Changes in Cytosolic Androgen Receptor after Administration of Testosterone of Androgen-Dependent Mouse Mammary Tumor (Shionogi Carcinoma) and Its Sublines of Altered Androgen Dependency

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Abstract

The effect of testosterone treatment on the cytosolic androgen receptor in an androgen-dependent tumor (Shionogi Carcinoma 115, SC 115) and its sublines of altered hormone-dependency (CS 1, less androgen-dependent, and CS 2, androgen-independent) was examined. In all tumors examined a single, high-affinity binding to ^3H -R 1881 was detected in cytosols. The size of the maximum binding sites was in the order SC 115, CS 1 and CS 2. Inhibition experiments revealed that the binding to ^3H -R 1881 in cytosols of these tumors was attributable to the androgen receptor. The amount of the androgen receptor in cytosols was decreased at 1 hr after testosterone injection, then replenished gradually towards the pretreatment level. Depletion of the androgen receptor was dependent on the dose of testosterone administered. Multiple injections of testosterone depressed the replenishment of the receptor. Treatment with actinomycin D or cycloheximide did not alter the dynamics of the androgen receptor. Therefore, it was concluded that the depletion and replenishment of the androgen receptor in cytosols of the tumors were caused by receptor recycling. Because a similar pattern of depletion and replenishment of the androgen receptor was also observed in the less androgen-dependent and -independent tumors, the growth of tumors was felt to be unrelated to the receptor dynamics.

It has been well discussed that receptors for androgen, estrogen or progestin in cytosols of target tissues enter nuclei after forming steroid-receptor complexes (O'Malley *et al.*, 1976). A part of the receptors which have been transferred into nuclei return to cytoplasm, so that depletion and replenishment of receptor in cytosols of the target tissue seem to be the first step in the steroid hormone action (Luu Thi *et al.*, 1975, Mešter and Baulieu, 1975, Isomaa *et al.*, 1979). Recently it was observed that

some of the receptors which enter nuclei remained as a nuclear-bound form for a rather long time, but the physiological meaning of the long retention of the receptors in nuclei is obscure (Kassis and Gorski, 1981).

A transplantable mouse mammary carcinoma (Shionogi Carcinoma 115: SC 115) was established by Minesita and Yamaguchi (1964), and this tumor is known to be androgen-dependent, since the SC 115 grows only in male mice, and not in female or castrated male mice. The androgen receptor which binds with testosterone and

dihydrotestosterone (Yamaguchi *et al.*, 1978) has been observed in this tumor. Incorporation of testosterone in nuclei of the tumor was also found, so that the effect of androgen on the growth of SC 115 seems to be mediated by the androgen receptors (Matsumoto *et al.*, 1972, Yamaguchi *et al.*, 1974). The present study was undertaken to examine changes in the cytoplasmic androgen receptors from the SC 115 after the administration of testosterone in an attempt to clarify the role of androgen receptors in the regulation of growth of androgen-dependent tumor by comparing them with those from less androgen-dependent and -independent sublines of the SC 115.

Materials and Methods

Animals

Adult male mice of DD/S strain weighing approximately 25 g at the time of tumor transplantation were used throughout the experiments. This strain was originally supplied by the Shionogi Research Laboratory and has been maintained by inbreeding in our laboratory. Castration was performed via the scrotal route when tumors grew to approximately 2 cm in diameter. To estimate the androgen binding in tumors of testosterone-uninjected mice (uninjected control), tumors were removed 48 hr after castration. Except where otherwise indicated, a single injection of testosterone was given subcutaneously with a dose of 100 μ g/animal dissolved in 0.2 ml of 30% ethanol-saline at 48 hr after castration, and the mice were sacrificed at appropriate intervals. When multiple testosterone injections were given, each 100 μ g of testosterone was injected at the indicated time. Actinomycin D (30 μ g/100 g body weight) or cycloheximide (2.5 mg/100 g body weight) was injected intraperitoneally at the same time of testosterone injection.

