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GROWTH INHIBITION BY DIETHYLSTILBESTROL AND RELAPSE OF THE NOBLE RAT PROSTATIC TUMOR

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The response of androgen-sensitive Noble (Nb) rat prostatic adenocarcinoma [2Pr-121D (1)] to varying doses (50～1,000 μg/kg body weight) of diethylstilbestrol (DES) was investigated and characterized with respect to cytosol and nuclear androgen binding profiles, histology and pattern of relapse. Inhibition of tumor growth was closely related to the dose of DES. Treatment at all but the lowest dose (50 μg/kg) initially caused tumor regression, whereas serum testosterone concentrations in all groups, including that receiving the lowest dose, were decreased to castrate levels. Histologically, while extensive cellluar destruction was clearly evident at higher doses of DES, some active tumor cells appeared to survive. Tumors eventually relapsed when higher doses of DES were stopped or with the continued administration of low doses. The cytosol dihydrotestosterone (DHT) receptor in this tumor line, as determined by sucrose density gradient, dextran charcoal and Sephadex column methods, was negative. Nuclear binding, however, was positive. Salt-extractable and salt-resistant fractions of nuclei derived from the untreated primary tumor and relapsed tumor following DES treatment contained high affinity androgen receptor. Comparison of binding constants revealed no significant differences. Our findings, based on the Nb rat prostatic tumor model, indicate that DES acts not only by eliminating circulating testosterone, but also by a direct cytotoxic effect on malignant cells. The results also suggest the lack of an apparent relationship between the loss of hormone responsiveness associated with recurrence of prostatic tumor growth and nuclear androgen binding parameters.

Key words: Diethylstilbestrol, Noble rat prostatic tumor, Relapse, Nuclear androgen receptor

INTRODUCTION

After an initial suppression of growth following the removal of androgen support, most prostatic cancers relapse[1]. This recurrence of growth has been attributed to the survival and subsequent multiplication of androgen-insensitive cells that resist androgen-ablation measures[2]. Clinically, diethylstilbestrol (DES) is an alternative form of therapy to metastatic prostatic cancer. The rationale for the endocrine treatment is mainly to lower circulating testosterone to castrate levels, thereby removing the stimulus to androgen dependent growth. However, the observation that the response to high-dose estrogen therapy achieved by patients no longer responsive to standard dose estrogen therapy would suggest that an additional cytotoxic effect at the cellular level is involved[3,4].

In the present study, the Noble (Nb) rat model of androgen-sensitive prostatic adenocarcinoma was chosen to investigate the effect of DES on tumor growth and the pattern of relapse subsequent to the discontinuation of high dose of DES. We have also investigated possible changes in nuclear androgen binding characteristics

associated with recurrent tumor growth.

MATERIALS AND METHODS

Animals and tumors—The animals used in this study were male Nb rats, originally obtained from Dr. J. Drago, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, PA. The animals were housed in polycarbonate cages and maintained on Purina rat chow and water. The Nb rat prostatic adenocarcinoma [2Pr-121D (1)], sensitive to androgens, was supplied by Dr. A.E. Bogden, Mason Research Institute, Worcester, MA. The tumor was maintained by serial passages in young (7~8 weeks old) male Nb rats.

Implantation of tumor (2 mm^3 pieces) was performed with a trocar needle into both flanks of the host animals. Treatment with DES was initiated when the average tumor diameter was approximately 10 mm. The rats were randomly distributed into the following groups:

1. Untreated controls
2. 50 μg/kg body weight
3. 100 〃 〃 〃 〃
4. 200 〃 〃 〃 〃
5. 300 〃 〃 〃 〃
6. 500 〃 〃 〃 〃 28 days→100 μg/kg
7. 1000 〃 〃 〃 〃 23 days→ treatment stopped

Each group contained 5 rats, bearing bilateral tumors. DES (Sigma Chemical Co., St. Louis, MO), in sesame oil-10% ethanol, was administered daily by subcutaneous injections. Tumor measurements (length and width) were conducted twice weekly with the use of a Vernier microcaliper.

