Transformation of Human Neonatal Prostate Epithelial Cells by Strontium Phosphate Transfection with a Plasmid Containing SV40

Early Region Genes

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ABSTRACT

Neonatal human prostatic epithelial cells (NP-2s) were transfected by strontium phosphate coprecipitation with a plasmid (pRSV-T) containing the SV40 early region genes. The cells transfected with pRSV-T, but not the sham-transfected controls, formed rapidly growing, multilayered colonies within 2 weeks at a frequency of 1 x 10^-4 in a serum-free medium (P4-8F). In all, 28 colonies of transformed cells were isolated. Three of these have been cultured for a sufficient length of time to show that their growth potentials are well beyond that of the normal progenitor cells (NP-2s). There is also little or no indication of the culture “crisis” commonly seen in SV40-transformed cells in these transfected lines. All contain cytokeratins and SV40 T-antigen as revealed by immunofluorescence, have ultrastructural features of epithelial cells, and are pseudodiploid. None have produced tumors within 1 year after s.c. injection into nude mice. The transformed as well as the parental NP-2s cells require bovine pituitary extract for growth in serum-free medium and are stimulated by transforming growth factor βi (TGF-βi) and epidermal growth factor in clonal growth assays. In contrast, a prostatic carcinoma cell line (PC-3) is inhibited by TGF-βi. This serum-free system and immortalized transfected clones will be useful for studying the action of putative prostatic carcinogens and tumor-promoting agents.

INTRODUCTION

In 1978, methods suitable for the isolation of replicative epithelial cells from human neonatal prostate were described (1). These cells, NP-2s, were later transformed by infection with SV40' virus (2). Several colonies with extended culture life-spans, chromosomal aberrations, reduced growth factor dependence, altered morphology, and anchorage-independent growth were isolated. However, they were not tumorigenic, nor did they develop into established “immortal” cell lines. Further attempts to induce immortality and tumorigenicity by infection or transfection by ras oncogenes, or by exposure to chemical carcinogens were unsuccessful (3).

Renewed interest in utilizing the NP-2s system for investigating the etiology of prostatic cancer was stimulated by recent technological advances in culturing human prostate cells (4, 5) and in introducing cloned oncogenes into human epithelial cells (6). Because of the difficulties in inducing tumorigenicity by SV40 infection, possibly due to the fact that a small fraction of cells remain capable of viral synthesis (7), we explored the use of transfection to introduce only the SV40-transforming sequence. Since calcium phosphate was toxic to NP-2s cells, we substituted the newly developed strontium phosphate transfection procedure.

Transformed cells are generally less dependent upon and less responsive to mitogenic factors than are their normal counterparts. Earlier, we found that NP-2s cells infected with SV40 virus had reduced mitogen requirements in serum-supplemented medium (2). During the past several years, the complexity of the role of hormones and growth factors in controlling both growth and differentiation pathways has received more attention. In particular, TGF-βi has been shown to have bi-functional regulatory activities, the nature of which varies with cell type and the total cellular environment (8). The availability of a serum-free medium simplified the study of the effect of TGF-βi and other factors on the growth of NP-2s cells and its T-antigen gene-transfected derivatives (9).

