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)

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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 finasterone 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-R25 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, F. 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).

Abbreviations: AR, androgen receptor; FBS, fetal bovine serum; PSA, prostate-specific antigen; TP, testosterone propionate.
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§A more detailed description of various properties of LNCaP 104-R2 cells will be included in a separate publication by J.K. and S.L. LNCaP 104-R previously reported from this laboratory (6) is now designated as LNCaP 104-R1. LNCaP 104-R1 cells were derived from androgen-dependent LNCaP 104S cells after 40 passages in DMEM containing charcoal-stripped FBS, whereas LNCaP 104-R2 cells were derived from LNCaP 104-R1 cells after 60 additional passages in the same androgen-depleted medium.
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 × width × height × 0.52 (15). 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, Merck) was administered to the mice. 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 KpnI–Sall fragment of human AR cDNA (6, 17), a 77-bp fragment of human PSA cDNA (6, 18), a 252-bp PstI–ClaI fragment of human c-myc cDNA (19), and a 144-bp PstI–HindII fragment at the 5′ terminus of human β2; microglobulin (20). Inclusion of a β2; microglobulin antisense RNA probe 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′-GGCCGATCTTCATTCAAGATGTC-3′ (AR nucleotide sequence numbers 2780–2799) and 5′-GGAAAGGTCCACGCCTCACCAT-3′ (AR nucleotide sequence numbers 3184–3203; ref. 17). Gel-purified PCR products (424 bp) were inserted into the EcoRI site of pBluescript SK(+), (Stratagene) and sequenced by a double-stranded DNA dyeoxy 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-R 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 ± 191 (mean ± SE) mm3, which was almost the same size as tumors found in castrated mice (884 ± 64 mm3) at this time. LNCaP cells have a point mutation from A to G (21, 23) at 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-R Cells. 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 regressed to 10% of the size of tumors developing in normal male nude mice. LNCaP 104-S tumors developed very slowly compared with LNCaP 104-R2 tumors. Growth data for 6-15 tumors were analyzed as described for 6-15 tumors. Each point represents data for 6-15 tumors. Control mice were castrated but did not receive a steroid pellet implant.

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

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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.

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 ± 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 (E2). 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.
was also both LNCaP 104-R2 and MCF-7 cells that grew and its reversal group received no additional treatment (C), and mice in the other group were implanted with TP (C + TP). Tumor size was measured every week. Each point represents data for five tumors. Continued to grow from 299 ± 27 mm³ to 965 ± 166 mm³ during the next 4 weeks. TP did not affect the growth of AR-negative PC-3 tumors. In female nude mice, the growth of MCF-7 tumors, which express both estrogen receptor and AR, was also not affected by TP. Therefore, the androgen-dependent suppression of LNCaP 104-R2 tumor growth was both tumor- and steroid-specific.

Androgen-Dependent Remission of LNCaP 104-R2 Tumors and Its Reversal by Removal of TP or Implantation of Finasteride. The LNCaP 104-R2 tumors in the control castrates grew to 884 ± 64 mm³ in castrated mice 7 weeks after injection of cells (Figs. 1, 3, and 4A). TP implantation in these mice resulted in a rapid reduction in tumor size. The effect of TP was clearly visible within 1 week; massive hemorrhage was seen in tumors (Fig. 4B). Four weeks after TP implantation, tumor size was reduced to 208 ± 33 mm³ (Figs. 3 and 4C). If TP was removed at the 7th week from LNCaP 104-R2 tumor-bearing mice that were originally implanted with TP at the 4th week (Fig. 1), tumors regrew from 96 ± 26 mm³ (Figs. 3 and 4D) to 641 ± 157 mm³ (Figs. 3 and 4E) within the next 4 weeks.

5α-Reductase inhibitors (24) such as finasteride can prevent testosterone action that is dependent on the conversion of testosterone to 5α-dihydrotestosterone (25, 26). Therefore, we studied whether finasteride can prevent the TP-dependent suppression of LNCaP 104-R2 tumors in nude mice. When finasteride (2.5 mg) pellets were implanted at the 7th week in mice originally implanted with TP at the 4th week, LNCaP 104-R2 tumor growth resumed from the TP repressed level of 84 ± 15 mm³ and reached 593 ± 144 mm³ within 4 weeks (Figs. 3 and 4F). The rate of this regrowth was about the same as that in nude mice from which implanted TP was removed (Figs. 3 and 4E). Thus, finasteride alleviated the testosterone suppression of tumor growth.

In contrast, finasteride treatment of LNCaP 104-S tumors, in normal nude mice, reduced tumor size by 45%, from 1387 ± 432 mm³ to 759 ± 136 mm³ within 4 weeks (Fig. 5). During this period, the tumor size in the control mice without finasteride implant increased by 280%. Finasteride did not affect the growth of human breast MCF-7 tumors in female nude mice.

