LNCaP Model of Human Prostatic Carcinoma

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ABSTRACT

The LNCaP cell line was established from a metastatic lesion of human prostatic adenocarcinoma. The LNCaP cells grow readily in vitro (up to 8 x 10^6 cells/sq cm; doubling time, 60 hr), form clones in semisolid media, are highly resistant to human fibroblast interferon, and show an aneuploid (modal number, 76 to 91) human male karyotype with several marker chromosomes. The malignant properties of LNCaP cells are maintained. Androgen nude mice develop tumors at the injection site (volume-doubling time, 86 hr). Functional differentiation is preserved; both cultures and tumor produce acid phosphatase. High-affinity specific androgen receptors are present in the cytosol and nuclear fractions of cells in culture and in tumors. Estrogen receptors are demonstrable in the cytosol. The model is hormonally responsive. In vitro, 5α-dihydrotestosterone modulates cell growth and stimulates acid phosphatase production. In vivo, the frequency of tumor development and the mean time of tumor appearance are significantly different for either sex. Male mice develop tumors earlier and at a greater frequency than do females. Hormonal manipulations show that, regardless of sex, the frequency of tumor development correlates with serum androgen levels. The earlier and at a greater frequency than do females. Hormonal manipulations show that, regardless of sex, the frequency of tumor development correlates with serum androgen levels. The rate of the tumor growth, however, is independent of the gender or hormonal status of the host.

INTRODUCTION

CaP is the second most frequent tumor of males in the United States (41). Unknown etiology, variable pathology, intricate relationship to endocrine factors, and anaplastic progression contribute to the complexity of this disease and confound the investigator. The progress toward establishing effective methods for early detection and successful management of CaP is predicated on both clinical studies and laboratory experimentation with appropriate models. Extensive studies on models of rodent prostate carcinoma (7, 9, 18, 23, 32, 40) have already provided a better understanding of the biology of prostatic neoplasia. These tumors, derived from rat prostate, are readily transplantable, and the tumor cells proliferate in vitro. Their value as models is based on the preservation of malignancy, biological and biochemical markers, hormonal responsiveness, and drug sensitivity. Their limitations stem from apparent restrictions imposed on extrapolating data derived from animal models to human neoplasia.

The development of a correspondingly wide array of appropriate models of human origin is hindered by difficulties in sustaining the propagation of human CaP cells outside its natural host. With few exceptions (12, 33), the athymic nude mouse fails to provide satisfactory growth environment for direct CaP xenografts from patients (10, 34). Although human prostate cancer cells grow readily as short-term explant cultures (6, 16, 24, 45), reports on established malignant cell lines with convincing pedigree are rare (14, 20, 27, 42). In addition, some of such cell lines fail to maintain markers characteristic of CaP: e.g., production of secretory human prostatic acid phosphatase (19, 42); organ-specific prostate antigen (30); responsiveness to sex hormones (19, 42); or the presence of the Y chromosome (19).

The subject of this report is the cell line LNCaP which we have isolated from a metastatic lesion of human prostatic cancer (14). The growth properties in vitro, clonogenic potential, karyology, androgen responsiveness with its attendant presence of specific receptor molecules, and tumorigenicity in intact and hormonally manipulated athymic nude mice are described. These data extend our knowledge toward a full characterization of a versatile new model suitable to study human prostatic cancer in the laboratory.

MATERIALS AND METHODS

Culture Medium. The LNCaP cell line was originally established (14) and subsequently propagated in RPMI-1640-GA (Grand Island Biological Co., Grand Island, N. Y.) and supplemented with 5% (v/v) heat-inactivated single-lot (No. 300010H) FBS (K.C. Biological, Inc., Lenexa, Kans.). For hormone responsiveness experiments, ZSD (AMF Immuno-Reagents Inc., Seguin, Texas) was utilized in place of FBS. ZSD is a commercially processed serum of bovine origin. The concentration of steroids is reduced by charcoal filtration by approximately 20-fold in comparison with regular FBS. Data provided by the manufacturer for the single lot (No. 050481) of serum used in our experiments indicated the following concentrations: testosterone, 72 pw; 17β-estradiol, 58 pw; cortisol, not detectable; DHT, not determined; cholesterol, 0.52 μM; insulin, 6 μunits/ml; and total protein, 45 mg/ml. The DHT was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell Propagation. Confluent monolayers (6 to 8 x 10^6 cells/sq cm) of LNCaP cells cultivated in 75-sq cm plastic flasks (Falcon Plastics, Oxnard, Calif.) were dispersed with trypsin (0.05%):EDTA (0.02%) solution (Grand Island Biological Co.) and counted. The cells were inoculated into new vessels at 3 to 8 x 10^5 cells/sq cm in RPMI-1640-GA with 5% (v/v) FBS (0.1 ml/sq cm) and left undisturbed for 48 hr in the incubator (37°, 5% CO₂ atmosphere) to facilitate attachment. The cultures were fed at 5- to 7-day intervals with fresh medium (0.3 ml/sq cm) of the same composition. LNCaP cells from between the 30th to the 55th passage in vitro were used to conduct these studies. This corresponds to between 100 and 300 population doublings after the original isolation. The cells are free of Mycoplasma.