MATERIALS AND METHODS

Cell Culture. Culture procedures were as previously reported (2) except that a serum-free growth medium (P4-8F) was used (10). This consisted of P4, a modified basal medium PFM4R4 (11) without the trace element concentrate and with the Ca2+ concentration reduced to 0.5 mM (Biological Research Facility & Facility, Inc., Minneapolis, MD). P4-8F consisted of P4 basal medium supplemented with 30 nM H2SeO3 (Johnson Matthey Chemicals, Ltd., Hertfordshire, England), 5 nM/μl epidermal growth factor (Collaborative Research), 5 μg/ml insulin, 0.28 μM hydrocortisone, 0.5 μM phosphoethanolamine, 0.1 mM chola toxin (Calbiochem), 250 μg/ml bovine serum albumin (Sigma), and 0.5% BPE prepared in this laboratory as previously described (12). TGF-βi was obtained from R & D Systems, Inc., Minneapolis, MN. Culture dish surfaces were treated with a coating mixture [100 μg/ml bovine serum albumin, 10 μg/ml bovine fibronectin (both from Calbiochem) and 20 μg/ml type I collagen (Vitrogen 100, Collagen Corp., Palo Alto, CA)] to promote attachment (13). PC-3, a prostatic carcinoma line (14), was also cultured in serum-free P4-8F. Adult human prostate cells were cultured as previously described (4, 5). Transfected cell lines, which were difficult to dissociate, were suspended by a sequential procedure. Either culture dishes were washed with HBS, then treated for 15 min at 37°C, with K-pass Solution A which contains (in g/liter): KCl, 8.4; NaCl, 7.1; NaH2PO4·7H2O, 1.9; KH2PO4, 0.005; polyvinyl-pyrrolidone (Calbiochem), 10; glucose, 3.4; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 4.8, pH 7.4. Serum which was present in the original Solution A (1, 11), was omitted. Solution A was removed and the cells were suspended with PET 1% polyvinylpyrrolidone, 0.02% EGTA, 0.02% crystalline trypsin (Sigma) in HBS, pH 7.4 at room temperature. When required, 0.4 mg/ml deoxyribonuclease-1 was added to further aid dissociation. Cultures were found to be free of mycoplasma by R. A. Del Giudice, Program Resources, Inc., NCI-FCTR, Frederick, MD (15).

Transfection. The plasmid, pRSV-T obtained from Dr. B. Howard, National Cancer Institute, is an SV40 ori' construct containing the SV40 early region genes and the Rous sarcoma virus long terminal repeat (6). NP-2s cells were recovered from frozen stocks, subcultured once, and were plated at the 25th PD level (1-2 x 10^6/100-mm dish)
in 10 ml of growth medium P4-8F. The plasmid DNA was coprecipitated with strontium phosphate as previously reported using the modification described in the addendum of Reference 6. After 4-h treatment, the precipitate was removed, and the cells were shocked by addition of 1 ml of 15% glycerol in HBS (w/v) for 30 s. The monolayer was washed three times with basal medium (P4), and incubated in P4-8F medium. Five days later, the plates were subcultured (2 x 10^5 cells/100-mm dish). The culture medium was replaced three times per week.

Isolation of Transformed Colonies. Colonies of tightly packed, multilayered cells which reached a diameter of 1 cm or more within 4–8 weeks were observed on dishes containing pRSV-T-transfected cells, but not on the control dishes. Colonies were ringed with glass cylinders and individually dissociated by sequential treatment as described in "Materials and Methods." The cells were cultured and expanded in P4-8F medium. The ability of the transfected cells to form colonies on a background of senescing normal cells was the only selective procedure used.

Clonal Growth Assays. Subconfluent cultures were suspended with PET and plated at 500 cells/60-mm dish in P4-8F; four dishes per variable were used. After overnight incubation, the medium was replaced with experimental media. After 6–7 days' incubation, plates were fixed with 10% buffered formalin and stained with Giemsa. Cells/colony were counted using an image analyzer. The mean clonal growth rate (PD/day) was based on counts of at least 18 colonies (2).

Tumorigenicity Assay. Cells were suspended with PET and injected s.c. in the mid-dorsal cranial region of athymic nude mice (nu/nu) (NCI-FCRF, Frederick, MD). A total of 2 x 10^6 cells in 0.1 ml of medium were used per animal. At least five animals per cell line were injected. Similar protocols were also used at Stanford (D.M.P.), but in addition one animal per cell line was used for histological examination of the inoculum site at 2 weeks. Animals were observed for tumor development up to 12 months.