Histology. There was no clear histological difference between LNCaP 104-R2 and LNCaP 104-S tumors grown in nude mice. For LNCaP 104-R2 tumors, no remarkable histological change was noted within 3 days after TP implantation (Fig. 6A). At 5–7 days after TP implantation, histological sections revealed extensive necrosis with severe hemorrhage (Fig. 6B). At the 4th week after TP treatment, tumor size was markedly decreased, and histological sections revealed fibrosis with infiltration of chronic inflammatory cells and scattered carcinoma cells in the process of degeneration (Fig. 6C).

Effect of Androgen on the Expression of AR, c-myc, and PSA by LNCaP 104-R2 Tumors. Immunocytochemical staining of LNCaP 104-R2 tumors localized AR to the nucleus (Fig. 6D) and PSA to the cytoplasm (Fig. 6E) in tumor cells but not in surrounding mouse cells. The level of mRNA for AR and c-myc in the LNCaP 104-R2 tumor was reduced by 50% to 70% within 3 days after TP implantation (Fig. 7). This initial rapid loss preceded the general loss of tumor cells. The level of PSA mRNA in tumor samples (Fig. 7) and serum PSA (results not shown) increased >10-fold after 1 week of TP treatment and remained at this high level for at least 1 more week. At this early stage of TP action, enhanced PSA expression indicates that some tumor cells are viable and still respond to androgenic stimulation.

Biological Effects of Androgen in Nude Mice. Various results described in this paper suggested that TP implants were biologically effective for at least 7 weeks. TP used in the experiments maintained the serum testosterone level at 20–28 ng/ml for at least 7 weeks. In comparison, the serum testosterone level was ≥5 ng/ml in normal and 0.3 ng/ml in castrated male mice without TP implants. Since TP stimulated the growth of tumors derived from LNCaP 104-S cells and had no effect on the growth of PC-3 and MCF-7 tumors in nude mice, it is unlikely that the growth suppression of LNCaP 104-R2 tumor by TP was due to a general toxicity of implanted androgen. This conclusion is supported by the fact that at the 4th week after androgen implantation, the seminal vesicle weight in the nude mice with either LNCaP 104-S or 104-R2 tumors increased ≥10 times (compared with that in castrates without TP treatment) and there was no loss in the body weight of these nude mice.
very high vector which with TP within and are necessaryulated by androgen (0.1 nM) concentrations of PC-3 cells transfected with lines, LNCaP back leg); (B) experiment summarized stimulatory LNCaP androgen-dependent. A mouse, suppression vector expression have found that mouse, finasteride cells 430 or point tumors MCF-7 compared the as E 300 o200 > \frac{M}{M}

FIG. 5. Effect of finasteride on the growth of LNCaP 104-S and MCF-7 tumors in nude mice. Human prostate cancer LNCaP 104-S cells or human breast cancer MCF-7 cells were injected into normal male or female nude mice, respectively. After tumors grew to 1400 ± 430 mm³, nude mice were divided into two groups. One group received finasteride (FS) implants, while the other group was kept as control. Each point represent data for four tumors.

FIG. 6. Histology of and immunocytochemical localization of AR and PSA in LNCaP tumors. Hematoxylin- and eosin-stained tissue sections for (A) LNCaP 104-R2 tumor from castrated male nude mouse shown in Fig. 4A; (B) LNCaP 104-R2 tumor from castrated male nude mouse 1 week after implantation of TP as shown in Fig. 4B; and (C) LNCaP 104-R2 tumor from a mouse 4 weeks after TP implantation as shown in Fig. 4C. Immunocytochemical staining (peroxidase-diaminobenzidine) for AR (D) in a LNCaP 104-R2 tumor from a castrated male nude mouse shown in Fig. 4A and for PSA (E) in the LNCaP 104-R2 tumor from a nude mouse implanted with TP for 1 week as shown in Fig. 4B.

DISCUSSION

Androgens are necessary for normal prostate development and function. Most newly diagnosed prostate cancers are also androgen-dependent. However, the human prostate cancer lines, LNCaP 104-R1 (9) and 104-R2 cells, which contain a very high level of AR (over 10-fold that of androgen-stimulatory LNCaP 104-S cells), are not proliferatively stimulated by androgen but are actually repressed by low concentrations (0.1 nM) of androgens. It has been reported that the proliferation of PC-3 cells transfected with an AR expression vector also is inhibited by androgen in culture (27). We also have found that PC-3 cells retrovirally infected with an AR expression vector do not survive well in culture.