The animals in Groups 2~5 were treated for 22 days, at which time they were killed, along with the untreated control rats (Group 1). Rats in Group 6 and 7 were treated for 54 and 62 days, respectively. At the time of sacrifice, blood was removed by cardiac puncture and tumor excised and cryopreserved for later biochemical analyses. In addition to the tumors, testes and prostate were removed and their weights recorded. Pieces of each tumor tissue were preserved for histology.

Nuclear preparation and fractionation—Two procedures for the purification of nuclei were tested. In the first, nuclei were prepared in isotonic sucrose, as previously described[5]. In the second procedure, minced tissue was homogenized in 0.5% citric acid, a procedure reported to be particularly suited for the preparation of nuclei from tumor tissue[6]. Following centrifugation at 800×g, crude nuclei obtained by both procedures were subjected to one hour centrifugation through 2.2 M sucrose at 70,000×g. Nuclei prepared by the sucrose method, as observed by light microscopy, were intact but contaminated with cytoplasmic debris. On the other hand, nuclei prepared in citric acid were relatively free of cytoplasmic tags but evidently damaged, as judged by irregularities in size and shape and some leakage of nuclear content.

Nuclei were sub-fractionated essentially according to the procedure outlined by Ekman et al[7]. All steps were carried out at 0°C. Briefly, the crude nuclear preparation was suspended in buffer consisting of 10 mM Tris, 1.5 mM ethylenediaminetetraacetate (EDTA), 1 mM dithiothreotol (DTT) and 10% (wt/vol) glycerol, pH 7.4[8] plus 1 mM freshly added phenylmethylsulfonyl fluoride (PMSF). Following extraction with 1% Triton-X-100 and two washes with the buffer, the nuclear pellet was digested with pancreatic deoxyribonuclease (20 μg/ml in 10 mM Tris, pH 7.4, 5 mM, MgCl, 1 mM DTT and 1 mM PMSF) for 30 min. Following centrifugation, the nuclear pellet was extracted with 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.6 M KCl and 1 mM PMSF. After 30 min., centrifugation at 1,000×g for 15 min., yielded the "salt-extractable" fraction and a residual pellet. The pellet, upon suspension in 10 mM Tris, pH 7.4, 1.5 mM EDTA, 1 mM DTT and 1 mM PMSF, constituted the "salt-resistant" fraction.

Androgen binding analysis—Assays to characterize cytoplasmic androgen receptor were previously described[5], as was the preparation of the cytosol fraction. In the nuclear binding assay, 0.5 ml of nuclear

subfraction was incubated in triplicate tubes with 5 concentrations (3~18 nM) of 1,2,5,6,7-^{3}H(N)-dihydrotestosterone (DHT), specific activity 123 Ci/mmole (New England Nuclear, Boston, MA) in the presence and absence of 200-fold excess unlabeled DHT (Steraloids, Inc., Wilton, NH). After 20 hours at 4°C, the incubation was terminated and the samples assayed by the Dextran charcoal method[7]. To each tube, 0.5 ml suspensions of 0.5% (wt/vol) charcoal and 0.025% dextran in incubation buffer was added. After 20 min., at 4°C, with gentle mixing, the charcoal was centrifuged at 10,000×g for 5 min, and the supernatant transferred to counting vials. Ten ml of scintillation fluid (ACS II, Amersham Corp., Arlington, IL.) was added and the radioactivity measured in a Packard 3,375 spectrometer, with a counting efficiency of approximately 45% for ^{3}H. Total binding and non-specific binding were estimated by saturation analysis. Specific binding was obtained from the difference.

Other methods—Protein content was measured by the method of Lowry et al.[9], serum testosterone concentrations were determined by radioimmunoassay, using a kit obtained from Endo-Tech, Inc., Louisville, KY. For histology, pieces of tissues were fixed in Bouin's solution and stained with hematoxylin and eosin.

