

# Enhanced Androgen Receptor Signaling Correlates with the Androgen-refractory Growth in a Newly Established MDA PCa 2b-hr Human Prostate Cancer Cell Subline

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## ABSTRACT

Bone metastasis is commonly found in prostate cancer (PC) patients. Although the mechanisms for the recurrence of bone metastasis-derived PC during medical or surgical castration therapy are still unclear because of the lack of suitable experimental models, one hypothesis is that enhanced androgen receptor (AR) signaling causes androgen-refractory PC growth. To test this hypothesis, we first established a novel androgen-refractory MDA PCa 2b cell subline, MDA PCa 2b-hr, which was generated *in vitro* from bone metastasis-derived, androgen-dependent MDA PCa 2b human PC cells after ~35 weeks of growth suppression by androgen-depletion treatment to mimic the clinical PC recurrence during androgen-ablation therapy. The changes of the androgen responsiveness of growth and the AR expression levels during the transition from an androgen-dependent to androgen-refractory proliferative phase through a temporal growth-suppressed phase precisely paralleled that of the basal growth rate. Furthermore, the androgen-refractory growth of MDA PCa 2b-hr cells in androgen-depleted medium was suppressed by an antiandrogen, bicalutamide. Next, we established nude mouse xenograft models to clarify whether AR signaling in MDA PCa 2b-hr cells is also enhanced *in vivo*. Both the MDA PCa 2b and MDA PCa 2b-hr tumors grew in gonadally intact mice, but only the MDA PCa 2b-hr tumors grew in castrated mice. The growth rate of MDA PCa 2b-hr tumors was significantly higher in gonadally intact mice than in castrated mice. Treatment with dehydroepiandrosterone pellets, which produced clinical castration levels of serum testosterone, accelerated the MDA PCa 2b-hr but not MDA PCa 2b tumor growth in castrated mice and increased blood prostate-specific antigen levels in castrated mice bearing MDA PCa 2b-hr tumors but not in mice bearing MDA PCa 2b tumors. Our data suggest that the enhanced AR signaling should be closely correlated with the androgen-refractory growth of human bone metastasis-derived PC, which might come to use adrenal androgens remaining in the blood even after castration therapy and warrant the continuation of hormone therapy for the recurrent PC.

## INTRODUCTION

PC<sup>2</sup> is the most common cancer and second leading cause of cancer deaths among men in the United States (1). Because PC growth is androgen-dependent at first, medical or surgical castration has been the standard treatment for metastatic PC. However, the castration therapy only causes a temporary regression of cancer, and most PCs become “androgen-refractory” (resistant to the androgen-ablation therapy) in several years, which is a major problem for the treatment of PC. The androgen-refractory phase was generally referred to as an androgen-independent phase in the clinical situation, because the PC growth was considered to be independent of androgen and have no

relation with AR signaling thus far. However, as described below, increasing evidence shows the close relationship between the “androgen-independent” phase and AR signaling. Therefore, in this study, we use the term “androgen-refractory” or “recurrent” for the PC starting to grow after androgen-ablation treatments, the term “androgen-independent” for the PC that does not need AR signaling for its growth, and the term “androgen-dependent” for the PC that needs AR signaling for its growth.

Most patients with androgen-refractory PC experience painful bone metastases, and ≤84% of PC patients are reported to have bone metastases at autopsy (2). Patients with recurrent PC generally continue to receive the androgen-ablation therapy. One study showed a superior survival effect of the maintenance of androgen-ablation therapy (3), but another study did not show an advantage of continued androgen blockade (4). Therefore, the continuation of androgen-ablation therapy in recurrent PC patients has never been conclusively justified. However, increased AR expression is found clinically in metastatic PC after androgen-ablation therapy (5–7), and serum levels of PSA, a marker of androgen action, increase in patients with androgen-refractory PC (7–9). These findings suggest that AR signaling might be still related with the androgen-refractory PC growth (5–7, 10).

DHEA is an androgen synthesized in adrenal glands and converted into active androgens, such as testosterone and dihydrotestosterone, in various tissues (11). Medical or surgical castration can suppress androgen derived from testes but cannot suppress androgen derived from adrenals. Therefore, the androgen derived from adrenals might activate AR signaling in recurrent PC during castration therapy (7).

At present, the lack of suitable experimental models hampered the evaluation of the mechanism for the androgen-refractory growth of PC. Several androgen-independent PC cell lines, such as PC-3 and DU145, lack AR and do not produce PSA, unlike the clinical androgen-refractory PC (12). Only LNCaP, MDA PCa 2a, and MDA PCa 2b cell lines express AR and PSA like clinical PC (12). The former cell line is derived from a lymph node metastasis (13), and the latter two cell lines are derived from a bone metastasis (14). Among them, only LNCaP cell sublines are reported to become androgen refractory during the culture in androgen-depleted medium (15–20). However, unlike most PCs in patients treated with androgen-ablation therapy, we found that LNCaP-FGC cells recurred only <4 weeks after androgen depletion without obvious and continued growth suppression (20). Because androgen-refractory PC cell lines generated from bone metastasis-derived, androgen-dependent PC cells have not been reported yet despite the fact that bone is the most common site for PC metastasis (21), we chose the bone metastasis-derived MDA PCa 2b cells as materials.

