Androgen-independent Cancer Progression and Bone Metastasis in the LNCaP Model of Human Prostate Cancer


Abstract

Our laboratory has previously reported on the derivation of LNCaP cell sublines from LNCaP tumors maintained in castrated and intact athymic male mice. These LNCaP sublines differ from the parental line in tumorigenicity and androgen dependence. This paper demonstrates that one of these sublines acquired metastatic potential. When inoculated either s.c. or orthotopically, the C4-2 subline metastasized to the lymph node and bone with an incidence of 11-50%. Interestingly, the incidence of osseous metastasis was higher in castrated than in intact male hosts. We evaluated the chromosomal, immunohistochemical, and biochemical characteristics of the LNCaP sublines derived from C4-2 tumors that metastasized to the lymph node and bone. Cytogenetic analysis showed that all sublines were human and shared common marker chromosomes with the parental LNCaP cells. This experimental human prostate cancer model may permit, for the first time, the study of the molecular mechanisms underlying human prostate cancer metastasis.

Introduction

With growing life expectancy, prostate cancer has become a significant health problem, recorded as the leading cancer diagnosed and the second cause of cancer death in North American men (1). Prostate cancer progression underlies both genetic and epigenetic factors. The multistep carcinogenesis leading from prostate epithelial transformation over an androgen-dependent, nonmetastatic phenotype to a more malignant, metastatic, androgen-independent phenotype also encompasses intrinsic genetic changes, predisposing tumor angiogenic factors, and circumventing the host immunological defense mechanisms.

Despite improvement in early detection, more refined diagnostic modalities, and a better understanding of the natural history of the disease prostate cancer remains somewhat unpredictable. Approximately one-third of men over the age of 50 years are affected, but not every one of these patients will eventually die from the disease (2). Because of the protracted natural history of prostate cancer progression, clinical and epidemiological studies alone may not provide the knowledge necessary to design strategies for the prevention, prediction, and treatment of metastatic disease. Therefore, to gain further insight into human prostate cancer progression and metastasis, well defined experimental in vivo models that mimic the different aspects of the natural course of the disease progression are essential. Early experimental models implanting athymic nude mice s.c. with human prostate cancer cells failed to yield metastatic disease (3). Recognizing the importance of androgen independence in prostate cancer progression (4) and using the androgen-independent PC-3 human prostate cancer cell line as a model, osseous metastasis could be induced in nude mice by delivering tumor cells i.v. with concomitant occlusion of the inferior vena cava (5). By implanting LNCaP human prostate cancer cells orthotopically to allow the interaction (6) between cancer cells ("seed") and the host microenvironment ("soil") in situ, Stephenson et al. (7) and Fu et al. (8) have observed prostate cancer metastasis but only in the lymph node.

We previously reported a cell-cell recombination model in which in vivo coinoculation of nontumorigenic epithelial and organ-specific mesenchymal cells (from the prostate and bone) promotes solid tumor formation (9). By coinoculating the LNCaP cell line with nontumorigenic MS fibroblasts derived from a human osteosarcoma, our laboratory has established an androgen-independent human LNCaP prostate cancer cell line, C4-2 (10).

In the present study we report for the first time on the development of an in vivo androgen-independent LNCaP metastatic model of human prostate cancer. Since this model mimics closely the natural history of human prostate cancer, it should be useful for future studies on the mechanisms and therapy of prostate tumor progression and metastasis.

Materials and Methods

Cell Lines and Cell Cultures. The C4 subline was derived by the coinoculation of two nontumorigenic cell lines, the human prostate cancer cell line LNCaP and the human osteosarcoma cell line MS (1.0 × 10⁶ cells each), into a male athymic nude mouse according to a procedure described previously (9, 10). The host was castrated at 8 weeks and a single tumor specimen was excised at 12 weeks (4 weeks after castration). This specimen was used as the source for the generation of a second generation cell line, C4-2, maintained in a castrated male host (Fig. 1). This subline was found to be androgen independent and capable of growing in castrated hosts.

C4-2 cells, passage 23, were grown in T-medium [80% Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY)-20% F12K (Irving Scientific, Santa Ana, CA)-3 g/liter NaHCO₃-100 units/liter penicillin G-100 μg/ml streptomycin-5 μg/ml insulin-13.6 pg/ml triiodothyronine-5 μg/ml transferrin-0.25 μg/ml biotin-25 μg/ml adenine] with 5% FBS. The cells were free of Mycoplasma.

