In vitro model of human prostate carcinogenesis

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Contents

aFGF	acidic fibroblast growth factor
AR	androgen receptor
bp	base pair
BPE	bovine pituitary extract
BPH	benign prostatic hyperplasia
DAB	3,3'-diaminobenzidine
DHT	5α-dihydrotestosterone
dmin	double minutes
DNA	deoxyribonucleïc acid
EGF	epidermal growth factor
E6-7	early region 6 or 7 of HPV genome
FISH	fluorescence in situ hybridization
HPV	human papillomavirus
hsr	homogeneously staining regions
IGF	insulin-like growth factor
KSFM	keratinocyte serumfree medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PAP	peroxidase anti-peroxidase
PCR	polymerase chain reaction
PIN	prostatic intraepithelial neoplasia
P53	tumorsuppressor gene
PSA	prostate-specific antigen
PSM	prostate-specific membrane antigen
RB1	retinoblastoma susceptibility gene
RNA	ribonucleïc acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	standard saline-citrate
SV40	simianvirus 40
TGF-ß	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha

General introduction

1.1 Scope of the thesis

In this thesis, *in vitro* transformation of epithelial human prostate cell cultures is described. Transformation can be defined as the uptake of any DNA molecule in any type of cell. Experimentally, transformation of cells may be achieved by the artificial introduction, also called transfection, of foreign genes. Genomic incorporation of specific DNA material may lead to the enhancement of cellular replication and growth. In prostate cancer research, a limiting factor has been the general inability of longterm *in vitro* growth of normal and malignant cells from the human prostate epithelium. By transformation, prostatic cells may become immortalized. Furthermore, the establishment of immortalized human epithelial prostate cell lines by transfection of different DNA sequences may be useful for the understanding of initiating and promoting factors in human prostate carcinogenesis. Inherent changes after the introduction of immortalizing agents may be determined by detailed characterization studies. This thesis describes the establishment of novel human prostate cell lines in an attempt to develop an experimental *in vitro* model of human prostate carcinogenesis. Current knowledge of several factors involved in prostate cancer and the implications of the experiments are presented.

1.2 Epidemiology and etiology in prostate cancer

Prostate cancer is one of the most common cancers in Western countries, with an estimated 317.000 newly diagnosed patients and 41.400 deaths in 1996 in the United States alone [1]. Similar to other Western countries, in the Netherlands, prostatic carcinoma is the second most diagnosed malignant tumor [2] and an average of 1% increase of the age-adjusted prostate cancer mortality rate has been registered between 1950 and 1989 [3]. However, whereas the age-adjusted cancer-specific mortality has shown a marginal increase and has remained relatively unchanged, the incidence has risen strongly over the last decade. This phenomenon may be attributed to generally improved detection methods, in particular the use of serological PSA determination, and increased awareness of the population at risk [4-6]. Currently, the question of over diagnosis and over treatment should be addressed [6-8]. From necropsy studies prior to the PSA era we know that, in more than one out of three men above age 50, evidence of "latent" invasive prostate carcinoma can be found [9,10], while a rather small subpopulation will have developed potentially lethal prostate cancer. It has been estimated that one out of eleven men will be diagnosed with prostate cancer, which in more than one fourth of the cases will lead to tumor progression and cancer death [1,11].

Despite continuous progress in understanding the nature of the disease, still the etiology and pathogenesis are unclear [12]. In several studies analyzing epidemiologic aspects of prostate cancer, many factors [13,14], including infectious sexually transmittable agents and sexual activity, have been proposed [15-23] and disputed [24-26]. A definite familial predispostion has been demonstrated, suggesting that, in some patients, an inherited gene or group of genes in a discrete location may be identified which are involved in either the initiation or in the progression of the disease [27,28]. Recently, linkage analysis of 91 high-risk prostate cancer families has provided strong evidence of a major prostate cancer susceptibility locus on chromosome 1 [29]. Other chromosomal abnormalities have been associated with prostate cancer, which may indicate a pattern of events leading to progressive disease [30-32, see chapter 1.4]. Regarding the etiology and pathogenesis of prostatic carcinoma, it seems clear that the multistep carcinogenic process is influenced by many factors. Several approaches to study this process, including transformation studies, should further clarify the relative importance of events which determine the course of the disease [33].

Regardless of the approach, the search for specific molecular events occurring at each stage of tumor development must eventually explain the multifocal origin [34], the relationship of prostatic intraepithelial neoplasia (PIN) or dysplasia to invasive adenocarcinoma [35,36], the correlation between volume, differentiation and malignant potential [37] and the development of androgen-refractory disease [38].

1.3 Human Papillomavirus in urogenital carcinogenesis

In general, viruses must be thought of as the second most important risk factor for cancer development in humans, exceeded only by tobacco consumption [39]. Human papillomaviruses (HPV) are small DNA viruses which infect a diversity of epithelial tissues. Figure 1 is an illustration of the structural configuration of the HPV type 18 genome. Over a hundred different HPV genotypes have been identified, and all seem to be associated with disease processes at distinct anatomic sites [40-44].

A subset of HPV types 6, 11, 16 and 18 are found most commonly in genital and mucosal lesions. Within the genital-mucosal HPV types, some appear to have greater oncogenic potential. These "high risk" HPV types 16, 18, 31 and 33 are implicated in the development of lower genital tract neoplasia. HPV 16 and 18 are found in a majority of cervical cancers [42-44] as well as in penile cancers [45-47]. Furthermore, HPV 16 and 18 DNA may be detected in squamous cell carcinoma of the urethra [48], but presumably also in transitional cell carcinoma of the urinary bladder [49,50]. The finding of HPV DNA in bladder cancer tissue was not confirmed in a large Finnish study using *in situ* hybridization and the method of PCR [51]. Notably, in this analysis,

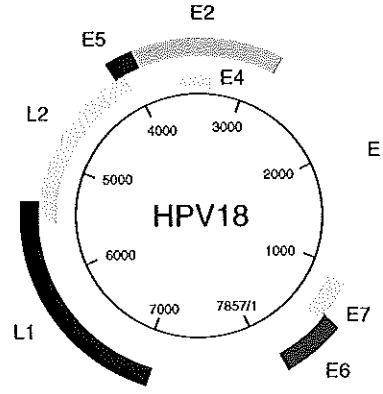

Figure 1. Structural configuration of the HPV type 18 genome. The early (E) and late (L) regions are subdived in a number a functional genes. The E6 and E7 gene products of "high risk" HPV types are important factors involved in the process of carcinogenesis.

PCR was performed with the use of consensus primers, although the sensitivity of detection could have been increased with the use of type specific primers. It is important to recognize the primer set used in the different studies, because consensus primers, in contrast to type specific primers, are designed to amplify DNA of several HPV types which sometimes may result in lower detection rates [62]. In anogenital lesions, HPV types 16, 18, 31, and 33 are most frequently associated with progression to malignancy [52,53]. It is clear that HPV has been recognized as a factor in anogenital carcinomas which are mainly of squamous differentiation.

The issue of HPV involvement in prostatic disease, including prostate cancer, was raised in the early nineties. However, histopathologically, prostate cancer is an adenocarcinoma in 95% of the cases. In view of the general association of HPV with squamous anogenital carcinoma, the involvement of HPV in adenocarcinoma of the prostate would seem rather exceptional. However, in a Canadian study, the detection of certain HPV DNAs in human prostate specimens, first by Southern blot analysis and later by PCR, pointed at a possible role of this viral factor in prostatic carcinoma [54-56]. With the use of PCR, "high risk" HPV DNAs have been detected in prostate cancers [55-62.] Furthermore, the detected HPV DNAs in these tissues were transcriptionally active [60], although not predominantly in cancers, suggesting that the prostate could serve as a reservoir for these HPVs. Moreover, in a study of Japanese prostate carcinoma patients, a high incidence of HPV type 18, as determined by PCR, correlated with both grade of differentiation by the Gleason score and clinical stage, with frequent detection (89%) of HPV type 18 DNA even in samples from bone metastases [59]. On the other hand, several other authors have not or only sporadically been able to detect HPV DNA in prostatic tissues [63-65]. However, as stated previously, the discrepancies in HPV detection rates measured

by PCR may possibly be explained by the use of different primer sets or consensus primers instead of HPV type specific primers [62]. Nevertheless, the evidence available to implicate an association of HPV with prostate cancer is limited. From a review of most of the available HPV detection rates, it is evident that the role of HPV in the prostate is unclear [66]. Currently, an association with prostate cancer seems unlikely. The role of the male urogenital tract as a reservoir for HPVs may be more evident [67], also illustrated by the presence of HPV DNA in urine sediments, demonstrating that HPV can be transported in exfoliated HPV-infected cells [68], and by the detection of HPV DNA in semen [69].

1.4 Cytogenetic correlates in prostate cancer

Characteristic cytogenetic aberrations may shed light on tumor specific mechanisms of oncogenesis. During the last decade, several chromosome abnormalities have been associated with prostate adenocarcinoma. The findings of specific karyotypic features of adenocarcinoma specimens have been rather diverse. Chromosomal gain and loss as well as structural anomalies reported in prostate cancer include almost all human chromosomes [30-32,70-92], which would seem to make the identification of prostate cancer specific events difficult. However, of all these chromosomal anomalies some seem to predominate and chromosomal deletions at specific sites seem to correlate with a poor prognosis [70]. Gains of 7, 8q, and X may be involved in prostate cancer progression [80-87]. Among the most commonly deleted regions implicated in prostate cancer are 8p [72,75-80,82-92], first suggested in 1988 by König *et al.* [88], 10q [71,74-77,82,87], 13q [87], 16q [71,75,82,87], and also anomalies of the Y chromosome [72-74,76-78,83-85].

Allelic loss or loss of heterozygosity and comparitive genomic hybridization studies would possibly uncover the location of tumor suppressor genes. The role of loss of 13q in the multistep process of carcinogenesis could be explained because this location harbors the retinoblastoma susceptibility gene *RB1*. LOH at the *RB1* locus 13q14 has been reported in 60% of primary prostate carcinomas [93]. However, whereas reduced expression of *RB1* has been documented in primary prostate tumors, relatively few *RB1* mutations have been detected in prostate cancer [32,94]. The presence of anomalies of 17p, which harbors the *P53* tumor suppressor gene, could be a factor in prostate carcinogenesis that may be explained by the evident role of *P53* in malignancies. LOH at the *P53* locus or mutations have been reported in only 10 to 30 % of prostate cancers, but an association of *P53* mutations and poor prognosis has been documented [95]. In view of 16q deletions, loss of cell-cell interaction mediated by E-cadherin has been studied and decreased E-cadherin expression has been associated with poor prognosis in prostate cancer [96].

Furthermore, almost 70% of patients with clinically localized prostate cancer have been found to harbor deletions of chromosome 8p22 [90], but the locus 8p12-21 is also frequently involved [92]. However, a derived prostate cancer susceptibility gene or human prostate cancer gene, proposed as *HPC1*, has not yet been elucidated. The currently implicated genes in prostate cancer are listed in table 1, abstracted and modified from the review of Kallioniemi and Visakorpi [32].

In addition to somatic genetic alterations, the clustering of prostate cancer in some families is indicative for the existance of a germline mutation. As stated previously, linkage analysis of 91 high-risk prostate cancer families provided strong evidence of a major prostate cancer supsceptibility locus on chromosome 1 [29]. The results of this study point to the long arm, 1q24-25, of chromosome 1, and may prove to be another significant finding in prostate cancer.

1.5 *In vitro* model of the human prostate epithelium

Development of a human prostate cell culture model: Since 1983, the work of Dr. Donna M. Peehl and her group at Stanford University has been devoted to the development of an optimal culture system for human prostatic epithelial cells. The efforts resulted first in the successful establishment of primary cultures and their serial passage [97], then in the development of a serum-free medium [98], and finally in clonal growth [99]. With the use of these techniques [100], cultures can be derived from normal, benign hyperplastic and malignant tissues obtained from radical prostatectomies, and even from ultrasound-guided needle biopsies using slightly modified methods [101]. This *in vitro* model system was subsequently used to characterize many biological properties of prostate cells and to compare normal, or benign, to malignant cells. The role of growth stimulatory and inhibitory factors was examined in detail [102-106], features of differentiation were analyzed [107], molecular and cytogenetic correlates of malignancy explored [72-74,108-110]. Notably, the Stanford cell culture system does not resolve the limited *in vitro* growth potential of prostatic epithelial cells. Also, the *in vitro* identification of normal versus malignant cells is not possible, although the histologic origin can be determined by accurate stepsectioning of the prostate specimen. Nevertheless, the Stanford methods have been extensively studied and should be recognized as one the best-characterized culture systems for epithelial human prostate cells. It was used as the cornerstone in developing an *in vitro* model of human prostate carcinogenesis.

Table I. Somatic genetic alterations and candidate genes implicated in prostate cancer

Chromosome arm (putative region)		Genes	Comments
Loss		***tumor suppressor***	
5q	-	*APC*, a-catenin	recurrent tumors
6q	-	-	-
7	(q31.1)	-	-
8p	(p11-p12/p22)	-	early lesions, PIN
9p	-	*MTS1*	cell lines
10q	-	*MXI1*	early lesions, PIN
11p	(p11.2)	*KAI1*	metastasis suppressor activity
11q	-	*GSTP1*	-
12	(pter-q13)	-	tumor suppressor activity
13q	-	*RB1*	possibly early event
16q	(q23-qter)	E-cadherin	decreased E-cadherin expression associated with poor prognosis
17p	-	*P53*	associated with advanced stage and poor prognosis
17q	-	*BRCA1*	-
18q	(cen-q22)	*DCC*	-
19q	-	*C-CAM*	BPH and PIN
Y	-	-	-
Gain		***oncogene***	
7		-	-
8q	(q24)	*MYC*	recurrent tumors and primary tumors with advanced stage
18q		*BCL-2*	recurrent tumors
X	(p11-q13)	*AR*	recurrent tumors
Several loci		*RAS* gene family	uncommon in Western countries

With modifications abstracted from the review of Kallioniemi O-P and Visakorpi T [32].

Transfection in human prostatic epithelial cells: Many ways to introduce foreign genes into mammalian cells have been described. However, only a limited number of successful transfection procedures in primary human prostate cells have been reported, suggesting that it is difficult to perform transfection experiments because of the limited *in vitro* growth potential of human epithelial prostate cells. The latter would make these cells rather sensitive to the toxic conditions imposed by the various transfection protocols. Calcium-phosphate [111] mediated transfection has not been reported in primary adult human prostatic cells. Neonatal prostatic cells were immortalized by strontium-phosphate [112] mediated introduction of a plasmid containing SV40 early region genes [113]. Later, adult prostatic epithelial cells were immortalized by transfection using liposomes [114] containing the large T-SV40 gene [115]. Currently, well defined culture conditions for human prostate cells should improve transfection efficiencies and possibilities to perform transformation studies.

1.6 Transformation

Transformation by viral DNA is associated with interaction of cell-cycle regulating proteins. For SV40 the virus encoded large T or tumor antigen confers the enhanced growth potential upon the cells. This 708 amino acid protein is able to form complexes with a 53 Kdalton protein, presently known as *P53*. In fact, it is by virtue of transformation studies using SV40 large T antigen that *P53* has been discovered [116]. For HPV the protein gene products of E6 and 7 are both essential mediators of the immortilizing capacity as well as the oncogenic potential [see also figure 1, 117-119]. The E6 and E7 proteins of transforming HPVs have been recognized to bind and inactivate tumor suppressive proteins *P53* [120] and *RB1* [121] respectively. The discovery of such mechanisms has been important to understand the HPV-mediated role in human carcinogenesis. Similar to *P53*, the retinoblastoma gene product *RB1* has been identified as an important mediator in the control of cell proliferation, acting like a tumor suppressor. The intact wild-type *RB1* protein forms complexes with several viral oncoproteins including the SV40 large T antigen and the E7 oncoprotein of "high risk" HPV. It is clear that these interactions may lead to enhanced replication. Consequently, HPV has been shown to immortalize a variety of human epithelial cells in culture, including primary rodent cells [122], fibroblasts, keratinocytes [123,124], as well as cervical cells [125], which are a natural target, and breast cells [126], which are not.

Transformation of normal cells is a common experimental approach to study the process of carcinogenesis. Either by viral infection or by DNA transfection, the intrinsic characteristics of cells are modified, which may resemble the process of tumor initiation. By transformation, it should be possible to establish valid models of

prostate carcinogenesis. Another motivation for this approach in prostate cancer research would be to prolong the limited *in vitro* life span of human prostatic epithelial cells per se. As stated previously [see chapter 1.5], few models of human prostate carcinogenesis have been established. Prostatic epithelial cells could not be transformed in vitro with *RAS* or *MYC* or adenovirus E1A and E1B, or combinations of these oncogenes [33]. In general, immortalization of human cells in culture can be achieved using whole genomic material of DNA viruses such as SV40 [116, 127] and HPV [117-126], which circumvents problems of maintenance and continuous viral replication associated with viral infection [128]. After the introduction of plasmid-constructs containing viral DNA, the enhanced ability to replicate can lead to an immortal state, which through genetic instability may result in a neoplastic phenotype. Notably, upon continuous passage of HPV immortalized foreskin derived keratinocytes, tumorigenic cell lines emerged [129]. Cytogenetic analysis of these cells at early, middle, and late passage levels, and cells cultured from tumors revealed that several chromosomal abnormalities segregated with the tumorigenic cell populations.

In general, however, transfection of SV40 or "high risk" HPV DNA is not sufficient for neoplastic transformation. Additional specific alterations are required to induce tumorigenicity, acting as tumor promoting factors. Co-transfection of *RAS* in HPV immortalized cells leads to a neoplastic state [130,131]. The development of such a stepwise model of carcinogenesis in the human prostate has not been described until recently [132]. In contrast, a multistep model for bladder cancer has been established since the late-eighties using SV40 immortalization of human urothelial cells followed by introduction of the *RAS* oncogene or by chemical mutagenesis [133,134]. Mechanisms involved with the different transforming events were analyzed, as well as the chromosomal losses that accompanied transformation. The latter were similar to the alterations found in transitional cell carcinomas. However, an interesting observation was that *RAS* transformation yielded transitional cell carcinoma, whereas chemical transformation induced squamous carcinoma.

Experimental *in vitro* transformation models of prostate carcinogenesis may be useful in several ways. Firstly, by transformation cell lines of different origin may become available. Secondly, after immortalization, the cell lines may serve as a target for the induction of additional changes. Ultimately, it may be possible to experimentally recreate some of the processes occurring during the development of prostate cancer, recognizing genetic changes at different stages of malignant progression.

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Characterization of adult human prostatic epithelial cells immortalized by polybrene-induced DNA transfection with a plasmid containing an origin-defective SV40 genome

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Abstract

Normal adult human prostatic epithelial cells were infected with an adenovirus 12-SV40 virus or transfected by polybrene-induced gene transfer with a plasmid (pRSV-T) containing the SV40 early region genes or with a plasmid (pRNS-1) containing an origin-defective SV40 genome and a plasmid carrying the neomycin resistance gene. Colonies of morphologically altered cells were isolated, cultured in a serum-free medium and characterized. These cells had extended lifespan in culture compared to normal adult human prostatic epithelial cells. Both Ad12-SV40-infected and pRSV-T-transfected cultures eventually underwent senescence. pRNS-1-transfected cells (pRNS-1-1), however, have now been grown for more than 50 passages. These cells contain the SV40 genome, express SV40 T-antigen, and are not tumorigenic in nude mice. They express cytokeratins 5 and 8, like the parent cells, and are pseudodiploid. Analysis of growth regulatory processes revealed that the growth of pRNS-1-1 cells was stimulated similarly to that of normal prostatic epithelial cells by epidermal growth factor, insulin like growth factor, and pituitary extract. The response of pRNS-1 cells to a growth-inhibitory factor, retinoic acid, was also similar to that of normal cells. However, pRNS-1-1 cells were less responsive than normal cells to growth inhibition by transforming growth factor-ß, and had lost altogether the ability of normal cells to be inhibited by tumor necrosis factor-α and 1,25 $(OH)_2$ vitamin D_3. Therefore, transformation appeared to alter growth-inhibitory but not growth-stimulatory mechanisms. These cells should be useful in elucidating the multistep mechanism of carcinogenesis of the prostate.