Tumors

The SC 115 was supplied by the Shionogi Research Laboratory and has been maintained in our laboratory. Chiba Subline 1 (CS 1) and 2 (CS 2) were derived from the original SC 115 during transplantation in 1976 (CS 1) and 1981 (CS 2). The CS 1 grows in male mice faster than in female mice, indicating that the androgen dependency of CS 1 is less prominent (Wakisaka *et al.*, 1980). The CS 2 grows equally in both male and female

mice, therefore, is referred to as androgen independent. The histological features of these three tumors are similar to each other and they are undifferentiated medullary adenocarcinomas. The SC 115 and CS 1 have been maintained in male host mice, and the CS 2 in female recipients. Tumors used in the present experiments were 295th-302th generations in the SC 115, 60th-70th in the CS 1 and 34th-40th in the CS 2.

For transplantation of tumors pieces of tumor tissues, approximately 5 mg in wet weight (approximately 10^7 cells) was implanted subcutaneously with a trocar in the dorsomedian region of the necks of recipient mice. Tumors became palpable on approximately the 8th day after transplantation, then exponential growth followed.

Tissue preparation

Animals were sacrificed by cervical dislocation, removed tumors were placed on ice, and necrotic tissues were separated. All procedures were performed at 0–4°C. The tumor tissues were minced with scissors, homogenized in 5 volumes of 0.01 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM β -mercaptoethanol and 10% (w/v) glycerol (TEMG buffer) using a glass homogenizer and filtered through nylon cloth. Cytosols were obtained after centrifuging the homogenate at $105,000 \times g$ for 60 min. Cytosols thus obtained were used for saturation analyses. When cytosols from mice injected with testosterone were assayed, one fourth volume of 0.25% dextran-coated 2.5% charcoal in TEMG buffer was poured into cytosols, mixed, centrifuged briefly, and the resulting supernatants were used.

Saturation analysis of the binding

Samples to be analyzed were incubated in 0.5 ml of TEMG buffer containing various concentrations (0.25–5 nM) of ^3H -R 1881 ([17 α -methyl- ^3H] 17 β -hydroxy-17 α -methyl-estra-4, 9, 11-trien-3-one, specific activity 87 Ci/mmol, New England Nuclear, Boston, USA) at 4°C for 16 hr. The ^3H -ligand was dissolved in 0.01 ml of ethanol and added to the incubation tube. After incubation, 0.05% dextran-coated 0.5% charcoal was added, centrifuged, and radioactivity in an aliquot of the supernatant was counted with 15 ml of toluene containing 0.4% PPO and 0.01% POPOP in a liquid spectrophotometer (LKB 1215 Rackbeta, Wallac OY, Finland). In parallel incubations, 1000-fold molar excess of unlabeled R 1881 was added to the incubation tube and nonspecific binding which was not displaced under these conditions was subtracted in all instances from the total binding to calculate specific binding. Binding capacity and affinity were analyzed by the method of Scatchard (1949).

When the effect of additive steroids on the R 1881 binding was examined, the additives were dissolved in another 0.01 ml of ethanol and added to the incubation mixture directly.

Fig. 1. Scatchard plot of binding to ^3H -R 1881 of cytosols from SC 115, CS 1 and CS 2. Cytosols of SC 115 (closed circle), CS 1 (open circle) and CS 2 (open triangle) were incubated with various concentrations of ^3H -R 1881 at 4°C for 16 hr. Dissociation constant (K_d) and maximum binding sites from the Fig. are 0.34 , 0.40 and $0.54 \times 10^{-9}\text{M}$ and 17 , 11 and 10 fmol/mg protein in SC 115, CS 1 and CS 2, respectively.

Protein determination

Protein was measured by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as the calibration standard.

Results

Binding to ^3H -R 1881 of cytosols from SC 115, CS 1 and CS 2

A single high-affinity binding to ^3H -R 1881 was observed in cytosols from the SC 115, CS 1 and CS 2 (Fig. 1). The dissociation constants (K_d) were almost identical in all three tumors (Table 1). Maximum binding sites were larger in the order SC 115, CS 1, and CS 2.

