A Metastatic and Androgen-sensitive Human Prostate Cancer Model Using Intraprostatic Inoculation of LNCaP Cells in SCID Mice

Naohide Sato, Martin E. Gleave, Nicholas Bruchovsky, Paul S. Rennie, Eliana Berakli, and Lorne D. Sullivan


ABSTRACT

Several metastasizing murine and human animal models for prostate cancer are available. However, these models are androgen-independent and lack differentiated features such as androgen receptor and androgen-regulated gene expression like prostate-specific antigen (PSA). The objective of this study was to develop a metastasizing prostate cancer model with differentiated features using the human LNCaP cell line. Athymic and SCID mice were injected either s.c. or intraprostatically with $1 \times 10^6$ LNCaP cells. Changes in serum and tumor PSA mRNA levels were determined before and after castration to assess time to androgen-independent progression. Local tumor and metastatic growth was assessed at sacrifice after 12 weeks. Reverse transcription-PCR (RT-PCR) was used to detect circulating LNCaP cells. LNCaP tumor incidence after s.c. injection was 100% (65 of 65) in SCID mice and 80% in athymic mice. No lymph node or distant metastases were observed with s.c. tumors, and RT-PCR for PSA transcripts was negative. Primary tumor incidence after intraprostatic injection was 89% (39 of 44) in SCID mice and 60% in athymic mice. In 10 SCID mice with primary tumors followed for 12 weeks, retroperitoneal or mediastinal lymph node metastases were found in 100%, and microscopic pulmonary metastases were identified in 40%. RT-PCR for PSA transcripts was positive in 3 of 10 mice tested. Serum PSA levels in mice with s.c. and intraprostastic tumors decreased by 65% to nadir levels at 7 and 4 days after castration, respectively. Serum PSA and LNCaP tumor PSA mRNA levels increased to precastration levels earlier in SCID mice with intraprostastic tumors compared to those with s.c. tumors. Intraprostatic injection of LNCaP cells in SCID mice provides a useful animal model to investigate mechanisms of metastasis and to evaluate therapies targeted toward inhibiting the metastatic cascade.

INTRODUCTION

Despite its common occurrence, for various reasons prostate cancer remains poorly characterized in terms of its biological and molecular basis. Prostate cancer rarely arises spontaneously in most animal species, which hinders research on the etiology and tumor biology of prostate cancer. Furthermore, it is difficult to obtain fresh human tissues for biochemical research because most tissues obtained through surgery are required for pathological staging and grading.

To date, an ideal animal model for the study of human prostate cancer does not exist. The LNCaP cell line is the only human prostate cell line established with functional androgen receptors and PSA expression (1), but it is weakly tumorigenic when inoculated s.c. in athymic mice. Using a cell-cell recombination technique, Gleave et al. (2, 3) reported that LNCaP tumor growth in athymic male mice is dependent on cooincoid tissue-specific (prostate or bone) fibroblasts. As in human prostate cancer, serum PSA levels in this model are regulated by androgen and are directly proportional to tumor volume. After castration, serum and tumor cell PSA levels decrease up to 80% and remain suppressed for 3–4 weeks (4). Beginning 4 weeks after castration, however, PSA production gradually increases above precastration levels in the absence of testicular androgens, signaling development of androgen-independent PSA gene expression.

A disadvantage of the s.c. LNCaP tumor model is that metastases do not occur, which limits assessment of tumor progression to local growth rates. It is difficult to evaluate therapies using survival as an end point in nonmetastasizing s.c. tumors. Appropriate in vivo metastatic models are necessary to investigate the steps in the metastatic cascade and to evaluate therapies that either specifically target metastatic mechanisms or attempt to prolong survival. Many investigators have noted that the site of inoculation is a critical determinant of the rate and pattern of metastases (5–11). We (3) and others (5) have previously demonstrated enhanced growth of LNCaP and PC-3M cells after orthotopic injection compared to ectopic implantation in nude mice and found that intraprostatic injection increases the metastatic potential of these prostate cancer cell lines. However, metastases remain uncommon and limited to small deposits in the retroperitoneal lymph nodes. In this report, we describe a reliable metastasizing model using SCID mice after intraprostatic inoculation of LNCaP cells. Intraprostatic tumors occurred in 89% of hosts, and 100% of these developed retroperitoneal lymph node metastases. Furthermore, hematogenous metastases occurred with positive peripheral blood RT-PCR for PSA and pulmonary metastases. Changes in PSA after castration and time to androgen-independent progression of PSA gene expression in intraprostatic, lymph node, and s.c. tumors were examined.