Cell Counts. An electronic particle counter (Model ZB-1 Coulter Counter) was used to enumerate formalin-fixed nuclei from detergent-
lysed cells according to the method of Butler et al. (4).

Karyology. For cytogenetic analysis, LNCaP cultures were incubated with Colcemid (0.015 μg/ml, 2 hr; Grand Island Biological Co.), harvested, treated with hypotonic solution (0.075 M KCl), fixed with methanol:acetic acid (3:1), and spread on dry slides. The G-banding method (36) of staining was used for chromosome identification and analysis.

Sex Hormone Receptors. Methods used for the preparation of cytosols, nuclear fractions, and measurements of steroid receptors were published (11, 25, 39). Briefly, for androgen receptor determinations, the competition binding assay with methyltrienolone (17β-hydroxy-17-methyl-estrof4,9,11-trien-3-one) in the presence of triamcinolone acetonide was used (11, 43). Estrogen receptor was quantified by the dextrancoated charcoal assay (25) as well as the sucrose density gradient analysis (39) including nafloxidine as the competitor for the binding of radioactive 17β-estradiol.

Where appropriate, the binding was analyzed using the Scatchard plot method (38). The concentrations of protein (22) and DNA (3, 35) were determined as published.

Methylation studies (specific activity, 87 Ci/mmol) and nonradioactive methyltrienolone as well as 17α-[2,4,6,7-3H]estriadiol (specific activity, 90 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). The trizma (free base) and triamcinolone acetonide were obtained from Sigma. Norit A charcoal was purchased from Matheson, Coleman and Bell Manufacturing Chemists (Norwood, Ohio); dextran (Grade C, M, 60,000 to 90,000) was obtained from Mann Research Laboratories (New York, N. Y.); and sucrose (RNase free) was purchased from Schwarz/Mann (Orangeburg, N. Y.). Dithiothreitol was obtained from Calbiochem (La Jolla, Calif.), and nafloxidine was a gift from the Upjohn Co., Kalamazoo, Mich.

AP, AP activity was determined by the method of Babson and Phillips (1) using 3 nm α-naphthyl acid phosphate sodium salt as the substrate, 0.02% Fast Red B salt (5-nitro-2-aminomethoxybenzene diazotate) as the color developer and 6 nm α-naphthol as the standard. All these reagents were obtained from Sigma.

HulFNβ. The highly purified (specific activity, 2 x 10^7 reference units/mg protein) HulFNβ used in these studies was produced in our laboratory for clinical trials (21). Interferon assays were carried out using published methods (13, 15). The International reference standard of human fibroblast interferon (G-023-902-527; Research Resources Branch, NIH) was included in each assay for calibration, and titers were expressed in reference units as geometric means of triplicate tests.

Athymic Nude Mice. A breeding nude mouse colony has been maintained in our laboratory since 1976 under pathogen-limited conditions. The animals bear a nu/nu genotype on super Swiss and C3H background, and their life span is in excess of 12 months.

Tumor Induction. Tumors were induced in athymic nude mice (6 to 8 weeks old) by injection of cultured LNCaP cells. The cells were dispersed by trypsin, washed (twice) in serum-free medium RPMI-1640 (10 min centrifugation, 200 x g), resuspended (2 x 10^7 cells/ml, same medium), and injected (0.2 ml) s.c. between the shoulder blades of each mouse.

The tumors were measured with calipers (2 perpendicular diameters), and the tumor volume was calculated using the formula W^2 x L/2 cu mm (W, shorter diameter; L, longer diameter (mm)).

Hormonal Manipulations. Castration or ovariecctomy were performed 3 days before inoculation of LNCaP cells. Pellets, containing either 2 mg testosterone propionate or 2 mg 17β-estradiol, with cholesterol, Emcompress (Edward Mendel, Carmel, N. Y.), and magnesium stearate as excipients were prepared by the Roswell Park Memorial Institute Drug Formulation and Development Laboratory and implanted s.c. 3 days before inoculation of LNCaP cells. Both hormones were purchased from Sigma.