Assessment of In Vivo Tumor Growth. Six- to 8-week-old athymic nude mice (BALB/c; Charles River Laboratories, Baltimore, MD) were used for all in vivo experiments. They were kept under pathogen-free conditions in laminar flow boxes in accordance with established institutional guidelines and approved protocols. Eleven intact and 10 castrated mice were given s.c. injections; 1.0 × 10⁶ C4-2 cells resuspended in 0.1 ml of RPMI 1640 and 10% FBS were injected (27-gauge needle, 1-ml disposable syringe) at 6 sites/animal. For orthotopic administration, tumor cells (1.0 × 10⁶) resuspended in the same...
Dorsolateral lobe of the prostate gland of athymic mice (20 intact animals and 14 castrated animals; 1 were routinely inspected for physical abnormalities and tumor growth. Tumor tissues were harvested from the primary tumor, lymph node, and bone for subsequent derivation of C4-2 sublines. In brief, tumors were minced into 1-mm³ cubes, placed on Falcon plastic dishes (Becton Dickinson, Lincoln Park, NJ), and immersed in tissue culture medium as defined above. Within 2 weeks, tumor cells were outgrown together with the host fibroblasts. LNCaP tumor cells, which adhere loosely to the plastic dishes, were enriched by washing the culture dishes with T-medium. Pure LNCaP sublines, as judged by morphological, cytogenetic, and immunohistochemical criteria, were obtained after 5–10 of these subculturing steps.

**Bone Scanning.** To confirm the location and extent of osseous metastasis, selected animals were subjected to a bone scan after i.v. infusion of 1 mCi of ⁹⁹ᵐéchnetium-methylene diphosphonate. Two h after radioisotope infusion, whole-body sagittal imaging was performed.

**Histology and Immunohistochemistry.** Specimens for routine histological examination were fixed in 4% paraformaldehyde and 5 mM MgCl₂. Six-μm paraffin-embedded tumor sections were cut and stained with hematoxylin and eosin.

Immunohistochemical staining was performed on deparaffinized tissue sections or cultured cells fixed in 3.7% formaldehyde and 0.9% NaCl. Specimens were treated with 3% H₂O₂ in methanol, blocked with Super Block (Scytek Laboratories, Logan, UT), and incubated with the monoclonal antibodies against cytokeratin 8 (generously provided by Dr. M. Normand, Laval University, Quebec City, Quebec, Canada) and PSA (Biogenex Laboratories, San Ramon, CA), which are present in benign and malignant prostate tissues, as well as in LNCaP cells (11). Monoclonal antibody raised against a bacterial TrpE protein (Oncogene Science, Inc., Uniondale, NY) and normal rabbit serum were used as primary antibodies to establish negative staining controls. The intensity of the staining was enhanced by the amplification of signals through a biotin-streptavidin system using the AEC Substrate Pack for the detection of peroxidase activity (Biogenex Laboratories, San Ramon, CA). The relative intensities of all specimens were scored by two independent observers.

**Hoechst Stain for Cellular DNA.** To differentiate the mouse cells from the human cells in tumor specimens, we stained the paraffin-embedded sections with the bisbenzimidazole dye, Hoechst 33258 (12). This dye was demonstrated previously to stain intensely for mouse satellite DNA, which is absent in human and rat cells. Mouse salivary gland and human benign prostatic hyperplasia tissue served as positive and negative controls, respectively.

**Cytogenetic Analysis.** Cultures from the C4-2 primary tumor (C4-2Pr), the lymph node (C4-2Ln) and the bone metastasis (C4-2B) lines, fed 24 h earlier with fresh medium, were exposed to Colcemid (final concentration, 0.02 μg/ml) for 40 min at 37°C. Cells from these cultures were dislodged by exposing them to 2 ml Hanks’ balanced salt solution containing 0.01% trypsin. Cells were then allowed to adhere loosely to the plastic dishes, washed twice with fresh medium, and fixed with 3.7% formaldehyde and 0.9% NaCl.

### Table 1. Tumorigenicity and metastatic behavior of C4-2 cells injected either orthotopically or s.c. in intact and castrated athymic male nude mice

<table>
<thead>
<tr>
<th>Status of host</th>
<th>Injection</th>
<th>Tumor formation</th>
<th>Paraplegia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>Orthotopic</td>
<td>20/20 (100)*</td>
<td>2/20 (10)</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>1/66 (1.5)</td>
<td>1/11 (18.2)</td>
</tr>
<tr>
<td>Castrated</td>
<td>Orthotopic</td>
<td>14/14 (100)</td>
<td>3/14 (21.5)</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>0/60 (0)</td>
<td>5/10 (50)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
trypsin. The single cell suspension in 5 ml of RPMI 1640 containing 10% FBS was centrifuged at 1700 rpm for 5 min. After discarding the supernatant, the cell pellet was disturbed and exposed to a hypotonic solution (0.06 M KCl) for 15–20 min at room temperature. After centrifugation, cells were fixed in acetic acid:methanol (1:3, v/v) for 15 min and then washed three times in the fixative. Conventional air-drying chromosome preparations were made following the routine laboratory technique (13).