Introduction

Prostate cancer is the most commonly diagnosed cancer in men and the second leading cause of male cancer death in America [1]. In 1992, 132,000 new prostate cancer cases were diagnosed with over 36,000 deaths [2]. The risk of developing prostate cancer increases dramatically with age, as does the occurrence of the proliferative disorder benign prostatic hyperplasia (BPH) [3]. Compared with all other cancers, prostate cancer increases most rapidly with age [4]. The study of adult prostatic epithelial cells is therefore of considerable interest. The basic mechanisms of prostate cancer growth and the cause of tumor formation are largely unknown despite the increasing prevalence of the disease in the United States. A major hindrance to research in this area has been the lack of a suitable cell culture system to investigate the process.

During the past several years remarkable progress has been made in the field of human epithelial cell transformation. Neoplastic transformation of human epithelial cells in culture has been achieved in a stepwise fashion - first immortalization and then conversion of the immortalized cells to tumorigenic cells [5]. With few exceptions, normal human epithelial cells require immortalization to provide a practical system for transformation studies *in vitro* [6]. However, immortalization of human epithelial cells is difficult to achieve compared to animal cells. Different cell types require different conditions and transforming agents to achieve a useful cell line. Immortalization of adult human prostatic epithelial cells has been difficult [7]. There has been only one report on the immortalization of such cells by liposomes containing the SV40 large T-antigen gene [7]. We report herein the establishment and characterization of adult human prostatic epithelial cells immortalized by polybrene-induced DNA transfection with a plasmid containing an origin-defective SV40 genome and a plasmid carrying the neomycin resistance gene.

Materials and methods

Cell cultures and media. Epithelial cells (8891) were derived from the normal peripheral zone of an adult human prostate. A small wedge of tissue was dissected from a specimen obtained after radical prostatectomy of a 53-year old male. The tissue was minced and digested overnight with collagenase. After rinsing and centrifugation to remove collagenase and most stromal cells, the digested tissue was inoculated into a 60-mm tissue culture dish coated with collagen type I and containing medium PFMR-4A supplemented with growth factors and hormones [8]. Cells which grew out in primary culture were aliquoted and stored frozen in liquid nitrogen. Secondary cultures derived from the frozen aliquots were grown in serum-free keratinocyte medium (Gibco, Grand Island, NY) and used for transformation. The epithelial nature of these cells was verified by immunocytochemical labeling of keratin and prostate-specific antigen. These cultures were 100% epithelial, with no contaminating stromal cells. To verify the histology of the tissue of origin, the prostate was inked after dissection, fixed and serially sectioned. The histology of sections immediately adjacent to and surrounding the portion removed for culture was reviewed. No malignant foci were seen and therefore, the corresponding cell culture was classified as normal.

Transformation assay. Secondary culture of 8891 cells was infected at multiplicity of infection of approximately 100 with the Ad12-SV40 virus [9]. The infected cultures were subcultured at a 1:2 ratio every 7-10 days and were observed for the appearance of morphological alterations.

Plasmids. The plasmids used were as follows: pRSV-T (a gift from Dr B. Howard, National Institutes of Health) contains the SV40 early region genes under the control of the Rous sarcoma virus long terminal repeat [10]. The plasmid pRNS-1 contains an origin-defective SV40 genome expressing a wildtype T antigen (gene) and the gene for neomycin resistance being driven by the long terminal repeat of the Rous sarcoma virus [11].

Transfection. Polybrene-induced DNA transfection was carried out as described previously [12]. Cells were transfected with 10 *ug* of DNA using polybrene at a concentration of 10 *ug*/ml and incubated at 37°C overnight. The cells were then shocked with 30% DMSO for 4 min. Five days after transfection the cells were subcultured at a 1:2 ratio approximately weekly. The cell culture medium was changed twice weekly.

Colony formation in soft agar. A cell suspension ($1x10^4$ cells per ml) in 5 ml of 0.35% Noble agar with serum-free keratinocyte medium was overlaid into a 60-mm dish containing a 0.6% agar base. Viable colonies were scored at 21 days.

Tumorigenicity in nude mice. Adult 129J nude mice were subcutaneously inoculated with $1x10^7$ freshly trypsinized cells to determine tumorigenicity.

Chromosomal analysis. Karyotypic analysis was caried out by Dr Joseph Kaplan Jr, Children's Hospital of Michigan, Detroit, MI by Giemsa banding. Chromosome counts of 30 to 31 metaphases per line were made for ploidy determination. For each culture, eight karyotypes were prepared.

Immunocytochemistry. Cells were fixed with 2% paraformaldehyde and permeabilized with cold 95% ethanol. Nonspecific binding was blocked by pre-incubation in phosphate-buffered saline containing 10% horse serum. Monoclonal antibodies against keratin 8 (902) and keratin 5 (903) were obtained from Enzo Diagnostics (New York, NY), and monoclonal antibodies against *P53* (0P09) and the large T-antigen of SV40 (DP02) were obtained from Oncogene Science (Uniondale, NY). Polyclonal antibody against prostate-specific antigen 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.

Growth assays. pRNS-1-1 cells were inoculated into 60-mm collagen-coated dishes containing 5 ml of control or experimental medium at 1000 cells per dish. After 14 days of incubation, growth was quantitated with an Artek image analyzer (Dynatech, Chantilly, VA), which measures the total area of the dish covered by cells.

Southern blot hybridization analysis. DNA blot analysis was performed as described [13]. High molecular weight DNA was digested with the appropriate restriction endonuclease and applied to a 1.0% agarose gel. After electrophoresis, the DNA fragments were transferred from the gel to a nitrocellulose membrane and hybridized to a random-primed ^{32}P-labeled DNA probe.

Western immunoblot analysis. Subconfluent cultures of the appropriate cell clones were rinsed with phosphate-buffered saline (pH 7.2) and lysed with a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100 and 1.0% Na deoxycholate. The protein concentration of the lysates was determined and equivalent amounts of protein from each cell line were immunoprecipitated with the appropriate antibody. The resulting immunoprecipitates were fractionated on a 12.5% polyacrylamide-SDS gel. After electrophoresis, proteins on the gel were electroeluted onto a nitrocellulose membrane which was then reacted against the relevant antibody and ^{125}I-labeled protein A [14].

Results

Transformation. Colonies of transformed cells were readily identifiable by cellular morphology. The transformed cells were more tightly packed than normal cells, and by the time colonies had grown to a diameter of 1 cm or more they were countable following staining (6 to 8 weeks from the time of infection or transfection), by which time most of the untransformed cells had clearly senesced (figure 1). Colonies were isolated using cloning cylinders and then serially passaged as clonally-derived cell strains. A number of clonal cell strains from the Ad12-SV40-infected and pRSV-T and pRNS-1-transfected cells were isolated and stored frozen in liquid nitrogen. One of each group was further characterized.

Increase in lifespan in culture. All of the three transformed cell strains studied had increased lifespans in culture compared to the untransformed parent cells (Figure 2). The control cultures had ceased to replicate by the time of colony selection. The Ad12-SV40 infected and pRNS-T transfected cells also eventually underwent senecence at 40-46 population doublings and 24-26 weeks following infection or transfection (figure 2). The pRNS-1 transfected cell clone designated pRNS-1-1 has an apparently unlimited lifespan and has been successfully subcultured for more than 50 passages over the course of 1 year with no evidence of decreased proliferative capacity. The pRNS-1-1 line was further characterized.

Characterization. The pRNS-1-1 cells had the typical polygonal arrangement of epithelial cells (figure 3) but were less polygonal than the original 8891 parent cells.

Using immunocytochemistry, we examined the expression of specific epithelial cytokeratins 5 and 8, prostate-specific antigen (PSA), SV40 T-antigen, and *P53*, a tumor suppressor protein (table I). The pRNS-1-1 cells expressed cytokeratin 8, a simple epithelial cell marker, and also cytokeratin 5, which is known to be exhibited intensely in adult prostatic cells but not in the fetal prostatic cells [15]. Intense nuclear staining was observed in pRNS-1-1 cells with antibody against SV40 T-antigen but not in normal 8891 cells. Similarly, there was a strong reaction with antibody against *p53* in the nuclei of pRNS-1-1 cells but not in 8891 cells. PSA was present in early passages of both normal 8891 and pRNS-1-1 cells, but progressively disappeared and was not present at later passages of the pRNS-1-1 cells.

When we analyzed biological properties, we observed that pRNS-1-1 cells neither grew in soft agar or produced tumors in 129J nude mice (male or female) even when the mice were inoculated with 10^7 cells (table I).

To confirm that pRNS-1-1 cells contain the SV40 early region genes, Southern blot hybridization analysis was carried out with genomic DNA that had been digested with *Hind*III. Detection of the two expected DNA fragments of 1.2- and 0.5-kb in pRNS-1-1 cells, which were also present in pSV_3*neo* transfected human fibroblast (positive control) but not in a nontransfected human fibroblast clone, is consistent with the integration of the T-antigen coding sequence in the transformants (figure 4).

Table I. Properties of SV40 Ori-transfected pRNS-1 and untransfected (8891) prostatic epithelial cells.

	8891	pRNS-1-1
Life span	<5 passages	>50 passages
Colony formation in soft agar	-	-
Immunochemical markers[a]		
Cytokeratin 8	±	++ (he)
Cytokeratin 5	+++	+++
SV40 T	-	+ (nu)
p53	±	++ (nu)
Prostate-specific antigen	+	-
Karyology		
Modal number	46 (94%)	45-52 (94%)
Marker	none	three (M_1,M_2,M_3)
Chromosome	human male (XY)	human male (XY)
Tumorigenicity in nude mice	ND	-(P-20,32,40)[b]

he = heterogenous; nu = nuclear; ND = not done; M1 = del(2) (q32 q37); M2* = 4 qt; M3* = 32 pt (HSR); [a]Antibody against keratin 8 was used at 1:1000, against keratin 5 at 1:2000, against SV40 T-antigen at 1:10, against P53 at 1:200 and against PSA undiluted; [b]Passage levels of the pRNS-1-1 cells tested.*

* Footnote: The description of markers may be a matter of discussion according to the current standards in cytogenetics.

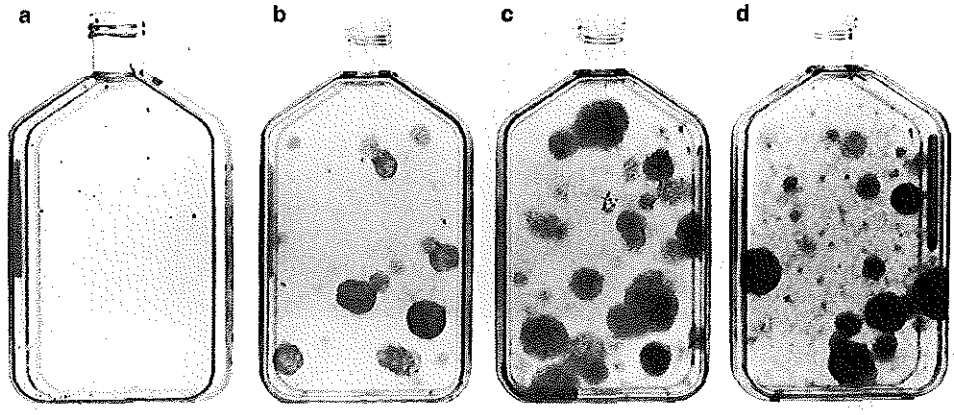

Figure 1. Transformation of normal adult human prostatic epithelial cells by infection with the Ad12-SV40 virus or by transfection with SV40 early region genes. Cultures of 8891 cells were fixed and stained 5 wks after infection or transfection. No colonies were visible in control cells (a), but tightly packed colonies of variable size were seen in the Ad12-SV40 virus infected (b), pRNS-1 (c) and pRSV-T transfected cells (d).

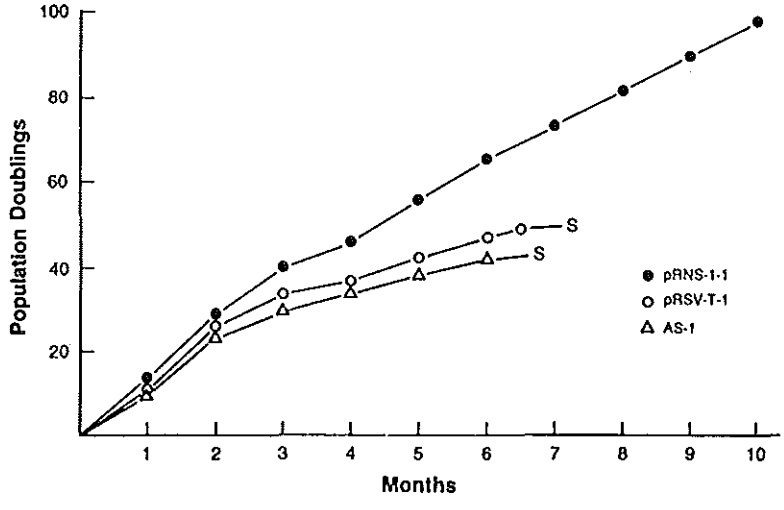

Figure 2. Growth of three transformed adult human prostatic epithelial cells. Each line was isolated from a single colony. The population doublings were not corrected for plating efficiency at each passage. S = senescence. AS = Ad12-SV40.

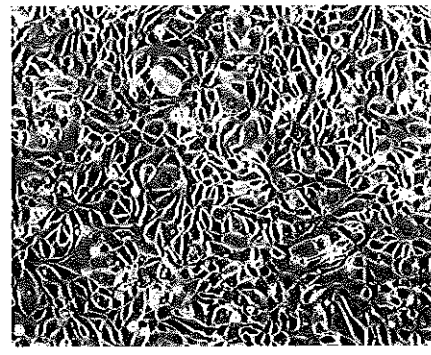

Figure 3. Morphology of pRNS-1-1 transfected adult human prostatic epithelial cell line. x 300.

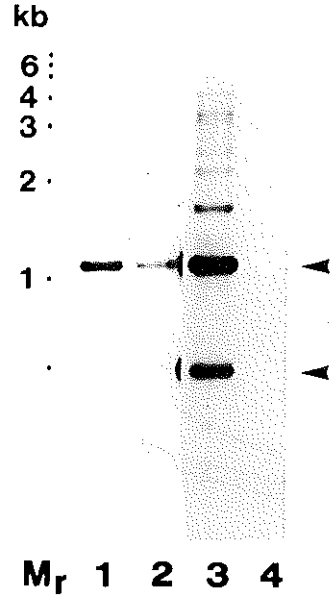

Figure 4. Southern blot hybridization analysis of HindIII-digested genomic DNA from transfected adult human prostatic epithelial (8891) cells, using an SV40 early region DNA probe that spans the T-antigen coding region. The arrowheads indicate the two expected DNA fragments of 1.2- and 0.5-kb which confirm the presence of the T-antigen coding sequence in the appropriate cells. Lane 1, pRNS-1 transfected 8891 cells; Lane 2, pRSV-T transfected 8891 cells; Lane 3, pSV$_2$neo transfected human fibroblasts (positive control); Lane 4, nontransfected human fibroblasts (negative control).

As evidence for the acquisition of the morphological change being the consequence of expression of SV40 functions, we determined by Western immunoblot analysis the presence of the SV40 early region gene product using a hamster antitumor serum. Identification of an approximately 90-kD component in the pRNS-1-1 cells, but not in early passage nontransfected 8891 cells, is consistent with transformation requiring at least the expression of the SV40 T-antigen (figure 5).

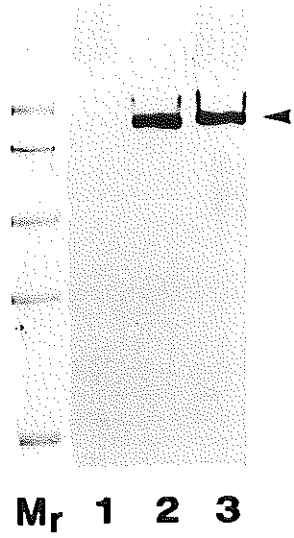

Figure 5. Western immunoblot analysis of SV40 T-antigen in transfected adult human prostatic epithelial (8891) cells. The appropriate cell extracts were analyzed using a hamster antitumor serum which detects the SV40 T-antigen. The molecular weight markers (Mr) used were 97.4, 69, 46, 30 and 14.3 kilodaltons. The arrowhead indicates the 90-kD T-antigen of SV40. Lane 1, early passage untransfected adult human prostatic epithelial cells (8891); Lane 2, pRNS-1-transfected adult human prostatic epithelial (8891) cells; Lane 3, pRSV-T transfected adult human prostatic epithelial (8891) cells.

Chromosomal analysis. Chromosomal study of 8891 and the pRNS-1-1 line was performed at passage 4 and 12 respectively. Evidence of the human origin of the cells was obtained by isoenzyme analysis and cell membrane, species-specific immunofluorescence. Moreover, both cells showed human karyotypes with a Y chromosome. The 8891 cells at passage 4 were essentially normal diploid; 94% of the chromosome counts were diploid (2n=46). There were some random cytogenetic abnormalities noted including monosomy 16 and monosomy 5. At passage 12, pRSN-1-1 cells showed three marker chromosomes and were pseudodiploid with most chromosomal counts (94%) in the diploid range (table I, figure 6).

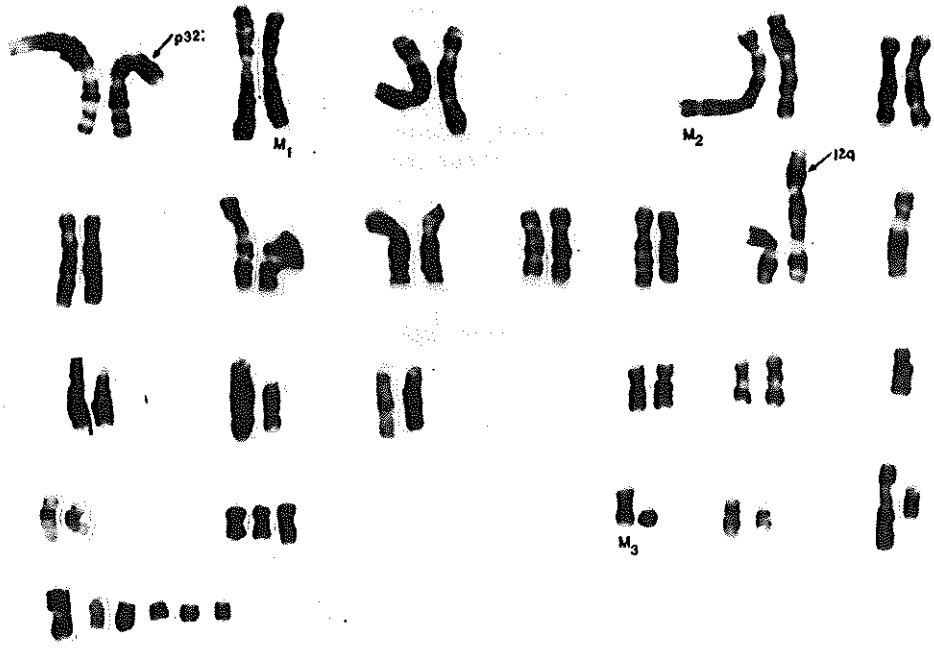

Figure 6. Karyotype of pRNS-1-1 line at passage 12. Three marker chromosomes (M1, M2, M3) and abnormal no. 1 and no. 11 chromosomes are shown.

Response to growth-stimulatory and -inhibitory factors. Normal prostatic epithelial cells require several growth factors for optimal proliferation in serum-free medium. Among these are epidermal growth factor, insulin or insulin-like growth factors, and bovine pituitary extract [16,17]. pRNS-1-1 cells were tested for their response to these factors using assays similar to those for which the growth requirements of normal cells had been determined. Figure 7 shows that pRNS-1-1 cells also require epidermal growth factor, insulin-like growth factor, and pituitary extract for proliferation in serum-free medium, and that each is a potent mitogen. Apparently transformation did not alter the response of pRNS-1-1 cells to factors which are mitogenic for normal prostatic epithelial cells.

