Lipofection-mediated Immortalization of Human Prostatic Epithelial Cells of Normal and Malignant Origin Using Human Papillomavirus Type 18 DNA

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ABSTRACT

Human papillomavirus (HPV) type 18 DNA was introduced into epithelial cell strains derived from normal and cancer tissues of human prostatectomy specimens by the lipofection transfection method. Two cell lines were established: PZ-HPV-7 (transfected normal cell) and CA-HPV-10 (transfected cancer-derived cell). These lines have been maintained for over 100 passages. Incorporation of HPV type 18 DNA was confirmed by polymerase chain reaction. Immunocytochemical analysis showed expression of keratins 5 and 8, similar to the cells of origin, and the early region 6 oncoprotein of HPV. PZ-HPV-7, derived from normal diploid cells, had a modal chromosome number of 46 in early passages but became tetraploid later. CA-HPV-10 cells were aneuploid and some retained the double minute chromosomes that were noted in the cancer-derived cells of origin. The cell lines showed a typical transformed morphology and were nontumorigenic in nude mice. We conclude that human prostatic epithelial cells derived from both normal and cancer tissues have been successfully transformed to immortality with HPV type 18 DNA. The establishment of these cell lines provides an opportunity for further development of an in vitro model of carcinogenesis for prostate cancer.

INTRODUCTION

The etiology of adenocarcinoma of the prostate, a leading cause of cancer deaths in males, is still unclear. Many factors have been proposed, including sexually transmittable infectious agents (1–3). Recently, detection of specific HPV DNA sequences in human prostate specimens by PCR has indicated a possible role for this viral factor in prostatic carcinoma (4–7).

HPVs are DNA viruses that infect a diversity of epithelial tissues at distinct anatomic sites. More than 60 different genotypes of HPV have been identified (8). Some types of HPV appear to have oncogenic potential, and of these “high risk” HPV, types 16, 18, 31, and 33 are implicated in the development of lower genital tract neoplasia. HPV types 16 and 18 are found in a majority of cervical cancers (9) as well as in penile cancers (10) and urinary bladder cancers (11). In uro- and anogenital lesions, HPV types 16, 18, 31, and 33 are most frequently implicated in the development of lower genital tract neoplasia. HPV DNA has been used to transform a variety of epithelial cells in culture, including cervical cells, which are a natural target (17), and breast cells, which are not (18). Generally, human epithelial cells are immortalized but not made tumorigenic by the introduction of HPV types 16 or 18 DNA (19). Chromosomal changes occur in HPV-transformed cells and additional specific changes are required to induce the tumorigenic phenotype (20).

In an attempt to develop an in vitro model of carcinogenesis of the prostate, we chose to introduce HPV DNA into human prostatic epithelial cells because of the immortalization potential of HPV and its possible link with cancer etiology. Previous attempts to immortalize human prostatic cells have been infrequent. Neonatal prostatic epithelial cells were immortalized by the introduction of SV40-DNA via strontium phosphate-mediated transfection (21). Adult prostatic epithelial cells have also been immortalized with SV40 by lipofection- or polybrene-mediated transfection (22, 23). The increased availability of prostatic epithelial cell cultures for transformation studies due to improved culture techniques (24, 25) contributes to the feasibility of immortalizing human prostatic cell cultures of different histological origin. We used adult human prostatic epithelial cell strains from normal and malignant prostatic tissues for our transformation studies. After transfections with HPV type 18 DNA, we isolated several cell populations with prolonged life spans, two of which were established as immortal cell lines and were further characterized.

MATERIALS AND METHODS

Cell Culture. Adult human prostatic epithelial cell strains were established according to previously published methodologies (25). Small wedges of tissue were dissected from radical prostatectomy specimens, minced, and digested overnight with collagenase. The digested tissues were inoculated into dishes coated with type I collagen and containing medium FFMR-4A (25) supplemented with cholera toxin (10 ng/ml), epidermal growth factor (10 ng/ml), bovine pituitary extract (10 µg/ml), phosphoethanolamine (0.1 mM), hydrocortisone (1 µg/ml), selenous acid (30 µM), insulin (4 µg/ml), α-tocopherol (2.3 µM), retinoic acid (0.03 nm), and gentamicin (100 µg/ml). The cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO2 until reaching semiconfluency. Aliquots of the primary cultures were then frozen and stored in liquid nitrogen until the cells were reestablished in secondary culture for transfection experiments. Cell strains established by this protocol consist of pure populations of epithelial cells (25).