Chromosomal Analysis. Karyotypic analyses were carried out by Dr. Ward D. Peterson, Jr., Children's Hospital of Michigan, Detroit, MI, by Giemsa banding (16). Exact chromosome counts of 30 metaphases per line were made for ploidy determination. For each culture, six to 10 karyotypes were prepared.

Immunostaining and Electron Microscopy. Cytokeratins and SV40 T-antigen were identified by indirect immunofluorescence as previously described (17). Cells were grown on coated Chamber-Tek (Miles) slides and fixed with acetone at −20°C. Mouse monoclonal antibodies were used to detect large and small T-antigens (Oncogene Science, Mineola, NY), and cytokeratins (Labsystems, Chicago, IL). Primary antibodies were subsequently stained with goat fluorescent anti-mouse immunoglobulin antibody. Additional immunoperoxidase labeling was carried out at Stanford (D.M.P.) using an avidin-biotin-peroxidase complex (18). Cells were prepared for transmission and scanning electron microscopy by standard procedures (17).

Southern Analysis. Cell lines were analyzed for the presence of integrated SV40 early region DNA. Genomic DNA (10 μg) was digested with restriction enzymes BamHI (which cleaves a single site in pRSV-T) and XhoI (to which pRSV-T is resistant). The DNA digests were electrophoresed on 1% agarose gel, and the DNA was transferred (19) to Gene Screen Plus membrane (New England Nuclear, Boston, MA). Membranes were prehybridized in 1% sodium dodecyl sulfate/1 M NaCl/10% dextran sulfate for 6 h at 65°C. The blots were then hybridized with 32P-labeled DNA probe overnight at 65°C in prehybridization solution containing 200 μg/ml denatured salmon sperm DNA. The probe, labeled by nick translation (20) was the 1.2-kilobase HindIII DNA fragment of the SV40 early region from plasmid pRSV-T. The membranes were washed twice for 5 min with 2 x SSC at room temperature, twice with 2 x SSC/1% sodium dodecyl sulfate for 30 min at 65°C, and twice with 0.1 x SSC for 30 min at room temperature. The membranes were autoradiographed with an intensifying screen at −70°C.

RESULTS

Transfection and Isolation of Transformed Cells. Preliminary experiments showed that calcium phosphate was toxic to NP-2s cells. However, colonies of transformed cells that developed in cultures transfectected with pRSV-T by the strontium phosphate technique could be seen by phase microscopy within 2 weeks and had reached a diameter of about 1 cm by 4–8 weeks. The transformation frequency in three separate experiments was approximately 1 x 10−4. No colonies developed in control dishes containing only the strontium phosphate precipitate (Fig. 1) or constructs carrying H-ras, v-myc, H-ras + v-myc, or...
the adenovirus 12 genes E1a and E1b. Initially, the colonies consisted of small aggregates that developed into tightly packed, multilayered colonies that were very difficult to reduce to a single-cell suspension. However, sequential treatment with a solution containing an elevated K+ concentration, followed by trypsinization and addition of deoxyribonuclease was effective in reducing the extent of clumping. The reduced ability of the transfected cells to attach to the culture dish surface was eliminated by coating the surface of the dishes ("Materials and Methods").

Escape from Senescence. The cumulative growth of three selected transformed lines is shown in Fig. 2. Two lines, 267B1 and 272E1, are approaching the 100th PD, whereas 272A9 has reached the 65th PD level. Except for a lag period in the case of 272E1, no indication of a traditional crisis has been seen. In contrast, the life expectancy of the normal cells at transfection was approximately 10–15 PDs (1).