Table 1. Binding of cytosols from SC 115, CS 1 and CS 2 to ^3H -R 1881

	No. of assays ^a	K_d $\times 10^{-9}\text{M}$	Maximum binding sites fmol/mg protein
SC 115	23	0.36 ± 0.06	17 ± 2
CS 1	10	0.43 ± 0.11	11 ± 2
CS 2	9	0.49 ± 0.15	$9 \pm 1^{\text{b}}$

Data are shown as Mean $\pm$ S.E.

a: Three or four mice were used in one determination.

b: Statistically significant differences from that of the SC 115 ($p < 0.05$).

Fig. 2. Inhibition of ^3H -R 1881 binding in cytosol from SC 115. Cytosol (5.6 mg as protein/tube) was incubated with 1 nM of ^3H -R 1881 in the presence of various concentrations (10–1000 nM) of radioinert steroids (closed circle; R 1881, closed square; dihydrotestosterone, open circle; estradiol- 17β , open square; R 5020) at 4°C for 16 hr. The binding is expressed as a percentage of the binding incubated in the absence of competitors (11 fmol/mg protein).

The cytosol from SC 115 was incubated with ^3H -R 1881 in the presence or absence of an excess amount of either dihydrotestosterone, R 1881, R 5020 or estradiol-17 β (Fig. 2). Dihydrotestosterone showed a potent inhibition which was similar to that by R 1881, indicating that binding of ^3H -R 1881 was of the androgen receptor. Inhibition by estrogen and progestin was rather weak.

Time course changes in the binding of ^3H -R 1881 in cytosols from SC 115, CS 1 and CS 2 after testosterone administration

To determine the rate of exchange of androgen-bound receptor with ^3H -R 1881, the cytosol was preincubated with 10 nM of radioinert testosterone or dihydrotestosterone at 4°C for 6 hr. After treatment with dextran-coated charcoal, the supernatant was again incubated with ^3H -R 1881. Maximum binding sites were calculated and compared with that of the cytosol which was not preincubated. ^3H -R 1881 bindings were approximately 73% and 48% of those of cytosol without preincubation in the cytosols preincubated with testosterone and dihy-

drotestosterone, respectively. Thus, appreciable amounts of the binding of cytosol to testosterone and dihydrotestosterone were exchangeable in the incubation conditions used in the present study.

Tumor-bearing castrated mice which received testosterone were sacrificed to observe time course changes in the binding of ^3H -R 1881 in cytosols (Fig. 3). One hour after testosterone administration, the binding of ^3H -R 1881 was significantly diminished when compared with that of the respective uninjected control, and rates of reduction were 70%, 68% and 60% in the SC 115, CS 1 and CS 2, respectively. Thereafter, the binding of ^3H -R 1881 was increased gradually, and six hours after testosterone injection the binding from the SC 115 and CS 2 reached two thirds of that of the uninjected controls, while at this time, the binding of ^3H -R 1881 in the CS 1 passed the uninjected control level.

Scatchard plots of the binding of ^3H -R 1881 in cytosols of the SC 115 from the testosterone-injected and -uninjected mice are shown in Fig. 4. Although the maximum binding sites in cytosols from animals

Fig. 3. Time course changes in ^3H -R 1881 binding of cytosols from SC 115, CS 1 and CS 2 after testosterone administration. Cytosols prepared from tumors of testosterone-uninjected mice (ordinate) or from tumors of castrated mice sacrificed at indicated time after testosterone injection were treated with dextran-coated charcoal, then incubated with ^3H -R 1881. SC 115 (closed circle), CS 1 (open circle), CS 2 (open triangle). Maximum binding sites are expressed as Mean $\pm$ S.E. (shown as bar) in experiments performed independently using 3-4 mice for one point (n; number of experiments). Statistically significant differences from values of the respective uninjected control are: SC 115 and CS 1 ($p < 0.02$), CS 2 ($p < 0.01$) at one hr after testosterone injection, SC 115 and CS 1 ($p < 0.05$) at 3 hr after testosterone injection.

