Advances in Brief

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第二段...

Model of Human Prostate Cancer

 oldest age...at 6 sites/animal. For infections; 1.0 X 10^6 C4-2 cells resuspended in 0.1 ml of RPMI 1640 and 10% 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 X 10^9 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 X 10^9) resuspended in the same...
OSSEOUS METASTASIS OF HUMAN PROSTATE CANCER MODEL

Fig. 1. Scheme of the development of C4-2 sublines from LNCaP tumors maintained in intact and castrated athymic male nude mice. MS, fibroblasts derived from a human bone osteosarcoma, as described previously (8).

medium (total volume, 20 μl) were delivered to the dorsolateral lobe of the prostate gland of athymic mice (20 intact animals and 14 castrated animals; 1 site/animal) by a 30-gauge needle using a calibrated push-button Hamilton syringe (Hamilton, Reno, NV). Orthotopic injections were performed under methoxyflurane anesthesia (Metofane) with the prostate lobe exposed following a lower midline abdominal incision. The wound was closed by metal clips (Autoclip; Clay Adams, Parsippany, NJ). In some cases, prior to tumor cell inoculation, some mice were bilaterally castrated by scrotal incision. Animals were routinely inspected for physical abnormalities and tumor growth.

LNCaP Tumor Cell Sublines. To examine the cytogenetic and immunohistochemical characteristics of the LNCaP sublines with a defined cell lineage relationship, we derived LNCaP sublines from the parental LNCaP cells (10) and one C4-2 subline each from the primary tumor, lymph node, and bone metastasis from a tumor maintained in a castrated host inoculated with C4-2 cells orthotopically (see Fig. 1). The animal was sacrificed at the time when paraplegia was detected. Tumor tissues were harvested from the primary tumor, lymph node, and bone for subsequent derivation of C4-2 sublines (10). 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 99mtechnetium-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. 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

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Fig. 2. Characterization of the metastatic LNCaP tumor model and the primary and metastatic C4-2 sublines C4-2Pr, C4-2Ln, and C4-2B. (a) H&E stain of a C4-2-derived tumor; note an example of the increased vascularisation of tumors grown at both s.c. and orthotopic sites. (b) Whole-body sagittal images show a focal area of markedly increased uptake of $^{99m}$technetium-methylene diphosphonate in the region of the lower thoracic spine (closed arrows), indicating osseous metastases of prostate cancer. Note normal spine (closed arrows) in the animal without metastatic disease (c); open arrow, bladder activities. (d) H & E stain of a tumor grown in the host prostate, (e) lymph node metastasis, and (f) bone metastasis; note that in the bone, prostate cancer cells were observed to elicit marked local osteoblastic responses. (g) Immunohistochemical staining of PSA associated with C4-2B cells; note heterogeneity of PSA staining in the C4-2B cells. (h) Immunohistochemical staining of cytokeratin 8 associated with C4-2B cells. (i) Hoechst stain of DNA of a LNCaP tumor grown in the host prostate. (j) Human epithelial cells detected in bone metastasis (closed arrows). (k) Mouse salivary gland staining served as a negative control (mouse cells, open arrows). (l) Hoechst stain in human benign prostate hyperplasia served as a positive control. A ×20 objective was used to photograph a, d, and i–l, whereas a ×10 objective was used to photograph a–h.

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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. 2a); 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. 2a) and human benign prostate hyperplasia (Fig. 2f).

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 karyotyping because of the presence of human (light and even staining) and mouse cells (sharp and granulated staining) in the primary tumor and bone metastases (Fig. 2f). The C4-2B subline did not show the presence of X and Y chromosomes (Fig. 2a); 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. 2a) and human benign prostate hyperplasia (Fig. 2f).

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**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) tumor 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 × 10⁶ 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 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 cognate ligands, thus possibly enhancing their proliferative and malignant potential in response 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 male 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.

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References

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