Optionally aged slides (5–6 days old) were treated in trypsin and stained with Giemsa solution for G-banding following the routine procedures described elsewhere (13). Ten to 15 G-banded metaphase spreads were photographed by the GENETISCAN (PSI, Houston, TX), and a minimum of 4 complete karyotypes were prepared from each sample. All slides were added at the time of chromosome analysis.

Results

Table 1 summarizes the incidences of tumor formation and paraplegia detected in intact and castrated hosts inoculated with $1.0 \times 10^6$ C4-2 cells. C4-2, when administered orthotopically in intact male hosts, resulted in the development of both primary tumor (20 of 20, or 100%) and osseous prostate cancer metastases (2 of 20, or 10%). In the castrated hosts, orthotopic administration of C4-2 cells also resulted in the development of both primary tumor (14 of 14, or 100%) and bone metastasis (3 of 14, or 21.5%).

When injected s.c. into intact male hosts, C4-2 cells induced a palpable tumor at only one of the primary sites of injection (1 of 66
sites, or 1.5%) but induced a substantially higher incidence of osseous metastases (2 of 11, or 18.2%). This general pattern of differences in the incidence of tumor formation and paraplegia between mice given orthotopic and s.c. injections was also observed in the castrated hosts. Notably, however, the incidence of paraplegia detected in the castrated hosts (5 of 10, or 50%) was 3-fold that found in the intact male hosts (2 of 11, or 18.2%). In general, animals found to harbor osseous metastases (i.e., injected either s.c. or orthotopically) do not necessarily contain lymph node metastasis. All animals that were found to have a primary prostate tumor (i.e., injected orthotopically), however, contained lymph node metastases. While para-aortal lymph node involvement was dominant in the orthotopically injected mice, axillary and inguinal lymph node involvement was common in the s.c. injected mice. The average latent period for paraplegia was 6.8 months (range, 4—10 months). Local invasiveness was evidenced by hydronephrosis and muscular infiltration. C4-2 tumors, like the parental LNCaP cell line-derived tumors, were hemorrhagic, indicating increased angiogenesis (Fig. 2a).

Bone scans of paraplegic animals showed focal lesions of high radioisotope uptake (Fig. 2b, closed arrows) in comparison with a negative control (Fig. 2c, closed arrows). Histological sections showed the presence of prostatic tumor cells in the primary tumor (Fig. 2d), the lymph node (Fig. 2e), and the bone (Fig. 2f); marked osteoblastic reactions were observed in the bone and were confirmed by immunohistochemical staining of markers characteristic for host osteoblasts (data not shown). The prostatic origin of the epithelial cells derived from the primary tumor, lymph node, and bone metastases in male hosts was confirmed by positive but scattered staining for PSA (Fig. 2g) and positive and uniform staining for cytokeratin 8 (Fig. 2h); all other metastatic sublines showed similar immunostaining (data not shown). Using the Hoechst dye to detect cellular DNA, we demonstrated the presence of both human (light and even staining) and mouse cells (sharp and granulated staining) in the primary tumor (Fig. 2i) and in the bone metastases (Fig. 2j); control samples demonstrated the respective differences of Hoechst dye staining patterns of the mouse salivary gland (Fig. 2k) and human benign prostate hyperplasia (Fig. 2l).

The chromosome numbers in all three C4-2 sublines varied between 72 and 90 with a peak at 85 (C4-2Pr), 85 (C4-2Ln), and 87 (C4-2B) chromosomes. Karyotypic analysis of the C4-2 sublines with a defined cell lineage relationship indicated that all three C4-2 sublines derived from the same host were of human origin and shared eight common marker chromosomes (M1—M8) with the parental LNCaP cell line (10). The C4-2B subline was not pure at the time of passage; it contained lymph node metastases. All C4-2 sublines derived from the same host were of human origin and shared eight characteristic marker chromosomes found in the parental cell line. All C4-2 sublines appeared clonal but exhibited metastatic site-specific chromosomal alterations. For example, in comparison with the C4-2 subline derived from the primary tumor, C4-2Ln contained a new translocation between 12p and 17q (Fig. 3b). This marker chromosome is designated as m1 for this subline. It is a translocation product between 12p and 17q. Similarly, C4-2B contained two new markers (m1 and m2) shown in Fig. 3c. Tentative identifications of these unique markers are: m1 = i(7q); m2 = der(11), t(11p+7). All three sublines showed the presence of X and Y chromosomes.