Characterization. The transformed colonies had the typical polygonal arrangement of epithelial cells by phase contrast microscopy (not shown). Transmission and scanning electron micrographs (Fig. 3) revealed features comparable to those originally reported for NP-2s (1, 11). Analyses of eight isozymes were carried out on NP-2s, 267B1, 272E1, and 272E4. All had the human lactic dehydrogenase and type B glucose-6-phosphate-dehydrogenase. These data support the conclusion that the transfected lines are derived from NP-2s (16). Cytokeratins 8 (Mr 52,000) and 18 (Mr 45,000), vimentin, actin, and fibronectin were demonstrated in all three transfected cultures by indirect immunoperoxidase staining (Table 1). Intense nuclear staining was observed in all three transfected cultures with anti-SV40 T-antigen, but not in normal cells. In many respects, the labeling pattern was similar to that of adult prostate epithelial cells. Labeling of structural filaments was less intense in the transfected lines. In 272E4, some cells were intensely stained whereas other were negative with anticytokeratin 8. Cytokeratins 1, 5, 10, and 11 stained intensely in the adult cells but not in the transfected lines (not shown). In cell line 272E4, keratin 8 is strongly expressed in denser cultures. In some cases antisera to a single antigen from different sources gave variable results. This was true of prostatic acid phosphatase, prostatic specific antigen (Table 1), collagen Type IV, and laminin (not shown). Factor VIII, an endothelial cell antigen, was not detected.

Chromosomal Alterations. Karyotypic analysis of several transformed lines revealed varying mixtures of normal and aneuploid populations. All lines had the Y-chromosome. The chromosome counts of four lines are summarized in Table 2. In most cases, 90% of the chromosome counts were in the diploid range (2n = 46). The line 267B1 (36 PDs) consisted mostly of normal male cells with random loss and gain of normal chromosomes. However, by the 53rd PD level, 267B1 had become 24% tetraploid and had a significantly higher proportion of abnormal metaphases, monosomy, trisomy, rearrangements, dicentrics, and gaps than were observed at 36 PDs. Cell lines 272E1 and 272E4 had normal as well as aneuploid cells. 272E1 had a higher proportion of aneuploid cells than did 272E4. Fig. 4 shows an abnormal 46 chromosome karyotype of 272E1 at the 36th PD level carrying a 4p+ translocation and an aberrant chromosome 14. No consistent losses, gains, or stable marker chromosomes were seen. In contrast, the parental line, NP-2s was predominantly normal male (XY) diploid even when approaching senescence at the 30th PD level. Two metaphases, one with a missing chromosome 12, the other with a missing chromosome 9 were seen, indicating an occasional lack of division fidelity as the normal NP-2s culture approached senescence (2). No marker or unassignable chromosomes were found.

Tumorigenicity. None of the transfected lines have formed tumors in nude mice 12 months after s.c. inoculation. This observation was confirmed at Stanford (D.M.P.) and at Northwestern University by Dr. E. W. Sherwood.4 Dr. Sherwood also

4 Personal communication.
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Fig. 4. Karyotype of transformed line, 272E1, at the 20th PD. A 4p+ translocation (arrow) and an aberrant no. 4 chromosome are shown.

Table 1 Antigenic markers in transfected prostatic epithelial cells

Cells were fixed and stained as described in "Materials and Methods." Labeling was assigned values from – (none) to +++ (very intense).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>267B1</th>
<th>272E1</th>
<th>272E4</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratin 8</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>+</td>
</tr>
<tr>
<td>Keratin 18</td>
<td>+*</td>
<td>+*</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>Vimentin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>SV40 T-antigen</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>PAP</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+++</td>
</tr>
<tr>
<td>PSA</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Heterogeneous staining.
* Boehringer Mannheim mouse antikeratin 18, 1:50.
* Enzo Biochemicals mouse antivimentin, 1:200.
* Miles rabbit antiactin, 1:40.
* ICN mouse antifibronectin, 1:200.
* ICN goat anti-factor VIII, 1:200.
* Oncogene mouse anti-SV40 T-antigen, 1:1000; nuclear staining only.
* Prostatic acid phosphatase was negative with Ortho rabbit anti-PAP, undiluted and positive with Miles rabbit anti-PAP, 1:300.
* Prostatic specific antigen was negative with Ortho rabbit anti-PSA, undiluted and positive with aberrant nuclear staining with Dako rabbit anti-PSA, 1:400.