MATERIALS AND METHODS

Animals and Cell Lines. Male 6–8-week-old SCID mice were obtained from the breeding program at the Joint Animal Facility of the British Columbia Cancer Agency. Male 6–8-week-old athymic nude mice (BALB/c strain) were purchased from Charles River Laboratory (Montreal, Canada). LNCaP cells were kindly provided by Dr. L. W. K. Chung (The University of Texas M. D. Anderson Cancer Center, Houston, TX) and maintained in RPMI 1640 supplemented with 5% FBS (Life Technologies, Inc., Burlington, Canada).

Inoculation of LNCaP Cells. All animals were anesthetized with methoxyflurane before inoculation of LNCaP cells. For s.c. tumor growth, $1 \times 10^6$ LNCaP cells were suspended in 75 $\mu$l of RPMI 1640 plus 5% FBS and 75 $\mu$l of Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected via a 27-gauge needle into the s.c. space of the right flank region. Tumors were measured twice weekly, and their volumes were calculated by the formula $L \times W \times H \times 0.5236$ (2). For intraprostatic tumor growth, a transverse incision was made in the lower abdomen. Abdominal wall muscles were incised, and the bladder and seminal vesicles were delivered through the incision to expose the dorsal prostate. The dorsal lobe of the prostate was identified, and $1 \times 10^6$ cells suspended in 20 $\mu$l of RPMI 1640 plus 5% FBS were carefully injected under the prostatic capsule via a 30-gauge needle. The incision was closed using a running suture of 4–0 silk.

Castration of Mice and Tumor Extirpation. Mice were anesthetized using methoxyflurane and castrated via abdominal approach. Mice were sacrificed either before castration or at various times after castration to assess the effect of androgen ablation on serum PSA and tumor mRNA levels and
time to progression to androgen-independence. At sacrifice, the prostate, retroperitoneal lymph nodes, liver, kidneys, axial skeleton, and lungs were examined grossly and histologically for the presence of tumors. The invasiveness of tumors and the presence of hydrophrosis were noted.

**Radionuclide Bone Scans.** Bone scans were performed using $^{99m}$Tc-methylene diphosphonate on five mice with large primary tumors and high serum PSA levels. Scans were reviewed by a certified nuclear medicine physician.

**Histology and Immunohistochemistry.** For routine histology, specimens were fixed in 10% neutral buffered formalin and embedded in paraffin, and fixed sections were cut and stained with H&E. For immunohistochemistry studies, sections were deparaffinized and incubated with anti-PSA polyclonal antibodies (Biogenex, Dublin, CA). The avidin-biotin complex method was used with Fast-red or AEC as chromogens (Biogenex). Slides were counterstained with aqueous hematoxylin and mounted with glycerol for visual inspection and photography.

**Determination of Serum PSA Levels.** Blood samples were obtained with tail vein incisions of mice before castration and at 4, 7, 14, and 28 days postcastration. Serum PSA levels were determined by an enzymatic immunoassay kit with a lower limit of sensitivity of 0.2 ng/ml (Abbott IMX, Montreal, Canada) according to the manufacturer’s protocol. Time to androgen-independent PSA regulation was defined as the duration of time required after castration for serum PSA levels to return to or increase above precastration levels.