Discussion

Experimental models of human neoplastic diseases are needed to reconstruct molecular events associated with prostatic carcinogenesis and acquisition of androgen-independent growth. In vitro systems provide much information on cellular events but are inadequate for studying mechanisms of progression and metastases. No animal model has been available for studying the progression of human prostate cancer from the primary tumor to dissemination to the lymph node and the bone. In this report we describe the establishment of the first such model, in which an androgen-independent LNCaP subline,
C4-2, when administered either s.c. or orthotopically, was observed to metastasize to lymph nodes and the axial skeleton.

The procedures used to establish this androgen-independent C4-2 subline were based on the cell-cell recombination model developed in this laboratory (14), as outlined in Fig. 1. We obtained the following evidence for the nonrandom dissemination of the C4-2 subline to the axial skeleton: (a) bone scans of animals that developed paraplegia demonstrated focally increased radioisotope uptake in thoracic and lumbar vertebrae; (b) histological, immunohistochemical, and cellular DNA staining profiles evidenced these findings; and (c) tumors cells derived from the primary tumor and the lymph node and bone metastases shared common cytogenetic marker chromosomes with the parental LNCaP and C4-2 cell lines.

The results of this study allow us to draw the following conclusions: (a), the LNCaP/C4-2 metastasis model has a striking resemblance to clinical human prostate cancer, in that tumor cells metastasize from the primary tumor to the lymph node to bony sites, where primarily osteoblastic lesions are observed; (b), since the androgen-independent phenotype of the C4-2 cells is stable and these cells are derived from LNCaP cells following cellular interaction with bone fibroblasts, it may be concluded that cellular interaction with mesenchymal bone fibroblasts facilitated androgen-independent LNCaP progression; (c), although tumor formation was more prevalent after orthotopic than after s.c. injection at a dose of 1.0 $\times$ 10^6 cells, the ability of the C4-2 cells to metastasize to the bone was consistently higher when injected s.c. than when injected orthotopically. These findings suggest that the organ-specific microenvironment might not necessarily be required for metastasis to occur but rather the status of differentiation and malignant potential of the tumor epithelial cells may be the critical determinant affecting local growth and dissemination (15, 16).

Finally, it is surprising to find that osseous metastases of the androgen-independent tumors are enhanced in the castrated host, suggesting a possible androgen suppression of androgen-independent dissemination, a finding that seemingly contradicts certain clinical observations. There are several possible explanations and potential implications for these findings. (a) androgen deprivation may be only beneficial to androgen-dependent tumors; its optimal role in the treatment of androgen-refractory prostate cancer has yet to be defined. Our results could be interpreted to mean that androgen deprivation may be beneficial to early stages of prostate cancer, which is commonly known to be more androgen-dependent. However, persistent androgen deprivation may facilitate androgen-independent progression and osseous metastasis in later stages of prostate cancer. (b) Some clinical observations, such as the clinical benefit of flutamide withdrawal (17) in a subset of patients with long-term treatment with flutamide and a rebound in PSA, may support the concept that androgen deprivation enhances androgen-independent prostate cancer progression. Such progression may be influenced by allowing the rebound of endogenous testicular androgens (e.g., flutamide withdrawal or discontinuation of luteinizing hormone-releasing hormone (17) in a subset of patients with long-term treatment with flutamide and a rebound in PSA, may support the concept that androgen deprivation enhances androgen-independent prostate cancer progression. Such progression may be influenced by allowing the rebound of endogenous testicular androgens (e.g., flutamide withdrawal or discontinuation of luteinizing hormone-releasing hormone antagonists) or the intermittent administration of exogenous testosterone (18). (c) LNCaP sublines presumably contain point mutations at the steroid-binding domain of androgen receptors that decrease steroid receptor specificity and may increase their responsiveness to other steroids (e.g., adrenal androgens).

In summary, an androgen-independent LNCaP subline, C4-2, was derived from LNCaP tumors maintained in castrated hosts. This tumor subline was found to acquire metastatic potential targeting the bone when inoculated s.c. or orthotopically into both castrated and intact athymic nude mice. Since it mimics the natural course of prostate cancer progression from androgen dependence to independence and developing regional as well as nonrandom osseous metastasis, this experimental model may permit for the first time the study of these mechanisms in vivo and lead to the design of new therapeutic concepts.

Acknowledgments

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References

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