RESULTS

Studies in Vitro

LNCaP Cell Cultures. The LNCaP cells form monolayers which are only weakly attached to the surface of plastic culture vessels. The cultures require gentle handling at all times because the cells could be easily dislodged by tapping, shaking, or pipeting. The reattachment of dissociated cells is slow, and only about 70% of cells from the original inoculum adhered to the growth surface within 40 hr. Varying the concentration of FBS between 1 and 15% (v/v) does not measurably affect this process (data not shown). The low anchoring potential is also responsible for the 10 to 20% cell loss during media changes in long-term experiments. When cell density in excess of 8 x 10^5 cells/sq cm is reached, the cell sheet tends to detach spontaneously. High concentrations of LNCaP cells, dispersed by routine trypsinization or by mechanical manipulations, form clumps which are difficult to dissociate or count reliably. Accurate cell counts can be obtained after lysing the cells and counting the free nuclei (4). To initiate growth in new cultures, a broad range of inocula (from 5 x 10^3 to 1 x 10^5 cells/sq cm) could be used (Chart 1).

Serum Requirement. Cultures of human cells in vitro require serum supplement for growth. The optimal concentration varies for different cells and is usually lower for cells derived from tumors than from normal tissues. To characterize further the LNCaP cells in vitro, their growth in RPMI-1640-GA supplemented with different concentrations of FBS from 0.01% to 15% (v/v) was measured. The results (Chart 2) show that LNCaP cells can proliferate in the presence of a broad range (0.1 to 15%) of FBS concentrations. Maximal growth was obtained using media supplemented with 2.5% to 15% FBS. Under these culture conditions, the mean population-doubling time was calculated to be approximately 60 hr. Lowering the serum concentration resulted in slower growth. At a FBS concentration of 0.01%, the number of cells in the culture slowly declined. For maintenance of cell stocks and experiments in vitro, 5% (v/v) FBS was routinely added to medium RPMI-1640-GA.

Cloning. Single malignant cells are capable of colonial growth in semisolid media. Table 1 shows that LNCaP cells have anchorage-independent proliferation and can be cloned easily and with good efficiency. From plates inoculated with the lowest number of cells, 13 individual clones were isolated, propagated, and cryopreserved (10% dimethyl sulfoxide, liquid N2 storage) for future studies.

Karyology. The LNCaP cells were examined as to their karyological characteristics at 12 and 32 months after initial isolation, i.e., after approximately 100 and over 300 population doublings in vitro, respectively. At both points in time, the LNCaP cells...
exhibited grossly aneuploid human male karyotype with several marker chromosomes present. At 32 months, the number of chromosomes ranged from 33 to 91 with a modal number of 76 to 91 (in 26 of 46 metaphases counted) (Table 2; Fig. 1). Some marker chromosomes were present consistently: \( m_1 \), long submetacentric marker chromosome of unknown origin; \( m_2 \) and \( m_6 \), identified as \( t(1;15) \); \( m_7 \) and \( m_9 \), identified as \( 10q- \). The last 4 \( (m_4 \) to \( m_7) \) were usually present in 2 copies. Other chromosome abnormalities have been observed with lesser regularity.

**Sex Hormone Receptors in Cultured LNCaP Cells.** Specific androgen- and estrogen-binding proteins were reported in normal and neoplastic human prostatic tissues (8, 11, 28, 43). To determine if these macromolecules are present in the LNCaP cells maintained *in vitro*, androgen receptor in both the cytosol and the nuclear extract and estrogen receptor in the cytosol were measured. Table 3 shows that both specific androgen and estrogen receptors are present in the cytosol. Nuclear androgen receptor was also detected, but at a relatively lower concentration. Scatchard plot analysis (Chart 3) of the results indicated the presence of a separate single class of specific, high-affinity, low-capacity, and saturable androgen-binding molecules.

**DHT Modulation of Cell Growth and AP Production.** Human prostatic epithelial cells in *in vivo* are responsive to male sex hormones. Therefore, both the cell proliferation and the AP production in LNCaP cultures in media supplemented with DHT were studied. The DHT concentrations tested (between 1 nw and 1 \( \mu \)m) include and exceed the normal range of DHT levels found in the serum of healthy males (0.8 to 4.1 nm) (37). Simultaneous and parallel experiments were conducted using media supplemented with 5% (v/v) of either ZSD or FBS. ZSD was used because it is depleted of steroid hormones by activated charcoal during the manufacturing process. The LNCaP cells in RPMI-1640-GA supplemented with 5% (v/v) ZSD grew at a slower rate with a mean population-doubling time of approximately 144 hr as compared with the 60 hr for cells cultivated in medium with 5% (v/v) FBS. ZSD, however, does not contain apparent inhibitors of LNCaP cell division, since its addition (up to 10% v/v) to media already containing FBS (at 5% or 10% v/v) did not diminish the growth rate of the cultures (data not shown).