**RT-PCR.** Two months after intraprostatic injection of LNCaP cells in SCID mice, blood samples from 10 animals were analyzed using RT-PCR to detect PSA mRNA in peripheral blood. Five animals without tumors were used as negative controls. Tail vein blood (80 μl) was collected, and RBCs were hemolyzed by adding a 5:1 volume of lysing buffer (0.15 M NaHCO$_3$, 0.09 mM EDTA). After 10 min, nucleated cells were collected by centrifugation, and total RNA was isolated using a TRIzol extraction kit (Life Technologies, Inc.) according to the manufacturer’s specifications. Five μg of total RNA were added to 100 pmol of random hexamer primers (Pharmacia/Biotech, Montreal, Canada) and brought to a final volume of 10 μl. After denaturation at 85°C for 10 min, samples were incubated for 1 h at 37°C in 25 μl of reaction mixture consisting of 5X first-strand buffer (0.25 M Tris-HCl (pH 8.3) and 0.375 mM KCl, Mg$_2$Cl$_2$, 10 mM dideoxynucleotide triphosphate mix, 0.1 mM DTT, and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The cDNA was successfully amplified by two rounds of PCR reactions using nested primers. The primers cover a portion of exons 3 (PSA outer sense) and 5 (PSA inner antisense and outer antisense) of the PSA gene. The upstream (a) and downstream (b) sequences of PSA outer and inner primers in the PSA cDNA sequence were as follows: PSA outer (a), 5'-GATGACTCCAGCCACGACCT-3' (starting at nucleotide 648); PSA outer (b), 5'-CACAGACACCCCATCCTATC-3' (starting at nucleotide 1338); PSA inner (a), 5'-GCAAGTTCACCCTCA-3' (starting at nucleotide 1269). Five μl of RT reaction product were amplified for the PCR reaction in a 50-μl mix containing 1X PCR buffer (20 mM Tris-HCl (pH 8.4) and 30 mM KCl (pH 8.8)), 1.5 mM MgCl$_2$, 0.2 mM of each dideoxynucleotide triphosphate, 0.2 μM of outer primers, and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). The program used requires 49 cycles with the following conditions: 95°C for 30 s (70 s for the first cycle); 60°C for 1 min; and 72°C for 1 min (10 min for the last cycle). A further amplification with the inner primer was successively performed under the same conditions using a 1:5 dilution of the first PCR product for 30 cycles. One aliquot (one of five) of the reaction was analyzed by electrophoresis on a 2.5% agarose gel and visualized by ethidium bromide staining. The sizes of the fragments produced by the PSA outer and inner primers were 710 and 455 bp, respectively. Each PCR reaction included RNA isolated from LNCaP cells as a positive control and water as a negative control. Sensitivity of the assay was determined using serial dilutions by adding $10^7$ to $10^8$ LNCaP cells to 100 μl of whole blood. Total RNA was extracted from the mixture and used for RT-PCR as described above.

**Northern Blot Analysis.** Frozen tumor tissues were ground into fine powder using liquid nitrogen, and their total RNA was extracted by acid guanidinium thiocyanate-phenol chloroform method as described in previous reports (2-4). The range of typical yields of total cellular RNA was 200–300 mg/100 mg tissue as quantified by spectrophotometry. Five mg of total cellular RNA were denatured in 50% formamide/2.2% formaldehyde at 60°C and fractionated by electrophoresis on a 1% agarose gel containing 6.7% formaldehyde. Samples were transferred onto a Nitran membrane (S&S, Inc., Keene, NH) using the capillary method in 20X SSC (1X SSC is 0.15 m NaCl and 0.015 m sodium citrate), and the membrane was baked at 80°C for 2 h. Hybridization was carried out at 42°C overnight in 6X SSC, 50% formamide, 5 mM EDTA, 0.1% SDS, 5X Denhardt’s solution, 10 mM sodium phosphate (pH 6.5), and 0.2 mg/ml sonicated salmon sperm DNA with a cDNA probe labeled with $[^{32}$P]-dCTP using an oligolabeling kit (Pharmacia). The DNA probe for PSA was a 1.4-kb EcoRI fragment of PSA cDNA (4). Films were scanned using a GS-300 densitometer (Hoefer Scientific Instruments, San Francisco, CA). The density of bands for PSA was normalized against that of β-actin and shown as a mean value of a relative PSA expression ratio from two Northern analyses that were carried out separately.

**RESULTS**

**Incidence of Intraprostatic and s.c. Tumors and Metastases.** The incidence of local tumor growth and metastases was compared between different hosts (athymic nude mice or SCID mice) and the site of inoculation (intraprostatic or s.c.). Table 1 summarizes tumor incidence in SCID mice, with a tumor take rate of 100% (65 of 65) at s.c. sites and 89% (39 of 44) at intraprostatic sites. No metastases were identified in SCID mice bearing s.c. tumors. In contrast, all SCID mice with intraprostatic tumors developed grossly evident retroperitoneal lymph node metastases after 3 months (Fig. 1). The viscera and axial skeleton were completely examined in 10 mice with intraprostatic tumors, and 40% (4 of 10) had microscopic pulmonary metastases. Bone scans were negative in the five mice examined (data not shown). One mouse developed paraliegia, suggesting possible vertebral bone metastasis, but no lesion was identified in histological sections of the spine.

**Histological Characterization of Intraprostatic and Metastatic Tumors.** LNCaP tumors incidence and metastases were less frequent in athymic nude mice compared to SCID mice. Tumor incidence was 60% (12 of 20) at both s.c. and intraprostatic sites (Table 2). Retroperitoneal lymph node metastases were found in 25% (3 of 12) of mice with intraprostatic tumors, whereas no metastases were identified in mice with s.c. tumors. No microscopic visceral metastases were found in athymic mice with s.c. or intraprostatic tumors.