After removal of wedges of tissue for culture, the prostatectomy specimens were fixed, blocked, and serially sectioned at 3-mm intervals (26). The histology of hematoxylin and eosin-stained sections surrounding the area of tissue removed for culture was reviewed by Dr. John McNeal (Department of Urology, Stanford University Medical Center). The cell strains used in this study were derived from a tissue of normal histology (i.e., no cancer or benign prostatic hyperplasia was present) from the peripheral zone, and from an adenocarcinoma of Gleason Grade 4/4.

Transfection. Secondary cultures were established according to previously described protocols (25). Briefly, frozen amplexus of primary cell strains were thawed and inoculated into collagen-coated 100-mm dishes containing medium MCDB 105 (Sigma Chemical Co., St. Louis, MO), supplemented with the same factors as described previously for FFMR-4A. After reaching semi...
confluency, the cells were treated with liposomes containing HPV type 18 DNA cloned into pBR322 (kindly provided by Dr. H. Zur Hausen, Heidelberg, Germany). The liposome mixture was prepared by incubating 50 μg of lipofectin (BRL Life Technologies, Gaithersburg, MD) with 1 to 10 μg of plasmid DNA at room temperature for 15 min. The liposome mixture was added to each 100-mm dish containing cells and 3 ml of fresh medium. After incubation for 8 to 24 h, the lipofection medium was removed, the cells were rinsed, and 10 ml of fresh medium were added per dish. Untreated cells were included in every experiment as a control. Subcultures were prepared within 1 week by inoculating limited numbers of cells (up to 50,000) into 60-mm dishes.

**PCR Analysis.** High molecular weight DNA was isolated from cells by phenol-chloroform extraction and ethanol precipitation. One μg of DNA was used for each sample to be amplified. Primers for the E6 region of HPV type 18 with the same sequence as described by McNicol and Dodd (14) were utilized. The PCR reaction mixture contained 50 mM KCl; 10 mM Tris-HCl (pH 9); 2.5 mM MgCl2; 200 μM concentrations each of dATP, dGTP, dCTP, and dTTP; 0.01% gelatin; 0.1% Triton X-100; 50 pm concentrations of each primer; and 1 unit/reaction of Taq DNA polymerase (Promega, Madison, WI). The substrate diaminobenzidine was used as a color reagent.

**Cytogenetic Analysis.** At low passage number, chromosome spreads of metaphase cells were prepared and stained according to the method of Nelson-Reese et al. (27). At high passage number, chromosome spreads were prepared and R-banded with acridine orange according to the method of König et al. (28). At least 20 metaphase spreads from each line were examined for each analysis.

**Growth Assays.** Cells were inoculated into 60-mm dishes containing supplemented MCDB 105 and grown until a density of approximately 106 cells/dish was attained. At that time, cells were fed either MCDB 105 or KSFM (Gibco, Grand Island, NY). Every 3–4 days, duplicate dishes were fed and counted in order to establish a growth curve.

**Tumorigenicity Assays.** Congenitally athymic BALB/c nude mice were used to determine the tumorigenic potential of the cell lines. For each cell population, at least three mice were inoculated s.c. with 5 to 10 million cells/site. All animals were examined for the presence of tumors at regular intervals. At autopsy, tissues were taken for histopathological evaluation.

**Immunocytochemistry.** Cells were fixed with 2% paraformaldehyde and permeabilized with cold 95% ethanol. Nonpecific binding was blocked by preincubation in phosphate-buffered saline containing 10% horse serum. Monoclonal antibodies against keratin 5 (903) and keratin 8 (902) were obtained from Enzo Diagnostics (New York, NY); antibody specific for the E6 protein of HPV 18 was obtained from PharMingen (San Diego, CA); antibody against the large T antigen of SV40 (DP02) was from Oncogene Science (Uniondale, NY). Polyclonal antibody against PSA was from Signet (Dedham, MA). Primary antibodies were detected with biotinylated anti-mouse IgG or anti-rabbit IgG and the ABC reagent (Vector Laboratories, Burlingame, CA). The substrate diaminobenzidine was used as a color reagent.