To study the involvement of AR signaling in bone metastasis-derived PC recurrence and clarify whether the continuation of androgen-ablation therapy is beneficial to recurrent PC patients, we have generated a novel androgen-refractory PC cell line, MDA PCa 2b-hr, from the androgen-dependent MDA PCa 2b cell line after as long as 35 weeks of growth suppression by androgen-depleted culture to mimic the clinical progression of PC during androgen-ablation

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<sup>2</sup>The abbreviations used are: PC, prostate cancer; AR, androgen receptor; PSA, prostate-specific antigen; DHEA, dehydroepiandrosterone; DCC, dextran charcoal-stripped; FBS, fetal bovine serum.

therapy. To our knowledge, this is the first report of a human bone metastasis-derived androgen-refractory PC cell line that was established after long periods of growth suppression by androgen-depletion treatment.

## MATERIALS AND METHODS

**Cell Culture.** The androgen-dependent human metastatic PC cell line MDA PCa 2b was obtained from the ATCC (Manassas, VA) and maintained in the ATCC-modified medium, which consists of Ham's F-12K (Sigma), 25 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, 5  $\mu$ M phosphoethanolamine, 100 pg/ml hydrocortisone, 45 nM selenious acid, 5  $\mu$ g/ml insulin, and 20% FBS (Trace Scientific Ltd., Melbourne, Australia), at 37°C in 5% CO<sub>2</sub>. MDA PCa 2b-hr cells were maintained in the DCC medium, which is the same with the ATCC-modified medium, except the usage of 20% DCC-FBS instead of 20% FBS. All culture media were supplemented with 5 units/ml penicillin and 5  $\mu$ g/ml streptomycin.

**Cell Proliferation and PSA Secretion Assays.** In the experiment studying the change of androgen sensitivity during androgen-depletion treatment, MDA PCa 2b cells which had been cultured in the ATCC-modified medium or DCC medium for 1, 2, and 4 weeks, and MDA PCa 2b-hr cells which had been cultured in the DCC medium for 48 and 111 weeks, were plated in 24-well plates at  $4 \times 10^4$  cells/well in the DCC medium. After incubation at 37°C in 5% CO<sub>2</sub> overnight, cells were treated with varying doses of testosterone (0.1–1000 ng/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan). After 3 days of incubation at 37°C in 5% CO<sub>2</sub>, the culture medium was removed for the measurement of PSA by ELISA (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), and cells were trypsinized and counted with a particle counter (Beckman Coulter, Tokyo, Japan).

In the experiment studying the effect of antiandrogen, MDA PCa 2b and MDA PCa 2b-hr cells which had been cultured in the DCC medium for 111 weeks were plated on 24-well plates at  $8 \times 10^4$  cells/well in the ATCC-modified and DCC medium, respectively. The effects of bicalutamide (purchased as Casodex tablets and extracted in our company) on the cell growth and PSA secretion were examined as described above.

**Western Blot Analyses.** After cells were lysed on ice in radioimmunoprecipitation assay buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM EGTA, and protease/phosphatase inhibitor cocktails (Sigma), the cell lysate was centrifuged at  $21,000 \times g$  for 10 min at 4°C. After the supernatant with 30  $\mu$ g of protein determined by bicinchoninate acid protein assay (Pierce, Rockford, IL) was dissolved in electrophoresis sample buffer consisting of 2% SDS, 5% 2-mercaptoethanol, 60 mM Tris-HCl (pH 6.8), 0.01% bromphenol blue, and 10% glycerol, the samples were fractionated in a 7.5% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) by electroblotting. The membranes were blocked in Blockace (Dainippon Pharmaceutical Co., Ltd.) and then probed with first antibody (AR; monoclonal mouse antihuman AR antibody, 2  $\mu$ g/ml, clone G122-434; BD PharMingen, San Diego, CA; actin; monoclonal mouse antibody, 1  $\mu$ g/ml, Actin (c-2); Santa Cruz Biotechnology, Santa Cruz, CA) in PBS containing 0.1% Tween 20 (PBST) and 10% Blockace. The membranes were then washed with PBST and incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) in PBST. After washing with PBST, the proteins were visualized using the enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech). The density of bands corresponding to AR and actin protein was quantitatively evaluated by Image Reader LAS-1000 (Fuji Film, Tokyo, Japan) with the software of Image Gauge V3.46 (Fuji Film).

**Animal Experiments.** The protocols of animal experiments were approved by the Takeda experimental animal use and care committee in accordance with NIH standards. Five-week-old male BALB/C athymic nude mice purchased from Charles River Japan (Kanagawa, Japan) were maintained on a 12/12 h light/dark cycle (lights on at 7 a.m.) with constant temperature (25°C). Food and water were available *ad libitum*. For the study of tumorigenicity and tumor growth in castrated and gonadally intact mice, MDA PCa 2b or MDA PCa 2b-hr cells which had been cultured in the DCC medium for 41 weeks ( $5 \times 10^6$  cells/100  $\mu$ l Matrigel; BD Biosciences, Bedford, MA) were injected s.c. into one flank of castrated and gonadally intact mice. The number of mice bearing

palpable tumors was counted, and the tumor size was measured weekly using a caliper and expressed in mm<sup>3</sup> using the formula  $0.5 \times a \times b^2$ , where  $a$  is the largest diameter and  $b$  is the largest diameter perpendicular to  $a$ . For the study of tumor growth by DHEA, 5 million MDA PCa 2b and MDA PCa 2b-hr cells which had been cultured in the DCC medium for 49 weeks were injected as described above in intact and castrated nude mice, respectively. After the size of MDA PCa 2b tumors had reached 260–420 mm<sup>3</sup>, surgical castration and s.c. implantation of a DHEA pellet (5, 25, and 100 mg, 21-day release; Innovative Research of America, Sarasota, FL) was performed under ether anesthesia. In MDA PCa 2b-hr cells, after the size of the tumors had reached 80–160 mm<sup>3</sup>, DHEA pellet implantation (1.5, 5, and 25 mg, 21-day release) was performed. DHEA pellets were supplemented after 21 days in both cases. The tumor size was measured weekly as described above. Blood samples were obtained weekly to measure the plasma PSA levels. At the end of the experiments, the animals were sacrificed by collecting blood by heart puncture under ether anesthesia to measure the serum DHEA, testosterone, and PSA levels.

