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Human prostate tumor growth in athymic mice: Inhibition by androgens and stimulation by finasteride

(cancer cell progression/tumor regression/androgen receptor/5α-dihydrotestosterone/5α-reductase inhibitor)

Yoshihisa Umekita, Richard A. Hiipakka, John M. Kokontis, and Shutsung Liao*

The Ben May Institute for Cancer Research, Department of Biochemistry and Molecular Biology, and Committee on Cancer Biology, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637

ABSTRACT   When the human prostate cancer cell line, LNCaP 104-S, the growth of which is stimulated by physiological levels of androgen, is cultured in androgen-depleted medium for >100 passages, the cells, now called LNCaP 104-R2, are proliferatively repressed by low concentrations of androgens. LNCaP 104-R2 cells formed tumors in castrated male athymic nude mice. Testosterone propionate (TP) treatment prevented LNCaP 104-R2 tumor growth and caused regression of established tumors in these mice. Such a tumor-suppressive effect was not observed with tumors derived from LNCaP 104-S cells or androgen receptor-negative human prostate cancer PC-3 cells. 5α-Dihydrotestosterone, but not 5β-dihydrotestosterone, 17β-estradiol, or medroxyprogesterone acetate, also inhibited LNCaP 104-R2 tumor growth. Removal of TP or implantation of finasteride, a 5α-reductase inhibitor, in nude mice bearing TP implants resulted in the regrowth of LNCaP 104-R2 tumors. Within 1 week after TP implantation, LNCaP 104-R2 tumors exhibited massive necrosis with severe hemorrhage. Three weeks later, these tumors showed fibrosis with infiltration of chronic inflammatory cells and scattered carcinoma cells exhibiting degeneration. TP treatment of mice with LNCaP 104-R2 tumors reduced tumor androgen receptor and c-myc mRNA levels but increased prostate-specific antigen in serum- and prostate-specific antigen mRNA in tumors. Although androgen ablation has been the standard treatment for metastatic prostate cancer for >50 years, our study shows that androgen supplementation therapy may be beneficial for treatment of certain types of human prostate cancer and that the use of 5α-reductase inhibitors, such as finasteride or anti-androgens, in the general treatment of metastatic prostate cancer may require careful assessment.

Prostate cancer is now the most commonly diagnosed cancer in American men. In 1996, 317,100 new prostate cancer cases are expected and 41,400 men may die from prostate cancer (1). The growth and development of prostate cancer is initially androgen-dependent, and androgen ablation therapies have been the standard treatment for metastatic prostate cancer since Charles Huggins published his classic report in 1941 (2). Prostate cancer patients treated with androgen ablation therapy often have remission of their prostate cancer, but within a few years, tumor regrowth occurs. The recurrence of prostate cancer is largely due to progression of initially androgen-dependent prostate cancer cells to tumor cells that do not depend on androgen for their proliferation (3–5). The reason for this loss of androgen dependency is not known, but human prostate cancer cells, including various LNCaP sublines, have been used to study the changes occurring during progression and tumorigenesis (6–9). Some androgen-independent prostate cancer cell lines, such as PC-3 and DU-145, lack androgen

receptor (AR; ref. 10). However, AR has been found in metastatic prostate cancer after ablation therapy (11), and progression to steroid insensitivity can occur irrespective of the presence of functional steroid receptors (12).

To mimic the natural course of human prostate cancer, we have derived LNCaP 104-R2 cells from the androgen-dependent LNCaP 104-S cells, after long-term culture in androgen-depleted medium (6). LNCaP 104-R2 cells contain AR, but their proliferation is not dependent on androgen. Instead, these cells are proliferatively repressed by very low concentrations of androgen in culture medium. We report here that testosterone prevents and suppresses the growth of LNCaP 104-R2 tumors in nude mice and that this effect was inhibited by finasteride, a 5α-reductase inhibitor.[†]

MATERIALS AND METHODS

Cell Lines. Androgen-dependent LNCaP 104-S (passage 37) and androgen-independent LNCaP 104-R2[‡] sublines were isolated as described (6). The characteristics of these cells in vitro were confirmed before injection into nude mice. Briefly, proliferation of LNCaP 104-S cells increased 10- to 13-fold in medium containing charcoal-treated fetal bovine serum (FBS) and 0.1 nM synthetic androgen R1881, compared with cells cultured in medium containing charcoal-treated FBS without added androgen. LNCaP 104-R2 cells grew in medium supplemented with charcoal-treated FBS without additional androgen. Their proliferation was not stimulated but was repressed by 0.1 nM R1881. LNCaP 104-S cells were maintained in DMEM (GIBCO) supplemented with 1 nM 5α-dihydrotestosterone and 10% FBS (Summit Biotechnology, Ft. Collins, CO), and LNCaP 104-R2 cells were maintained in DMEM supplemented with 10% FBS treated with charcoal to remove steroid (6). PC-3 and MCF-7 cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in DMEM supplemented with 10% FBS.