**RESULTS**

**Transfection.** Several conditions for transfection of human prostatic epithelial cells were explored within the lipofection protocol. Ultimately, prolonged in vitro growth, a first criterion for transformation, was noted when cells were treated with liposomes containing 10 μg of HPV type 18 plasmid DNA for 8 h. Nontransfected cells, which were included as a control in all experiments, typically senesced after 4 passages. However, after the 5th passage of transfected cell populations, several colonies of cells with transformed morphology were recognized on separate dishes following subculture at low cell density. The frequency of colony formation at this stage was about 1/106 cells/site. All animals were examined for the presence of tumors at regular intervals. For each cell population, at least three mice were inoculated s.c. with 5 to 10 million cells/site. All animals were examined for the presence of tumors at regular intervals. At autopsy, tissues were taken for histopathological evaluation.

**PCR Analysis.** HPV type 18 DNA sequences amplified from genomic DNA of the transformed cell lines are shown in Fig. 2. Specific amplification of a 160-base pair fragment of the HPV type 18 E6-transforming region was noted in DNA from PZ-HPV-7, CA-
Cytochemistry (Table 1). Cytokeratins 5 and 8 were expressed in medium.

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were observed in 10% of the metaphase spreads. At passage 89, 

Lane 9, negative control (no DNA added); Lane 10, DU 145 (HPV-negative prostate cell 

Lanes I and 11, molecular weight ladder; Lane 2, normal prostatic epithelial cells 

precursor to PZ-HPV-7); Lane 3, cancer-derived prostatic epithelial cells (precursor to 

CA-HPV-10 at passage 26 was aneuploid with an average chromo 

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PZ-HPV-7 revealed keratin-forming, squamous epithelia arranged in 

a nodular pattern (Fig. 4). CA-HPV-10 formed lumps that were less 

well-differentiated but still squamous (Fig. 4). Injection of both pa-

HPV-10, and HeLa cells (known to contain multiple copies of the HPV type 18 genome). Hybridization of an HPV E6-specific probe to a Southern blot of the PCR-amplified products confirmed the presence of HPV sequences in PZ-HPV-7, CA-HPV-10, and HeLa cells but not in the two parental cell strains or in another HPV-negative control cell line (Fig. 2).

Cytogenetic Analysis. Chromosome counts of metaphase spreads prepared from PZ-HPV-7 at passage 38 showed a modal number of 46 (range, 35 to 105). At passage 99, the modal number had shifted to near-tetraploid (106), with a range of 103 to 108. The karyotype of 

banded chromosomes showed 4–5 copies of most chromosomes.

CA-HPV-10 at passage 26 was aneuploid with an average chromosomal number of 73 (range, 40–132). Double minute chromosomes were observed in 10% of the metaphase spreads. At passage 89, CA-HPV-10 still had an average number of 72 chromosomes (range, 69 to 75). Double minute chromosomes or homogeneously staining regions were then present in 43% of the metaphases examined.

Growth Requirements. In order to determine whether MCDB 105, the medium in which the transfected cells originated, was an optimal growth medium, we compared growth in other media. KSFM, a serum-free medium developed for keratinocytes, was found to support growth of both PZ-HPV-7 and CA-HPV-10 better than MCDB 105. This was true especially as the cells reached higher densities. From the exponential parts of the growth curves, we calculated a doubling time of about 24 h for each cell line in KSFM medium.

Immunocytochemistry. Expression of prostate- and HPV-associated antigens in the established cell lines was assessed by immunocytochemistry (Table 1). Cytokeratins 5 and 8 were expressed in PZ-HPV-7 and CA-HPV-10 and in the parental cell strains, demonstrating the epithelial origin of the cell lines. PSA, expressed at a low but detectable level in the parental cells, appeared to be absent from the cell lines. As expected, neither the cell lines nor the parental cells were labeled by antibody against the large T antigen of the SV40 virus, whereas PZ-HPV-7 and CA-HPV-10 cells were exclusively labeled by antibody against the E6 oncoprotein of HPV 18 (Fig. 3).