RESULTS

Effect of DES on body and tissue weights—

Fig. 1 shows the changes in body and tissue weights produced by DES treatment. The untreated rats showed an average gain in body weight of 17%. At the highest dose (1,000 μg/kg body wt.), there was a 17% loss in body weight. The decrease in tumor size and weight at day 22 were moderate at 50 μg/kg DES (75% and 55% of untreated control value, respectively). Higher doses produced further inhibition, although 200 and 300 μg/kg doses were only slightly more effective, but not significantly different from the 100 μg/kg dose. Tumor dimensions at 100, 200 and 300 μg/kg were 55, 46 and 45% of control,

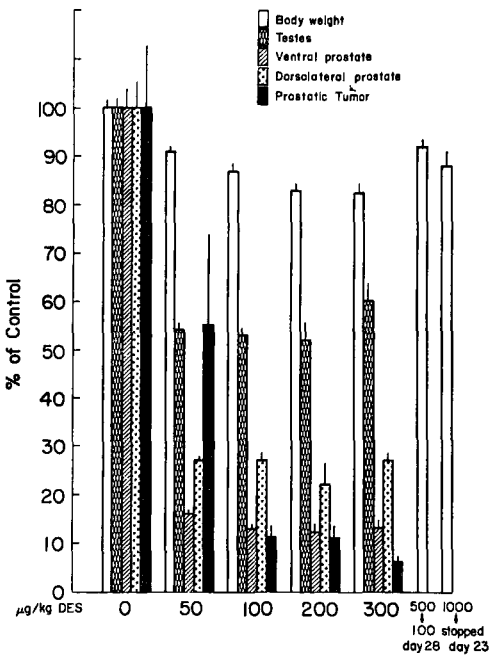

Fig. 1. Effect of DES treatment on final body and tissue weights. Open bar, body weight; cross-hatched bar, testes; dashed bar, ventral prostate; dotted bar, dorsolateral prostate; closed bar, prostatic tumor. N=5 for body weight and all tissue weights, except for tumor weight, N for which was 10.

respectively. Corresponding values in average final tumor weight were 12, 11 and 5% of the control, respectively. The actual values of mean tumor diameter (in mm) at day 22 were as follows: Untreated, 18.5±0.9; 50 μg/kg DES, 13.9±1.6 ($p<.025$); 100 μg/kg, 10.1±0.5 ($p<.001$); 200 μg/kg, 8.6±0.3 ($p<.001$); 300 μg/kg, 8.4±0.8 ($p<.001$).

Growth curves—

All tumors were approximately 1 cm in diameter at the beginning of the experiment. The tumors in the untreated rats (Group 1) increased 85% in mean tumor diameter in 3 weeks (Fig. 2). Based on the value on day 22, all of the DES-treated groups showed inhibition of growth, the degree of which was in the order of increasing dose. With exception of the lowest dose (50 μg/kg, Group 2), tumor regression was observed at all doses. In animals treated with 100, 200 and 300 μg/kg DES

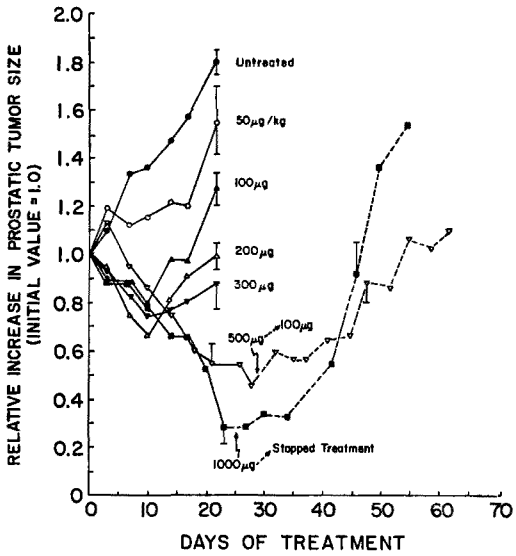

Fig. 2. Growth inhibitory response of prostatic tumor to DES. Average tumor diameter was determined from length and width and the mean initial average tumor diameter within each group was normalized to 1.0. Closed circle, untreated control group; open circle, 50 μg/kg; closed triangle, 100 μg/kg; open triangle, 200 μg/kg; closed inverted triangle, 300 μg/kg; open inverted triangle, 500 μg/kg reduced to 100 μg/kg at day 28; closed square, 1,000 μg/kg for 23 days. Each group consisted of 5 rats, with each animal bearing bilateral tumors. Bars represent standard error.