**Table 1**

<table>
<thead>
<tr>
<th>No. of cells/plate</th>
<th>Cloning efficiency</th>
<th>Clumping rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1 \times 10^6 )</td>
<td>TMTC*</td>
<td>TMTC</td>
</tr>
<tr>
<td>( 1 \times 10^5 )</td>
<td>TMTC</td>
<td>TMTC</td>
</tr>
<tr>
<td>( 5 \times 10^4 )</td>
<td>15.0</td>
<td>3.6</td>
</tr>
<tr>
<td>( 1 \times 10^4 )</td>
<td>12.0</td>
<td>1.8</td>
</tr>
<tr>
<td>( 5 \times 10^3 )</td>
<td>10.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*TMTC, too many to count.

**Chart 2.** Growth of LNCaP cells *in vitro* in the presence of different concentrations of FBS. Dispersed LNCaP cells were seeded at 4 x 10^4 cells/sq cm in 6-well plates and grown (37°.5% CO2) in RPMI-1640-GA with different concentrations of FBS. Cell counts (in triplicate from 3 wells) were carried out after 7, 10, and 14 days without medium change. The number of cells/sq cm is plotted against time in hr after seeding. FBS concentrations (%, v/v): \( ^{V} \), 0.01; \( ^{\circ} \), 0.1; \( ^{\circ} \), 0.5; \( ^{\Delta} \), 1.0; \( ^{\Delta} \), 2.5; \( ^{\Delta} \), 7.5; \( ^{\Delta} \), 15.

**Table 2**

Chromosome number distribution of LNCaP cells after 12 and 32 months of growth *in vitro*

<table>
<thead>
<tr>
<th>Distribution of cells with following numbers of chromosomes</th>
<th>Total No. of cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>40</td>
</tr>
<tr>
<td>12 mos. <em>in vitro</em></td>
<td>3</td>
</tr>
<tr>
<td>32 mos. <em>in vitro</em></td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3**

Androgen and estrogen receptors in cultured LNCaP cells

For each of 2 experiments shown, 2 x 10^6 LNCaP cells were mechanically removed from culture vessels, centrifuged (200 x g, 10 min, 0°), and washed twice in ice-cold serum-free RPMI-1640. The cell pellets were rapidly frozen and stored in liquid N\(_2\) until used (within 30 days). Competition binding assays of cytosol and nuclear extracts by the dextran-coated charcoal procedure were used to determine androgen and estrogen receptor content. \( K_d \) values were derived from Scatchard plots. Protein was measured according to the method of Lowry et al. (22) and DNA according to Richards' modification (35) of the method of Burton (3)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Androgen receptor</th>
<th>Estrogen receptor (in cytosol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg DNA/g cells</td>
<td>mg cytosol protein/g cells</td>
<td>fmoles/mg DNA</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>5.5</td>
<td>31.5</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>3.6</td>
<td>36.5</td>
</tr>
</tbody>
</table>

* For Scatchard plot, see Chart 3.

* ND, not determined.
The mixtures contained also triamcinolone acetonide (1000-fold excess).

Tory effects over the entire range of the tested concentrations by the addition of DHT. DHT was found to have potent stimulation (Chart 4B).

nM (Chart 4A); and (b) DHT addition to media supplemented with addition at all concentrations tested, with an optimum of about 10 (ZSD or FBS) was present in the medium: (a) the addition of apparently different effects of DHT depending on which serum (ZSD or FBS) was present in the medium: (a) the addition of

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The AP activity in the LNCaP culture medium is also influenced by the addition of DHT. DHT was found to have potent stimulatory effects over the entire range of the tested concentrations (Chart 5) regardless of which serum, ZSD or FBS, was used.

Resistance to Interferon. Interferons are glycoproteins with antiviral as well as antiproliferative activities. Both activities are readily demonstrable on a wide range of normal and neoplastic cell types. Since interferons are being tested as potential therapeutic agents in a variety of human tumors, including prostatic neoplasia, the in vitro susceptibility of LNCaP cells to HulFNβ was measured. HulFNβ, at concentrations as high as 10,000 reference units/ml, was found to have only a negligible effect on the proliferation of LNCaP cells during the 7-day period of exposure (Table 4). The presence of interferon did not affect the cell viability or influence the levels of AP production. The interferon levels detected at 24 and 96 hr postaddition (Table 4) indicate that the expected decay of interferon activity in LNCaP cultures is not greater than either the decay observed during incubation in cell-free medium (data not shown) or the decay reported for other cells (13).

To determine whether interferon could induce an antiviral state in LNCaP cells and thus prevent the killing action of vesicular stomatitis virus on these cells, HulFNβ (10 to 10,000 reference units/ml) was preincubated with the cells for 24 hr. Such treat-

The addition of DHT to growth medium has a distinct modulating effect on the proliferation of LNCaP cells (Chart 4). The orderly and dose-dependent curves obtained demonstrated 2 apparently different effects of DHT depending on which serum (ZSD or FBS) was present in the medium: (a) the addition of DHT to media supplemented with ZSD stimulated cell proliferation at all concentrations tested, with an optimum of about 10 nm (Chart 4A); and (b) DHT addition to media supplemented with FBS resulted in a dose-dependent suppression of cell proliferation (Chart 4B).