Animals. BALB/c athymic (nude) male (LNCaP, PC-3 cell lines) and female (MCF-7 cell line) mice (Taconic, Germantown, NY), 5 to 7 weeks old, were used. Mice were housed in a pathogen-free environment, four to five mice per cage. Cages (filter top), bedding, and water were autoclaved before use. Feed was irradiated Pico Lab Mouse Chow 20 5058 (Purina).

All procedures involving animals were approved by the University of Chicago Institutional Animal Care and Use Committee. For the tumor growth studies, 1 million cells in 0.25 ml of culture medium were mixed with 0.25 ml of Matrigel (Collaborative Research) and were injected subcutaneously into one or both flanks of the mice as described (9). Tumor size was measured weekly and tumor volume was calculated using the formula length $\times$ width $\times$ height $\times$ 0.52 (13). Bilateral orchiectomy and subcutaneous implantation or removal of pellets were performed under Metofane (Pitman–Moore, Mundelein, IL) anesthesia. Blood samples were obtained by heart puncture or from the orbital plexus while mice were under anesthesia and analyzed for testosterone levels by radioimmunoassay or prostate-specific antigen (PSA) levels by dual-site reactive enzymatic immunoassay (Tandem-E PSA; Hybritech, San Diego). All steroid hormone (20 mg) pellets were purchased from Hormone Pellet Press (Westwood, KS). Finasteride (Proscar, 5 mg; Merck) was obtained from The University of Chicago hospital pharmacy. All numerical data are expressed as the average of the values obtained from four to six tumors and the standard error.

RNA Analysis. Total RNA was isolated from tumor tissue using the acid-guanidium thiocyanate phenol-chloroform extraction method (14). Ribonuclease protection assay (15, 16) was performed using probes generated from a 210-bp *Kpn*I–*Sac*I fragment of human AR cDNA (6, 17), a 77-bp fragment of human PSA cDNA (6, 18), a 252-bp *Pst*I–*Cla*I fragment of human c-*myc* cDNA (19), and a 144-bp *Pst*I–*Hinc*II fragment at the 5' terminus of human β_2 microglobulin (20). Inclusion of a β_2 microglobulin antisense RNA probe in hybridizations served as an internal standard for normalization of samples containing different levels of total RNA.

Sequencing of LNCaP AR mRNA from Tumors. cDNA encoding LNCaP AR (21) androgen-binding domain was amplified by reverse transcriptase-PCR using the primers 5'-GGCGATCCTTCACCAATGTC-3' (AR nucleotide sequence numbers 2780–2799) and 5'-GGAAAGGTCCACG-CTCACCAT-3' (AR nucleotide sequence numbers 3184–3203; ref. 17). Gel-purified PCR products (424 bp) were inserted into the *Eco*RV site of pBluescript SK(+) (Stratagene) and sequenced by a double-stranded DNA dideoxy sequencing method using Sequenase (Amersham).

Histology and Immunocytochemistry. For histological examination, resected tumor tissues were fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. Immunolocalization studies on paraffin sections used a rabbit polyclonal anti-human AR antibody (AN-15, 5 μg of protein/ml) that is directed against amino acids 1–15 of the amino terminus of AR and polyclonal anti-human PSA antibody (15 μg of protein/ml; Dako, Carpenteria, CA). Nude mice tumors originating from PC-3 cells were used as negative controls. Immunostaining was carried out using a streptavidin–biotin–peroxidase protocol (22). For AR immunostaining, deparaffinized tissue sections were pretreated with microwave irradiation in citrate buffer for 5 min (11).