**Measurement of DHEA and Testosterone.** The serum DHEA and testosterone levels in nude mice were determined with DHEA (Diagnostic Systems Laboratories, Webster, TX) and testosterone (Dia Sorin, Saluggia, Italy) RIA kits, respectively, according to the manufacturer's directions.

**Statistical Analysis.** In the cell growth study with bicalutamide, differences between means of the control group and bicalutamide-treated groups were analyzed by Dunnett's test. In the tumor growth study in castrated and gonadally intact mice, differences between means of the castrated and gonadally intact groups on day 97 were analyzed by unpaired Student's  $t$  test with Holm's correction for repeated tests. In the tumor growth study with DHEA pellets, differences between means of the control and DHEA-treated groups on the day of autopsy were analyzed by Dunnett's test where the variances among groups were homogeneous or Steel's test where those were heterogeneous. A value of  $P \leq 0.05$  was considered significant.

## RESULTS

**Establishment of the MDA PCa 2b-hr Cell Line.** MDA PCa 2b cells did not grow in the DCC medium at first but started to grow slowly after ~35 weeks of culture. After MDA PCa 2b cells started growing in the DCC medium, the cell line was designated as MDA PCa 2b-hr. MDA PCa 2b cells only continued to be fed with fresh medium at 2–3-day intervals and could not be propagated after the first 4 weeks of androgen-depletion treatment until the establishment of MDA PCa 2b-hr cells, because MDA PCa 2b cells became extinct when they were propagated during the period.

**Change of Androgen Sensitivity during Androgen-depletion Treatment.** The growth of MDA PCa 2b cells before the androgen-depletion treatment was stimulated by testosterone in a dose-dependent manner (Fig. 1A). As described in the legend of Fig. 1A, the basal growth rate in the androgen-depleted medium decreased gradually, and in parallel with that, the testosterone responsiveness diminished gradually during the first 4 weeks of the androgen-depletion treatment (Fig. 1A). However, the basal growth rate in the androgen-depleted medium increased, and in parallel with that, the response to low levels of testosterone reemerged in androgen-refractory MDA PCa 2b-hr cells, which had been treated with androgen depletion for 48 weeks (Fig. 1A). The basal growth rate of MDA PCa 2b-hr cells in the androgen-depleted medium increased after an additional 63 weeks of androgen-depletion treatment (Fig. 1A). The growth rate of androgen-refractory MDA PCa 2b-hr cells (111 weeks) by testosterone at concentrations  $< 3$  ng/ml was higher than that of androgen-dependent MDA PCa 2b cells (Fig. 1A). Interestingly, the growth curve of MDA PCa 2b-hr cells declined at testosterone  $> 1$  ng/ml (Fig. 1A).

The PSA secretion per cell was not stimulated by testosterone of 0.1–1000 ng/ml in MDA PCa 2b cells before the androgen-depletion treatment (Fig. 1B), but the PSA secretion showed tendency to be slightly stimulated by testosterone during the first 4 weeks of the androgen-depletion treatment (Fig. 1B). In androgen-refractory MDA PCa 2b-hr cells, which had been treated with androgen-depletion for

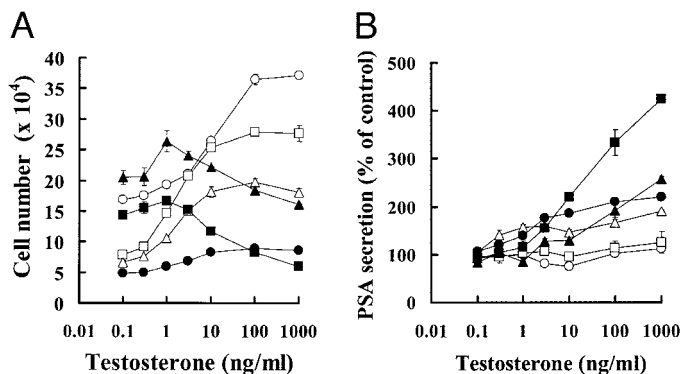


Fig. 1. Change of androgen sensitivity on the growth and PSA production during androgen depletion in MDA PCa 2b cell lines. MDA PCa 2b cells before (○) and after 1–4 weeks (□, 1w; △, 2w; ●, 4w) of androgen depletion and MDA PCa 2b-hr cells which had been treated with androgen depletion for 48 and 111 weeks (■, 48w; ▲, 111w) were plated in 24-well plates ( $4 \times 10^4$  cells/well) in the DCC medium. On the next day, the indicated concentrations of testosterone were added. After 3 days, cells were counted (A), and the culture medium was collected for the measurement of PSA levels (B). B, ordinate values were calculated by dividing the PSA concentration in the medium by the mean of the plated and final cell numbers and expressed by setting the PSA production of the vehicle control to 100. The cell numbers of the vehicle control (no testosterone) in cells before and after 1, 2, 4, 48, and 111 weeks of the androgen depletion treatment were  $160,000 \pm 3,400$ ;  $67,000 \pm 170$ ;  $64,000 \pm 750$ ;  $47,000 \pm 670$ ;  $120,000 \pm 6,300$ ; and  $180,000 \pm 9,400$ , respectively. Data are expressed as the mean of triplicate determinations. Bars,  $\pm$ SE.