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Table 5

<table>
<thead>
<tr>
<th>LNCaP tumors in male and female athymic nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice (6 to 8 weeks old) were given s.c. injections of 4 x 10^6 cultured LNCaP cells and observed for 12 weeks. Tumors were measured with calipers at 5- to 7-day intervals. Data were combined from 2 to 6 experiments.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean time of tumor appearance (days postinjection)</th>
<th>Tumor incidence (%)</th>
<th>Calculated mean tumor volume-doubling time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>23.79 ± 0.85(a)</td>
<td>58(^b)</td>
<td>85.5 ± 6.5(f)</td>
</tr>
<tr>
<td>Females</td>
<td>33.64 ± 2.02(a)</td>
<td>36(^d)</td>
<td>87.7 ± 5.1(f)</td>
</tr>
</tbody>
</table>

**Significance of difference**

\[ p < 0.0001\] \(d\) \(p < 0.0005\) Not significant\(n\)

\(a n = 28\)

\(b\) Mean ± S.E.

\(c n = 153\)

\(d n = 15\)

\(e n = 58\)

\(f n = 162\)

\(g\) Students' \(t\) test.

\(h\) \(\chi^2\) test.

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**Studies in Athymic Nude Mice**

Inoculum. The frequency of tumor formation following s.c. injection of 3.3 x 10^6 and 16.5 x 10^6 cells/animal did not significantly differ: 5 of 10 and 6 of 10 animals, respectively, developed tumors. No tumors grew after injection of 0.65 x 10^6 cells (10 mice) for up to 4 months of observation. For further studies, a tumor-inducing inoculum of 4 x 10^6 cells, injected s.c. in 0.2 ml, was chosen.

Tumor Formation and Growth. Nude mice given injections of LNCaP cells grown in vitro developed at the injection site poorly differentiated adenocarcinomas (14). Palpable tumors appeared between 14 and 56 days postinoculation. Once formed, the tumors grew rapidly (Chart 7). During the exponential phase of growth, many tumors doubled in volume within 3 to 4 days.

**Table 6**

AP activity in the plasma and LNCaP tumors from nude mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Sex</th>
<th>Tumor wt (g)</th>
<th>Plasma AP (milliunits/ml)</th>
<th>Total milliunits/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>6.7</td>
<td>506</td>
<td>23,879</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>1.05</td>
<td>39</td>
<td>3,791</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>6.4</td>
<td>18</td>
<td>12,115</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>6.4</td>
<td>26</td>
<td>15,577</td>
</tr>
</tbody>
</table>

**Cytosol AP**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Sex</th>
<th>Tumor wt (g)</th>
<th>Plasma AP (milliunits/ml)</th>
<th>Total milliunits/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>4</td>
<td>Female</td>
<td>6.4</td>
<td>26</td>
<td>15,577</td>
</tr>
</tbody>
</table>

**Table 7**

LNCaP tumor development in intact and hormonally manipulated athymic nude mice

Mice (6 to 8 weeks old) were castrated, ovariectomized, or implanted with hormone (2-mg) pellets 3 days before s.c. injection with 4 x 10^6 cultured LNCaP cells. Data from 2 experiments were combined: Experiment 1 involved Groups A, B, C, F, G, and H; Experiment 2 involved Groups A, B, D, E, F, G, I, and J (see also Chart 7).

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Plasma testosterone (ng/ml)</th>
<th>Animals with tumors/animals inoculated</th>
<th>% with tumors</th>
<th>p &lt; 0.01 from Group</th>
<th>p &lt; 0.05 from Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Normal Controls</td>
<td>1.9</td>
<td>18/29</td>
<td>62</td>
<td>B, G</td>
<td>F</td>
</tr>
<tr>
<td>B. Castrated</td>
<td>&lt;0.1</td>
<td>7/32</td>
<td>22</td>
<td>A, H</td>
<td>C</td>
</tr>
<tr>
<td>C. Castrated + testosterone(^d)</td>
<td>0.45</td>
<td>8/14</td>
<td>57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. Castrated + estradiol(^d)</td>
<td>ND(^c)</td>
<td>7/14</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. Normal + estradiol(^d)</td>
<td>ND</td>
<td>7/14</td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Females             |                             |                                       |               |                     |                     |
| F. Normal controls  | <0.1                       | 10/30                                 | 33            | H                   | A                   |
| G. Ovariectomized   | <0.1                       | 7/28                                  | 25            | A, H                | I                   |
| H. Ovariectomized + testosterone\(^d\) | 0.49 | 13/15                                 | 87            | B, F, G             |                     |
| I. Ovariectomized + estradiol\(^d\) | ND | 6/14                                  | 43            |                     | H                   |
| J. Normal + estradiol\(^d\) | ND | 8/15                                  | 53            |                     |                     |

* Measured by radioimmunoassay (Bioscience Laboratories, Van Nuys, Calif.) on plasma samples pooled from 10 animals.