Figure 7. (next page) Response of pRNS-1-1 cells to growth stimulatory factors. pRNS-1-1 cells (closed circles) were inoculated at 1000 cells per dish into collagencoated dishes containing 5 ml of medium pr dish. After 14 days of incubation, growth was measured with an Artek image analyzer. Growth in control medium (MCDB 105 with 10 ng/ml of cholera toxin, 10 ng/ml of epidermal growth factor, 10 ug/ml of bovine pituitary extract, 0.1 mM phosphoethanolamine, 1 ug/ml of hydrocortisone, $3x10^{-8}$ M selenous acid, 4 ug/ml of insulin, $2.3xl0^{6}$ M alpha-tocopherol, and $3xl0^{-11}$ M retinoic acid) was set as 100% in each expriment. Epidermal growth factor, insulin, or bovine pituitary extract were individually deleted

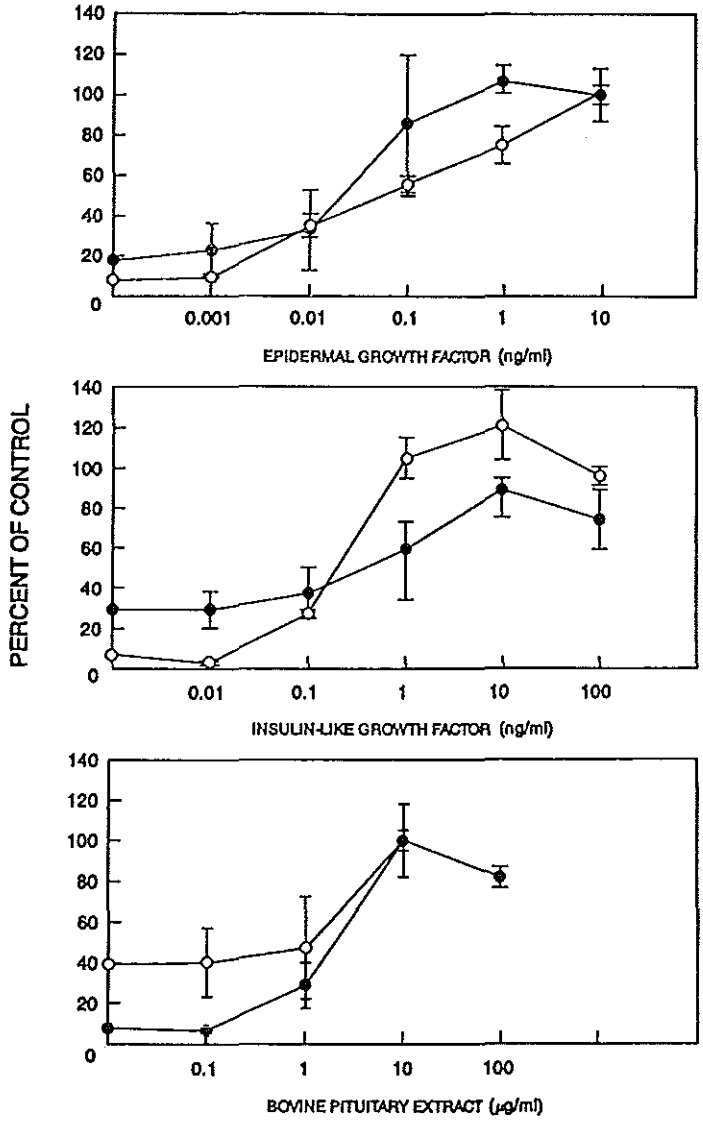

Figure 7. (continued page 26) and replaced at the indicated concentrations (insulin-like growth factor-I was used to replace insulin). Duplicate dishes were tested in each expriment, and each point represents the average of duplicate expriments ± S.E.M. Representative responses of normal prostatic epithelial cells (open circles), tested under the same conditions except that 200 cells were inoculated per dish, are shown in each panel for comparative purposes.

Growth-inhibitory factors for normal prostatic epithelial cells have also been identified. These include retinoic acid [18], 1,25 $(OH)_2$ vitamin D_3 [19], tumor necrosis factor-α [20], and transforming growth factor-ß [16]. The response of pRNS-1-1 cells to retinoic acid was similar to that of normal cells in that retinoic acid at doses of 0.1 ng/ml or higher was growth-inhibitory. However, the growth-stimulatory effect of 0.01 ng/ml of retinoic acid on normal cells was not observed on pRNS-1-1 cells, and retinoic acid at this concentration remained growth-inhibitory for pRNS-1-1 cells (figure 8). Growth of pRNS-1-1 cells was also inhibited by transforming growth factor-ß, but not to the extent of inhibition of normal cells (figure 8). Whereas the growth of normal prostatic epithelial cells was completely inhibited by 1 ng/ml of transforming growth factor-ß, growth of pRNS-1-1 was only inhibited by about 60% with this level of transforming growth factor-ß, and growth was not further inhibited even with 10 ng/ml of transforming growth factor-ß.

The response of pRNS-1-1 cells to tumor necrosis factor-α and to 1,25 $(OH)_2$ vitamin D_3 was also very different from that of normal cells. The growth of normal prostatic epithelial cells was half-maximally inhibited by approximately 1 ng/ml of tumor necrosis factor-α and 1 nM of 1,25 $(OH)_2$ vitamin D_3. In contrast, growth of pRNS-1-1 cells was not at all inhibited even by 10-fold higher levels of either of these factors (figure 8). pRNS-1-1 cells appear to have become resistant to tumor necrosis factor-α and 1,25 $(OH)_2$ vitamin D_3 as a result of transformation.

Discussion

Prostate cancer, the most common adult male malignancy, is a multistep disease with a typical onset late in life and is a serious medical problem which will only become more serious in the future [2]. According to present research, it is generally thought that BPH, the most prevalent form of benign prostate disease, does not undergo direct transformation into clinically manifest prostate cancer [21]. However, little is known about factors in the progression from the early stages of prostate cancer to the clinically manifest form. The main objective of this study was to develop and characterize adult normal human prostatic epithelial cell models suitable for investigating the etiology and progression of prostate cancer. Increased lifespan of human adult prostatic epithelial cells was achieved by a variety of techniques for introducing SV40 T-antigen into the cells, namely infection with Ad12-SV40 virus or transfection by polybrene-induced DNA transfer with either of two plasmids (pRSV-T and pRNS-1) containing the SV40 early region genes. In addition, normal adult human prostatic epithelial cells transfected with a plasmid (pRNS-1) containing an origin-defective SV40 genome and a plasmid carrying the neomycin resistance gene became stably immortalized and were further characterized.

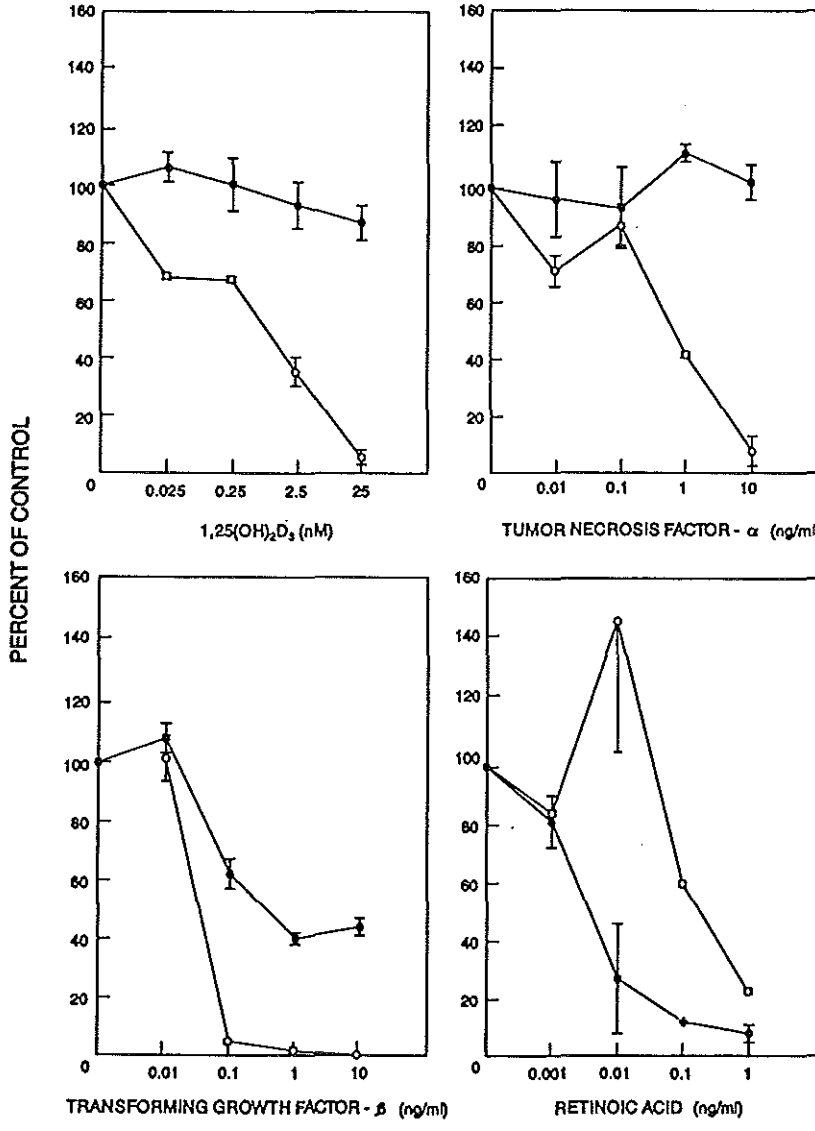

Figure 8. Response of pRNS-1-1 cells to growth-inhibitory factors. Growth responses of pRNS-1-1 cells (closed circles) and normal prostatic epithelial cells (open circles) were tested as described in figure 7. Control medium was described in figure 7 and 1,25 $(OH)_2$ vitamin D_3 , tumor necrosis factor-α, transforming growth factor-ß, and all-trans retinoic acid were added at the indicated concentrations.

Previously attempts to immortalize adult human prostate epithelial cells have been difficult [7]. Strontium phosphate transfection has been successfully used to immortalize neonatal prostatic epithelial cells using a plasmid containing the SV40 early region genes [15] but has failed for adult cells [7]. Immortalization of human adult prostatic epithelial cells has been achieved by using liposomes with encapsulated DNA [7]. As described we have also succeeded in immortalizing adult human epithelial cells by polybrene induced DNA transfection of a plasmid containing an origin defective SV40 genome together with a plasmid carrying the neomycin resistance gene. Polybrene, in conjunction with dimethyl sulfoxide (DMSO) shock, has been shown to increase the frequency of DNA transfection of mammalian cells including human epidermal keratinocytes, as compared with the frequency obtained with calcium phosphate-mediated transfection [12,22,23]. It has recently been shown that three of the transfection methods tested were adequate for transfection of primary human keratinocytes: calcium phosphate co-precipitation, lipofection, and polybrene-mediated transfection. Of the three, the polybrene method was found to be the best since it was effective both in subconfluent and in post-confluent cultures, but lipofection was expensive and calcium in the co-precipitation procedure induced keratinocytes to differentiate [24].

Normal human cells in culture have a limited lifespan beyond which the cells cease to proliferate, enlarge in size, and undergo cellular senescence [25]. Immortalization of human cells has been achieved in cells infected or transfected with specific viral genes; however, it occurs infrequently, and only after continuous cell passaging [6,26]. While immortality is not sufficient for neoplastic transformation, most immortalized cells have an increased sensitivity for further carcinogen-induced neoplastic progression. Therefore, escape from senescence can be a preneoplastic change that predisposes a cell to neoplastic conversion. Although immortalization of human cells is an initial key step in neoplastic progression, the mechanisms underlying this event are poorly understood [26]. In contrast to rodent cells, normal human cells in culture have generally proven difficult to immortalize. As we have shown, different cell types require different conditions and transforming agents to achieve a useful immortalized cell line.

Growth regulatory mechanisms are often altered by transformation with oncogenic viruses. In the case of pRNS-1-1 cells, the ability of the growth of normal prostatic epithelial cells to be regulated by the mitogens epidermal growth factor, insulin-like growth factor, and pituitary extract was retained after transformation. Our results do not rule out the possibility, however, that pRNS-1-1 cells make autocrine growth stimulatory factors, because the growth assays were performed under conditions of low-cell density in order to eliminate effects of autocrine factors. Whether the production of autocrine factors was a consequence of transformation, though, could only be determined after a careful evaluation of the expression of autocrine factors by normal prostatic epithelial cells. Secretion of autocrine factors is not confined to transformed or malignant cells, and we have found that normal prostatic epithelial cells secrete and respond to epidermal growth factor-like autocrine factors (Nickas and Peehl, submitted).

In contrast to the absence of dramatic changes in the response of pRNS-1-1 cells to growth-stimulatory factors, responses to several growth-inhibitory factors were appreciably altered. Although normal prostatic epithelial cells are inhibited by tumor necrosis factor-α, pRNS-1-1 cells were unresponsive. A survey by Spriggs *et al.*[27] of human epithelial tumor cell lines revealed that 11 of 14 were resistant to tumor necrosis factor, so resistance may be rather common among transformed or malignant cell lines. On the other hand, the pattern of response of prostate cells to tumor necrosis factor is very different from that of cells from a somewhat similar organ, the breast. Normal breast cells are resistant to tumor necrosis factor, whereas breast cancer cell lines are sensitive [28]. So responsiveness to tumor necrosis factor may be very tissue-specific and the role of transformation in altering tumor necrosis factor responsiveness must be considered with that possibility in mind.

pRNS-1-1 cells were also unresponsive to 1,25 $(OH)_2$vitamin D_3. We have recently demonstrated the presence of vitamin D receptors on normal prostatic epithelial cells and the potent growth-inhibitory effects of 1,25 $(OH)_2$ vitamin D_3 on these cells [19]. We plan to determine whether lack of response is simply due to loss of the vitamin D receptor or to some perturbation in the vitamin D signaling mechanism. Whatever the mechanism, loss of response to a physiologic growth inhibitor such as vitamin D could have important consequences for progression to a malignant phenotype.

Similar to most other normal epithelial cells, normal prostatic epithelial cells are inhibited by transforming growth factor-ß. The growth of pRNS-1-1 cells was partially inhibited by 1 ng/ml of transforming growth factor-ß, a concentration which completely inhibits the growth of normal prostatic cells, but increasing the concentration of transforming growth factor-ß did not further decrease growth. A possible explanation for such a response could be the presence of a resistant subpopulation. Prostate cancer cell lines have also exhibited a rather unusual response to transforming growth factor-ß. The cell lines PC-3 and DU-145 are initially inhibited by transforming growth factor-ß, but their rates of growth eventually return to normal in the continuous presence of transforming growth factor-ß [29]. Further information regarding the mechanism of action of transforming growth factor-ß will be required in order to understand the nature of the response of prostate cell lines.

Of the growth-inhibitory factors tested, retinoic acid was the only one to which pRNS-1-1 cells appeared to retain normal responsiveness. Our previous studies of retinoic acid and normal prostatic cells indicated that retinoic acid is an important regulator of growth as well as differentiation [18]. Since growth of pRNS-1-1 cells was inhibited by retinoic acid, our future studies will aim to determine whether the differentiation which accompanies growth inhibition of normal cells by retinoic acid is retained by pRNS-1-1 cells.

Acknowledgements

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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 associated with progression to malignancy [12,13]. An association of HPV with prostate cancer has also been suggested. By the application of PCR, "high risk" HPV DNAs were detected in

prostate tissues [14,15]. Furthermore, the detected HPV DNAs in these tissues were transcriptionally active [16], although not predominantly in cancers, suggesting that the prostate could serve as a reservoir for HPV. Moreover, in a study of Japanese prostate carcinoma specimens, a high incidence of HPV type 18, as determined by PCR, correlated with both grade of differentiation by the Gleason score and clinical stage, with frequent detection (89%) of HPV DNA even in samples from bone metastases [5]. Thus far, however, a definitive role for HPV as an active effector of prostatic carcinoma remains to be further established.

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 PFMR-4A (25) supplemented with cholera toxin (10 ng/ml), epidermal growth factor (10 ng/ml), bovine pituitary extract (10 *ug*/ml),

phosphoethanolamine (0.1 mM), hydrocortisone (1 *ug*/ml), selenous acid (30 nM), insulin (4 ug/ml), alpha-tocopherol (2.3 uM), retinoic acid (0.03 nM), and gentamicin (100 *ug*/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 ampules 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 PFMR-4A. After reaching semiconfluency, 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 ug of lipofectin (BRL Life Technologies, Gaithersburg, MD) with 1 to 10 *ug* of plasmid DNA at room temperature for 15 minutes. The liposome mixture was added to each 100-mm dish containing cells and 3 ml of fresh medium. After incubation for 8 to 24 hours, 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 *ug* 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 $MgCl_2$; 200 *uM* 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 DNA was initially denatured for 4 minutes at 94°C followed by 40 cycles of amplification with primer annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes.

Analysis of amplified DNA. The amplification products were separated through a 2% agarose gel and stained with ethidium bromide. A 100-base pair molecular weight ladder was included on the gel for reference. DNA was electroblotted from the gel to a nylon membrane. Hybridization was performed at 37°C in 5x SSC, 5x Denhardt's solution, 0.5% SDS, and 100 ug/ml denatured herring sperm DNA, with 10^6 cpm of 32P-end labeled probe (5'CAGACTCTGTGTATGGAGACAC) per ml of hybridization mix. The membrane was washed twice for 15 minutes each time at 37°C in 2x SSC/0.1% SDS and once for 15 minutes at 56°C m 1x SSC/0.1% SDS. The blot was exposed to Kodak X-OMAT film with intensifying screens for 4 hours at -80°C.

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 10^4 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 subcutaneously 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. Nonspecific 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 *ug* of HPV type 18 plasmid DNA for 8 hours. 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 $1/10^6$ cells originally exposed to the transfection protocol. Of 6 colonies that were isolated, 2 continued to proliferate and became established cell lines. The line PZ-HPV-7 was derived from normal epithelial cells from the peripheral zone of the prostate, and CA-HPV-10 was derived from cells cultured from an adenocarcinoma of Gleason Grade 4/4. The two lines were morphologically distinct from each other and from the parental cells (figure 1).

PCR Analysis. HPV type 18 DNA sequences amplified from genomic DNA of the transformed cell lines are shown in Figure 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-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 (figure 2).

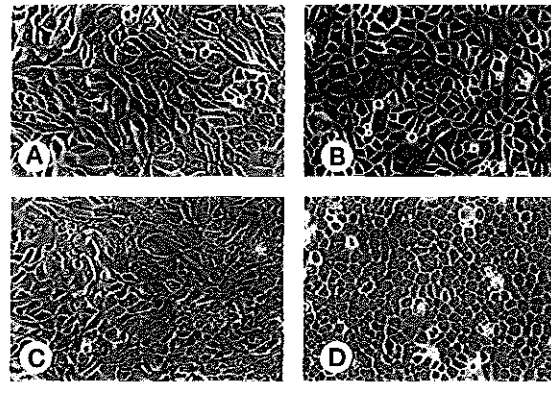

Figure 1. Morphology of parental and transformed cells. Normal prostatic epithelial cells (A), HPV-immortalized normal cells (PZ-HPV-7) (B), cancer-derived prostatic epithelial cells (C), and HPV-immortalized cancer-derived cells (CA-HPV-10) (D), were grown to confluency and photographed. x 200.

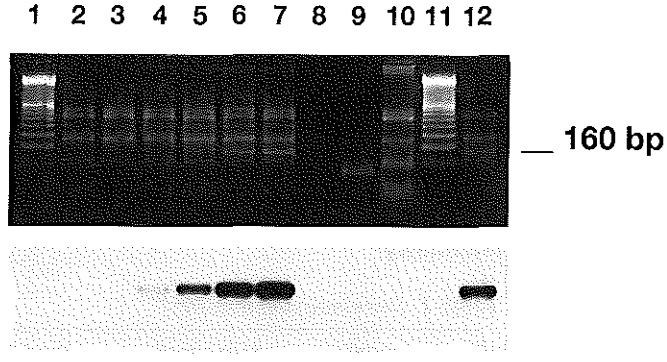

Figure 2. HPV DNA in transformed cells. (Top) DNA from each cell population was amplified with HPV 18-specific primers by PCR as described in "Materials and Methods." Amplification products were separated in a gel and photographed after staining with ethidium bromide. (Bottom) A blot was prepared from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

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 hours 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 (figure 3).

Table I. Immunocytochemistry

Table 1 *Immunocytochemistry*[a]

Cells	902[b]	903[c]	HPV-E6[d]	Tag[e]	PSA[f]
Normal	+	+	–	–	+
Cancer-derived	+	+	–	–	+
PZ-HPV-7	+	+	+	–	–
CA-HPV-10	+	+	+	–	–

[a] Immunocytochemistry 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 –.

[b] Antibody 902, against keratin 8, was used at 1:1000.

[c] Antibody 903, against keratin 5, was used at 1:2000.

[d] Antibody against the E6 protein of HPV was used at 1:500.