Table 2 Chromosome numbers of normal and transfected prostatic epithelial cells

Data represent the number of metaphases in the specified range. 100 metaphases per culture were scored.

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Population doubling level</th>
<th>Near diploid (42-48)</th>
<th>Near tetraploid (85-92)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP-2s</td>
<td>30</td>
<td>86</td>
<td>11†</td>
</tr>
<tr>
<td>267B1</td>
<td>36</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>267B1</td>
<td>53</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>272E1</td>
<td>25</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>272E4</td>
<td>25</td>
<td>91</td>
<td>9</td>
</tr>
</tbody>
</table>

* Estimated population doublings since isolation (NP-2s) and since transfection of NP-2s.
* Range of chromosome numbers.
* This analysis contained three of 100 metaphases with >150 chromosomes.

observed no tumors following intrasplenic inoculation. The histology of the inocula at the s.c. injection sites was examined 2 weeks after injection. In all three lines, small nodules, 2–5 mm in diameter, were observed. They were formed by clusters of cells with largely mostly oval nuclei, prominent nucleoli and scanty pale cytoplasm sometimes forming acinar structures. There were frequent mitoses and evidence of infiltration into the surrounding stromal, blood vessels, and muscle tissue. The histological pattern was consistent with carcinoma in all three lines. Observation at subsequent times showed that the nodules never increased in size and eventually regressed. Line 272E4 showed more irregular nuclear sizes and shapes and evidence of pyknosis.

Response to Growth Factors. TGF-β1 has been reported to inhibit growth or to stimulate terminal differentiation in epithelial cells (21). The effect of TGF-β1 on the growth rate of the transformed lines was tested by clonal growth assay (Fig. 5). For comparison the parental line, NP-2s, and the prostatic carcinoma line, PC-3, were included. TGF-β1 stimulated the growth rate of the normal as well as the transformed lines, but inhibited the growth of PC-3 at high levels. Bovine pituitary extract was found to be both essential for serum-free growth and, at higher levels, slightly inhibitory (Fig. 6).

Southern Analysis. For each of three cell lines analyzed for the presence of SV40 early region DNA, hybridization to the T-antigen gene probe revealed single bands in genomic DNA digested with either XbaI (for which there is no restriction site in pRSV-T) or BamHI (which cleaves pRSV-T once outside of the sequence corresponding to the probe) (Fig. 7). There was unequally intense hybridization to the probe. However, inspection of the gel after electrophoresis by ethidium bromide staining showed a corresponding inequality of DNA loading, and prolonged autoradiography revealed no additional bands (not shown). It is therefore likely the pRSV-T has integrated at only one site in each of these three cell lines. The SV40 probe did not hybridize with the control DNA from PC-3 cells.

DISCUSSION

Prostatic cancer, now the third most prevalent form of cancer in men and the second leading cause of male cancer-related deaths, is becoming more significant as life expectancy is extended (22). Despite the dimensions of this problem, little is known about the etiology of prostatic carcinogenesis (23). The main purpose of this work was to develop a culture model
suitable for investigating the etiology and progression of prostatic cancer. Since normal prostatic epithelial cells have a short lifespan, it was important to find a way of extending their replicative potential without introducing genes allowing viral synthesis. Efforts to introduce the SV40 early region genes into NP-2s by the standard calcium phosphate transfection method were unsuccessful due to the toxicity of the calcium phosphate. However, the newly developed strontium phosphate procedure (6) yielded stable transfectants with an efficiency of $1 - 2 \times 10^{-4}$. This frequency of transformation is approximately the same with both the calcium and strontium procedures providing that the cells can withstand both. The observed transformation frequency is identical to that achieved in this laboratory using the same plasmid with human cells from the bronchial epithelium (21), mesothelium (24), and esophagus.5

All types of normal human cells appear to have finite life-spans in culture (25). Human cells infected with SV40 virus have extended lifespans but, in most cases, undergo a "crisis" during which the rate of proliferation markedly decreases or ceases entirely (26). More recent reports indicate that crisis does not always occur and its incidence and severity may vary with cell type, for example, human keratinocytes often become immortalized without the intervention of crisis (26).