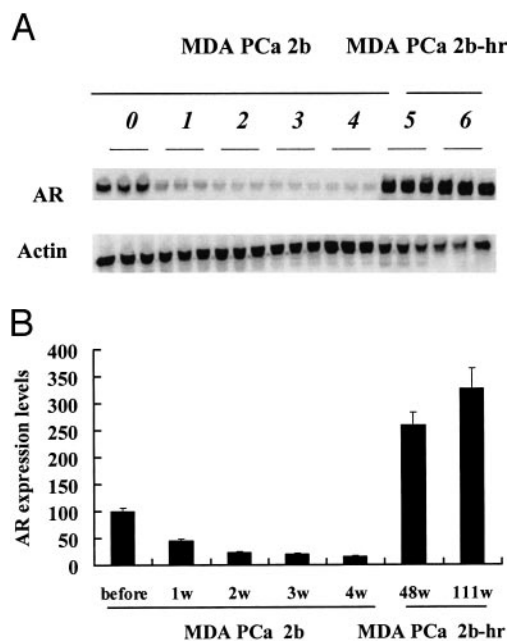


Fig. 2. Change of AR expression levels during androgen depletion in MDA PCa 2b cell lines. A, AR and actin expression levels in MDA PCa 2b cells before (0) and after 1–4 weeks (1, 1w; 2, 2w; 3, 3w; 4, 4w) of androgen depletion and MDA PCa 2b-hr cells which had been treated with androgen depletion for 48 (5) and 111 (6) weeks were determined by immunoblot analysis using  $30 \mu\text{g}$  of proteins. B, graphical representation of AR staining intensity normalized to the amount of actin. Ordinate values were calculated by setting the AR protein level in MDA PCa 2b cells before the androgen depletion treatment to an arbitrary value of 100. Data are expressed as the mean of triplicate determinations. Bars,  $\pm$ SE.

48 weeks, the PSA secretion was stimulated remarkably by testosterone in a dose-dependent manner (Fig. 1B). However, the response to testosterone decreased after an additional 63 weeks of androgen-depletion treatment (Fig. 1B).

**Change of AR Expression Levels during Androgen-depletion Treatment.** To study the involvement of AR levels in the change of androgen sensitivities, we examined the change of AR expression

levels during the androgen-depletion treatment by immunoblot analysis. The AR levels decreased gradually during 4 weeks of the androgen-depletion treatment (Fig. 2). However, the AR levels recovered in MDA PCa 2b-hr cells (Fig. 2). The AR levels in MDA PCa 2b-hr cells which had been treated with androgen depletion for 48 and 111 weeks were 2.6- and 3.3-fold higher than those in MDA PCa 2b cells (Fig. 2B).

**Suppression of the Androgen-refractory Growth of MDA PCa 2b-hr Cells by Antiandrogen.** To study the role of AR signaling on the androgen-refractory growth of PC, we examined the effect of an antiandrogen, bicalutamide, on the growth of androgen-refractory MDA PCa 2b-hr cells in androgen-depleted medium. Bicalutamide significantly inhibited the growth of MDA PCa 2b-hr cells (Fig. 3A). In addition, bicalutamide significantly suppressed the PSA secretion in MDA PCa 2b-hr cells in androgen-depleted medium (Fig. 3B). The growth and PSA secretion of MDA PCa 2b cells in the propagation medium were also suppressed in the same way (Fig. 3, C and D).

**Tumorigenicity and Tumor Growth of MDA PCa 2b and MDA PCa 2b-hr Cells in Castrated and Gonadally Intact Nude Mice.** Palpable tumors of MDA PCa 2b were detected in 100% of gonadally intact mice but 0% of castrated mice 34 days after injection of MDA PCa 2b cells (Table 1). In contrast, palpable tumors of MDA PCa 2b-hr were detected in 100% of gonadally intact mice and 89% of castrated mice 34 days after injection of MDA PCa 2b-hr cells (Table 1). This tendency was shown to continue by 97 days after injection (Table 1). Both the MDA PCa 2b and MDA PCa 2b-hr tumors grew in gonadally intact mice, and the MDA PCa 2b-hr tumors also grew in castrated mice (Fig. 4). The growth rate of MDA PCa 2b-hr tumors was significantly higher in gonadally intact mice than in castrated mice (Fig. 4).