\(^{a}\) Students' \(t\) test.

\(^{b}\) Animals implanted with 2-mg testosterone propionate pellets.

\(^{c}\) Animals implanted with 2-mg 17\(\beta\)-estradiol pellets.

\(^{d}\) ND, not determined.
tumor enlargement, the calculated mean tumor volume-doubling time was about 86 hr (Table 5), which is only 1.5 times longer than the mean population-doubling time of LNCaP cells grown in vitro. Within 6 weeks after inoculation, the majority of tumors reached the weight of 1 g. The largest tumor observed weighed 28 g at 9 weeks postinjection. No apparent distal metastases were found upon autopsy and subsequent histological examination of the internal organs.

Tumors in Males versus Females. The frequency of tumor development and the mean time of tumor appearance are significantly different for males versus females (Table 5). Male mice develop tumors earlier and at a greater frequency than did females. The rate of tumor growth, however, is independent of the gender of the host (Chart 7).

AP. Prostatic cancer in humans is frequently associated with elevation of prostatic acid phosphatase in the plasma. Since prostatic acid phosphatase is produced in vitro by LNCaP cells (14), the AP activity in LNCaP tumor cytosol and in the plasma of tumor-bearing animals was measured. In nude mice, the plasma AP level was found to increase concomitantly with the size of the tumors (Chart 6), but a considerable variation was encountered among individual animals. Ninety-six % of animals with tumors over 100 cu mm (33 of 35 males and 16 of 16 females) had plasma AP above 10 milliunits/ml (i.e., higher than the upper range in control mice); however, the magnitude of the plasma AP elevation did not reflect tumor volume on an individual
basis. These observations could not be accounted for either by the total content of AP per tumor or by the relative AP activity per mg of cytosol protein. Table 6 shows that a 28-fold difference in plasma AP level was observed between 2 animals (Animals 1 and 3) while the total AP content per tumor differed only by 2-fold in these same animals.

**LNCaP Tumors and Hormonal Manipulations.** Prostatic cancer in humans, as well as animal models of prostatic neoplasia, have shown responses to hormonal manipulation of the host. In the LNCaP tumor model system, this was examined using 10 groups of athymic nude mice, each with a different hormonal status. Following injection of LNCaP cells, each group of animals was observed for time and incidence of tumor development (Table 7) as well as kinetics of tumor growth (Chart 7). Table 7 shows that, under conditions of androgen deficiency, the percentage of animals which developed tumors was significantly reduced. When the androgen deficiency was corrected by s.c. testosterone propionate implants, the tumor incidence returned to levels similar to those in intact males (Tables 5 and 7). Estrogen treatment did not result in a statistically significant modulation of tumor development. The graphic representations of individual tumor growth in each of the 10 experimental groups (Chart 7, A to J) illustrate that the rate of increase of tumor volume was essentially independent of the hormonal status of the animals. These results demonstrate that the formation of LNCaP tumors is androgen responsive but that tumor growth is independent of intact gonadal function.

**Sex Hormone Receptors in LNCaP Tumors.** High-affinity specific androgen receptor (Chart 8) was found in the cytosol from all 38 nude mouse tumors examined (Table 8). No significant differences were observed in receptor content between tumors from intact and hormonally manipulated animals of both sexes (Table 8), regardless of whether the results are expressed as per g of tissue, per mg protein, or per mg DNA. No correlation emerged between the tumor size and the cytosol androgen receptor concentration (Chart 9).

Although the concentration of the nuclear androgen receptor was low [118 ± 23 (S.E.) fmol/mg DNA and 453 ± 92 fmol/g of tumor], it was detected in 12 of 38 tumors examined. Nonquantifiable trace amounts of nuclear androgen receptor were found in 20 additional tumors. In the remaining 6 tumors, no nuclear androgen receptor was detected.

Estrogen receptor (Table 9) was present in the cytosol from all 14 tumors examined from nude mice (intact, castrated, or ovariectomized). The specific and high-affinity (K<sub>app</sub> from 1.2 to 5.5 nm) macromolecules sedimented in the 8S region using the sucrose density gradient centrifugation procedure.