[e] Antibody DP02, against large T antigen of SV40, was used at 1:10.

[f] Antibody against PSA was used undiluted.

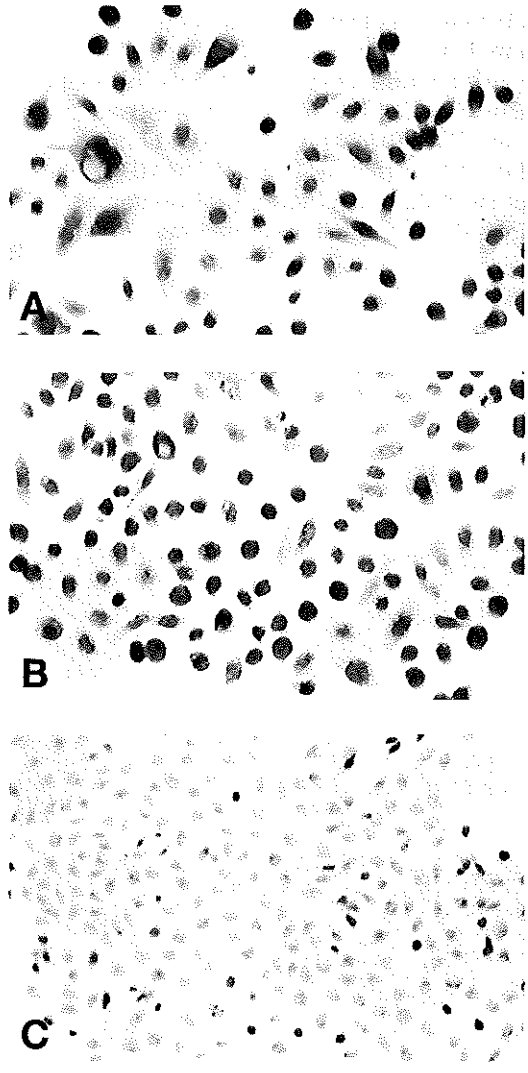

Figure 3. Immunocytochemical detection of HPV E6-oncoprotein in transformed cells. PZ-HPV-7 (top) CA-HPV-10 (middle) and pRNS-1-1, a line of SV40 virus-transformed cells (bottom) were labeled with antibody (1:500) against the E6 protein of HPV 18 by an indirect immunoperoxidase technique. Positive labeling was seen in the two HPV transformed cell lines but not in the SV40 virus-transformed line. x 800.

Tumorigenicity. Subcutaneous injection 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 (figure 4). CA-HPV-10 formed lumps that were less well-differentiated but still squamous (figure 4). Injection of both parental cell strains under similar conditions did not result in formation of lumps.

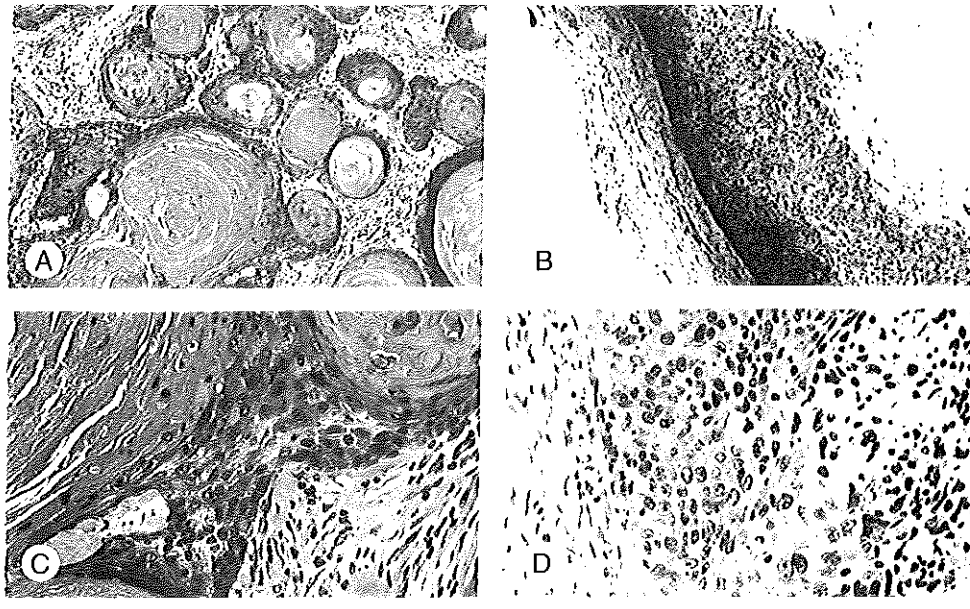

Figure 4. Histology of injected cells. CA-HPV-10 (right) and PZ-HPV-7 (left) cells were injected s.c. into nude mice. Small lumps formed which were removed after 3 months, fixed, and sectioned. Examination of hematoxylin and eosin-stained sections revealed keratinizing, squamous nodules. Top, X 100; bottom, X 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 PZHPV-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 subcutaneously 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 uroepithelial cells by Reznikoff *et al.* [35]. Normal uroepithelial 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.

Acknowledgements

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Specific cytogenetic aberrations in two novel human prostatic cell lines immortalized by human papillomavirus type 18 DNA

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Abstract

Using chromosome banding and fluorescence in situ hybridization (FISH) with painting probes, sequential cytogenetic analysis was performed of two novel prostate cell lines, PZ-HPV-7 and CA-HPV-10, established by human papillomavirus (HPV) 18 DNA transformation.

PZ-HPV-7 originates from a normal diploid prostate epithelial cell strain. PZ-HPV-7 progressed from an initial diploid to a hypertetraploid chromosome number with a relative gain of chromosomes 5 and 20 (7 to 8 copies each). Structural changes were limited; 3p- (2 copies), 3q- (1 copy), and possibly a der (16p;12q). CA-HPV-10 originates from an epithelial cell strain derived from a high grade human prostate cancer specimen, which showed several karyotypic abnormalities including an extra Y chromosome and double minutes (dmin). In early passage, the karyotype of CA-HPV-10 appeared unstable with a decreasing number of cells exhibiting dmin. In late passage, the dmin were replaced by a large homogeneously staining region (hsr) on 9p+ marker. The hsr was shown by FISH to be of chromosome 1 origin. The modal number was mainly hypertriploid (72, range 69 to 75). Loss of Y was remarkable (0 to 1 copy). Consistent markers included 2 copies each of del(1)(q12q31) and der(9)t(1;9)(?;p22), and 1 der(11)t(4;11) (?,q21). HPV type 18 genomic integration sites were identified on 1p for PZ-HPV-7 and on the 9p+ marker for CA-HPV-10.

In conclusion, both PZ-HPV-7 and CA-HPV-10 showed clonal cytogenetic changes. These two cell lines constitute a novel in vitro model to study the mechanisms involved in human prostate carcinogenesis.

Introduction

During the last decade in Western countries, the apparent incidence of prostate cancer has shown a tremendous rise mainly because of improved diagnostic modalities, such as the tumor marker prostate-specific antigen [1]. In comparison, the age adjusted cancer-specific mortality has not increased significantly, suggesting that prostate cancers may be diagnosed in the latent stage and that the risk of over treatment should be considered [2]. Nevertheless, prostate carcinoma is one of the leading causes of cancer mortality in the male. Therefore, because of the uncertain behavior and heterogeneous nature of the disease, the establishment of methods to distinguish clinically insignificant from potentially lethal cancers has become the future challenge for prostate cancer research. The development of human models for prostate carcinogenesis may allow for the identification of specific tumorigenic

features. Even with continuous progress [3], the possibilities to study primary cells from the human prostate epithelium are limited by their short lifespan in culture. In search for an in vitro model of human prostate carcinogenesis, we used HPV type 18 DNA to immortalize epithelial cell cultures. Two cell lines were established: PZ-HPV-7, epithelial precursor cell derived from normal prostatic peripheral zone tissue, and CA-HPV-10, epithelial precursor cell derived from a high grade adenocarcinoma of the prostate [4].

Characteristic cytogenetic aberrations may shed some light on tumor specific mechanisms of oncogenesis. During the last decade, several chromosomal abnormalities have been associated with prostate adenocarcinoma. The findings of specific karyotypic features of adenocarcinoma specimens have been significant. Chromosomal gain and loss as well as structural anomalies reported in prostate cancer include almost all human chromosomes i.e., 1, 2, 3, 5, 6, 7, 8, 9, 10, 13, 14, 15, 16, 17, 18, 22, X and Y [5-18]. Of these chromosomal anomalies some seem to predominate. Gains of chromosome X and 7, and 8q may be involved in prostate cancer progression [11-17]. Among the most commonly deleted regions implicated in prostate cancer we find 8p [6-10,13-17], first suggested in 1988 by König et al. [19], 10q [5-8,13,17], 16q [5,6,13,17], and also anomalies of the Y chromosome [7,8,10,14-16]. Furthermore, linkage analysis of 91 high-risk prostate cancer families recently provided strong evidence of a major prostate cancer supsceptibility locus on chromosome 1 [18]. Interestingly, one of the newly established HPV transformed cell lines, CA-HPV-10, was derived from a human prostate cancer cell strain with several karyotypic abnormalities, including an extra Y chromosome and double minutes (dmin) [7].

In this study, we report on the sequential analysis of both prostate cell lines and a detailed characterization of CA-HPV-10 marker chromosomes using whole chromosome paints and site specific probes. The HPV integration sites were also investigated using fluorescence *in situ* hybridization (FISH) methodology.

Materials and Methods

Cell lines. As reported previously [4] plasmids containing the full genome of HPV type 18 incubated with lipofectin (BRL Life Technologies, Gaithersburg, MD) were used for immortalization of two primary epithelial human prostate cell strains LJpz and HSca. The two established cell lines, PZ-HPV-7, precursor LJpz derived from normal prostatic peripheral zone epithelium, and CA-HPV-10, precursor HSca derived from a high grade (Gleason grade 4+4) prostatic adenocarcinoma specimen, were

analyzed at different passages ranging from early (15 and 14) to late (99 and 94) numbers. Keratinocyte serum free media KSFM (Gibco, Grand Island, NY), added with bovine pituitary extract (50 ug/ml), epidermal growth factor (5 ng/ml) and antibiotics was required for optimal growth.

Cytogenetics. Monolayers of in log phase growing cultures were incubated with colcemid (0.015 μg/ml) for 30 min to one hour. Longer incubation was required when limited numbers of rounded or mitotic cells were observed. Subsequently, metaphase cells were harvested by trypsinization, swollen in KCl-EGTA (0.075M), fixed in methanol:acetic acid (3:1), and spread on slides [19]. Chromosomes were identified by the R-banding and Q-banding technique using acridine-orange and atebrine, respectively. At least 20 metaphases from each cell line were karyotyped.

Fluorescence in situ hybridization. The DNA probes were labeled with Biotin-16-dUTP by standard nick translation, precipitated, and resuspended in hybridization mixture (50% formamide, 10% dextran sulfate in 2 x SSCP) with 50 ug salmon sperm DNA and 50 ug yeast t-RNA when competitive hybridization was necessary. The probe concentration was as follows: 0.5 ng/ul for centromeric probes, 7.5 ng/ul for the HPV probe, and 10 ng/ul for chromosome-specific libraries. After probe denaturation (4 minutes at 72°C), the repeat sequences were allowed to preanneal with 10 ug Cot-1 DNA for 1 hour at 37°C. Metaphase spreads on glass were pretreated with RNAse and 0.2% pepsin (0.01 N HCl), postfixed, and denatured at 72°C for 2.5 minutes in 70% formamide. Probes were hybridized overnight at 37°C in a humified box. After washing, the hybridization sites were visualised by immunochemistry using two layers of FITC in three steps with avidin-FITC, biotinylated goat-anti-avidin, and avidin-FITC. Results were observed in fluorescence using DAPI banding counterstaining and sometimes cohybridization with a known probe relevant for chromosome identification.

Probes. Various probes were used, single or in combination, in single or double color experiments.

HPV type 18: The full plasmid of HPV type 18 DNA cloned into pB322 (kindly received from Dr. E-M. de Villiers, Heidelberg, Germany) was used as a probe.

Whole chromosome paint: Chromosome specific libraries pBS 1 to 22, X and Y, were prepared and made available to us by J. Gray (University of California, San Francisco, USA). Separate experiments were performed using a commercially available chromosome 11 probe (Cambio LTD) as well as chromosomes 4 and 9 probes prepared by degenerate oligonucleotide-primed-polymerase chain reaction (DOP-PCR)- amplification [20].

Centromere probes: Probe PUC1.77 [21] for chromosome 1, pYAM11-39 [22] for 4, D8Z2 [23] for 8, pHUR98 [24] for 9, pLC11A [25] for 11, pHUR195 [24] for 16, L1.84 [26] for 18 and p3.4 [27] for 20 were used.

Gene loci specific probes: Site or gene loci- specific probe p1.79 (received from H. van Dekken, Rotterdam, The Netherlands) was used for identification of 1p36; oct 7a (received from R. de Zwart, Rotterdam, The Netherlands) was used for 1p32/2q23; ERCC3 [28] was used for 2q; 10.62/10.63 *P16* tumorsuppressor gene [29] was used for 9p21; and Cos-ABL-18 [30] was used for 9q34.

Results

Cytogenetic analysis. The PZ-HPV-7 cell line, precursor originating from a normal human prostatic epithelial cell strain, was studied at passage 15, 38, and 99. PZ-HPV-7 progressed from an initial diploid chromosome number at passage 15 and 38 to a modal number of 106 chromosomes, range 103 to 108. Analysis showed 3 to 5 copies of all chromosomes except X and Y (2 copies each) and chromosomes 5 and 20 (7 to 8 copies each). There were only a few structural changes: 3p- (M1, 2 copies), 3q- (M2, 1 copy) and a third marker possibly a der(16p;12q)(M3). In figure 1A the full karyotype is shown representing the late passage findings as described above.

The CA-HPV-10 parental cell strain HSca originated from a core of pure Gleason grade 4+4 prostatic adenocarcinoma obtained from a radical prostatectomy specimen. The previously reported karyotype of HSca [7] showed several abnormalities including an extra Y chromosome and dmin. At passages 14 and 38, the karyotypes of cell line CA-HPV-10 varied from hypodiploid to hypotetraploid with a slowly rising mode in the hypertriploid range. The karyotype was unstable with mainly telomeric associations, di- and tricentric chromosomes, isochromosomes, and a decreasing number of cells exhibiting dmin. At passage 89 the karyotype was mainly hypertriploid (modal number 72, range 69 to 75) with a few cells hyperhexaploid and hyper-12 ploid. Consistent markers included two copies each of del(1)(q12q31)(m1) and der(9)t(1;9)(?;p22)(m2) with probably a large hsr, one der (11)t(4;11)(?;q21)(m3) and one structurally abnormal der(16)(m4). Chromosomes 20 and 5 were overrepresented with an average of 4 and 5 copies, respectively. Loss of Y (0 to 1 copies), as well as the relative loss of chromosomes 6, 13 (2 to 3 copies), and 22 (2 copies) was remarkable. Figure 1B shows the full karyotype of the CA-HPV-10 cell line in late passage as described above.

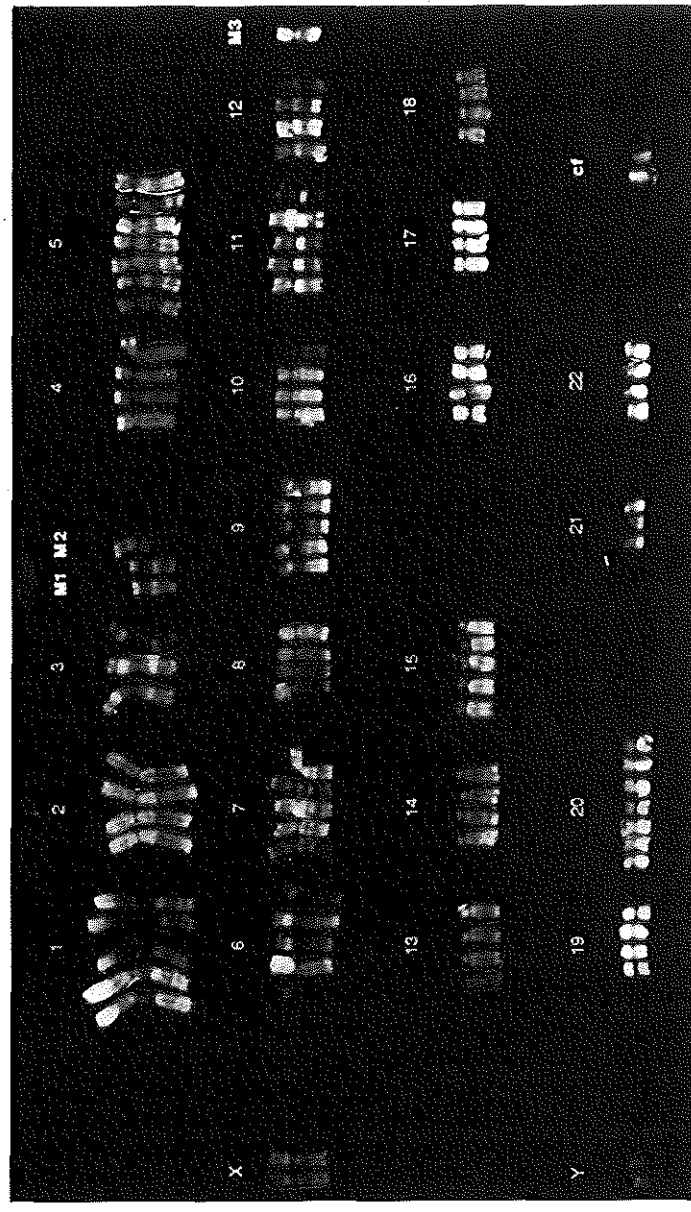

Figure 1A. Karyotype by R-banding of cell line PZ-HPV-7 at passage 99. Small chromosomal markers mainly composed of a centromere are marked as cf.

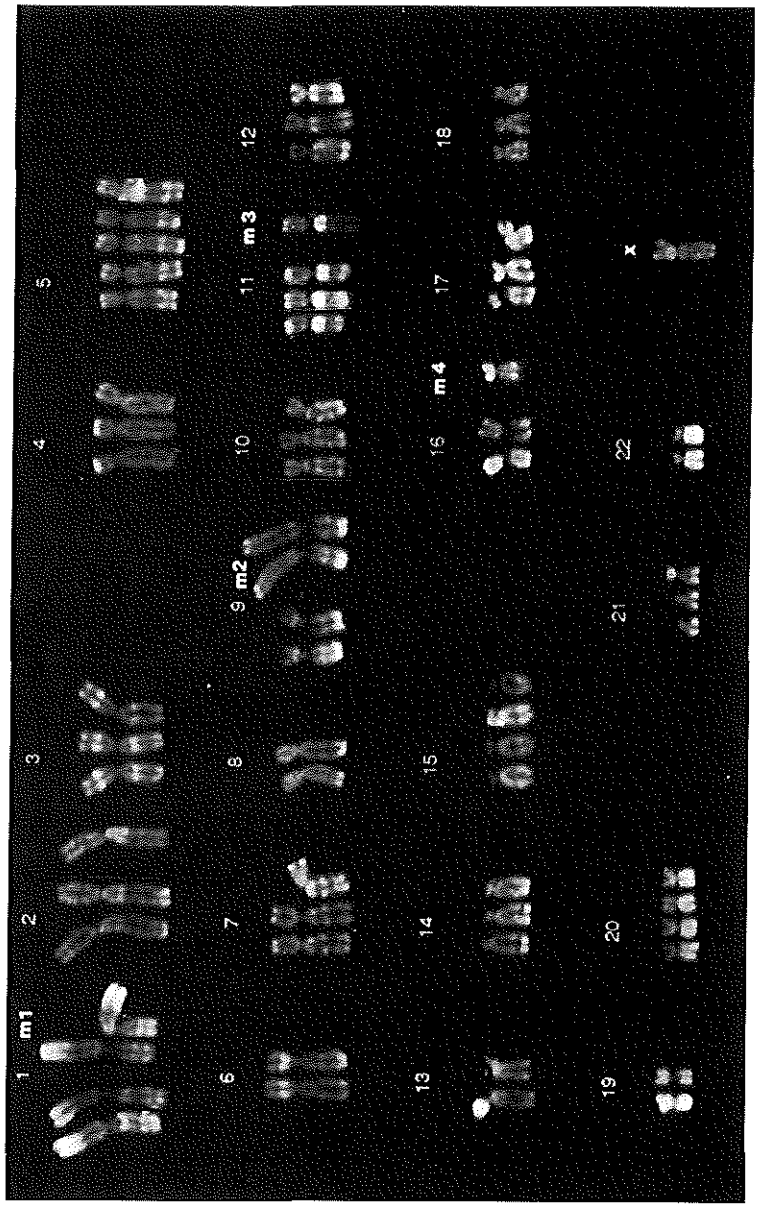

Figure 1B. Karyotype by R-banding of cell line CA-HPV-10 at passage 89.