(groups 3 to 5) the tumor begn to relapse 10 days after the initiation of treatment. When DES at 1,000 μg/kg was stopped, tumors immediately relapsed (Group 7). Similarly, when the 500 μg/kg dose was reduced to 100 μg/kg, relapse occurred (Group 6) although at a slower rate. Some atypical observations in Group 6 included the complete disappearance (undetectable by palpation) of 3 tumors, 2 of which reappeared and grew. The remaining tumor did not recur during the observation period of 120 days.

In rats treated with 500 and 1,000 μg/kg DES, the reduction in tumor size was significantly accelerated, such that at day 22, the values were 30 and 16% of the control, respectively (Fig. 2).

Serum testosterone concentration—

Table 1. Serum testosterone levels in DES-treated Nb rats

Group	DES,ug/kg	ng/100ml
Intact	0	422.7±83.3
Castrated	0	2.4± 0.5
Intact	50	1.7± 0.5
Intact	100	0.9± 0.5
Intact	200	2.9± 1.0
Intact	300	1.4± 0.4

Values are means for samples from 4 rats per group ± s.e.

Table 1 shows the effect of DES treatment on serum concentration of testosterone. Orchiectomy decreased circulating testosterone to less than 1% of control, as expected. A strong anti-gonadal effect of DES, as reflected by the greater than 40% reduction of testes weight by day 22 (Fig. 1) was confirmed in all the treated groups. Even at the lowest dose (50 μg/kg), serum testosterone fell to a value similar to that measured in castrated animals.

Cytosol androgen receptor analysis—

Fig. 3A shows the results of the assay for the presence of an androgen receptor in the cytosol fraction of prostatic and tumor tissues by sucrose density gradient centrifugation. Cytosol prepared from ventral prostates of 1-day castrated Nb rats contained an 8S DHT binding peak. By contrast, no such peak was detected when the cytosol preparation from the androgen-sensitive prostatic tumor was incubated with radiolabeled DHT. In view of this negative finding, two other conventional methods of discriminate specific, high affinity receptor binding sites were utilized. Fig. 3B shows the results of quantitation by the dextran-coated charcoal method. In these experiments, molybdate was added to ensure stabilization of receptor protein and PMSF was added to minimize proteolytic degradation. Compared to the ventral prostate cytosol, which served as a control for the presence of specific DHT receptor, the binding activity present in the tumor cytosol was not displaced by excess non-radioactive DHT ligand. The third methods of analysis, by gel filtration through a Sephadex G-25 column (not shown), confirmed the results obtained

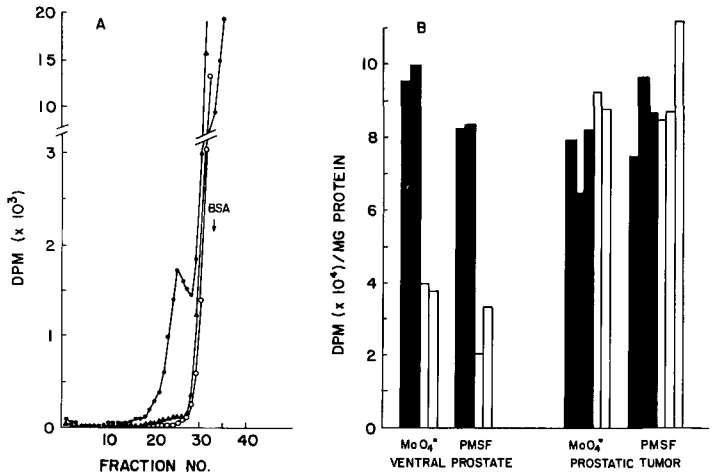

Fig. 3. Cytosol androgen receptor determinations. DHT binding was measured by sucrose density gradient (A) and dextran-charcoal method (B). Closed circle, prostate cytosol incubated with ^{3}H-DHT; open circle, prostate cytosol incubated with ^{3}H-DHT plus excess unlabeled DHT; closed triangle, prostatic tumor cytosol incubated with ^{3}H-DHT; BSA, bovine serum albumin. Closed bar, ^{3}H-DHT; open bar, ^{3}H-DHT plus excess unlabeled DHT; $MoO_4^=$, molybdate; PMSF, phenylmethylsulfonyl fluoride.

with the charcoal method. Thus, the high affinity cytosol receptor, specific for DHT, was not detectable in the Nb rat prostatic adenocarcinoma used in this study.