**Table 8**

<table>
<thead>
<tr>
<th>Tumor host</th>
<th>No. of tumors</th>
<th>Cytosol protein content (mg/g tumor)</th>
<th>DNA content (mg/g tumor)</th>
<th>Cytosol androgen receptor concentration</th>
<th>K&lt;sub&gt;app&lt;/sub&gt; (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>10</td>
<td>50.6 ± 5.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.2 ± 0.37</td>
<td>129 ± 16&lt;sup&gt;±&lt;/sup&gt;</td>
<td>1.04 ± 0.16</td>
</tr>
<tr>
<td>Castrated</td>
<td>4</td>
<td>60.9 ± 1.3</td>
<td>4.0 ± 0.05</td>
<td>126 ± 16</td>
<td>0.85 ± 0.03</td>
</tr>
<tr>
<td>Castrated male with testosterone implant</td>
<td>5</td>
<td>52.0 ± 3.6</td>
<td>3.3 ± 0.19</td>
<td>117 ± 12</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>52.7 ± 4.2</td>
<td>4.6 ± 0.33</td>
<td>208 ± 31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.44 ± 0.22</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>2</td>
<td>77.1</td>
<td>4.5</td>
<td>194</td>
<td>0.90</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>56.0</td>
<td>4.4</td>
<td>125</td>
<td>0.60</td>
</tr>
<tr>
<td>Ovariectomized female with testosterone implant</td>
<td>7</td>
<td>40.8 ± 1.6</td>
<td>3.1 ± 0.16</td>
<td>177 ± 21</td>
<td>0.65 ± 0.004</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.E.
<sup>b</sup> Significant difference analyzed by Students' t test at 0.05 > p > 0.02. None of the remaining differences between groups is significant at p < 0.05.
The LNCaP cells are aneuploid. They have a full complement of human chromosomes, including the Y-chromosome as well as several marker chromosomes. Their karyotype is clearly different from that of HeLa cells which in the past were frequently mistaken for some established malignant cell lines (29). The LNCaP cells are maintained. After injection of the uncloned LNCaP cells into nude mice, the time and frequency of tumor development is favored by androgens. However, once the tumors are established, their growth rates are similar, regardless of the gender or hormonal manipulation of the animals suggesting that the LNCaP cultures may consist of cells which are heterogeneous in their responsiveness to sex hormones. This is supported by published evidence (17) that in the castrated rats the progression of the Dunning prostatic adenocarcinoma to an androgen-independent state is due to the basic heterogeneity of the original inoculum and a rapid selection process in vivo. Future experiments with single-cell-derived clones could resolve this issue.

The resistance of the LNCaP cell lines to the antiproliferative as well as the antiviral effects of HuIFNβ implies, but does not prove conclusively, the existence of a defect in the cell receptor for type I interferon (2). Such defects are rare among human cells (5). It may therefore be of value to assess, before large-scale clinical trials with interferon are undertaken in this disease, whether LNCaP cells are unique in this respect or whether interferon resistance is a common property of human CaP.

Recent observations (unpublished) in our laboratory on the sensitivity of LNCaP tumors in nude mice to chemotherapeutic regimens currently used in the treatment of CaP suggest the probable utility of this model for screening new drugs or combinations of drugs for potential activity against prostatic neoplasia. The complexity of CaP requires several model systems available to the investigator to study this disease (26). The well-characterized LNCaP cells should find its proper place in research aimed at finding relevant answers concerning etiology, genetic stability, hormonal regulation, immunological properties, and the detection (30, 31) and therapy of human prostatic carcinoma.

ACKNOWLEDGMENTS

The authors thank Dr. Avery A. Sandberg and Dr. Zenon Gibas for helpful advice and discussion in karyological studies; Darlene Denzen, Sharon Mingus, Dr. Robert J. S. Horoszewicz et al.

Table 9

Cytosol estrogen receptors in LNCaP tumors from athymic nude mice

Tumors were induced by s.c. injection of 4 × 10^6 cultured LNCaP cells in 6- to 8-week-old nude mice. Ovariectomy or castration was performed 3 days before injection of LNCaP cells. Ten weeks after inoculation, the tumors were removed and stored in liquid N2. Estrogen receptor in the cytosol prepared from individual tumors was measured both by the competition binding assay with DCC (25) and by the SDGC analysis (39) with nafoxidine as the competitor.

<table>
<thead>
<tr>
<th>Receptor concentration (fmol/mg cytosol protein)</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact</td>
<td>Castrated</td>
</tr>
<tr>
<td>SDGC</td>
<td>DCC</td>
<td>SDGC</td>
</tr>
<tr>
<td>1.9</td>
<td>7.7</td>
<td>7.0</td>
</tr>
<tr>
<td>2.7</td>
<td>14.3</td>
<td>ND</td>
</tr>
<tr>
<td>2.0</td>
<td>21.0</td>
<td>ND</td>
</tr>
<tr>
<td>4.9</td>
<td>22.9</td>
<td>19.5</td>
</tr>
</tbody>
</table>

* DCC, dextran-coated charcoal after 4-hr incubation: SDGC, su挫e density gradient centrifugation after 4-hr incubation (85 peak); ND, not determined.