Tumorigenicity. Injection s.c. into nude mice of 5 to 10 million cells/site gave rise to occasional lumps with diameters of less than 1 cm when PZ-HPV-7 or CA-HPV-10 cells were tested. These lumps were nonprogressive and slowly regressed. Histological examination of hematoxylin and eosin-stained sections of recovered lumps from PZ-HPV-7 revealed keratin-forming, squamous epithelia arranged in a nodular pattern (Fig. 4). CA-HPV-10 formed lumps that were less well-differentiated but still squamous (Fig. 4). Injection of both pa-

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\begin{array}{cccccc}
\text{Cells} & 902^b & 903^c & \text{HPV-E6}^d & \text{Tag}^e & \text{PSA}^f \\
\text{Normal} & + & + & - & - & + \\
\text{Cancer-derived} & + & + & - & - & + \\
PZ-HPV-7 & + & + & - & - & - \\
CA-HPV-10 & + & + & - & - & - \\
\end{array}
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\^ Immunochemistry was performed as described in "Materials and Methods." If stained cells were present, staining was indicated as +. If no stained cells were present, staining was indicated as —.

\^ Antibody 902, against keratin 8, was used at 1:1000.

\^ Antibody 903, against keratin 5, was used at 1:2000.

\^ Antibody against the E6 protein of HPV was used at 1:500.

\^ Antibody against the large T antigen of SV40, was used at 1:10.

\^ Antibody against PSA was used undiluted.

Fig. 2. HPV DNA in transformed cells. (Top) DNA from each cell population was amplified with HPV 18-specific primers by PCR as described in "Materials and Methods." Amplification products were separated in a gel and photographed after staining with ethidium bromide. (Bottom) A blot was prepared from the gel shown in the top section and hybridized with HPV 18-specific oligonucleotide probe. The autoradiogram is shown.

Lanes 1 and 11, molecular weight ladder; Lane 2, normal prostatic epithelial cells (precursor to PZ-HPV-7); Lane 3, cancer-derived prostatic epithelial cells (precursor to CA-HPV-10); Lanes 4 and 5, PZ-HPV-7; Lanes 6 and 7, CA-HPV-10; Lane 8, blank; Lane 9, negative control (no DNA added); Lane 10, DU 145 (HPV-negative prostate cell line); Lane 12, HeLa (HPV-positive cell line). bp, base pairs.

Fig. 3. Immunocytochemical detection of HPV E6-oncoprotein in transformed cells. PZ-HPV-7 (top), CA-HPV-10 (middle) and pRNS-1—1, a line of SV4O virus-transformed cells (bottom), were labeled with antibody (1:200) against the E6 protein of HPV 18 by an indirect immunoperoxidase technique. Positive labeling was seen in the two HPV-transformed cell lines but not in the SV40 virus-transformed line. × 800.
Fig. 4. Histology of injected cells. CA-HPV-10 (right) and PZ-HPV-7 (left) cells were injected s.c. into nude mice. Small lumps formed which were removed after 3 months, fixed, and sectioned. Examination of hematoxylin and eosin-stained sections revealed keratinizing, squamous nodules. Top, × 100; bottom, × 400.

DISCUSSION

Cell transformation studies provide information about the involvement of oncogenes and other genetic changes in the initiation and progression of cancer. Transformation of normal cells by viral infection or DNA transfection is a frequent approach to inducing early events in the development of a malignant phenotype. The transforming abilities of HPV are well established and HPV has been implicated in human carcinogenesis. Therefore, we introduced HPV type 18 DNA into normal and cancer-derived human prostatic epithelial cells via lipofection-mediated transfection. This transfection method was chosen because other methods, such as calcium phosphate-mediated DNA transfection (29), often induce considerable toxicity in primary cell cultures, especially those grown in serum-free media.