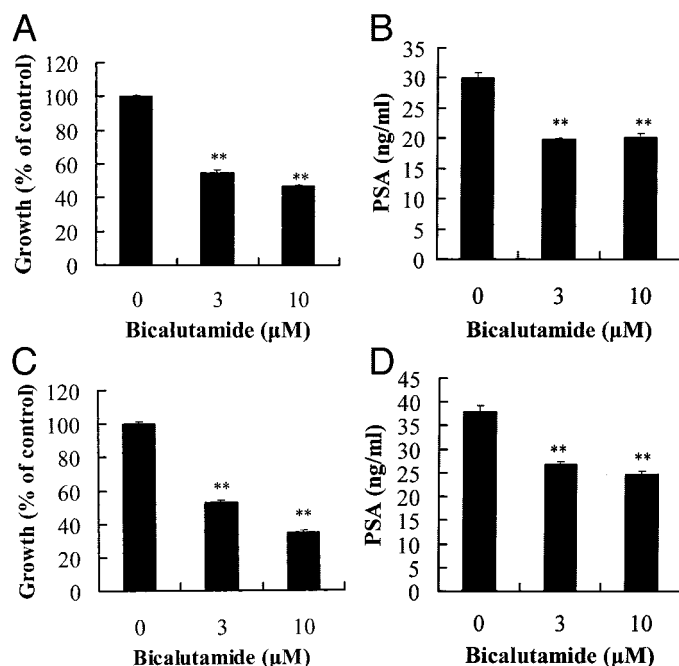


Fig. 3. Suppression of androgen-independent growth of MDA PCa 2b-hr cells by bicalutamide. MDA PCa 2b-hr (A and B) and MDA PCa 2b (C and D) cells were plated at  $8 \times 10^4$  cells/well in the DCC and ATCC-modified medium in 24-well plates, respectively. On the next day, the indicated concentrations of bicalutamide were added. After 3 days, cells were counted (A and C), and the culture medium was collected for the measurement of PSA levels (B and D). A and C, ordinate values were expressed by setting the plated cell number and mean of final cell numbers in the vehicle control group to 0 and 100%, respectively. The final cell numbers of the control in MDA PCa 2b-hr and MDA PCa 2b cells were  $560,000 \pm 4,200$  and  $410,000 \pm 4,300$ , respectively. Data represent the mean. Bars,  $\pm$ SE.  $n = 6$ . \*\*,  $P < 0.01$  versus vehicle control by Dunnett's test.

Table 1 Tumorigenicity of MDA PCa 2b and MDA PCa 2b-hr in castrated and gonadally intact nude mice

MDA PCa 2b and MDA PCa 2b-hr cells ( $5 \times 10^6$  cells) were injected into castrated and gonadally intact male nude mice. Mice with palpable tumors were identified every week. No palpable tumors were detected until day 27 ( $n = 11-18$ ).

Days	MDA PCa 2b				MDA PCa 2b-hr			
	Castrated		Intact		Castrated		Intact	
	Incidence/n	%	Incidence/n	%	Incidence/n	%	Incidence/n	%
27	0/12	0	15/18	83	1/18	6	17/17	100
34	0/12	0	18/18	100	16/18	89	17/17	100
41	1/12	8	18/18	100	15/18	83	15/17	88
48	1/11	9	18/18	100	17/18	94	15/17	88
55	1/11	9	18/18	100	17/18	94	15/17	88
62	2/11	18	18/18	100	17/18	94	15/17	88
69	0/11	0	18/18	100	17/18	94	15/17	88
76	0/11	0	18/18	100	17/18	94	15/17	88
83	1/11	9	18/18	100	17/18	94	15/17	88
90	0/11	0	18/18	100	17/18	94	15/17	88
97	1/11	9	18/18	100	17/18	94	13/15	87

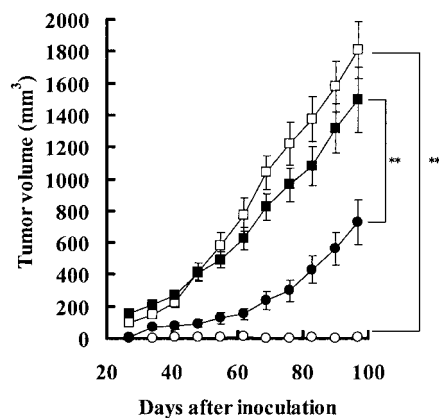


Fig. 4. Growth of MDA PCa 2b and MDA PCa 2b-hr tumors in castrated and gonadally intact male nude mice. MDA PCa 2b (○, castrated; □, intact) and MDA PCa 2b-hr cells (●, castrated; ■, intact) were injected into castrated and gonadally intact male nude mice. Tumor size was measured every week. Data represent the mean. Bars,  $\pm$  SE.  $n = 11-18$ . \*\*,  $P < 0.01$  on day 97 by unpaired Student's  $t$  test with Holm's correction for repeated tests.

**DHEA Accelerates the Growth of MDA PCa 2b-hr Tumors in Castrated Nude Mice.** The growth of MDA PCa 2b tumors was slightly accelerated by the treatment with 100-mg DHEA pellets but not with 5- nor 25-mg DHEA pellets either (Fig. 5A). The blood PSA levels were also increased only by the treatment with a 100-mg DHEA pellet in mice bearing MDA PCa 2b tumors (Fig. 5B). The serum DHEA levels in nude mice bearing MDA PCa 2b tumors implanted with a 100-mg DHEA pellet were 380.5 ng/ml (Table 2), which are much higher than the physiological levels in men (22). The serum testosterone levels in these nude mice were 12.4 ng/ml (Table 2), which are comparable with the physiological levels in men not treated by castration (23). In contrast, the growth of MDA PCa 2b-hr tumors was accelerated by the treatment with 1.5–25-mg DHEA pellets in a dose-dependent manner (Fig. 6A). The blood PSA levels were also increased by the treatment with 1.5–25-mg DHEA pellets in a dose-dependent manner in mice bearing MDA PCa 2b-hr tumors (Fig. 6B). The serum DHEA and testosterone levels in nude mice bearing MDA PCa 2b-hr tumors implanted with a 5-mg DHEA pellet were 17.5 and 0.22 ng/ml, respectively (Table 2), which are both comparable with the physiological levels in castrated men (22, 23).