For identification of CA-HPV-10 markers, detailed FISH analyses were performed. In initial experiments DOP-PCR- amplified flow-sorted chromosomes 4 and 9, and a commercially available chromosome 11 probe was used on metaphase spreads of cell line CA-HPV-10 (passage 89) to identify the 11q+ and 9p+ markers. These single chromosomal paints of chromosomes 4, 9, and 11, as well as cohybridization experiments of 4 and 11 revealed a der(11)t(4;11), whereas the hsr on 9p was investigated. Subsequently, for each chromosome, whole libraries were used as paints, sometimes in combination with specific centromeric probes. Eight to 31 metaphases were analyzed. Each paint showed numerical changes consistent with the cytogenetic findings (table I) as well as a number of marker chromosomes (table II). The 9p+ hsr is painted with chromosome 1 specific DNA, which is shown in figure 2 [page 62]. In double staining experiments using loci specific probes for 1p36, 1p32/2q23, 2q, and 9p21, the hsr was studied in detail. However, none of these probes were located on the hsr. The studied probes exhibited an otherwise expected staining pattern on the respective chromosomes 1 and 2, whereas the 9p21 probe for the *P16* tumor suppressor gene revealed no deletion.

Table I. FISH analysis of CA-HPV-10 (passage 89) using painting probes for chromosomes with numerical changes only[a]

Chromosome	Number of copies
Y	0 or 1
X,22	2
6,13	2 or 3
2,7,10,12,14,17,21	3
15	3 or 4
20	4
5	5

[a]Eight metaphases analyzed per probe.

Table II. FISH analysis of CA-HPV-10 (passage 89) using painting probes for chromosomes exhibiting numerical and structural changes

Probe		Chromosomes (partially) painted		
Chromosome Specific Paint	Number of Metaphases Analyzed	Number of Intact Copies	Markers[a]	
1	9	2	del(1)(q12q31) x2	(m1)
			add(9)(p22) x2	(m2)
	7	3	del(1)(q12q31)	(m1)
			add(9)(p22) x2	(m2)
			marker(E size)	
3	15	3 or 4		
	7	3	marker(A size)	
	6	3	marker(D size)	
	3	2 or 3	marker(G size)	
4	8	2 or 3	der(11)t(4;11)(?;q21)	(m3)
8	7	3		
	3	2 or 3	der(16)	(m4)
9	8	2	add(9)(p22) x2	(m2)
11	5	3		
	16	2 or 3	der(11)t(4;11)(?;q21)	(m3)
16	4	3		
	4	2	der(16)	(m4)
18	3	3		
	5	3	small marker	
19	4	2 or 3		
	4	2 or 3	small marker	

[a]Markers are described by their appearance, DAPI banding and (partial) paint. (m1-4) markers indicated in karyotype (Figure 1B).

FISH analysis of HPV 18 incorporation sites. Preparation of HPV 18 DNA specific probes was established using the original purified plasmid. Metaphase chromosomal spreads of Hela cells, containing several genomic copies of integrated HPV 18 DNA, were used as positive controls. At least 20 metaphases of each cell line were analyzed. Using the full HPV 18 plasmid as a probe, we obtained a strong hybridization signal in the HeLa cell line and a weak signal in our cell lines. The weaker signal was probably because of to a low number of copies of the HPV 18 genome in the PZ-HPV-7 and CA-HPV-10 cell lines. Both cell lines showed single HPV type 18 genomic integration sites. For the PZ-HPV-7 cell line, the integration site was identified on 1p, whereas for the CA-HPV-10 cell line, it was identified on the 9p+ marker. The latter is demonstrated in figure 3 [page 62] in a double staining experiment with the centromeric 8 probe.

Discussion

As a model for the study of prostate carcinogenesis, two epithelial cell lines, PZ-HPV-7 and CA-HPV-10, have been established using HPV type 18 DNA. Immortalization with the HPV type 18 genome was attempted because HPV DNA sequences have been detected in human prostatic tissues of different histologies [31-36], and the detected HPV DNAs were shown to be transcriptionally active [37], although the role of HPV in the prostate remains unclear [38]. Another reason to perform HPV transformation studies was to prolong the limited in vitro lifespan of human epithelial prostate cells per se, which had been accomplished previously using SV40 DNA [39-41]. The resulting cytogenetic changes were evaluated in the described analysis. The PZ-HPV-7 cell line, originating from a normal epithelial human prostate cell strain, was diploid and relatively unchanged at early (15 and 36) passage. PZ-HPV-7 progressed in culture to a modal number of 106 chromosomes with mostly numeric changes and only a few structural markers. In contrast, CA-HPV-10 showed a hypertriploid mode in both analyses of early and late passages, with a number of characteristic markers, which were not fully present in the earlier passages. Loss of chromosome 22 was of interest, because it was not observed in HPV immortalized keratinocyte cell lines [42] nor in HPV immortalized cell lines from other organ systems [39-42]. Furthermore, the relative gain of both chromosomes 5 and 20 in the two cell lines, PZ-HPV-7 and CA-HPV-10, was remarkable. This combination has not specifically been described before as a HPV transformation related phenomenon, although a gain of chromosome 20 has been reported [43-45].

After HPV transfection triploidization is rather commonly described as well as structural genomic rearrangements [45,46]. Also, single site HPV DNA integration was observed in cervical carcinoma cell lines [47]. HPV sequences of cervical carcinoma cell lines integrated on normal and abnormal chromosomes. The staining pattern of HPV 16 integration sites showed evidence of replication together with the heterochromatic regions of chromosomes 1, 9 and 16 [48]. Reznikoff *et al.* [44] showed how the initial genetic alterations may direct late genetic changes and how different combinations of genetic alterations work together to block cellular senescence, initiate tumorigenesis, and lead to tumor progression in transitional cell carcinoma. After transfection of HPV type 16 E6, which represents one of the early gene regions involved in transformation, a specific association of 3p loss with chromosome 9 instability was found. These karyotypic changes would seem to fit the general cytogenetic features of the PZ-HPV-7 and CA-HPV-10 cell lines.

Loss of the Y chromosome is of interest in view of recent studies. Gains as well as deletions of the Y chromosome were found in prostatic carcinomas using *in situ* hybridization [10,14-16]. Recently, a loss of the Y chromosome was found in metastatic prostate carcinoma specimen, whereas the primary carcinoma and adjacent prostatic intraepithelial neoplasia lesions did not show this deletion [15]. However, it is unclear whether this loss of the Y chromosome is a relatively nonspecific or possibly significant cytogenetic correlate of progression in prostate cancer. It has been found in other urinary tract tumors [49,50]. Our findings could be significant although cytogenetic phenomena associated with the HPV immortalization should be considered. Relative marked loss of the Y chromosome was found in the cell line CA-HPV-10, which was derived from a high grade adenocarcinoma precursor cell strain HSca, that had an extra Y chromosome before transfection [7]. The PZ-HPV-7 cell line did not show such a prominent loss of the Y chromosome.

In addition to an abnormal karyotype of 47,XY,+Y, the precursor cell strain of CA-HPV-10 contained dmin chromosomes. The presence of both dmin and hsr is suggestive of gene amplification events possibly representing oncogene amplification [51], as has been reported in other solid tumors [52,53]. The cytogenetic evaluation with banding techniques of cell line CA-HPV-10 showed an hsr on chromosome 9p. Instead of a possible complex translocation, additional painting experiments revealed the configuration of a translocation, der(11)t(4;11)(?;q21). The hsr on 9p was studied using whole chromosome paints, which showed its chromosome 1 origin. The determination of HPV integration sites on 1p in the PZ-HPV-7 cell line and 9p+ in chromatin of 1 on the add(9)(p22) in the CA-HPV-10 cell line does raise the question of whether there is a preferential integration site following HPV transfection in prostate epithelial cells. Using several loci specific probes, the 9p+ marker was targeted; however, because none were found to be located on the hsr, the conclusions from these experiments were limited. A probe for the *P16* tumor

suppressor gene located on 9p21 [29] showed no deletion. The question of whether the hsr and dmin were retained in CA-HPV-10 or if they resulted from the HPV immortalization remains open. The formation of hsr as a reflection of DNA amplification has been described after HPV immortalization of keratinocytes [48]. Unfortunately, the original metaphase spreads of the CA-HPV-10 precursor HSca were not available for additional chromosomal painting, but it would seem appropriate to assume that the dmin and hsr of the CA-HPV-10 cell line have been retained from the precursor cell. Efforts to isolate these particular hsr and dmin using flow sorting techniques [20] have been unsuccessful because of the high viscosity of chromosome suspensions from these cells. Nevertheless, further studies for isolation of these aberrant chromosomal parts are warranted, because building a physical map of the involved regions could provide new insights of mechanisms involved in human prostate carcinogenesis.

The cytogenetic evaluation of cell lines PZ-HPV-7 and CA-HPV-10 revealed several karyotypic abnormalities that can be of interest in the pursuit of a multistep in vitro model of human prostate carcinogenesis. The cytogenetic modifications that were identified represent a range of HPV transfection related phenomena within the residual pattern of the original karyotype.

Acknowledgements

We thank Bert J. Eussen for technical assistance with initial painting studies of CA-HPV-10 chromosomes 4, 9 and 11. This work was in part supported by grant IKR 90-12 from the Dutch Cancer Society.

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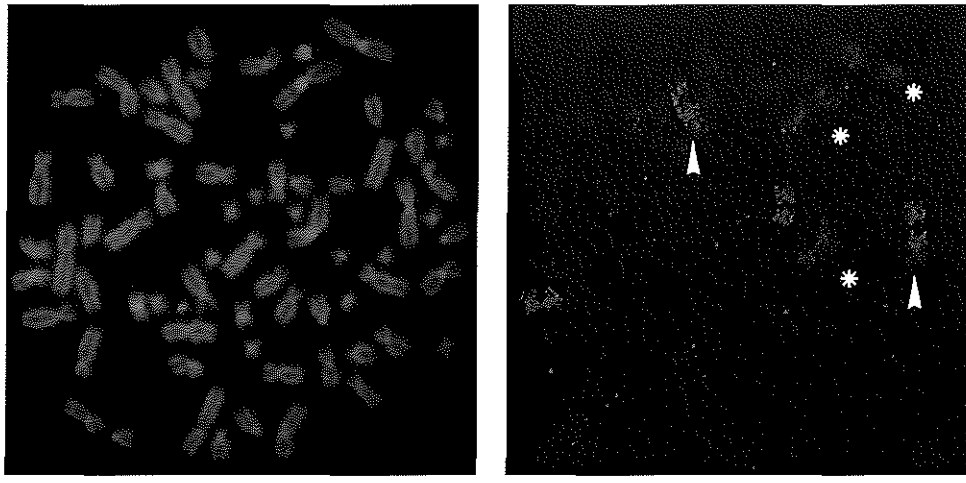

Figure 2. CA-HPV-10 cell line metaphase spread using DAPI counterstaining (left side) for chromosome identification and whole chromosome paints (right side) for 1 (staining red) and 9 (staining green). Two copies of the 9p+ hsr are stained with chromosome 1 specific DNA (arrow head). Three copies of 1 are indicated with an asterix.

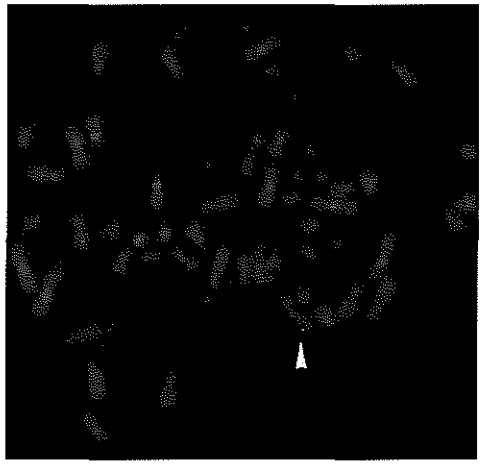

Figure 3. The HPV type 18 DNA integration site (green) of the CA-HPV-10 cell line is shown on 9p+ (arrowhead). In this experiment double staining with a centromere 8 probe was used to distinquish C sized chromosomes 8 and 9.

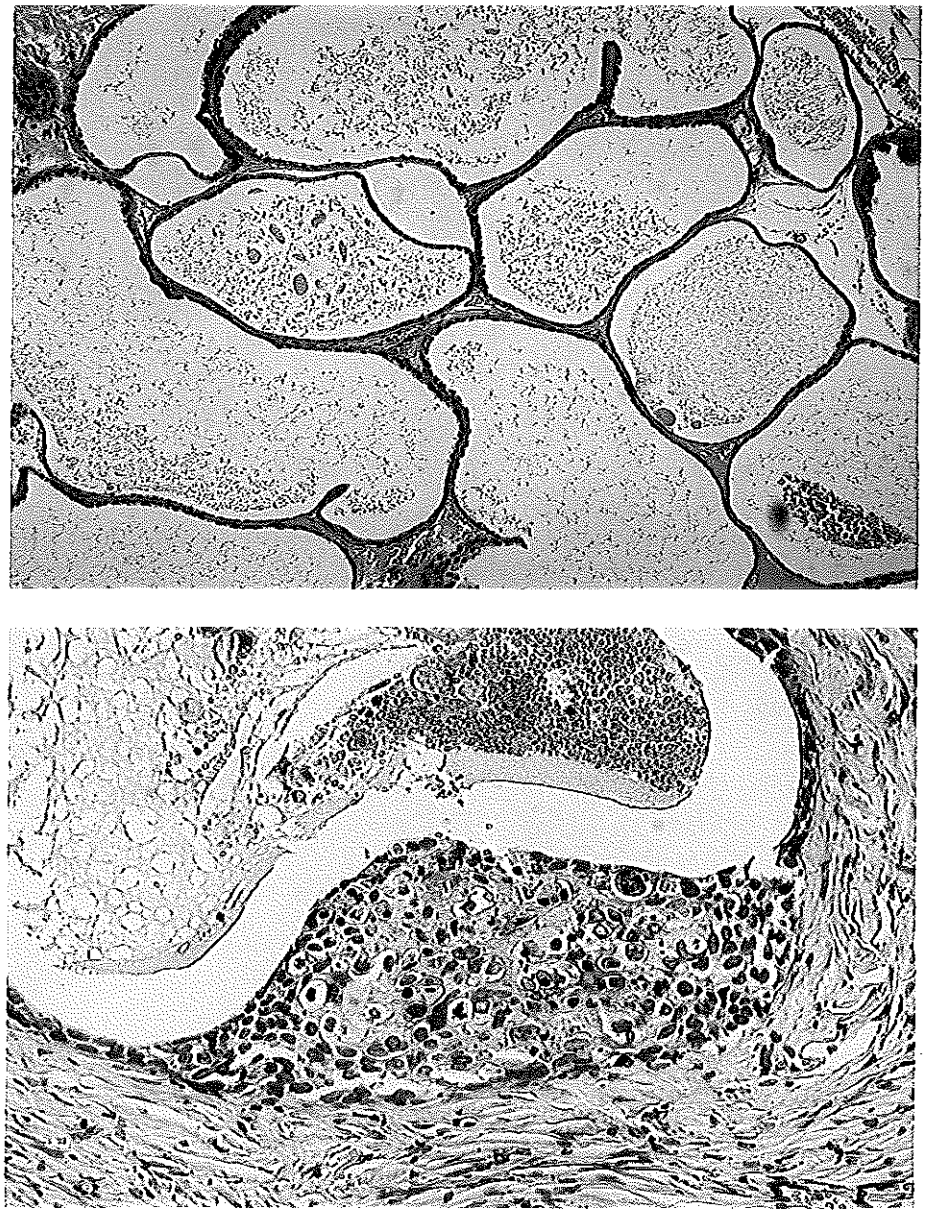

Figure 1. Histology of normal mouse prostate x100 (top) and squamoid outgrowth x400 of HPV immortalized human prostate cell line (bottom).

Expression of prostatic factors measured by RT-PCR in human papillomavirus type 18 DNA immortalized prostate cell lines

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Urology, submitted

Abstract

Objectives. To investigate expression of prostatic markers prostate-specific antigen (PSA), prostate-specific membrane antigen (PSM), and the androgen receptor (AR) after human papillomavirus (HPV) type 18 DNA transfection and subsequent immortalization of human prostatic epithelial cells.

Methods. Recently, two human prostatic epithelial cell lines were established by HPV transformation: PZ-HPV-7, derived from normal peripheral zone tissue, and CA-HPV-10, derived from high Gleason grade adenocarcinoma. Expression of PSA was studied by RT-PCR, because in preliminary studies using immunocytochemistry and Nothern blotting no PSA expression was found. PSM was analyzed by RT-PCR and nested RT-PCR. These analyses included primary human prostate cell strains. Furthermore, androgen supplemented MTT growth assays were performed and expression of AR was studied by immunocytochemistry. Prostate carcinoma cell lines LNCaP and PC-346C were included as positive controls and breast carcinoma cell line MCF-7 as a negative control.

Results. Both cell lines exhibited low levels of RNA for PSA and PSM in comparison with cell lines LNCaP and PC-346C. AR expression by immunocytochemistry was negative using monoclonal antibody F39.4 and polyclonal antibody SP-197. In an androgen supplemented environment, growth rates of both HPV immortalized cell lines were not stimulated in contrast to LNCaP.

Conclusions. RNA transcripts of PSA and PSM were detected by RT-PCR in HPV immortalized prostate epithelial cell lines PZ-HPV-7 and CA-HPV-10. The maintenance of expression of prostate-specific markers further validates the utility of this stepwise transformation model of human prostate carcinogenesis.

Introduction

Prostate cancer is one of the most prevalent diseases among older aged men. The rising incidence and still limited therapeutic modalities in metastatic disease stimulate a continuous search for a better understanding of the oncogenic process. Experimental approaches to elucidate this process include *in vitro* transformation studies which are currently applied more often in prostate cancer research, since technical improvements have been made [1]. Neonatal [2] as well as adult prostatic epithelial cells have been immortalized using simian virus 40 (SV40) DNA [3-6]. We initiated transfection experiments using HPV type 18 to establish prolonged

growth of primary prostatic epithelial cells and to study the effect of an immortalizing agent that has been detected in the human prostate [7-13]. Analysis of these cell lines revealed differential *in vitro* responses to several growth inhibitory and stimulatory factors, distinguishing the HPV-immortalized phenotype from the precursor cells and from SV40-immortalized prostate epithelial cell lines [14-16].

Initial immunocytochemical analyses of the HPV-transformed cell lines (PZ-HPV-7 and CA-HPV-10) did not reveal PSA expression [17]. Here we describe RT-PCR detection of prostatic markers PSA and PSM in human prostatic epithelial cell lines PZ-HPV-7 and CA-HPV-10. We also used immunocytochemical techniques to examine expression of the AR and performed growth assays to evaluate responsiveness to androgen.

Material and methods

Cell Lines. Two previously established human prostate cell lines were investigated: PZ-HPV-7 and CA-HPV-10. These immortalized cell lines were obtained after lipofection-mediated transformation with HPV type 18 DNA of two primary epithelial human prostate cell strains derived from normal peripheral zone epithelium and from a high grade prostate adenocarcinoma, respectively [17]. The cell lines were studied at passage, 93-111, and cultured according to previously published methodologies [17] in medium KSFM (Gibco, Grand Island, NY). Primary human prostatic epithelial cell strains were cultured serumfree in MCDB 105 medium with additives as described previously [1]. The prostate cancer cell lines LNCaP [18] and the mammary tumor cell line MCF-7 [19], used as control cells, were cultured in RPMI 1640 culture medium supplemented with 7.5 % fetal calf serum (Gibco, Grand Island, NY). PC-346C, a human prostate cancer cell line recently established from a xenograft cell line in nude mice [20], was cultured in DMEM/F12 medium supplemented with 2 % fetal calf serum and various growth and attachment factors as described by Limon *et al.* [21].