Nuclear androgen receptor analysis—

Preliminary nuclear binding assay was carried out on high salt-extractable and -resistant fractions on nuclei prepared by isotonic sucrose or citric acid method, each followed by centrifugation through 2.2 M sucrose. Nuclei prepared with isotonic sucrose were intact, but attached with cytoplasmic tags, whereas those prepared with citric acid were relatively free of cytoplasmic contamination, but showed some damage and extrusion of nuclear material (not shown). Fig. 4 shows that high affinity DHT binding sites could be demonstrated in subfractions of nuclei prepared by both methods. An exception was the salt-resistant fraction of nuclear obtained in citric acid. Essentially similar results were obtained when the subnuclear fractions were extracted for crude preparations of nuclei and examined for DHT binding.

The apparent absence of specific androgen receptor in the cytosol fraction, at least in our androgen-sensitive Nb rat prostatic tumor line and under our conditions of assay (Fig. 3), precluded the

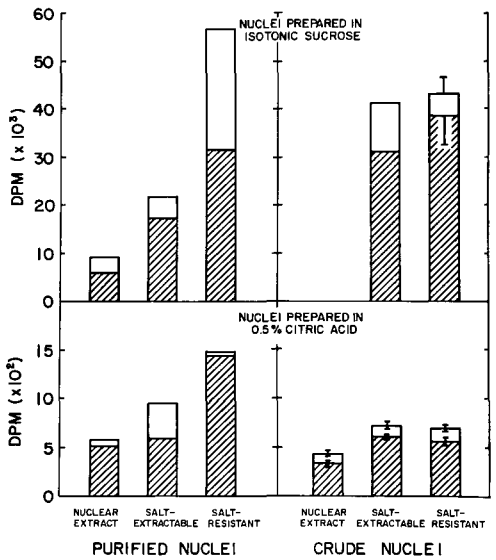

Fig. 4. Measurement of ^{3}H-DHT binding in unclear fractions of prostatic tumor. Nuclei were prepared in isotonic sucrose (top) or citric acid (botton). Dashed portion of bars represents non-specific binding; open portion of bars indicates specific binding.

Table 2. Androgen binding characteristics in nuclear sub-fractions of untreated and DES-treated recurrent Nb rat prostatic tumor

Nuclear sub-fraction	Exp. No.	Primary Tumor		Relapsed Tumor	
		K_d (nM)	B_{max} (pM)	K_d (nM)	B_{max} (pM)
Salt-extractable	1	7.2	80.0	4.3	18.0
(0.6 M KCl-soluble)	2.	1.1	16.8	8.7	248.4
	3.	3.4	26.8	4.2	53.2
Salt-resistant	1	–	–	7.4	44.4
(matrix-bound)	2.	6.7	83.0	15.9	137.0
	3.	0.7	36.6	10.4	33.2

Results from 3 separate determinations are reported for each of untreated (primary) tumor and relapsed tumor following DES treatment.

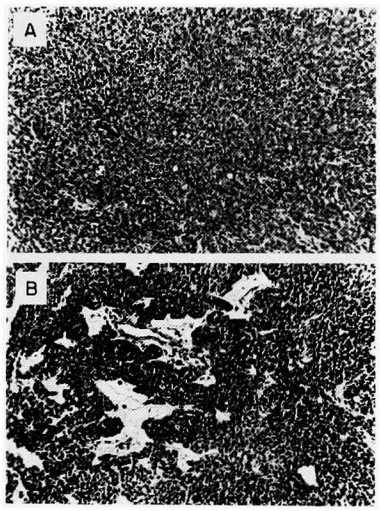

Fig. 5. Representative histology of prostatic tumor in untreated Nb rat. Sections characteristic of control tumor included (A) solid sheet of malignant cells and (B) other areas with glandular elements. Hematoxylin and Eosin, ×240.