## DISCUSSION

Currently, we have no effective treatment for the recurrent PC during hormone therapy. Most patients dying of PC experience pain-

ful and sometimes crippling osseous metastases, and  $\leq 84\%$  of PC patients are reported to have bone metastases at autopsy (2). However, the lack of suitable experimental models of bone metastasis-derived androgen-refractory PC prevents the development of effective drugs. In this study, we showed for the first time that an androgen-refractory cell line MDA PCa 2b-hr could be established from bone metastasis-derived, androgen-dependent MDA PCa 2b cells after as long as 35 weeks of complete growth suppression by androgen-depletion culture. MDA PCa 2b-hr cells express higher levels of AR than their parental cells and still secrete PSA. These observations are consistent with the findings that most recurrent PCs during androgen-ablation therapy show increased AR expression (5–7), and the serum PSA levels in PC patients reascend when PC recurs (8, 9). Therefore, MDA PCa 2b-hr seems to be a very useful *in vitro* model for bone metastasis-derived, androgen-refractory PC, and analyses of the process of transition from an androgen-dependent to -refractory state in MDA PCa 2b cells may clarify the mechanisms of bone metastasis-derived PC recurrence during hormone therapy.

On the other hand, several cell sublines of LNCaP, which is derived from a lymph node metastasis (13), are reported to become androgen refractory during the culture in androgen-depleted medium (15–20). However, unlike most PCs in patients treated with androgen-ablation therapy, we found that LNCaP-FGC cells became androgen refractory during only  $< 4$  weeks of androgen-depletion treatment without obvious and continued growth suppression (20). Therefore, the model of

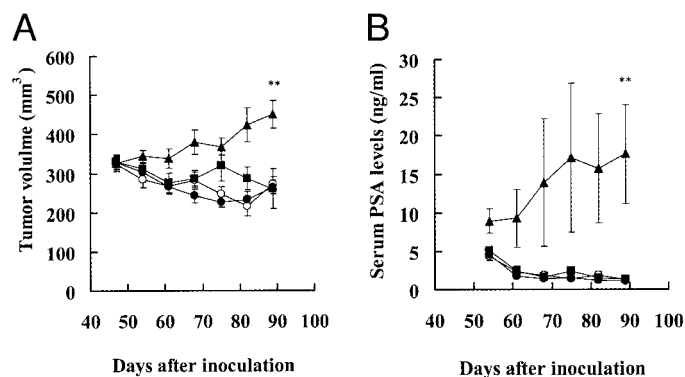


Fig. 5. Effect of DHEA on the growth of MDA PCa 2b tumors and serum PSA levels in castrated male nude mice. MDA PCa 2b cells were injected into gonadally intact male nude mice, and, 47 days later, nude mice bearing tumors ( $327 \pm 8$  mm<sup>3</sup>) were castrated and divided into four groups. On the day of castration, implantation of a DHEA pellet (5, 25, and 100 mg; 21-day release) was performed. The tumor size was measured weekly (A), and blood samples were also obtained weekly for the measurement of PSA levels (B). ○, placebo; ●, 5 mg of DHEA/21days; ■, 25 mg of DHEA/21days; ▲, 100 mg of DHEA/21days. DHEA pellets were supplemented 21 days after the first implantation. Data represent the mean. Bars,  $\pm$  SE.  $n = 6-7$ . \*\*,  $P < 0.01$  versus the placebo group on day 89 by Dunnett's (A) or Steel's (B) test.

Table 2 Serum DHEA and testosterone levels in nude mice implanted with a DHEA pellet

Implantation of DHEA pellets was performed in castrated nude mice bearing MDA PCa 2b and MDA PCa 2b-hr cells, and blood samples were obtained 6 and 4 weeks after the first implantation of DHEA pellets, respectively. The serum DHEA and testosterone levels were determined by RIA. Results are expressed as mean  $\pm$  SE ( $n = 6-7$ ).

	DHEA (ng/ml)	Testosterone (ng/ml)
MDA PCa 2b		
Placebo	0.0 $\pm$ 0.0	0.01 $\pm$ 0.01
DHEA 5 mg/3w	4.3 $\pm$ 0.8	0.14 $\pm$ 0.05
DHEA 25 mg/3w	20.5 $\pm$ 4.1	0.68 $\pm$ 0.12
DHEA 100 mg/3w	380.5 $\pm$ 85.6	12.40 $\pm$ 2.70
MDA PCa 2b-hr		
Placebo	0.4 $\pm$ 0.1	0.00 $\pm$ 0.00
DHEA 1.5 mg/3w	6.2 $\pm$ 0.6	0.04 $\pm$ 0.02
DHEA 5 mg/3w	17.5 $\pm$ 2.8	0.22 $\pm$ 0.05
DHEA 25 mg/3w	86.3 $\pm$ 9.6	1.24 $\pm$ 0.10