<table>
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<th>Table 1 Comparison of primary and metastatic tumors in SCID mice with S.C. or intraprostatic inoculation of LNCaP cells</th>
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<td><strong>Route of injection</strong></td>
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<td>-----------------------</td>
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<tr>
<td><strong>Tumor take</strong> (%)</td>
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<td><strong>Metastasis</strong> (%)</td>
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<td><strong>RPL</strong></td>
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*Viscera and axial skeleton were completely examined in 10 mice 3 months after injection.

* RPL, retroperitoneal lymph node.
Metastatic and primary LNCaP tumors stained strongly positive for PSA, whereas lymphocytes and pulmonary alveolar cells were negative (Fig. 3, B and C). After castration, PSA staining was stronger and more homogeneous in metastatic lymph node and pulmonary lesions compared to primary prostatic tumors (Fig. 3).

RT-PCR. Serial dilutions of LNCaP cells in 100 μl of whole blood from SCID mice showed that this method can detect as few as 10 tumor cells/100 μl before the isolation of nucleated cells (Fig. 4A). RT-PCR amplification using PSA nested primers produces a 455-bp fragment. RT-PCR evaluation of peripheral blood samples of SCID mice with intraprostatic tumors was positive in 3 of 10 animals tested (Fig. 4B). The three mice with positive RT-PCR had the highest serum PSA levels (146, 83, and 78 μg/liter). Only one mouse with a PSA >50 μg/liter had a negative RT-PCR. All mice with intraprostatic tumors and lower PSA levels (<50 μg/liter), as well as all five mice with s.c. tumors, were RT-PCR-negative.

Changes in Serum PSA after Castration. Serum PSA levels in tumor-bearing SCID mice became detectable beginning at 4 weeks after inoculation. Serum PSA levels increased over time and in the easily measurable s.c. tumors were proportional to tumor volume before castration (data not shown), as reported previously (4).

Table 2 Comparison of primary and metastatic tumors in athymic mice with s.c. or intraprostatic inoculation of LNCaP cells

<table>
<thead>
<tr>
<th>Route of injection</th>
<th>S.C.</th>
<th>Intraprostatic</th>
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<tr>
<td>Tumor take (%)</td>
<td>12/20 (60)</td>
<td>12/20 (60)</td>
</tr>
<tr>
<td>Metastasis (%)</td>
<td>0/12 (0)</td>
<td>3/12 (25)</td>
</tr>
<tr>
<td>RPL (%)</td>
<td>0/12 (0)</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td>Other organ (%)</td>
<td>0/12 (0)</td>
<td>0/12 (0)</td>
</tr>
</tbody>
</table>

* a Viscera and axial skeleton were completely examined in 12 mice 3 months after injection.
* b RPL, retroperitoneal lymph node.
METASTATIC HUMAN PROSTATE CANCER MODEL

days postcastration (Fig. 6). Differences in PSA mRNA expression may partially account for the faster rise in serum PSA of mice with intraprostatic (and metastatic) tumors compared to those with s.c. tumors (shown in Fig. 5).

DISCUSSION

Insights into and understanding of the nature of human prostate cancer and therapeutic advances will come from developing useful concepts through careful extrapolation from appropriate animal models. Several metastasizing animal models of murine and human prostate cancer are available for study, including the Dunning Mat-LyLu and Mat-Lu (12), Pollard (13), and PC3 cell lines (5, 14). However, these cell lines do not possess any differentiated cell features characteristic of prostate cancer, making clinical extrapolation of experimental results more tenuous. Because metastasis, not the primary tumor, is the threat of cancer, it is important to develop a reliable metastasizing human prostate cancer model that possesses differentiated features such as an androgen receptor and androgen-regulated growth and gene expression and tumor markers such as PSA and prostatic acid phosphatase.

The LNCaP cell line is a useful and important cell line because it is the only human prostate cancer cell line established with functional androgen receptor and PSA expression. Horoszewicz et al. (1) reported that s.c. inoculations of $1 \times 10^6$ LNCaP cells in athymic nude mice produced a nonmetastasizing adenocarcinoma. However, other laboratories using later passages were unsuccessful in inducing LNCaP tumor growth with s.c. inoculation. Using a cell-cell recombination technique, Gleave et al. (2—4) observed that tissue-specific mesenchymal or stromal cells were potent inducers capable of stim-

higher than nadir levels in both intraprostatic and s.c. tumors. Although the levels of PSA mRNA expression were identical in primary and metastatic tumors before castration, they were approximately 20% higher in lymph node metastasis than in primary prostate tumors at 28 days postcastration (Fig. 6). Differences in PSA mRNA expression may partially account for the faster rise in serum PSA of mice with intraprostatic (and metastatic) tumors compared to those with s.c. tumors (shown in Fig. 5).