RNA Isolation. Isolation of total cellular RNA was carried out using RNAzolB according to the manufacturer's directions (Cinna/Biotecx Bulletin). RNA concentration and purity were determined by UV spectroscopy at 260 nm. The integrity of the preparation was checked by agarose gel electrophoresis.

Reverse Transcriptase Reaction. cDNA was synthesized from 1 ug of total RNA in a 20 uL reaction mixture containing 5.0 mM $MgCl_2$, PCR buffer, 1.0 mM dNTPs, 1.0 U RNase inhibitor, 2.5 uM of random hexamer primers and 2.5 U murine leukemia virus reverse transcriptase (Perkin-Elmer). The samples were placed at 22°C for 10 minutes, 42°C for 15 minutes, 99°C for 5 minutes, 5°C for 5 minutes and then chilled on ice. The cDNA quality was tested using GAPDH primers as an internal control [22] before continuing with the PCR protocols as described below.

PCR Amplification for PSM. The method of PCR was followed as described previously [23]. Primer pairs spanned an intron to insure the amplification of complementary DNA and not genomic DNA. The outer primer set consisted of a sense primer identical to nucleotides 1368-1390 (5'-CAG ATA TGT CAT TCT GGG AGG TC-3') and an antisense primer corresponding to nucleotides 1995-2015 (5'-AAC ACC ATC CCT CCT CGA ACC-3'), yielding a 647 base pair product. If needed, a nested PCR regimen was used with an inner primer set, 5'-CCT AAC AAA AGA GCT GAA AAG CCC-3' (nucleotides 1689-1713), and 5'-ACT GTG ATA CAG TGG ATA GCC GCT-3' (nucleotides 1899-1923) yielding a PCR product of 234 base pairs. 10 uL of cDNA was used as the starting DNA template. The 50 uL PCR mixture included 0.125 U Super Taq polymerase (Sphaero Q, Leiden, The Netherlands) in appropriate Super Taq buffer solution, 1.0 mM dNTPs, and 1.0 uM of each outer primer. The samples were transferred to a Perkin Cetus 9600 DNA thermal cycler and the following PCR profile was used: incubation at 94°C for 4 minutes followed by 35 cycles of 60 seconds at 94°C, 30 seconds at 55°C and 60 seconds at 72°C. Then, after 10 minutes at 72°C the samples were put on ice.

In some experiments, a second PCR was performed to increase sensitivity of detection. In this nested PCR protocol 5 uL of the first PCR product was used as the starting template. After the addition of 1.0 mM dNTPs and 1.0 uM of each inner primer, another 35 cycles were performed at the same conditions as described above.

PCR Amplification for PSA. A PCR protocol was performed using 10 uL of cDNA as the template in a 50 uL PCR mixture containing 5 uL of 10x Super Taq buffer, 0.125 U Super Taq polymerase in buffer, 1 uL of 10mM dNTP's and 1.0 uM of each primer. The primers used (kindly received from C.B.J.M Cleutjens, Department of Pathology, Erasmus University Rotterdam, The Netherlands) were 5'-TGC GGA TCC TCA GGC TGG GGC AGC A-3' (exon 3), and 5'-TGT CAG ATC TCC TGC ACA CAA CAT-3' (exon 4) spanning an intron and yielding a 140 base pair product. PCR conditions were 60 seconds at 94°C, 2 minutes at 50°C, and 3 minutes at 72°C, for 30 cycles.

Southern blot. DNA was separated by electrophoresis on a 1% agarose gel and then transferred onto a nylon membrane (Hybond N+, Amersham, Buckinghamshire, UK) with 0.4N NaOH as a transfer solution overnight. Hybridization was carried out in 14% SDS and 1.0 mM EDTA at 60°C for 20 hours with a ^{32}P ATP labelled cDNA probe prepared from the original cDNA [24]. The PSM cDNA was kindly received from Dr. W. D. W. Heston, Memorial-Sloan Kettering, New York. As a probe for PSA, a 320 bp EcoRI ClaI fragment from PSA cDNA [25] was used (kindly received from C.B.J.M Cleutjens). The blot was washed with 1x SSC/0.1% SDS, 0.3x SSC/ 0.1% SDS, and 0.1x SSC/0.1% SDS at 60°C for 15 minutes each, and subsequently exposed to Fuji X-film.

Growth Assay. To examine the effect of androgen supplementation on growth, cells were seeded in 96-well microtiter plates at a density of 3000 cells in 0.1 mL per well. After cellular attachment, experimental media with or without 5α-dihydrotestosterone (DHT) or synthetic androgen R1881 were added to a final volume of 0.2 mL. The growth rate was assessed after an exposure time of 4 to 5 days using a modified MTT assay [26].

Immunocytochemistry. Monoclonal antibody (MAb) F39.4 directed against a fragment of the N-terminal of the androgen receptor [27] and polyclonal SP-197 antibody (PAb) against the androgen receptor [28] were used. Cells were fixed with 10% formalin for 10 minutes at room temperature (Rt), methanol for 2 minutes and acetone for 4 minutes at -18°C. The staining protocol was initiated with 3% hydrogen peroxide in methanol for 20 minutes followed by rinsing in water and phosphate buffered saline (PBS). Nonspecific binding was blocked using normal goat serum (NGS) 1:10 in PBS for 15 minutes at Rt. Overnight incubation at 4°C was performed in a 1:400 PBS dilution for MAb F39.4 and a 1:1000 PBS dilution for SP-197. After rinsing in PBS goat anti-mouse immunoglobulin 1:50 in the presence of 3% NGS and 3% normal horse serum was added for 30 minutes at Rt. Complex formation with peroxidase anti-peroxidase (Sigma, Munchen, Germany) 1:300 in PBS for 30 minutes was followed by 3,3'- diaminobenzidine containing 0.03% hydrogen peroxide for 7 minutes, both at Rt.

Results

PSA Expression. Because previous attempts to detect PSA in PZ-HPV-7 and CA-HPV-10 at the RNA level using Northern blot analysis or at the protein level by immunocytochemistry were unsuccessful, we sought to increase the sensitivity of detection by use of the RT-PCR method. Figure 1 shows the expression of PSA by RT-PCR in PZ-HPV-7 and CA-HPV-10 cell lines in comparison to cell lines LNCaP and PC 346C (positive controls) and MCF-7 (negative control). Primary cell strains were included as well. PSA mRNA was detected in PZ-HPV-7 and CA-HPV-10, although the level of expression was consistently lower than in LNCaP and PC-346C cells. RNA from primary human prostatic cell strains derived from normal peripheral zone and another cell strain derived from a human prostatic adenocarcinoma specimen showed a signal for PSA at the detection limit. These results were confirmed by hybridization with a ^{32}P-labelled probe.

PSM Expression. Using a standard RT-PCR procedure, PSM expression was found in both cell lines PZ-HPV-7 and CA-HPV-10, but not in the RNA isolated from the primary cell strains. These results are shown in figure 2. The positive signal for PSM in the transformed cell lines was confirmed by hybridization with a ^{32}P-labelled probe. Since PSM expression was near the detection limit in PZ-HPV-7 and CA-HPV-10 and not detectable in the two primary cell strains, a nested PCR procedure was applied as well. However, also by this more sensitive method, expression of PSM could not be detected in the two primary prostatic cell strains.

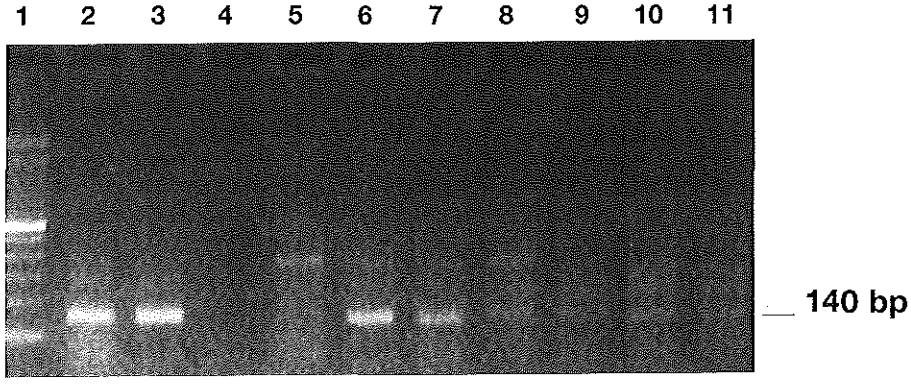

Figure 1. PSA in HPV type 18 DNA transformed cells.
PSA mRNA transcripts were detected using RT-PCR as described in Material and Methods. cDNA amplification products were separated in a gel and photographed after staining with ethidium bromide. Lane 1, 100 base pairs molecular weight ladder; Lane 2, LNCaP (positive control 1); Lane 3, PC-346C (positive control 2); Lane 4, H2O (blank); Lane 5, MCF-7 (negative control); Lane 6, CA-HPV-10; Lane 7, PZ-HPV-7; Lane 8, cancer-derived prostatic epithelial cell strain E-CA-21; Lane 9, normal prostatic epithelial cell strain E-PZ-13; Lane 10, cancer-derived prostatic epithelial cell strain E-CA-21 in KSFM; Lane 11, normal prostatic epithelial cell strain E-PZ-13 in KSFM. bp, base pairs.

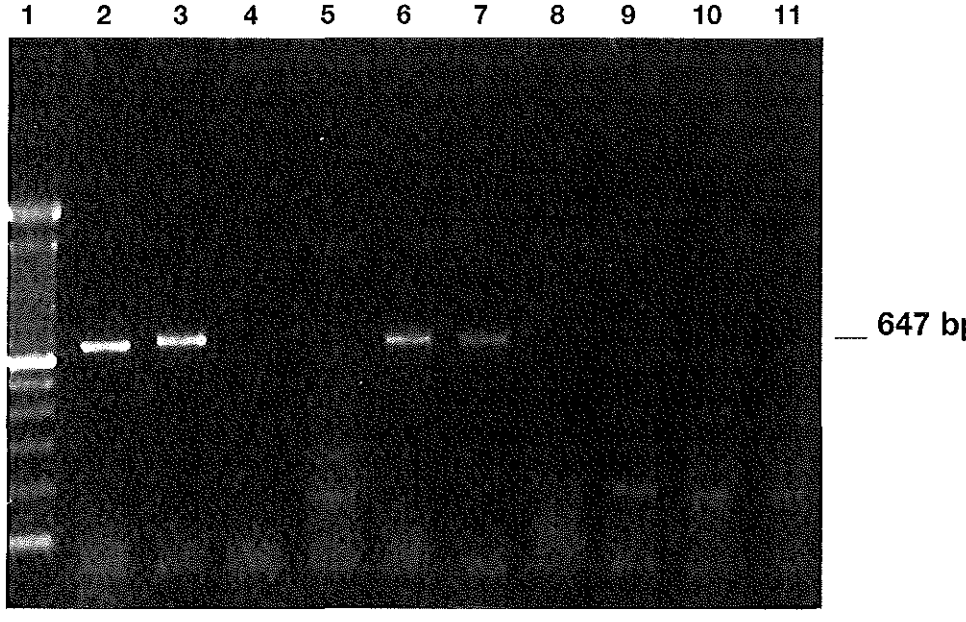

Figure 2. PSM in HPV type 18 DNA transformed cells.
PSM mRNA transcripts were detected using RT-PCR as described in Material and Methods. cDNA amplification products were separated in a gel and photographed after staining with ethidium bromide. Lane 1, 100 base pairs molecular weight ladder; Lane 2, LNCaP (positive control 1); Lane 3, PC-346C (positive control 2); Lane 4, H2O (blank); Lane 5, MCF-7 (negative control); Lane 6, CA-HPV-10; Lane 7, PZ-HPV-7; Lane 8, cancer-derived prostatic epithelial cell strain E-CA-21; Lane 9, normal prostatic epithelial cell strain E-PZ-13; Lane 10, cancer-derived prostatic epithelial cell strain E-CA-21 in KSFM; Lane 11, normal prostatic epithelial cell strain E-PZ-13 in KSFM. bp, base pairs.

AR Expression. Immunocytochemical studies failed to show androgen receptor expression in the cell lines PZ-HPV-7 and CA-HPV-10, whereas positive staining results were obtained with LNCaP. The responsiveness of the two HPV-transformed cell lines to androgen was tested by MTT assays. Using DHT or R1881 up to 10^{-7}M we observed no growth stimulation of either cell line. Under the same conditions, i.e. using KSFM with the standard additives EGF and BPE as experimental media, increased growth rates could be demonstrated for LNCaP cells, which were used as positive control. In the presence of appropriate androgen concentrations of 10^{-10}M R1881, LNCaP showed maximal stimulation of growth (figure 3).

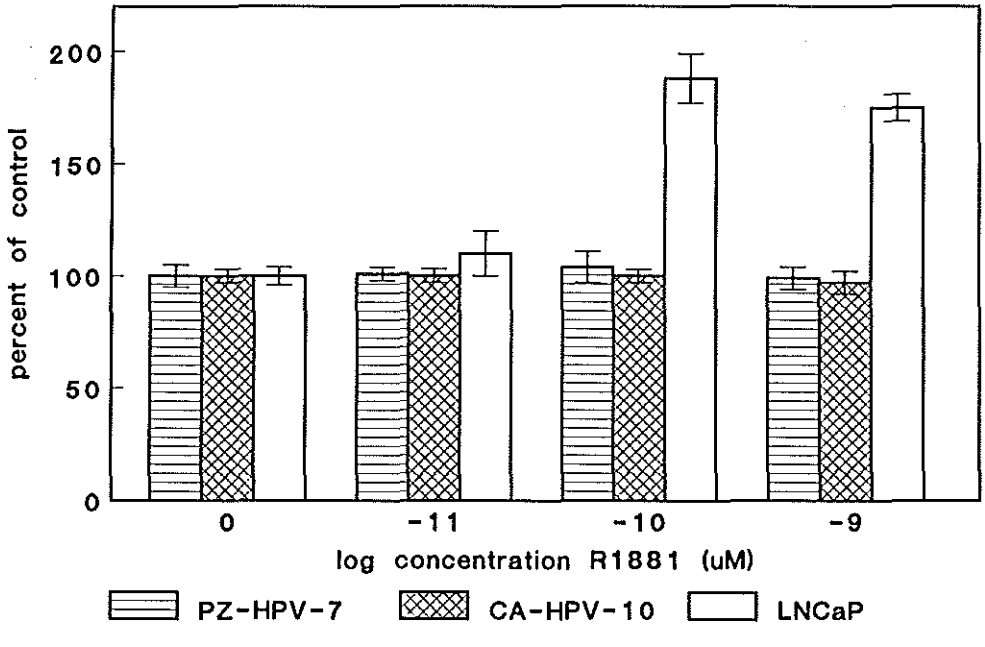

Figure 3. Results of MTT assay showing growth stimulation of LNCaP cells by synthetic androgen R1881, whereas no increase of growth rates of HPV-transformed cell lines PZ-HPV-7 and CA-HPV-10 was observed.

Comment

In order to establish a model for prostate carcinogenesis, primary prostatic epithelial cells derived from tissues of different histologic patterns were transfected with HPV type 18 DNA [17]. HPV DNA was used to prolong the limited *in vitro* lifespan of adult human prostatic epithelial cells because of its immortalizing potential. This approach resulted in the establishment of cell lines PZ-HPV-7 and CA-HPV-10 [17]. Almost simultaneously, Rhim *et al.* reported the stepwise immortalization and transformation of prostatic epithelial cells using HPV-18 DNA and an activated Ki-*ras* oncogene [29]. Rationale for using HPV DNA was that DNA sequences of several oncogenic HPV types were detected in the human prostate [7-13]. This suggested a possible correlation of HPV infection with prostatic disease in general or with prostatic adenocarcinoma specifically. However, the currently available evidence does not support an etiologic role for HPV in prostate cancer [30].

PSA [31-33] and also PSM [23,24] are unique markers of the human prostate. Therefore, the measurement of PSA and PSM expression should be included in the characterization of novel prostate cell lines. However, ex vivo expression levels of both markers may be difficult to determine because of variable conditions in culture. Initial immunocytochemical studies using a polyclonal antibody did not reveal the presence of PSA in the cell lines PZ-HPV-7 and CA-HPV-10 [17], whereas a low level of positive staining has been noted in primary cultures [1,17]. In fact, only few prostate cancer cell lines such as LNCaP [18,34], clearly show PSA expression. Human prostate epithelial cell lines established after SV40 immortalization have not been reported to express either PSA or PSM [2-4] although recently PSA expression was found in a prostatic epithelial cell line immortalized by an adenovirus 12-SV40 hybrid virus [35]. Using RT-PCR, mRNA for PSA as well as for PSM was detected in both cell lines PZ-HPV-7 and CA-HPV-10, although the level was low in comparison with LNCaP and PC-346C. In RNA isolates from primary cell cultures, expression of PSA was at the detection limit. PSM expression was not found by standard RT-PCR in the primary cell strains. Further enhancement of the sensitivity of detection by application of a nested RT-PCR procedure, which has been developed to detect low numbers of circulating prostate cells in the human blood stream, did not conclusively show the presence of PSM mRNA in the primary epithelial prostatic cells.

It is not evident why the level of PSA and PSM expression is lower in the precursor primary cells than in the HPV-transformed cell lines. Possibly, the expression is influenced by the culture media, which are different for the two cell types. Another possibility might be that cells with slightly elevated marker expression had been selectively transformed and/or preferentially cultured resulting in a relative enrichment of marker-positive cell populations.

The absence of AR expression by immunocytochemical analysis in both HPV-transformed cell lines PZ-HPV-7 and CA-HPV-10 and the non-responsiveness to androgens *in vitro* is in accordance with the findings in other immortalized human prostatic cell lines [2,4,26]. However, using the method of ^{3}H-DHT ligand binding, the presence of AR was noted in a SV40 immortalized prostatic epithelial cell line [3]. In an epithelial cell line from the human prostate immortalized by an adenovirus 12-SV40 hybrid virus [35] AR expression was found by immunocytochemistry. Moreover, in contrast to other studies growth enhancement was achieved by androgen supplementation.

In conclusion, after HPV type 18 DNA-immortalization, expression of prostate-specific factors PSA and PSM can be measured by RT-PCR. Expression levels of these factors are low compared to androgen-responsive cell lines LNCaP and PC-346c. *In vitro* expression of PSA and PSM have been found to be absent or low in most prostate cell lines and could represent a down regulation in epithelial cultures. Although cellular expression levels of these factors are lower than in androgen-responsive cell lines LNCaP and PC-346C, their presence further validates the utility of this stepwise transformation model of human prostate carcinogenesis.

Acknowledgement

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General discussion

6.1 Model of prostate carcinogenesis ?

Prostatic carcinoma is a heterogeneous disease which is currently diagnosed at an early stage, while the clinical behavior is unpredictable. The establishment of methods to distinguish clinically insignificant from potentially lethal cancers has become the future challenge for prostate cancer research. The different steps in prostate carcinogenesis are still poorly understood, which is in contrast to other organ systems. For colorectal carcinoma, the multistep process has been described and specific genetic alterations, as well as the relative timing and contribution of events, have been clarified in detail [1]. However, recent encouraging results from studies of the molecular and cellular biology of the prostate should provide for a more detailed understanding of human prostate carcinogenesis. Even with the current fragmented knowledge of prostate cancer development, it is possible to use genetic biomarkers for predicting the risk of prostate carcinoma recurrence after radical prostatectomy [2]. In the near future, a genetic test to diagnose cancer-prone individuals may be feasible if the identification of an inherited prostate cancer susceptibility gene is confirmed. Strategies for early screening and treatment may benefit the population carrying this germline genetic aberration. While this will only directly apply to 5-10% of prostate cancer patients [3], investigations into the hereditary predisposition could lead to new information on the pathogenesis and etiology of prostate cancer. In the meantime, prostate cancer research must be focussed on elucidating the carcinogenic process. The long latency period of the disease makes it difficult to determine the factors that govern prostate carcinogenesis by epidemiologic analysis. Therefore, in the last decades, we may appreciate a continuous search for useful experimental models to clarify the different steps in prostate cancer development. In this respect, *in vitro* transformation studies may be instrumental in order to recognize the relative importance of events in the process of malignant differentiation. However, as discussed previously [see also chapter 1.6], few studies have been reported on transformation in human prostatic epithelial cells. The authors who contributed to the studies in this thesis had a common goal of exploring the possibility of successful *in vitro* transfection of epithelial cells derived from the adult human prostate, using viral DNA as a first transforming step.