DISCUSSION

This report characterizes the cell line LNCaP and demonstrates its utility as a model for laboratory studies on human prostatic cancer in vitro and in the athymic nude mice.

The LNCaP cells established from human CaP (14) could be readily propagated in the laboratory by routine cell culture methods if appropriate measures are taken to prevent the weakly attached cells from being dislodged from the plastic growth surface. High growth saturation densities of monolayers, adequacy of low serum concentrations to promote cell division, anchorage-independent proliferation in semisolid media, and excellent cloning efficiency of the LNCaP cells are consistent with the generally recognized properties of neoplastic cells in vitro. The LNCaP cells are aneuploid. They have a full complement of human chromosomes, including the Y-chromosome as well as several marker chromosomes. Their karyotype is clearly different from that of HeLa cells which in the past were frequently mistaken for some established malignant cell lines (29). The continuously maintained high degree of polyploidy, as well as the major karyological characteristics in LNCaP cultures, examined at 20 months or approximately 200 population doublings apart, suggest that the selection pressure from growth conditions in vitro has not eliminated the apparent heterogeneity of the LNCaP cells.

Evidence that the LNCaP cell line originated from human prostatic cancer tissue is provided by: their morphology (14); preservation of functional differentiation; and maintenance of malignant properties in the athymic nude mice. Organ-specific glycoproteins, such as human prostatic acid phosphatase (14) and prostastic antigen (30), are present in cultured cells, in cell culture fluids, in tumors induced in the nude mice, and in the plasma of tumor-bearing animals.

Functional differentiation, consistent with human prostatic epithelial derivation, is also reflected by the responsiveness of the LNCaP cells to androgens. The cellular proliferation in vitro is modulated in a dose-dependent manner by the presence of DHT in the culture medium. In addition, DHT has an enhancing influence on AP production by the LNCaP cultures. This effect is apparently independent of the mitotic stimulation induced by DHT. Under conditions when DHT suppresses cell growth, the level of secretory AP in medium continues to rise. These observations suggest that the influence of androgen on cell division and AP synthesis may follow different regulatory pathways in human CaP. Recently, Hudson (16) reported an increase in the level of AP when short-term primary cultures of human CaP were maintained in media with DHT or testosterone. The detection of a specific, high-affinity, low-capacity, saturable androgen receptor in the cytosol and nuclear extracts from both cultured LNCaP cells, as well as in nude mice tumors, provides the molecular basis for the observed androgen responsiveness in vitro and in vivo. LNCaP cells also contain specific estrogen receptor in the cytosol, a finding consistent with the previously described presence of estrogen receptor in normal and malignant human prostatic epithelium (28).

The presence of complete FBS in culture medium was found to prevent the demonstration of DHT growth-stimulatory effects in LNCaP cells. Such effects became apparent when steroid-depleted, charcoal-treated serum, e.g., ZSD, is used as the growth medium supplement. The reasons for this are unclear, but these findings imply the existence of a significant interplay between androgens and charcoal-removable serum components in the regulation of CaP cell proliferation. Several conflicting observations (19, 24, 42, 44) on the effects of androgens on the in vitro growth of normal and malignant human prostatic epithelium are perhaps due, in part, to the use of a variety of complete mammalian sera as medium supplement.

Malignant properties and hormonal responsiveness in vivo of the LNCaP cells are maintained. After injection of the uncloned LNCaP cells into nude mice, the time and frequency of tumor development is favored by androgens. However, once the tumors are established, their growth rates are similar, regardless of the gender or hormonal manipulation of the animals suggesting that the LNCaP cultures may consist of cells which are heterogeneous in their responsiveness to sex hormones. This is supported by published evidence (17) that in the castrated rats the progression of the Dunning prostatic adenocarcinoma to an androgen-independent state is due to the basic heterogeneity of the original inoculum and a rapid selection process in vivo. Future experiments with single-cell-derived clones could resolve this issue.

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REFERENCES

10. Drury, and Joyce Romano for excellent technical assistance; Helen Biro for careful
11. Drury, and Joyce Romano for excellent technical assistance; Helen Biro for careful
Fig. 1. G-banded karyotype of a LNCaP cell from a culture maintained for 32 months in vitro after isolation. One set of normal human chromosomes is present in addition to multiple copies and marker chromosomes (m₁ to m₈) for a total of 88 chromosomes.
LNCaP Model of Human Prostatic Carcinoma

Julius S. Horoszewicz, Susan S. Leong, Elzbieta Kawinski, et al.


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