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Fig. 6. Castration-induced changes in PSA mRNA of LNCaP tumors in SCID mice. Northern blot analyses for PSA mRNA. Total RNA was isolated from tumors of intraprostatically (A) and s.c. (B) injected mice before castration (day 0) and at 4, 7, and 28 days postcastration (day 4, 7, and 28, respectively). P, primary prostatic tumors; L, metastatic lymph node tumors. C, densitometric analyses of PSA mRNA. The densities of PSA mRNA expressions shown in A and B were normalized by β-actin. Data, mean ± SD.
injection of LNCaP cells was associated with 100% lymph node and frequent hematogenous metastases. Lower tumor incidence with intraprostatic injections is likely due to technical difficulties with intraprostatic injection. However, it is important to emphasize that s.c. LNCaP tumor formation in both SCID and athymic mice requires coinoculation with either Matrigel or bone fibroblasts (2, 3), whereas intraprostatic LNCaP tumor growth can occur with LNCaP inoculation alone. The pattern of soft tissue (lymph node, lung) in this SCID mouse model, in contrast to osseous metastases, may partly be a selection factor because LNCaP was originally established from a lymph node metastasis of a patient with endocrine therapy-resistant prostate cancer. The absence of metastases with s.c. tumors is consistent with previous reports emphasizing the importance of orthotopic tumor growth (5—11).

Serum PSA levels in nude mice bearing s.c. LNCaP tumors are directly proportional to tumor volume and decrease by 80% by 2 weeks after castration before autonomously increasing again 21—28 days postcastration (4, 17). Regardless of tumor location or host, changes in serum PSA levels after castration reflect changes in PSA mRNA expression rather than tumor volume. The present study indicates that serum PSA increases earlier after castration in intraprostastically injected SCID mice compared to SCID or nude mice with s.c. tumors. PSA mRNA in the intraprostatically injected SCID model started to increase 7 days postcastration in the absence of androgen, at least 7 days earlier than that of s.c. tumors in SCID mice (Fig. 5) and 14 days earlier than that in nude mice (17). This difference may be due to the earlier onset of androgen-independent PSA regulation in LNCaP tumors at intraprostatic or lymph node sites compared to that of s.c. tumors. Differences in time to androgen-independence between s.c. and intraprostatic tumors suggest that epigenetic factors influence PSA gene regulation, which may vary depending on tumor site and microenvironment as well as the androgen milieu (18).

Recently, two groups reported rodent models of metastatic prostate cancer. Vieweg et al. (19) used the orthotopic approach with the highly metastatic rat Dunning R3327 subcell line, Mat-LyLu. All rats receiving intraprostatic implantation had gross intrapelvic lymph nodes metastases, whereas none of the s.c. implanted rats developed macroscopic intrapelvic lymph node metastases. The Mat-LyLu subline also metastasizes from the s.c. location and lacks differentiated prostate-specific characteristics such as the androgen receptor and PSA. Thalmann et al. (20) established an androgen-independent LNCaP cell subline, C4—2, with a high frequency of bone metastases after s.c. inoculation in castrated nude mice. However, serum PSA levels are undetectable or very low in this model. The advantages of the orthotopic LNCaP SCID mouse model include the availability of parental LNCaP cells, high incidence of primary and metastatic tumors, high circulating PSA levels that decrease and progress after castration, and documentation of circulating LNCaP tumor cells in peripheral blood. Although the number of cases analyzed was small, an association between positive RT-PCR and high serum PSA concentrations was observed. This is the first report documenting positive RT-PCR for PSA mRNA in an animal model, and it illustrates that metastasis after orthotropic injection is an active rather than passive process involving lymphatic and hematogenous dissemination.

In summary, we report in this study the establishment of a metastatic prostate tumor model using orthotopic injection of LNCaP cells in SCID mice. The SCID mice develop both retroperitoneal and pulmonary metastases with high levels of serum PSA that are initially androgen-regulated, with androgen-independent progression of PSA gene expression after castration. This animal model will permit the study of mechanisms of metastasis and factors influencing PSA RT-PCR and an evaluation of treatments focusing on suppression of the metastatic process.

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REFERENCES


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