From these transfection attempts, two immortal cell lines were established. One of these, PZ-HPV-7, was derived from epithelial cells cultured from normal tissue of the peripheral zone of the prostate. Normal epithelial cell strains derived from prostate tissues by our methods have been extensively characterized. They have a typical epithelial morphology consisting of small, cuboidal cells in a cobblestone pattern. Keratins present in the prostatic epithelium (keratins 5 and 8) continue to be expressed in vitro. PSA is also expressed, but at very low levels compared to tissues. These cultures have a limited life span and typically undergo 20 to 30 population doublings before senescence.

In many ways, the cell line PZ-HPV-7 resembled the cells of origin. An epithelial morphology was maintained, although features typical of transformed cells (rounded, loosely attached cells) were apparent. Keratin expression continued, demonstrating the epithelial origin of the transformed cells. It is interesting that keratin 5, found in the basal cells of the prostatic epithelium (30), continued to be expressed by the transformed cells. When mammary epithelial cells were transformed by HPV, they lost expression of basal cell-associated keratins and expressed keratins associated with the luminal cells of the mammary epithelium (18). PZ-HPV-7 expressed luminal cell-associated keratin 8 in addition to keratin 5. PSA expression, already at a low level in the parental cell strain, was undetectable in PZ-HPV-7. The diploid karyotype of the normal parental cells was maintained in low-passage PZ-HPV-7 cells, but by passage 99 the karyotype had changed to near-tetraploid. It is perhaps relevant to the development of HPV-transformed cells such as these as a model of in vitro carcinogenesis that flow cytometry studies of prostate cancers have also shown a prevalence of near-tetraploid amounts of DNA (31).

The other line, CA-HPV-10, was derived from cells cultured from a prostatic adenocarcinoma of Gleason Grade 4/4. Cancer-derived cell strains obtained by our methods have many traits similar to strains derived from normal tissues, including a mortal life span. We have, however, noted chromosomal abnormalities in approximately 30% of our cancer-derived cell strains by standard G-banding analysis (32, 33) and in about 90% by fluorescence in situ hybridization analysis (34). The parental cell strain from which CA-HPV-10 was obtained had several abnormalities, including an extra Y chromosome and double minute chromosomes (33). CA-HPV-10, in contrast to PZ-HPV-7, was aneuploid with an average chromosomal number of 72–73 at both low and high passages. Genetic structures that contain...
amplified DNA (double minutes and homogeneously staining regions) were present in almost one-half of the transformed cells. 

Characterization of HPV-transformed keratinocytes (19) or mammary epithelial cells (18) has indicated that introduction of HPV typically leads to an immortal phenotype but not to tumorigenic potential. Although PZ-HPV-7 and CA-HPV-10 cells formed small lumps when injected s.c. into nude mice, these lumps did not enlarge progressively and were found to be composed of squamous cells. The development of nonprogressive small lumps was also noted for SV40-transformed neonatal prostate cells when injected into mice (21).

The creation of immortalized, HPV-transformed human prostatic epithelial cells is a first step in the development of an in vitro model of prostatic carcinogenesis. We propose that further genetic changes can be induced by mutagenesis or the introduction of additional oncogenes which will lead to a tumorigenic phenotype. The possibility of developing such a model has been elegantly demonstrated with urethelial cells by Reznikoff et al. (35). Normal urethelial cells were transformed to immortality by SV40; then further exposure to chemical mutagens led to tumorigenicity. Nonrandom chromosomal losses were seen during this stepwise transformation which were also linked to bladder cancer (36). Therefore, the processes involved in experimental carcinogenesis in vitro may well be relevant to actual cancer etiology. In choosing to transform normal as well as cancer-derived prostatic cells with HPV, we thought that the cancer-derived cells might already have undergone genetic changes which would render them immediately tumorigenic upon immortalization by HPV. This did not turn out to be the case, although CA-HPV-10 cells were more genetically abnormal than PZ-HPV-7 and formed less well-differentiated nodules after injection into mice. It will be interesting to see whether CA-HPV-10 may spontaneously develop into a tumorigenic line, perhaps because of genetic instability related to its origin from malignant tissue. Study of PZ-HPV-7 and CA-HPV-10 cell lines and subsequent derivatives should give clues about genetic changes involved in prostatic carcinogenesis.

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REFERENCES

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