Transfection of SV40 DNA led to the establishment of a novel epithelial human prostate cell line pRNS-1 [chapter 2] without tumorigenic properties. Since then, Bae *et al.* reported the development of tumorigenic sublines after reinjection of *in vitro* recovered sporadic outgrowths of SV40 T antigen immortalized adult human prostate epithelial cells [4]. Furthermore, cell line BPH-1 was established after primary epithelial cell cultures obtained by transurethral resection were immortalized by SV40 large T antigen [5]. In an effort to develop a model of human prostate carcinogenesis, the use of HPV DNA rather than SV-40 DNA may be more appropriate, because HPV has been recognized as an important factor in human carcinogenesis, in contrast to

SV-40, which is not directly implicated in human cancer. Furthermore, at the time these experiments were anticipated, there was some evidence of a possible role of HPV in cancer of the human prostate. However, in view of the currently available data, HPV infection does not seem to play a major role in prostate pathogenesis [chapter 1.3].

The immortalizing capacity of HPV type 18 DNA was used to expand the limited in vitro growth potential of human prostatic epithelial cells [chapter 3]. In addition, the possibility of an emerging tumorigenic phenotype was analyzed. In vivo studies at different passage numbers did not reveal neoplastic behavior in both cell lines PZ-HPV-7 and CA-HPV-10. Although HPV-immortalization in general is not sufficient for tumorigenic transformation, neoplastic properties were anticipated especially in the carcinoma derived cell line CA-HPV-10. Moreover, in keratinocytes chromosome fragility and instability after the incorporation of HPV DNA was sufficient to induce neoplastic differentiation upon continuous passaging [6]. In general, however, the neoplastic phenotype only arises after additional events, such as the introduction of the v-Ha-*ras* oncogene [7]. At low passage both cell lines PZ-HPV-7 and CA-HPV-10 occasionally showed slowly regressing small nodules of squamous differentiation after subcutaneous injection into BALB-C athymic nude mice. In contrast, the precursor cell strains did not show any tumor formation. The propensity to form tumors was further studied in high passage by subcutaneous and orthotopic implantation in NMRI nude mice. Only occasionally a small outgrowth [figure 1] was found. Moreover, the outgrowth appeared as a squamoid nodule consistent with early passage findings [chapter 3, figure 4]. Rhim *et al.* [8] have demonstrated that superinfection of early passage HPV-18 immortalized adult human prostatic epithelial cells with Ki-MuSV, a virus with an activated Ki-*ras* oncogene, was sufficient to induce progression to a neoplastic phenotype. Although the morphology was that of a poorly differentiated carcinoma, the cell type in culture was squamous. The latter would be similar to the histology of cell lines PZ-HPV-7 and CA-HPV-10, which for the study of the carcinogenic process in prostate cancer would not seem to be optimal. However, the finding of a squamous epithelial appearance rather than a secretory epithelial growth pattern is also seen after subcutaneous injection of normal prostate cells, and also of somatic epithelial prostate cell hybrids [9]. This could indicate that the squamous phenotype might be related to the methodology of subcutaneous injection. In tumorigenicity assays using subcutaneous injection, the resulting histology may not be a representation of the actual phenotype [10]. Regarding this issue, one would expect that orthotopic implantation would be more appropriate [11], although with the use of HPV immortalized cell lines a similar squamous differentiation was found [figure 1, page 63].

Whether the squamous phenotype is caused by the HPV transformation, which would seem obvious, or otherwise caused by the general growth requirements of *in vivo* studies remains to be established. *In vitro*, the observation is that differentiation may be modulated, depending on the growth conditions. For instance, the growth

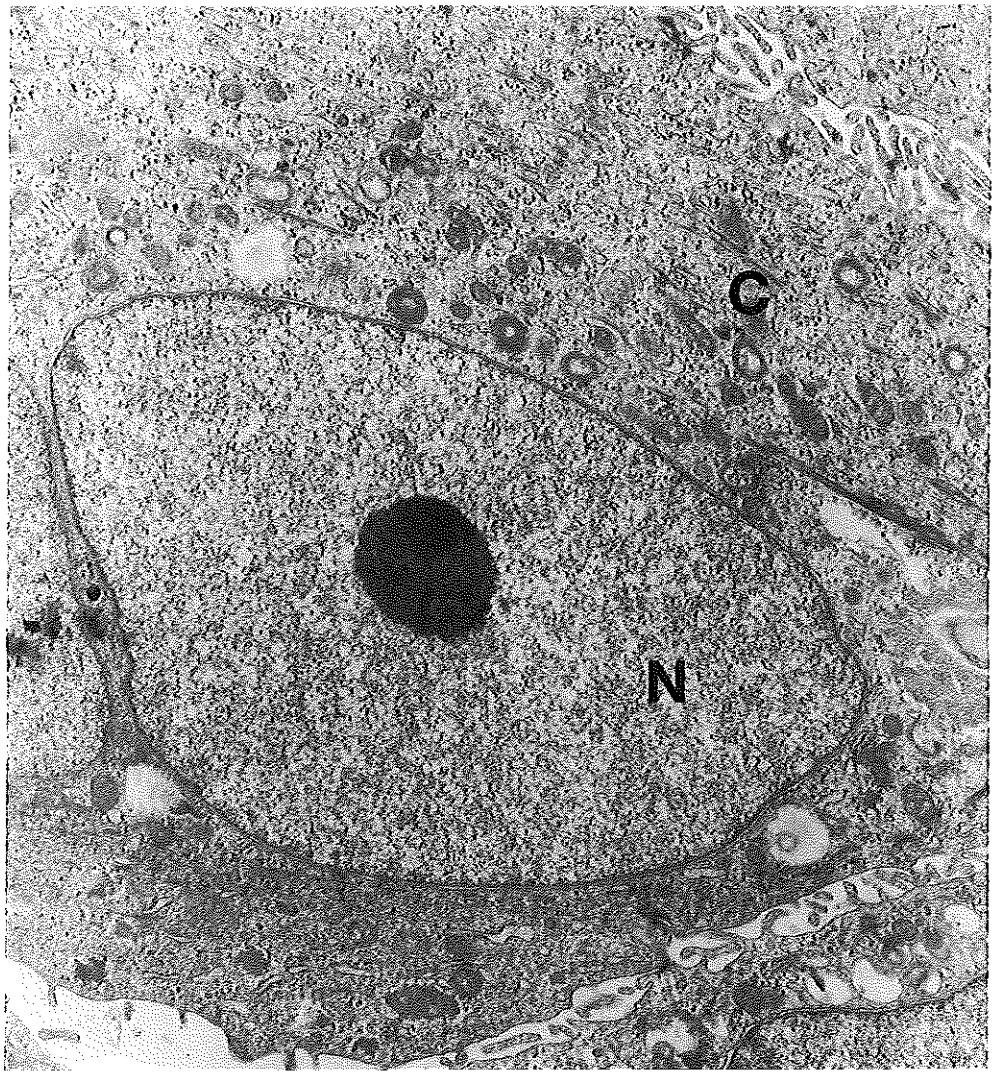
patterns of the HPV transformed cell lines PZ-HPV-7 and CA-HPV-10 were altered depending on the calcium concentration. High calcium concentration induced a flat squamous-like phenotype and lower growth rates as opposed to a rounded appearance and a loosely attached cell layer in the low-calcium Keratinocyte Serum-Free Medium (KSFM).

To further investigate the *in vitro* phenotype of the HPV immortalized cell lines, detailed microscopic studies were performed. Electronmicroscopic imaging shows a microcellular aspect that may be in line with the histologic differentiation *in vivo* i.e. keratinizing, squamous nodules [figure 2].

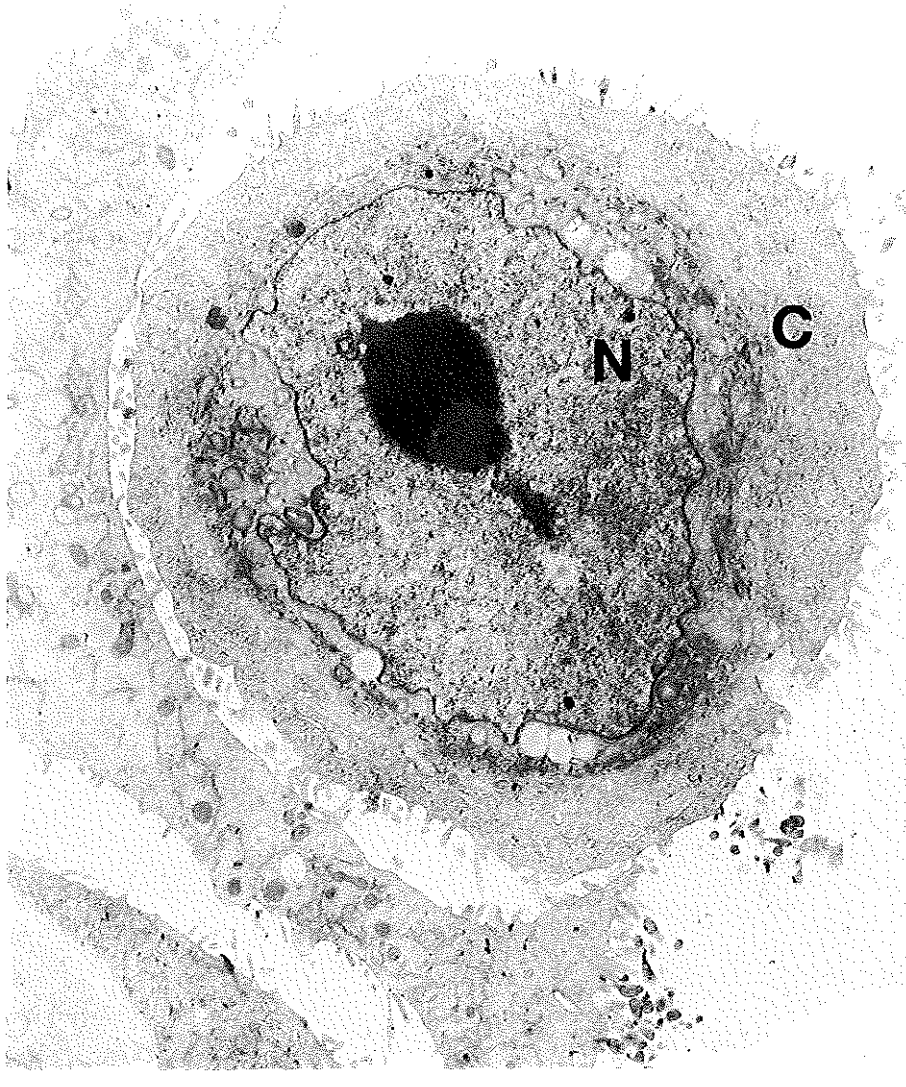
6.2 Modulation of growth

Transformation of cells from the human prostate epithelium by HPV and SV40 modulates growth requirements in several ways. EGF and BPE are important stimulatory factors as in primary cultures [12], but IGF does not increase clonal growth at least for the cell lines PZ-HPV-7 and CA-HPV-10 [13,14]. While IGF type I did not stimulate growth of cell lines PZ-HPV-7 and CA-HPV-10 in median density (2000 cells in a 60-mm collagen coated dish for 10 days) growth assays, in MTT assays a stimulatory effect was found. Other factors that have been studied include cholera toxin, hydrocortison and aFGF. While aFGF acts as a potent mitogen for primary cultures, this was not so for the HPV immortalized cell lines. Also, growth-inhibition was achieved by transforming growth factor-ß and 1,25 $(OH)_2$ vitamin D_3 , but not by tumor necrosis factor-α. The lack of growth inhibition by TNF-α is a rather common finding after HPV transformation [15]. Furthermore, both HPV immortalized prostate cell lines PZ-HPV-7 and CA-HPV-10 are growth-inhibited by 1,25 $(OH)_2$ vitamin D_3, whereas SV-40 transformed cell lines and the DU-145 cell line are not at concentrations as high as 100nM [16].

By immunocytochemical analysis, AR expression in PZ-HPV-7 and CA-HPV-10 was not detected [chapter 5]. Furthermore, in an androgen supplemented environment using DHT and synthetic androgen R1881, growth rates of both cell lines were unchanged, whereas increased proliferation of LNCaP was induced. Thus, the HPV immortalized cell lines are not stimulated by androgens, which is a common finding in most immortalized human prostatic cell lines. However, by adenovirus 12-SV40 hybrid virus transformation androgen responsiveness as well as AR expression may be conserved [17]. One of the few human prostate cell lines that has retained androgen responsiveness is LNCaP derived from metastatic prostate cancer [18]. In view of the fact that supplementation of androgens does not stimulate growth, human prostate derived immortalized epithelial cell lines, such as PZ-HPV-7 and CA-HPV-10, may have additional value in research aimed at the androgen-refractory stage of



PZ-HPV-7 cell, EM x 5000, n=nucleus, c=cytoplasm.



CA-HPV-10 cell, EM x 5000, n=nucleus, c=cytoplasm.

prostate cancer. Also, because a low-level of PSA expression in PZ-HPV-7 and CA-HPV-10 has been detected, which will be discussed further in the following paragraph.

6.3 Measurement of PSA and PSM

RT-PCR protocols have been developed to increase the sensitivity of detection of RNA putatively as a semi-quantitative measurement. In this thesis it has been shown that detection limits indeed can be shifted by the use of the RT-PCR method. We were able to determine PSA and PSM expression in human prostate derived HPV immortalized cell lines, whereas with regular Nothern blotting and immunocytochemistry no signal was found. However, an inherent problem is the interpretation of results, because, with the increased sensitivity, the risk of contamination may become a crucial factor. In this respect, we have found the nested PCR protocol [19] to be of rather limited value, because the results are variable.

The absence of AR expression in cell lines PZ-HPV-7 and CA-HPV-10 may seem to be in obvious contradiction with the presence of PSA expression, because of the interaction of androgens with PSA expression [12]. However, prelimary RT-PCR based analysis has indicated a low level of AR expression at least in one of the two HPV immortalized cell lines, which would be an argument against the aforementioned contradiction. It has been shown that the luminal rather than the basal prostatic epithelial cells express PSA and AR [12]. Probably primary cultures are mainly composed of basal cells. The matter of cellular origin, basal versus luminal cell, cannot be resolved, although by immunocytochemical analysis the pattern of expression for keratin 5 and 8 remains similar to that in primary cultures. This would be indicative for a basal as well as a luminal cell origin [20].

The fact that in the RNA extracted from primary cells PSA and PSM expression levels were below detection limits [chapter 5] cannot be readily explained. However, the discrepancy might be explained by a process of selective transformation and/or preferential growth of cell populations that still expressed low levels of PSA. The level of PSA expression in the primary cells and the different immortalized cell lines may also vary depending on *in vitro* growth conditions. In this regard, additional studies using RT-PCR analysis of RNA isolated after androgen stimulation might contribute to the explanation of our findings.

6.4 Limitations of human prostate cell cultures

Notwithstanding continuous improvements in defining the best *in vitro* growth conditions of human prostatic epithelial cells, there are still some inherent difficulties. Limitations of human prostate primary cell culture systems include: 1) The short lifespan and relatively long population doubling-times. 2) The absence of phenotypical features for the identification of benign and malignant epithelial cells. The histopathological diagnosis which can be determined in the stepsectioned radical prostatectomy specimen is until now the only way to distinguish different primary cell cultures. Other direct methods to identify the origin of the cells are not yet available to eliminate this problem. Indirectly, however, a promising method has been demonstrated using loss of heterozygosity on chromosome 8p as an alternative method for characterizing tumor cell lines originating from primary carcinomas after transformation by using a recombinant retrovirus encoding the E6 and E7 transforming proteins of HPV type 16 [21]. 3) The risk of selective isolation and preferential growth of normal, or benign, cells, which may be related to several factors. Collagenase treatment of prostatic tissue prior to the establishment of primary cultures [22,23] has been indicated as a possible artefact in preparation protocols. 4) The use of undefined factors such as serum in culture media. By the standardized methods as developed by Peehl *et al.* [24] serum-free culture media can be used with specified additives. However, using these methods one of the essential factors required for *in vitro* growth is BPE, which is a crude extract. The use of fully defined serum-free media for primary epithelial human prostatic cells has been reported recently [25].

The current spectrum of commonly used human prostate cell lines include LNCaP, DU145 and PC-3. Of these cell lines, LNCaP is the only PSA producing androgen responsive cell line. Other experimental models of human prostate cancer include heterotransplantable cell lines. These cell lines derived from human adenocarcinoma specimen represent well-characterized *in vivo* models for prostate cancer. One of these cell lines PC-82 [26-28] is well established and is permanently maintained in nude mice. However, a major disadvantage in the process of characterization has been the inability to establish these cell lines in culture. Despite the absence of *in vitro* growth, many aspects such as oncogene expression have been investigated [29]. Recently, the continuing pursuit of methods to establish *in vitro* growth has been successful resulting in novel cell lines such as PC-346C [30], which obviously increases the value of the heterotransplantable tumor model because *in vivo* studies [31] may be translated to *in vitro* conditions and vice versa.

6.5 Conclusion and perspectives

As stated in the introduction, experimental *in vitro* transformation models of prostate carcinogenesis may be useful in several ways. By transformation, cell lines of different origin may become available, which should allow for the identification of common denominators in prostate cancer. In an attempt to create a model of human prostate carcinogenesis, cells derived from different histologies of the human prostate epithelium have been immortalized. The SV40 and HPV type 18 DNA transformed prostate cell lines were extensively characterized. However, to what extent have these immortalized epithelial cells derived from the adult human prostate provided insight into the processes that may relate to prostate carcinogenesis ? Many different aspects of the cell lines have already been highlighted in this last chapter. However, the implications of the chromosomal abnormalities, which were seen in cell lines PZ-HPV-7 and CA-HPV-10, should be added to this discussion. As mentioned in chapter 4, we have found a pattern of chromosomal alterations that may relate to the process of transformation by viral DNA. This includes numerical alterations such as triploidization, as well as structeral alterations, such as the appearance of hsr. However, to some extent several chromosomal abnormalities are certainly of interest and may represent specific alterations related to prostate carcinogenesis. These include numerical anomalies such as the loss of chromosome Y in the cancer derived cell line, CA-HPV-10, as well as structural anomalies, such as the presence of dmin in the parent cell and in early passage of CA-HPV-10. The appearance of a hsr on 9p in late passage of CA-HPV-10 is another remarkable feature. With regard to the dmin and hsr, it may have been of additional value if the use of flow-sorting techniques would have been successful. This would have enabled the isolation and further identification of these aberrant chromosomal parts. Other specific genetic alterations that are rather frequently found in prostate cancer, such as 8p anomalies, have not been identified in the described cell lines. As stated above [chapter 6.4], in a recent study, multiple human prostatic cell lines derived from primary cancer specimens were generated by transformation using a retrovirus encoding E6 and E7 [21]. This study was remarkable for the fact that many cell lines were created, moreover, after transformation, analysis of chromosomal anomalies of 8p was performed as a possible selection marker for prostatic carcinomas. In conclusion, the establishment of transformed cell lines, as presented in this thesis, has provided results which may lead to a more detailed understanding of the multistep process of carcinogenesis. It shows that immortalization of human prostatic cells by viral DNA is feasible.

Another reason for the approach of transformation would be to establish a stepwise model of prostate carcinogenesis. After the initial immortalizing step, the cell lines may serve as a target for the induction of additional changes. Presently, several consistent genetic changes have been associated with prostate cancer. It would seem

evident that new molecular markers will be identified in prostate carcinogenesis. Genes capable of inducing a tumorigenic phenotype may be studied after extending the lifespan of prostatic cells to immortality. Ultimately, we could recreate some of the processes occurring during the development of prostate cancer, recognizing genetic changes at different stages of malignant progression. The resulting malignant phenotype may be the basis of studies defining new treatment strategies. In this respect, the cell lines may be instrumental and serve as targets for additional genomic alterations.

Lastly, within the spectrum of existing experimental models of human prostate cancer, these novel cell lines, pRNS-1-1, PZ-HPV-7 and CA-HPV-10 should have additional value.