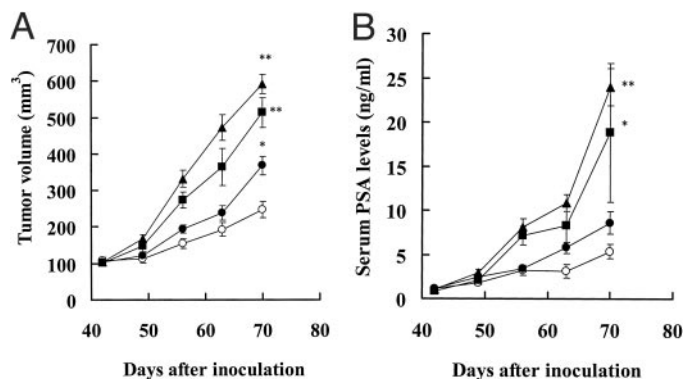


Fig. 6. Effect of DHEA on the growth of MDA PCa 2b-hr tumors and serum PSA levels in castrated male nude mice. MDA PCa 2b-hr cells were injected into castrated male nude mice, and, 42 days later, nude mice bearing tumors ( $104 \pm 3 \text{ mm}^3$ ) were divided into four groups, and implantation of a DHEA pellet (1.5, 5, and 25 mg; 21-day release) was performed. The tumor size was measured weekly (A), and blood samples were also obtained weekly for the measurement of PSA levels (B). ○, placebo; ●, 1.5 mg of DHEA/21days; ■, 5 mg of DHEA/21days; ▲, 25 mg of DHEA/21days. DHEA pellets were supplemented 21 days after the first implantation. Data represent the mean. Bars,  $\pm$ SE.  $n = 7$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus the placebo group on day 70 by Dunnett's (A) or Steel's (B) test.

PC recurrence with MDA PCa 2b cells seems to reflect clinical PC recurrence more precisely than that with LNCaP cells. Currently, there is no explanation for this difference of period required for becoming androgen refractory between MDA PCa 2b and LNCaP cells.

Our data obviously show that AR expression levels were decreased when the growth of MDA PCa 2b cells remained suppressed by androgen-depletion treatment and recovered when the growth of the cells recurred even in the androgen-depleted condition. Consistently, the close relationship between immunostained AR and Ki-67 expression has been reported recently in the CWR22 xenograft tumors, which is derived from primary human PC, during the transition from androgen-dependent to -refractory growth (24). The two different models of PC recurrence, one of which is an *in vitro* model with bone metastasis-derived PC cells and the other is an *in vivo* model with primary PC tumors, demonstrate the common feature of the close correlation between AR expression levels and PC growth rate. These findings suggest that AR signaling might generally be involved in androgen-refractory PC growth. In addition, our data show that the changes of AR levels and basal growth rate during androgen-depletion treatment also paralleled that of androgen sensitivity of growth in MDA PCa 2b and MDA PCa 2b-hr cells. Furthermore, we demonstrated that the androgen-refractory growth of MDA PCa 2b-hr cells was suppressed by inhibiting the AR function with an antiandrogen bicalutamide. Together with the fact that AR has a capacity to activate transcription in a ligand-independent manner (25), these findings suggest that AR signaling might be indispensable to recurrent growth of bone metastasis-derived PC during hormone therapy. To our knowledge, this is the first study showing the close correlation between basal growth rate, AR expression levels, and androgen sensitivity during the entire transition period from an androgen-dependent to -refractory proliferative phase through a temporal growth-suppressed phase.

Because MDA PCa 2b cells harbor the L701H/T877A double mutant AR (26), it is interesting that bicalutamide suppressed the growth and PSA secretion of MDA PCa 2b cells. The results suggest that bicalutamide could work as an antagonist for the double mutant AR. Consistently, bicalutamide demonstrated antagonistic action for the L701H/T877A double mutant AR in transactivation assays using COS-7 cells (data not shown). MDA PCa 2b-hr cells also harbor the

L701H/T877A double mutant AR (data not shown). These findings suggest that in MDA PCa 2b-hr cells, bicalutamide might suppress the growth and PSA secretion in androgen-depleted medium by blocking the AR signaling activated in a ligand-independent manner.

The growth of bone metastasis-derived, androgen-refractory MDA PCa 2b-hr cells became stimulated by low levels of testosterone, and the PSA secretion by testosterone became elevated in MDA PCa 2b-hr cells compared with their parental cells. This finding of acquired hypersensitivity to androgen during androgen-depletion treatment suggests that adaptation to low-androgen environment by enhancing AR signaling is one mechanism for transition from an androgen-dependent to -refractory state in bone metastasis-derived human PC. These findings are consistent with those in LNCaP cell sublines (data not shown; Refs. 16 and 17), although the periods required for becoming hypersensitive to androgen are quite different. The fact that these two independent PC cell lines, one of which is derived from a bone metastasis (14) and the other from a lymph node metastasis (13), demonstrate the common feature of becoming hypersensitive to androgen during androgen-depletion treatment strongly supports the hypothesis that the enhanced AR signaling causes recurrent PC growth during hormone therapy. It is speculated that the enhanced AR signaling in androgen-refractory PC cells is partly caused by the increased expression of AR. However, additional study is required to clarify the mechanisms involved in the enhancement of AR signaling.

The growth curves by androgen are different between androgen-dependent MDA PCa 2b and LNCaP cells. The former shows a dose-dependent increase between 0.1 and 1000 ng/ml testosterone, and the latter shows a bell-shaped increase pattern with decline at concentrations  $> 1 \text{ ng/ml}$  (data not shown; Refs. 16 and 17). Surprisingly, however, the growth of not only androgen-refractory LNCaP (data not shown; Refs. 16 and 17) but also androgen-refractory MDA PCa 2b-hr cells was suppressed by high concentrations of testosterone. Because the expression of cyclin-dependent kinase inhibitor p21 or p27 has been reported to be up-regulated by androgen in LNCaP cells (27, 28), the growth inhibition by androgen in MDA PCa 2b-hr cells might also be caused by the enhanced expression of these cyclin-dependent kinase inhibitors.