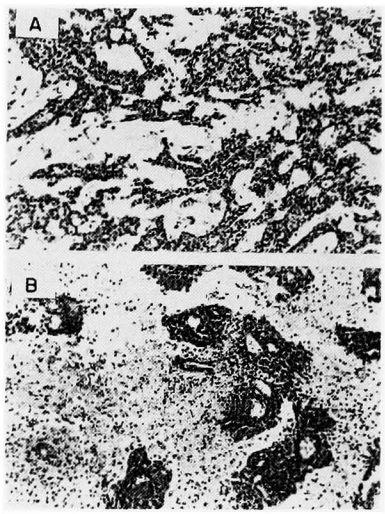

Fig. 6. Representative histology of prostatic tumor in DES-treated rat. Sections were of regressed tissue following 500 μg/kg DES for 22 days. A, reduction in cellular mass with relative increase in edematous stroma; B, degenerative changes showing cell debris and pyknotic nuclei. Hematoxylin and Eosin, ×240.

need for highly purified nuclei. All subsequent nuclear binding assays were performed on sub-fractions on relatively crude preparations of nuclei. The validity of the assay method was confirmed by saturation analysis. High affinity, low capacity binding for DHT ligand was demonstrated in both high salt-extractable and salt-resistant fractions.

Table 2 shows the binding constants derived from Scatchard plots. Even though the mean K_d and B_{max} values tended to be slightly higher for tumor tissues that had undergone relapse, the individual values among groups were highly variable. Consequently, no significance could be attached to differences between untreated tumors and tumors of recurrent gowth in either the high salt-extractable or -resistant nuclear fraction.

Histology—

Figs. 5 and 6 show the histological patterns of Nb rat prostatic adenocarcinoma. The androgen-sensitive tumor from an untreated rat (Fig. 5) was composed of a mixture of dense mass of poorly differentiated cells with scant stroma (5A) and areas of small glandular and pseudoglan-

dular formations (5B). At 50 μg/kg of DES, a dose which lowered the serum testosterone to castrate levels but did not suppress tumor growth, the histological appearance of the tumor was virtually unchanged (not shown). At 500 μg/kg, DES treatment for 22 days caused marked changes in the regressed tumor (Fig. 6). In some regions, the histology was characterized by small and diffuse groups of cells, widely separated by extensive areas of fibrous and mucoidal stroma (6A). In other areas, extensive regressive changes were apparent (6B). Remnants of cellular fragments and pyknotic nuclei were clearly visible. It was also evident that not all malignant cells were destroyed in this regressed tissue. Irregular nests of surviving cells remained. In the relapsed tumor, the histological appearance reverted to that of the untreated tumor, with the cells arranged in tightly packed cords separated by thin intervening stroma (not shown.)

DISCUSSION

The beneficial effect of DES in the treatment of cancer of the prostate is well known[3,4,10~12]. While it probably has several modes of action, DES acts primarily by suppressing the release of pituitary LH, thereby interfering with the gonadal production of testosterone[13~15]. Among other effect of DES, a direct cytostatic action at the cellular level cannot be ruled out. In the present study, even the lowest (50 μg/kg) dose of DES was sufficient to maximally lower serum testosterone to castrate levels. Since lower doses of DES failed to arrest tumor progression, it is apparent that DES, at high doses, caused tumor regression by a mechanism other than by simply abolishing circulating testosterone.

The Nb rat prostatic tumor line was partially dependent on androgen, as indicated by its sub-optimal growth in the female and castrated male rats[16]. In the present study the reduction in the overall tumor mass with DES treatment would suggest that androgen-insensitive cells as well as androgen-sensitive cells responded. This observation provides further evidence that the fall in serum testosterone levels via the anti-gonadal effect cannot solely account for the growth inhibitory action of DES.

