insulin, penicillin/streptomycin, and 10−8 M dihydrotestosterone.

To determine the doubling time in vitro, the cells were plated at 20,000 cells/well, and triplicate wells were counted every other day for 21 days. In culture, TRAMP-C1 and TRAMP-C2 had a similar doubling time of 24.4 and 25.4 h, respectively. TRAMP-C3 had a slower doubling time of 37.0 h. The growth of the three lines did not appear to be contact inhibited. When grown beyond confluency, the cells were observed to pile up on top of each other, and although they did not stop growing, their growth appeared to slow.

To characterize the pattern of cytokeratin expression, TRAMP-C1, TRAMP-C2, and TRAMP-C3 were grown on chamber slides in the presence of androgen and examined by immunohistochemistry. The cells grown on chamber slides appeared to spread and flatten, as indicated by the greatly enlarged size of the cell and nucleus. All three cell lines were positive for cytokeratin (Fig. 1, a–e), indicating that the cell lines were epithelial in origin. Staining was comparable to normal prostatic epithelial cells stained with the same antibody (Fig. 3F). It should be noted that the pan-cytokeratin antibody used in these studies heterogeneously stained normal prostate, with basal cells demonstrating the most intense staining (Fig. 3F). The cytokeratin staining of the cell lines was heterogeneous and was most intense in cells that were piled up on each other and not attached to the chamber slide, probably due to the thin cytoplasm in the flattened cells.

The AR status of the cell lines was determined by immunohistochemical staining using AR antibody PG-21. The three cell lines demonstrated intense nuclear and diffuse cytoplasmic AR staining (Fig. 1, f–j). The p53 status of the cell lines was also determined by immunohistochemical staining. In the three cell lines, p53 was essentially undetectable (Fig. 1, k–o), suggesting that no selection for p53 mutations had occurred. E-cadherin expression, known to be lost with prostate cancer progression, was determined by immunohistochemical analysis. E-cadherin staining was detectable in the three cell lines (Fig. 1, p–t), providing further evidence that the cell lines were epithelial in origin. E-cadherin staining was most intense near the nucleus, where the cytoplasm was the thickest. Tag protein expression was examined by immunohistochemical analysis. Staining for Tag was undetectable in the three cell lines, indicating that the transgene was not expressed in cells in culture (Fig. 2, B–E).

To determine the expression of Tag, AR and probasin mRNA, total RNA was isolated from confluent plates of TRAMP-C1, TRAMP-C2, and TRAMP-C3 cultured in the presence of androgen and analyzed by RT-PCR. Tag mRNA was not detected in the cell lines by RT-PCR analysis (Fig. 2A, Tag) and confirmed by blotting the RT-PCR gel and probing with an internal radiolabeled Tag oligonucleotide (Fig. 2A, Tag*). The absence of Tag expression was also confirmed by immunohistochemical analysis (Fig. 2, B–E). AR was detectable in the three cell lines by RT-PCR analysis (Fig. 2A, AR) in agreement with the immunohistochemical analysis (Fig. 1, F–H). Probasin mRNA, encoding a prostatic epithelium-specific gene product associated with terminal differentiation, was undetectable by RT-PCR analysis (Fig. 2A, Pb) and confirmed by blotting the RT-PCR gel and probing with an internal radiolabeled oligonucleotide (Fig. 2A, Pb*).

To determine the ability for anchorage-independent growth, 5 × 105 cells were grown in basic media containing 0.3% agar for 10 days on a layer of 0.6% agar. A positive colony contained 16 or more cells. TRAMP-C1 had very minimal growth in soft agar, forming less than 20 colonies after 10 days of growth. TRAMP-C2 did not form...
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colonies in soft agar. PC-3 formed colonies too numerous to count after 10 days of culture.

To determine if TRAMP-C lines were tumorigenic, cell suspensions (5 x 10^6 cells) of TRAMP-C1, TRAMP-C2, and TRAMP-C3 were s.c. injected into the flank of an intact adult C57BL/6 male host. TRAMP-C1 and TRAMP-C2 were observed to be tumorigenic (18 of 18 and 15 of 15, respectively). In contrast, TRAMP-C3 was not tumorigenic (0 of 15). To determine the doubling time of tumors in vivo, growth rates were calculated by two dimensional measurement of the tumors. The doubling time was calculated to be 271 h (11.3 days) for TRAMP-C1 tumors and 374.4 h (15.6 days) for TRAMP-C2 tumors.

To further characterize the tumors in vivo, samples were fixed and processed for histological analysis. TRAMP-C1 and TRAMP-C2 formed solid firm tumors that on gross examination were not hemorrhagic. Histological examination of the tumors showed poorly differentiated, unorganized sheets of cells (Fig. 3, A and B). The nuclei were irregular in size, and many nuclei had a punctate appearance. The tumors were well vascularized, with numerous capillaries and microvessels apparent. TRAMP-C1 and TRAMP-C2 tumors were cytokeratin positive, indicating that the cells were epithelial in origin (Fig. 3, C and E), and the pattern was consistent with luminal cells of the dorsolateral prostate (Fig. 3F).