The tumorigenicity study demonstrates that MDA PCa 2b tumors are suitable models of androgen-dependent PC, because MDA PCa 2b cells grew into palpable tumors only in gonadally intact mice but not in castrated mice. These results are inconsistent with the finding that the growth of MDA PCa 2b cells, which harbor the L701H/T877A double mutant AR, is stimulated not only by androgen but also by glucocorticoids (26). Because we confirmed that the growth of MDA PCa 2b cells was stimulated by corticosterone in the *in vitro* cell growth assay (data not shown), this discrepancy may be stemmed from the plasma corticosterone levels in the host castrated mice that were not high enough to activate the L701H/T877A double mutant AR in MDA PCa 2b tumors. On the other hand, MDA PCa 2b-hr cells grew into palpable tumors not only in gonadally intact nude mice but also in castrated nude mice. The high tumorigenicity and ability to grow in castrated mice indicate that MDA PCa 2b-hr tumors are suitable models of androgen-refractory PC. What must be emphasized is that MDA PCa 2b-hr tumors grew more rapidly in gonadally intact mice than in castrated mice, suggesting that androgen could still play a critical role in the acceleration of androgen-refractory PC growth. Furthermore, we confirmed that the growth of MDA PCa 2b-hr tumors in castrated nude mice was suppressed by the treatment with bicalutamide (data not shown), suggesting that AR signaling causes the MDA PCa 2b-hr tumor growth. To our knowledge, this is the first experimental model showing that testicular androgen exacerbates the growth of bone metastasis-derived, androgen-refractory human PC. Although patients with recurrent PC generally continue to receive the

androgen-ablation therapy, one clinical study showed no advantage of continued androgen blockade (4). Our data are consistent with the result of another clinical study showing a superior survival effect of the continuation of androgen-ablation therapy in recurrent PC patients (3) and warrant the maintenance of hormone therapy for recurrent PC.

It has been reported that DHEA itself can activate both the T877A and H874Y mutant ARs (29). However, in our tumor growth study, not DHEA itself but testosterone converted from DHEA is considered to stimulate the growth and PSA secretion of the MDA PCa 2b and MDA PCa 2b-hr tumors, because the addition of DHEA to these cells *in vitro* did not accelerate the growth nor elevate the PSA secretion, but testosterone did (data not shown). The L701H/T877A double mutant AR, which both MDA PCa 2b and MDA PCa 2b-hr cells harbor, might not be activated by DHEA.

The treatment with a 100-mg DHEA pellet but not with a 25-mg DHEA pellet stimulated the MDA PCa 2b tumor growth and elevated the blood PSA levels in mice bearing MDA PCa 2b tumors significantly. Because the serum testosterone level of 0.68 ng/ml by a 25-mg DHEA pellet is comparable with the clinical castration level and that of 12.4 ng/ml by a 100-mg DHEA pellet is comparable with the physiological levels in uncastrated men (23), the response of MDA PCa 2b tumors to androgen closely resembles that of clinical androgen-dependent PC. In contrast to MDA PCa 2b tumors, the treatment with 1.5-, 5-, and 25-mg DHEA pellets accelerated the growth of MDA PCa 2b-hr tumors and elevated the blood PSA levels in mice bearing MDA PCa 2b-hr tumors significantly in a dose-dependent manner. Because the DHEA level of 17.5 ng/ml and testosterone level of 0.22 ng/ml by a 5-mg DHEA pellet are both comparable with the castration levels in men (22, 23), our results clearly show that the growth of MDA PCa 2b-hr tumors was stimulated by the castration level of testosterone converted from DHEA. These findings suggest that AR signaling might be enhanced in MDA PCa 2b-hr tumors as compared with MDA PCa 2b tumors. To our knowledge, this is the first study showing the sharp contrast of adrenal androgen dependence between the growth of androgen-dependent and -refractory PC. Our data strongly support the hypothesis that some clinical PCs come to use adrenal androgens, such as DHEA, whose production cannot be suppressed by castration therapy, for their growth during long-term castration therapy. Usually, we use antiandrogens, such as flutamide and bicalutamide, in combination with castration to block the action of adrenal androgens. However, the treatment with the antiandrogens might lead to the emergence of mutated AR, which would exacerbate PC with the antiandrogens themselves (20, 30). These findings encourage us to develop new forms of endocrine therapy.

In conclusion, we presented in this study one novel model of recurrence of human bone metastasis-derived PC. Because MDA PCa 2b-hr cells were generated after long-term growth suppression under androgen-ablated conditions to mimic the clinical recurrence of human bone metastasis-derived PC treated with androgen-ablation therapy, this cell line should be a useful tool for investigating the mechanisms of androgen-refractory growth and testing drugs against androgen-refractory PC. Our results suggest that: (a) AR signaling might be indispensable not only to androgen-dependent but also androgen-refractory growth of bone metastasis-derived PC; (b) PC might come to use adrenal androgens, which remain in the blood even after medical or surgical castration, for the recurrent growth by becoming hypersensitive to androgen during androgen-ablation therapy; and (c) the continuation of hormone therapy and, beyond that, more complete blockade of AR signaling could be beneficial to patients with recurrent PC.

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