While our data indicate that the efficacy of DES cannot be due exclusively to an indirect effect via the pituitary-gonadal axis, a direct cytotoxic effect was clearly apparent in some histological sections. Moreover, the histology of regressed tissues showed some malignant cells resisted DES treatment. These remaining active cells may well represent clones from which malignancy is renewed and thereby account for the eventual recurrence of prostatic tumor growth[2,10].

The high variability in the response of prostatic cancer to anti-androgen therapy and the eventual progression to a state of hormone-insensitivity have been attributed to tumor cell heterogeneity[2]. Beginning with a mixture of pre-existing androgen-dependent and -independent cells, tumor regression during DES treatment thus reflects the death of androgen dependent cells. The androgen-independent malignant cells that survive the testosterone-depleted environment and/or cytotoxic action of DES and comprise the recurrent tumor might be expected to possess altered growth regulatory characteristics related to androgen sensitivity.

Growth regulation by steroid hormones involves an interaction with cytoplasmic receptors. The translocation of steroid-receptor complex to nuclear sites is prerequisite for the ultimate gene expression in the target cell[17,18]. In animal models of androgen-dependent carcinoma, comparative studies of dependent and independent tumor lines revealed no correlation between tumor growth and cytosol androgen receptor[19,20]. On the other hand, a relationship between nuclear salt-resistant receptor content and responsiveness to androgens was demonstrated[20]. The clinical response of prostatic adenocarcinoma to endocrine therapy was also found to correlate with both salt-extractable and matrix-bound nuclear androgen binding[21].

Despite these previous findings, our attempts to demonstrate significant diffe-

rences in the nuclear androgen binding parameters between primary (androgen-dependent) and relapsed (androgen-independent) tumors in the Nb rat model were unsuccessful. The lack of absolute androgen dependency in this prostatic tumor, in contrast to the Dunning R3327H tumors[2] minimized any differences that may exist between the two growth phases. It is also possible that the secondary growth in these experiments does not represent a true relapse phenomenon, such as that observed in a clinical situation. Since a natural progression to a state of relapse would outlast the life span of the animal, DES treatment in this experimental system was either discontinued or reduced in dosage to allow the recurrence of the tumor. Under these condition, the probability that the relapsed tumor still contained a heterogenous population of cells similar to the DES-untreated tumor cannot be ruled out.

The biochemical changes associated with the progression of initially androgen-sensitive prostatic cancer cells to an androgen-insentivite state remain unclear. An understanding of the mechanism for this relapse following androgen ablation measures would be of great value in the design of clinical treatment and eradication of prostatic cancer.

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和文抄録

Noble ラット前立腺癌に対するジエチルスチルベステロールの影響

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アンドロゲン反応性 Noble ラット前立腺癌 (2Pr-121D(1)) を用いて，これに種々の量 (50～1,000 μg/kg) のジエチルスチルベステロール (DES) を投与し，その影響をみた．血清テストステロン値は，すべての実験群において去勢レベルに下降していたが，腫瘍は DES の量が多いほど強く抑制された．腫瘍は DES 投与中に再燃を開始し，また DES を中止すると直ちに再燃を起こした．DES 投与にて抑制された腫瘍の組織像は，癌細胞のほとんどが死滅していたが，なお一部に active な腫瘍細胞が残存していた．無処置の腫瘍のサイトゾールの dihydrotestosterone (DHT) レセプターは蔗糖密度勾配法，dextran-coated charcoal 吸着法，ゲル沪過クロマトグラフィー法にて測定したが，いずれの方法でもサイトゾール DHT レセプターは存在しなかった．しかし核の DHT レセプターを測定すると DHT に対し高親和性結合がみられた．無処置の腫瘍と再燃した腫瘍の核 DHT レセプターでは，salt-extractable fraction と salt-resistant fraction の Kd. Bmax はいずれにおいても差はなかった．

以上のことから，この Noble ラット前立腺癌では，DES は血清テストステロンを去勢レベルまで低下させるのみならず，前立腺癌細胞に対して直接の殺細胞作用のあることが示唆された．DES 投与中の再燃癌の核アンドロゲンレセプターを測定したが，ホルモン依存性消失との関係はみいだせなかった．