RESULTS

Tumorigenicity of LNCaP 104-S and LNCaP 104-R2 Cells in Nude Mice. Palpable tumors were detected in 83% of normal mice, but 0% of castrated mice (Table 1) 4 weeks after injection of LNCaP 104-S cells. In contrast, palpable tumors were detected in 75% of castrated mice, but in 0% of normal mice, 4 weeks after injection of LNCaP-R2 cells. However, 7 weeks after injection, palpable LNCaP 104-R2 tumors were detected in 50% of normal mice, and their average size was 831 $\pm$ 191 (mean $\pm$ SE) mm³, which was almost the same size as tumors found in castrated mice (884 $\pm$ 64 mm³) at this time. LNCaP cells have a point mutation from A to G (21, 23) at

Table 1. Tumorigenicity of LNCaP 104-S and LNCaP 104-R in nude mice

	Tumor incidence							
	LNCaP 104-S				LNCaP 104-R2			
	Normal		Castrated		Normal		Castrated	
Week	No.	%	No.	%	No.	%	No.	%
3	0	0	0	0	0	0	0	0
4	10	83	0	0	0	0	9	75
5	10	83	0	0	1	8	9	75
6	10	83	0	0	4	33	9	75
7	11	91	0	0	6	50	10	83

LNCaP cells were injected into 12 normal male nude mice and 12 nude mice castrated 24 h before cell injection. Mice with palpable tumors were identified every week. No tumors were found 3 weeks after cancer cell injection.

nucleotide position 3157 (17) in the DNA coding for the androgen-binding domain of AR. We found that AR cDNA derived from LNCaP 104-S or 104-R2 tumors also have this mutation, which is consistent with these tumors originating from the injected LNCaP cells.

Effect of Androgens and Other Steroid Hormones on the Growth of LNCaP 104-R2 Tumors. If a testosterone propionate (TP) pellet was implanted at the 4th week in castrated nude mice with growing LNCaP 104-R2 tumors, further tumor growth was inhibited and tumor size was significantly reduced to 100 mm³ or less at the 7th week (Fig. 1). A similar tumor suppressive effect was observed when testosterone or 5α-dihydrotestosterone pellets were implanted. 5β-Dihydrotestosterone, a nonandrogenic stereoisomer of 5α-dihydrotestosterone, was not effective, suggesting that the suppressive effect required androgenic steroids. 17β-Estradiol and medroxyprogesterone acetate were not suppressive and actually showed some growth stimulatory activity.

Effects of TP on the Growth of Other Tumors. In contrast to LNCaP 104-R2 tumors, proliferation of LNCaP 104-S tumors was stimulated by androgens (Fig. 2). If tumor-bearing nude mice were castrated 4 weeks after injection of cells, growth of LNCaP 104-S tumors stopped and, during the next 4 weeks, tumors regressed to 10% of their size before castration. If TP was implanted at the time of castration, the tumors
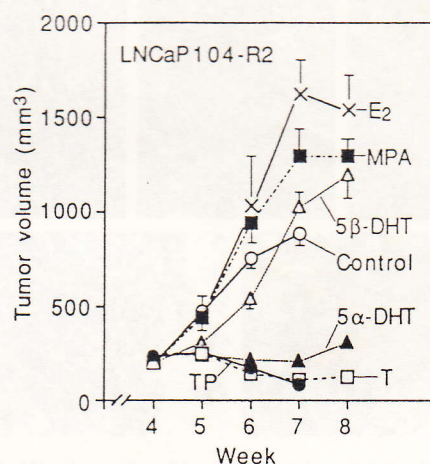

FIG. 1. Androgen-specific suppression of the growth of LNCaP 104-R2 tumors in castrated male nude mice. Nude mice were castrated and injected with LNCaP 104-R2 cells. Four weeks later, mice with tumors (240 $\pm$ 20 mm³) were implanted with a 20-mg pellet of testosterone (T), TP, 5α-dihydrotestosterone (5α-DHT), 5β-dihydrotestosterone (5β-DHT), medroxyprogesterone acetate (MPA), or 17β-estradiol (E$_2$). Tumor size was measured every week. Each point represents data for 6–15 tumors. Control mice were castrated but did not receive a steroid pellet implant.

〒606
京都市左京区吉田近衛町

京都大学

放射性同位元素総合センター

電話京都(〇七五)—七五一—二二一一

学内 七五〇〇

直通 七六一—六六三二

606
京都市左京区
岩倉中大鷺町
清水様

Tel: 075-711-4827
Fax: 075-711-0633三
五

711-0633