To compare expression patterns of Tag, AR, and probasin mRNA in vitro and in vivo, total RNA prepared from TRAMP-C1 tumors and TRAMP-C2 tumors was analyzed by RT-PCR. None of the TRAMP-C1 (0/6) and TRAMP-C2 (0/6) tumors expressed Tag mRNA (Fig. 4, Tag). This was confirmed by blotting the RT-PCR gels and probing with an internal radiolabeled oligonucleotide (Fig. 4, Tag*). In contrast, all TRAMP-C1 and TRAMP-C2 tumors expressed AR (Fig. 4, AR). Interestingly, although endogenous probasin mRNA was not detected in RNA from cells grown in vitro, two of six TRAMP-C1 tumors and two of six TRAMP-C2 tumors expressed endogenous probasin mRNA in vivo (Fig. 4, Pb). Expression was confirmed by probing the RT-PCR blots with an internal radiolabeled probasin oligonucleotide (Fig. 4, Pb*).

Discussion

The rationale for the current studies was that epithelial cell lines representing different stages in tumor progression could be isolated from the inbred TRAMP model of prostate cancer. Here we report the establishment and characterization of three parental cell lines having varying degrees of tumorigenicity isolated from a prostatic tumor of a 32-week-old TRAMP mouse. Because the cell lines were isolated from a pure C57BL/6 mouse, it was anticipated that these cells could
Fig. 2. RT-PCR analysis of Tag, AR, and probasin mRNA expression and immunohistochemical analysis of Tag expression by TRAMP-C lines in vitro. A, RT-PCR of cell lines in vitro for Tag, AR, and probasin (Pb) mRNA expression. Lane 1, TRAMP-C1; Lane 2, TRAMP-C2; Lane 3, TRAMP-C3; Lane 4, normal prostate; Lane 5, no RNA in the reaction; Lane 6, late-stage TRAMP tumor. Tag* and Pb* are RT-PCR gels transferred to membrane and probed with 32P-labeled internal oligonucleotides. B–E, immunohistochemical staining for Tag expression. B, TRAMP-C1; C, TRAMP-C2; D, TRAMP-C3; E, late-stage TRAMP tumor. All slides are shown at ×40.

be grafted into immune intact animals and thereby, in conjunction with TRAMP animals, facilitate studies relating to immunotherapy for prostate cancer.

The cell lines and the tumors derived from the cell lines were epithelial in origin by immunohistochemical staining for epithelial markers. Cells grown on chamber slides were positive for cytokeratins, as well as E-cadherin. The heterogeneous cytokeratin staining observed in the cell lines in culture could be due to a mixed cell population having different states of differentiation. Alternatively, because the cell lines grown on the chamber slides appear to attach and spread more than the positive control LNCaP cells, the variability in staining could be due in part to differences in cell attachment and the thickness of the cytoplasm of the cell. E-cadherin staining was also observed to be less intense than in LNCaP, but this was not surprising because E-cadherin expression is lost in poorly differentiated TRAMP tumors (10) and is reduced or lost in late-stage human prostate cancer.
Tumors derived from TRAMP-Cl and TRAMP-C2 were also cytokeratin positive, confirming that the tumors were epithelial in origin. The detection of probasin, a prostatic epithelial specific marker, in 4 of 12 of the tumors from the cell lines further confirmed that the cell lines were of epithelial origin.

It was interesting to note that the soft agar assay was not a predictive indicator of cell line tumorigenicity. None of the cell lines demonstrated appreciable growth in soft agar, although two of the cell lines (TRAMP-C1 and TRAMP-C2) were tumorigenic in vivo. However, the doubling times of the cell lines in culture did correlate with the doubling time of the tumors. TRAMP-C1 grew the fastest in vitro and in vivo, whereas TRAMP-C3 grew the slowest in vitro and was not tumorigenic.

Because the cell lines were derived from a TRAMP tumor and TRAMP tumors express Tag (9, 10), cells were analyzed for Tag expression by immunohistochemical staining and RT-PCR. Because Tag can act as a potent immunogen, cell lines expressing Tag would not be as useful a tool in immunotherapeutic approaches to the
treatment of prostate cancer. The fact that TRAMP-C1 and TRAMP-C2 were observed to grow in nontransgenic syngeneic C57BL/6 hosts indirectly indicated that Tag was not expressed. Direct proof that Tag was not expressed was the inability to detect Tag oncoprotein in cells grown on chamber slides by immunohistochemical analysis, and that Tag mRNA was not detectable in cells grown in culture, nor in tumors derived from the cell lines. Because Tag is known to bind and cause nuclear accumulation of p53 (16), immunohistochemical analysis indicating that p53 was not concentrated in the nucleus supports the observation that Tag is not expressed in the cells in culture. These data are consistent with previous reports that although Tag expression may be necessary to initiate transformation, continual Tag expression is not required for the maintenance of the transformed state in vitro or in vivo (17).

The recent establishment of syngeneic cell lines from the well-characterized TRAMP model provides a complete animal model system in which to study the progression of prostate cancer and for preclinical tests of new innovative therapeutic approaches. The cell lines provide a tool to develop and to test candidate immune therapeutics, as well as gene therapeutics. It is anticipated that the cell lines can be manipulated and transfected in culture and then introduced into a syngeneic C57BL/6 host to test the effect of treatment on the tumorigenicity of the cell line. Having both tumorigenic and nontumorigenic cell lines allows for the testing of both tumor suppressing activities, as well as tumor-promoting activities. The cell lines can also be used in conjunction with the TRAMP model to test the effect of cell line manipulation on the development and progression of the autochthonous tumor when the cell lines are transplanted into TRAMP hosts. One such example has been to use the cell lines to introduce cytokines to manipulate the immune system and to assay the effects on TRAMP tumor progression and metastasis (19). The cell lines will also be useful to identify pathways that may be important in tumorigenesis, androgen independence, and metastasis of prostate cancer.

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References

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