The molecular mechanism involved in androgen-dependent suppression of prostate tumor cell growth is not clear. Since androgens inhibited the growth of LNCaP 104-R cells in culture (6), androgen may exert its effect directly on the tumor cells in nude mice. We showed previously that overexpression of c-Myc effectively blocked repression by androgen (6) in LNCaP 104-S and 104-R1 cells. Therefore, excessive expression of androgen-induced gene(s) may result in an imbalance in coordination of various cellular functions or a change in the production of factors that affect cell cycling or apoptosis. For example, transforming growth factor β1 mRNA level in the rat ventral prostate is negatively controlled by androgen (28), whereas inhibition of LNCaP cell proliferation by transforming growth factor β1 in culture (29) is dependent on the presence.
of an appropriate concentration of androgen (30). Androgen also suppresses the expression of prostatic sulfated glycoprotein-2 (clusterin) (refs. 31 and 32), which prevents LNCaP cell death induced by tumor necrosis factor α (33). Tumor growth is dependent on tumor angiogenesis (34). However, our histological analysis did not reveal a clear effect of testosterone on vesselization in the LNCaP 104-R2 tumor during the initial weeks of tumor growth suppression. A more detailed analysis is needed to rule out androgen effects on tumor angiogenesis.

In this study, androgen-repressed LNCaP 104-R2 tumors slowly adapted to growth in the presence of androgens. In normal male mice, LNCaP 104-R2 cells did not grow into palpable tumors in 4 weeks. However, in 50% of these mice, they slowly adapted to the presence of androgen over a 7-week period and grew to a size equivalent to LNCaP 104-R2 cells grown in castrated nude mice for 7 weeks (Table 1). It has been suggested that intermittent use of androgen may delay prostate cancer cell progression (35). Our present observations indicated that some prostate tumors that would be considered androgen-independent may revert to an androgen-sensitive phenotype. These tumors may then be responsive to androgen-ablation therapy.

The derivation of LNCaP 104-R2 cells from LNCaP 104-S cells after a long period (2 years) of culture in androgen-depleted culture medium may mimic the situation in prostate cancer patients who receive androgen ablation therapy (orchiectomy or chemical castration; refs. 3–5). Prostatic tumors in these patients initially respond to androgen ablation therapy, but prostate cancer often reappears as an androgen-independent cancer. No agent is currently available to prevent this progression. For the development of new therapeutic methods for treatment of recurrent prostate cancer, it is important to elucidate the molecular mechanism involved in cancer cell progression and systematically analyze the properties of various cancer cells derived from androgen-dependent prostate cancer cells. A recent report showed that distant metastases in patients with prostatic carcinoma who have undergone various kinds of endocrine therapy contain AR (11). Some of these metastatic prostate tumor cells may behave like LNCaP 104-R2 cells and respond to androgen suppression or revert to androgen-dependent tumors as shown in our present study.

The 5α-reductase inhibitor finasteride has been found to be effective in the treatment of benign prostatic hyperplasia in some patients (36). Finasteride is also being tested for the chemoprevention of prostate cancer (37). Our present findings suggest, if the behavior of LNCaP 104-R2 cells is not unique, that it may be important to consider potential adverse effects of finasteride if it is to be used in prostate cancer chemotherapy. Flutamide (an anti-androgen being used for prostate cancer therapy) stimulates the growth of LNCaP cells (38), because the AR in these cells has a point mutation in the ligand-binding domain and can utilize anti-androgenic hydroxyflutamide as an androgen to transactivate target genes (21, 23). In preliminary studies we have also found that Casodex (Zeneica, Wilmington, DE) [bicalutamide; (2RS)-4-[(cyanooxy)methyl]-2-hydroxy-2-methyl-3-trifluoromethyl]-propionanilide; ref. 39], another anti-androgen, can inhibit androgen-dependent suppression of proliferation of LNCaP 104-R2 cells in culture. Effective use of anti-androgens and 5α-reductase inhibitors for prostate cancer therapy, therefore, may need careful assessment of the type of prostate cancer cells in the patient.

The response of LNCaP 104-R2 tumors in nude mice to androgens may be analogous to the effect of estrogens on metastatic breast cancer in postmenopausal women, where treatment with high doses of estrogens such as diethylstilbestrol causes regression of some tumors (40). The effect of androgen therapy on metastatic cancer of the prostate in a limited number of patients has been reported (41). Although no objective evidence of tumor remission was found, a limited number of patients experienced relief of symptoms. Optimization of dosage and better patient selection (long-term androgen ablation without adjuvant therapies) may be needed to enhance the therapeutic results of androgen therapy.

This article is dedicated to Professor Charles B. Huggins, who pioneered hormone ablation therapy. This research was supported by National Institute of Health Grants CA 58073 and DK 41670.