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Summary

Chapter 1. In Western countries, prostate cancer is one of the most common malignant tumors and a major cause of cancer mortality. Prostate cancer is a heterogeneous disease which is currently diagnosed at an early stage, while the clinical behavior is unpredictable. The establishment of methods to distinguish clinically insignificant from potentially lethal cancers has become the future challenge for prostate cancer research. The multistep process of prostate carcinogenesis is influenced by many factors. A short review of some factors implicated in prostatic carcinoma is included in chapter 1. Chromosomal alterations that are found in prostate cancer are described in chapter 1.4, which includes an overview of genetic aberrations, putative tumorsuppressor genes and oncogenes that are commonly implicated in prostate cancer. Furthermore, the role of human papillomaviruses (HPV) in urogenital disease is discussed in the introduction for two reasons: Firstly, because we used HPV DNA in our experiments, and secondly, because in the early nineties genetic material of several oncogenic types of the HPV was detected in human prostatic tissue. The latter suggested a possible association of prostatic disease with viral infection, which however has not been confirmed. The role of the human prostate as a reservoir for certain oncogenic viruses can not be ruled out, and may be more important than currently acknowledged.

The approach of transformation of epithelial cells derived from radical prostatectomy specimens is described in this thesis. In general, epithelial cells from the adult human prostate cannot be maintained in longterm culture. However, even with the limited growth potential, well-defined methods have been developed to study these cells in primary cultures [chapter 1.5]. With the use of viral DNA, enhancement of cellular growth was anticipated possibly leading to an immortal state. Based on the results of transformation studies in other organ systems, the experiments were performed with DNA of the simianvirus (SV40), which is not directly implicated in human carcinogenesis, and human papillomavirus (HPV type 18), which is implicated in anogenital carcinogenesis.

Chapter 2. Normal adult human prostatic epithelial cells were immortalized by polybrene-induced gene transfer with a plasmid containing an origin-defective SV40 genome. The cells had extended lifespan in culture compared to normal adult human prostatic epithelial cells. The cells pRNS-1-1 contain the SV40 genome, express SV40 T-antigen, and are not tumorigenic in nude mice. They express cytokeratins 5 and 8, like the parent cells, and are pseudodiploid. Analysis of growth regulatory processes revealed that the growth of pRNS-1-1 cells was stimulated similarly to that of normal prostatic epithelial cells by epidermal growth factor, insulin like growth factor, and pituitary extract. The response of pRNS-1 cells to a growth-inhibitory factor, retinoic acid, was also similar to that of normal cells. However, pRNS-1-1 cells were less responsive than normal cells to growth inhibition by transforming growth factor-ß, and had lost altogether the ability of normal cells to be inhibited by tumornecrosis factor-α and 1,25 $(OH)_2$ vitamin D_3. Therefore transformation appeared

to alter growth-inhibitory but not growth-stimulatory mechanisms.

Chapter 3. Human papillomavirus type 18 DNA was introduced into epithelial cell strains derived from normal and cancer tissues of human radical prostatectomy specimens, by the lipofection transfection method. Two cell lines were established: PZ-HPV-7, derived from normal peripheral zone tissue, and CA-HPV-10, derived from high Gleason grade adenocarcinoma. Incorporation of HPV type 18 DNA was confirmed by the polymerase chain reaction (PCR). Immunocytochemical analysis showed expression of cytokeratins 5 and 8, similar to the cells of origin, and the E6 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 (dmin) that were noted in the cells of origin. The cell lines showed a typical transformed morphology and were nontumorigenic in nude mice. With the establishment of these two cell lines, for the first time, human prostatic epithelial cells derived from both normal and cancer tissues were successfully transformed to immortality with HPV type 18 DNA.

Chapter 4. Using chromosome banding and fluorescence in situ hybridization with painting probes, sequential cytogenetic analysis was performed of two epithelial human prostate-derived cell lines, PZ-HPV-7 and CA-HPV-10, established by human papillomavirus type 18 DNA transformation. PZ-HPV-7, originating from a normal diploid prostate epithelial cell strain, progressed from an initial diploid to a hypertetraploid chromosome number with a relative gain of chromosomes 5 and 20. Structural changes were limited. CA-HPV-10 originated from an epithelial cell strain derived from a high grade human prostate cancer specimen, which showed several karyotypic abnormalities including an extra Y chromosome and double minutes (dmin). The modal number of CA-HPV-10 was mainly hypertriploid. In early passage, the karyotype of CA-HPV-10 appeared unstable with a decreasing number of cells exhibiting dmin. In late passage, the dmin were replaced by a large homogeneously staining (hsr) region on the short arm of chromosome 9, the 9p+ marker. By fluorescence in situ hybridization the hsr was shown to be of chromosome 1 origin. Loss of the Y chromosome was prominent. A number of other consistent markers were present. HPV type 18 genomic integration sites were identified on 1p for PZ-HPV-7, and on the 9p+ marker for CA-HPV-10.

In conclusion, both PZ-HPV-7 and CA-HPV-10 showed clonal cytogenetic changes. The cytogenetic evaluation of PZ-HPV-7 and CA-HPV-10 revealed several karyotypic abnormalities that can be of interest in the pursuit of a multistep *in vitro* model of human prostate carcinogenesis. The cytogenetic modifications that were identified represent a range of HPV transfection related phenomena within the residual pattern of the original karyotype.

Chapter 5. Expression of the prostatic markers prostate-specific antigen (PSA), prostate-specific membrane antigen (PSM), and the androgen receptor was analyzed in two human prostatic epithelial cell lines established by HPV transformation, PZ-HPV-7 and CA-HPV-10. PSA and PSM expression was studied with the use of the reverse transcription polymerase chain reaction (RT-PCR). Furthermore, androgen supplemented growth assays were performed. Expression of the androgen receptor was studied by immunocytochemistry. Both cell lines exhibited low levels of RNA for PSA and PSM in comparison with the prostate carcinoma cell lines LNCaP and PC-346C. Androgen receptor expression by immunocytochemistry was negative using monoclonal antibody F39.4 and polyclonal antibody SP-197. In an androgen supplemented environment, growth rates of both HPV immortalized cell lines were not stimulated.

In conclusion, RNA transcripts of PSA and PSM were detected by RT-PCR in HPV immortalized prostate epithelial cell lines PZ-HPV-7 and CA-HPV-10. The maintenance of expression of prostate-specific markers further validates the utility of this stepwise transformation model of human prostate carcinogenesis

Chapter 6. In this last chapter, the limitations and characteristics of the above mentioned cell lines are further evaluated. In short, in an attempt to create a model of human prostate carcinogenesis, cells derived from different histologies of the human prostate epithelium have been immortalized. The SV40 and HPV type 18 DNA transformed epithelial cells derived from the adult human prostate may provide insight into the processes that may relate to prostate carcinogenesis. However, although some of the *in vitro* characteristics may be useful, the *in vivo* phenotype, squamous rather than secretory, does not on one hand seem to favor the approach of viral transformation as a first transforming step in human prostate carcinogenesis. Specific genetic alterations that are rather frequently found in prostate cancer, such as 8p anomalies, were not present in the described prostate cancer derived cell line CA-HPV-10. On the other hand, within a pattern of chromosomal alterations that may relate to the process of transformation by viral DNA, several chromosomal abnormalities are of interest and may represent specific alterations related to prostate carcinogenesis. These include numerical anomalies as well as structural anomalies. The fact that low levels of PSA as well as PSM are expressed in the HPV transformed cell lines CA-HPV-10 and PZ-HPV-7 may further validate the approach of transformation using viral DNA. To establish a stepwise model of prostate carcinogenesis, after the initial immortalizing step, the cell lines may serve as a target for the induction of additional changes. The establishment of epithelial human prostate-derived cell lines pRNS-1-1, PZ-HPV-7 and CA-HPV-10 provides an opportunity for the further development of an in vitro model of carcinogenesis for prostate cancer.

Samenvatting

Hoofdstuk 1. Prostaatkanker is in de Westerse landen één van de meest voorkomende maligne tumoren en heeft een relatief groot aandeel in de totale kankersterfte. Het is een heterogene ziekte die heden ten dagen in een vroeg stadium kan worden gediagnostiseerd, terwijl het klinisch beloop onvoorspelbaar blijkt te zijn. Het ontwikkelen van methoden die een onderscheid mogelijk maken tussen klinisch minder relevante en potentieel lethale carcinomen, is de uitdaging van het huidige prostaatkanker gericht wetenschappelijk onderzoek. Het stapsgewijze proces van de prostaatcarcinogenese wordt bepaald door vele factoren. Een korte beschrijving van enkele van deze factoren kan in hoofdstuk 1 worden teruggevonden. Chromosomale veranderingen die vooral in weefsels van prostaatcarcinomen kunnen worden aangetroffen zijn in hoofdstuk 1.4 in kort bestek weergegeven, alsmede een overzicht van genetische afwijkingen, mogelijke tumorsuppressor genen en oncogenen, die met prostaatkanker in verband worden gebracht. In dit inleidende hoofdstuk wordt verder nog de rol van het humaan papillomavirus (HPV) in urogenitale ziekte processen besproken, om twee redenen: ten eerste, omdat DNA van het HPV in enkele van onze experimenten werd gebruikt, en ten tweede, omdat in het begin van de negentiger jaren genetisch materiaal van verschillende oncogene HPV typen gedetecteerd werd in humaan prostaatweefsel. Dit laatste suggereerde een mogelijk oorzakelijk verband van ziekteprocessen in de prostaat met dergelijke virale infecties, wat evenwel vooralsnog niet is bevestigd. De rol van de menselijke prostaat als mogelijk reservoir van potentieel oncogene virussen kan niet worden uitgesloten en wellicht is deze rol van groter belang dan op dit moment kan worden verondersteld.

In dit proefschrift wordt het experimentele concept beschreven, waarbij de transformatie van epitheliale cellen afkomstig van radicale prostatectomie preparaten, wordt beschreven. In het algemeen kunnen epitheliale cellen van de volgroeide menselijke prostaat niet gedurende een lange termijn in kweek worden gehouden. Evenwel zijn er, ondanks het gelimiteerde groeipotentieel, methoden ontwikkeld om deze cellen in primaire celkweken te bestuderen [hoofdstuk 1.5]. Gebruikmakend van viraal DNA werd geanticipeerd op een mogelijke stimulatie van de cellulaire groei, waarbij een staat van onbeperkte groei cq immortaliteit zou kunnen worden bereikt. Gebaseerd op de resultaten van transformatie studies in andere orgaansystemen werden de experimenten uitgevoerd met DNA van het simiaanvirus (SV40), welke niet direkt in verband wordt gebracht met kankerontwikkeling bij de mens, en het humaan papillomavirus (HPV type 18), welke nochtans wel in verband wordt gebracht met kankerontwikkeling bij de mens, in het geval van HPV type 18 in het anogenitale gebied.

Hoofdstuk 2. Normale volwassen menselijke epitheliale prostaatcellen werden geimmortaliseerd middels polybreen-geinduceerde genintroductie van een SV40 DNA bevattend plasmide. De levensduur van de cellen in kweek was verlengd vergeleken met normale epitheliale prostaatcellen. Deze cellen, pRNS-1-1, bevatten het SV40

genoom, brengen het SV40 T-antigen tot expressie en zijn niet tumorigeen in naakte muizen. De cellen vertonen expressie van cytokeratinen 5 en 8, daarmee gelijkend op de oorspronkelijke cel, en zijn pseudodiploid. Analyse van groei regulerende processen toont aan dat de groei van pRNS-1-1 cellen op eenzelfde wijze kan worden gestimuleerd als bij normale epitheliale prostaatcellen, door epidermale groeifactor, insuline-achtige groeifactor, en hypofyse extract. De respons van pRNS-1-1 cellen op een groei-inhiberende factor, retinoïne zuur, was ook overeenkomstig de responscurve van normale cellen. Evenwel waren pRNS-1-1 cellen minder gevoelig voor groeiinhibitie door transformerende groei factor-ß, dan normale cellen. Tumor necrose factor-α en 1,25 $(OH)_2$ vitamine D_3 hadden in het geheel geen groeiremmend effect op de getransformeerde cellen. Hierdoor wordt de indruk gewekt dat door het transformatieproces groei-inhiberende maar niet groei-stimulerende mechanismen worden veranderd.

Hoofdstuk 3. Het humaan papillomavirus type 18 DNA werd geintroduceerd middels de lipofectie transfectie methode in epitheliale primaire celkweken, afkomstig van normaal en maligne weefsel van menselijke radicale prostatectomie preparaten. Twee cellijnen werden ontwikkeld: PZ-HPV-7, oorspronkelijke cel afkomstig van normaal weefsel van de perifere zone, en CA-HPV-10, oorspronkelijke cel afkomstig van slecht gedifferentieerd adenocarcinoom. Incorporatie van het HPV type 18 DNA werd bevestigd door middel van de polymerase chain reaction (PCR). Bij immunocytochemische analyse werd expressie van cytokeratinen 5 en 8 aangetoond, in overeenstemming met het expressiepatroon in de oorspronkelijke cellen. Tevens werd expressie van het E6 oncoproteine van het HPV type 18 aangetoond. PZ-HPV-7 cellen, afkomstig van normale diploide cellen, hadden een modaal chromosoom nummer van 46 in vroege passage, maar bleken later tetraploid te zijn geworden. CA-HPV-10 cellen waren aneuploid, en enkele cellen bleken nog double minute (dmin) chromosomen te bevatten, die ook werden aangetroffen in de oorspronkelijk cellen afkomstig van een prostaatcarcinoom. De cellijnen bleken niet uitdrukkelijk tumorigeen te zijn in naakte muizen. Met de ontwikkeling van bovengenoemde cellijnen werden voor het eerst menselijke epitheliale prostaatcellen afkomstig van zowel normaal als maligne weefsel, met succes getransformeerd en geimmortaliseerd middels de introductie van HPV type 18 DNA.

Hoofdstuk 4. Middels band karyotypering en fluorescentie in situ hybridisatie (FISH) met painting probes werd sequentieele cytogenetische analyse verricht van twee epitheliale menselijke prostaatcellijnen, PZ-HPV-7 en CA-HPV-10, ontwikkeld na transformatie door introductie van het humaan papillomavirus type 18 DNA [hoofdstuk 3]. PZ-HPV-7, oorspronkelijk afkomstig van normale diploide epitheliale prostaatcellen, had initieel een diploide chromosoom aantal maar werd uiteindelijk hypertetraploid, met een relatieve toename van chromosomen 5 en 20. Structurele chromosomale veranderingen waren nochtans beperkt. De cellijn CA-HPV-10 was in

oorsprong afkomstig van epitheliale cellen van een slecht gedifferentieerd adenocarcinoom van de prostaat, waarbij verschillende karyotypische afwijkingen waren vastgesteld, zoals het bezit van een extra Y chromosoom en double minute (dmin) chromosomen. CA-HPV-10 was voornamelijk hypertriploid. In vroege passage leek het karyotype van CA-HPV-10 instabiel te zijn, met een afnemend aantal cellen die nog dmin bevatten. De dmin werden in latere passage vervangen door een groot homogeen gebied, een zogenaamd homogeneously staining region (hsr). Deze hsr was gelegen op de korte arm van chromosoom 9, de 9p+ marker. Bij FISH analyse bleek de hsr van chromosoom 1 origine te zijn. Er werden een aantal consistente markers vastgesteld. Bovendien was verlies van het Y chromosoom een opvallende bevinding. Integratie van het HPV type 18 genoom was gelokaliseerd op 1p voor PZ-HPV-7, en op de 9p+ marker voor CA-HPV-10.

Concluderend werden zowel bij PZ-HPV-7, als bij CA-HPV-10, klonale cytogenetische veranderingen aangetoond. Bij de cytogenetische evaluatie van PZ-HPV-7 en CA-HPV-10 werden enkele karyotypische bevindingen gedaan die mogelijk van belang kunnen zijn bij de ontwikkeling van een stapsgewijs *in vitro* model van carcinogenese van de menselijke prostaat. De cytogenetische modificaties blijken te bestaan uit een spectrum van HPV transfectie gerelateerde phenomenen binnen een resterend kader van het oorspronkelijke karyotype.

Hoofdstuk 5. Expressie van prostaatmarkers prostaat-specifiek antigeen (PSA), prostaat-specifiek membraan antigeen (PSM), en de androgeen receptor werd geanalyseerd in de twee HPV getransformeerde menselijke epitheliale prostaat-cellijnen PZ-HPV-7 en CA-HPV-10. PSA en PSM expressie werd met behulp van de reverse transcription polymerase chain reaction (RT-PCR) bestudeerd. Tevens werd het effect van androgeen supplementatie op de groei onderzocht. Expressie van de androgeen receptor werd bestudeerd middels immunocytochemie. In beide cellijnen kon, vergeleken met prostaatcarcinoom cellijnen LNCaP en PC-346C, een laag niveau van RNA expressie voor PSA en PSM worden aangetoond. Bij immunocyto-chemische analyse met het monoclonale antilichaam F39.4 en het policlonale antilichaam SP-197 werd expressie van de androgeen receptor niet aangetoond. *In vitro* androgeen suppletie bleek de groei van beide HPV geimmortaliseerde cellijnen niet te stimuleren, terwijl dit voor LNCaP wel het geval was. Concluderend werden in HPV geimmortaliseerde epitheliale prostaat-cellijnen PZ-HPV-7 en CA-HPV-10 RNA transcripten van PSA en PSM gedetecteerd middels RT-PCR. Het feit dat een lage expressie van deze prostaat specifieke markers kan worden aangetoond verhoogt de validiteit voor het gebruik van dit stapsgewijze transformatie model van prostaatcarcinogenese bij de mens.

Hoofdstuk 6. De beperkingen en eigenschappen van de bovengenoemde cellijnen worden in dit laatste hoofdstuk nader beschouwd. In kort bestek kan worden geconcludeerd dat, in een poging om een model te creëren voor carcinogenese in de menselijke prostaat, epitheliale prostaat cellen van verschillende histologische origine werden geimmortaliseerd. Deze SV40 en HPV type 18 DNA getransformeerde cellen, afkomstig van prostaatweefsel van de volwassen mens, kunnen een verder inzicht verschaffen in de processen die mogelijk een rol spelen bij de ontwikkeling van prostaatkanker. Echter, ondanks het feit dat enkele *in vitro* eigenschappen van het boven beschreven model bruikbaar kunnen zijn, pleit het *in vivo* phenotype, plaveiselcelachtig in plaats van secretoir, in eerste instantie niet voor de benadering van transformatie middels viraal DNA als een eerste stap bij de ontwikkeling van een model voor prostaatcarcinogenese. Specifieke genetische veranderingen die vrij frequent bij prostaatkanker worden gevonden bleken niet aanwezig in de van prostaatcarcinoom afkomstige cellijn CA-HPV-10. Echter, enkele van de cytogenetische bevindingen in CA-HPV-10 zijn mogelijk wel van belang voor een verder inzicht in het proces van prostaatcarcinogenese. Deze omvatten numerieke alsmede structurele chromosoom afwijkingen van CA-HPV-10. De bevindingen moeten worden beschouwd binnen het kader van transformatie gerelateerde veranderingen. Verder is het van belang dat PSA- en PSM expressie in de HPV getransformeerde cellijnen CA-HPV-10 en PZ-HPV-7 werd aangetoond. Hierdoor kan het gebruik van dit stapsgewijze transformatie model van prostaatcarcinogenese aan validiteit hebben gewonnen. Met dit laatste gegeven als uitgangspunt is het mogelijk om het model verder gestalte te geven. Na de initiële stap van immortalisatie kunnen bovengenoemde cellijnen worden gebruikt voor de introductie van additionele genetische modificaties. De waarde van de nieuw ontwikkelde epitheliale menselijke prostaat cellijnen pRNS-1-1, PZ-HPV-7 en CA-HPV-10 zal moeten blijken binnen het kader van een verdere ontwikkeling van dit *in vitro* model voor carcinogenese in de menselijke prostaat.

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Publications related to thesis

- *Human papillomavirus type 18 DNA immortalized cell lines from the human prostate epithelium.*
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- *Lipofection-mediated immortalization of human prostate epithelial cells of normal and malignant origin using human papillomavirus type 18 DNA.*
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  Submitted Urology, 1997.

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- *Lipofection-mediated transformation of human prostatic epithelial cells of normal and malignant origin using Human Papillomavirus 18 DNA.*
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- *Human papillomavirus immortalized cell lines: A novel in vitro model of human prostate carcinogenesis.*
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- *Cytogenetic analysis of HPV transformed epithelial human prostate cell lines. A new model for prostate cancer research.*
  Philip C. Weijerman, Josee J. König, Bert J. Eussen, Wilma Teubel, Johannes C. Romijn, Fritz H. Schröder, Anne Hagemeijer. Ist world congress on basic urological research ESUOE & SBUR, Rotterdam, The Netherlands, September 1995.
  In: Urological Research, 23,4: 260, 1995.

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