Three other types of human epithelial cells have been transfected with pRSV-T. Four of four clonally derived transfected bronchial lines senesced (21), and two survived crisis and became immortalized.6 Similarly, four pRSV-T-transformed mesothelial lines senesced and only one recovered from crisis (24). Two pRSV-T-transformed esophageal lines also have survived crisis.5 In contrast, the 3 pRSV-T-transfected NP-2s lines that have been in culture long enough to permit a prediction of their growth potential appear to have become immortalized without a crisis, with only a short (approximately 40-day) lag occurring in the 272E1 population. Whether immortalization without crisis is a general characteristic of prostatic epithelial cells transfected with pRSV-T awaits further study. In the previous study where NP-2s cells were infected with SV40 virus, all of the transformed isolates had senesced by the 45th PD (2) and none developed into immortal lines. It is unclear whether the contrasting result obtained in the present study is due to the different method of introducing the SV40 early region and

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3 G. D. Stoner, unpublished.
4 R. R. Reddel et al., unpublished.
the absence of late region genes, or due to the substantially improved culture techniques used in the present study.

The transformed cultures express T-antigen by immunoperoxidase nuclear staining. This is consistent with the Southern blotting data indicating that each line contains a single copy of integrated SV40 early region DNA. The antigenic profile of the transfected lines is quite similar to that of the adult prostatic epithelial cells (Table 1). They express cytokeratins as expected for cells of epithelial origin. All three lines are similar to benign prostatic adenomas and have nearly identical two-dimensional gel electrophoresis patterns of [35S]methionine-labeled cell extracts. They contain acidic (no. 19, M, 40,000) and basic (no. 6, M, 56,000) cytokeratins.7

Although the finding that TGF- ß stimulated the growth rate of the normal NP-2s and of T-antigen-transfected cells might appear in contrast with the known inhibitory effect of TGF- ß on several types of epithelial cells (27–30), TGF- ß is not always inhibitory for epithelial cells (8). Human mesothelial cells are also stimulated by TGF- ß1 (31). Since NP-2s, its SV40-transformed sublines, and the mesothelial cells were isolated and cultured in media containing serum, it might be expected that these lines could grow in the presence of TGF- ß1, a factor present in serum (27). The prostatic carcinoma line (PC-3) was inhibited by TGF- ß1, confirming a report concerning the androgen-independent prostatic cancer lines, PC-3 and DU 145 (32). Furthermore, the closely related TGF- ß2 has been isolated and purified from PC-3-conditioned medium (33). The mechanism by which normal prostatic cells are stimulated by TGF- ß1 whereas neoplastic cells is inhibited is unknown. However, these findings suggest that an autocrine mechanism of growth control may be involved. The availability of a serum-free system and the present T-antigen gene-immortalized lines will facilitate investigation of this hypothesis.

The karyotypic alterations in the transfected prostatic epithelial cells are similar to those previously reported after infection with SV40 virus (2). They are also comparable to those in the immortalized human bronchial epithelial line, BEAS-2B (21). Most transfected lines were near diploid with random loss and gain of chromosomes. With continued culture, an increase in chromosome number and other aberrations developed. Instability of the karyotype also appears to be a characteristic of virally transformed cells (26, 34).

None of the tested transfected lines were tumorigenic in nude mice, although a transient growth of carcinoma-like cells was observed at 2 weeks. Stanbridge et al. (34) have formulated a model for the progression of a normal to a neoplastic cell via a transformed but nontumorigenic intermediate. The transfected lines may represent such an intermediate in which altered growth control may be involved. The availability of a serum-free system and the present T-antigen gene-immortalized lines will facilitate investigation of this hypothesis.

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