Fig. 4. Scatchard plot of binding to ^3H -R 1881 of cytosols from the uninjected control and the tumors removed from testosterone-injected mice. Open circle (the uninjected control), open square (one hr after testosterone injection), open triangle (six hr after testosterone injection). K_d 's and maximum binding sites from the Fig. are 0.41, 0.56 and $0.40 \times 10^{-9}\text{M}$ and 17, 5 and 14 fmol/mg protein in the uninjected control, one hr and 6 hr treated tumors, respectively.

Fig. 5. Relationship between dose of testosterone administered and depletion of ^3H -R 1881-binding in cytosols of SC 115. Various amounts of testosterone were injected and tumor-bearing mice were sacrificed at the indicated time. Results of one of the typical experiments are shown. Dose of testosterone/mouse ($10\text{ }\mu\text{g}$; closed circle, $50\text{ }\mu\text{g}$; closed square, $100\text{ }\mu\text{g}$; open circle, $200\text{ }\mu\text{g}$; open square).

which received testosterone injection one hour before sacrifice were small, K_d was almost identical to that of the uninjected control. The replenished receptor observed in tissues of animals which received the injection 6 hr before also exhibited a similar K_d . K_d 's of cytosols in the CS 1 and CS 2 were not influenced by the testosterone injection (data not shown).

The time course changes in the binding of ^3H -R 1881 was studied after the injection of various amounts of testosterone to SC 115-bearing castrated mice (Fig. 5). At one hour after testosterone injection, maximum binding sites in cytosols of the tumors were reduced to 43%, 50%, 79% and 86% of those of the uninjected control in 10, 50, 100 and 200 μg injected animals, respectively. Moreover, injection with larger amounts of testosterone sustained low binding for a longer time period. A similar dose-related depletion of the binding was also observed in the CS 1 and CS 2 (data not shown).

Multiple injections of testosterone to castrated animals bearing the SC 115, CS 1 and CS 2 caused a persistent depletion of the binding of ^3H -R 1881 throughout the experimental periods (Fig. 6).

Time course changes in the binding of ^3H -R 1881 were also determined in the SC 115-bearing castrated mice which received

Fig. 6. Effect of multiple injections of testosterone on binding of ^3H -R 1881 to cytosols of SC 115, CS 1 and CS 2. Tumor-bearing mice each received serial injections of $100\text{ }\mu\text{g}$ of testosterone and binding of ^3H -R 1881 in cytosols was determined. Results of a typical experiment are depicted. SC 115 (closed circle), CS 1 (open circle), CS 2 (open triangle). Arrows indicate testosterone injection.

Fig. 7. Effect of actinomycin D and cycloheximide on testosterone-induced depletion of ^3H -R 1881 binding in cytosol of SC 115. Actinomycin D (closed circle, $30\text{ }\mu\text{g}/100\text{ g}$ body weight) or cycloheximide (open circle, $2.5\text{ mg}/100\text{ g}$ body weight) were injected intraperitoneally at the same time of testosterone injection. Values on the ordinate are those obtained from the uninjected control. Results of one of typical experiments are shown.

testosterone and actinomycin D concomitantly (Fig. 7). It was observed that depletion and replenishment of the binding to ^3H -R 1881 in the cytosol were not influenced by simultaneous injection of actinomycin D. Treatment of testosterone-injected tumor-bearing mice with cycloheximide did not appear to have any effect on depletion and replenishment of the binding. These results suggest that depletion and replenishment of the binding after testosterone injection did not require new RNA and protein syntheses.

Discussion

The presence of the androgen receptor has been proved in the cytosol of SC 115 by many investigators using dihydrotestosterone or R 1881 as the ligand (Jung-Testas *et al.*, 1976, King *et al.*, 1976, Kitamura *et al.*, 1978, Nohno, 1981). Binding to androgen of the androgen receptor and subsequent translocation of the hormone-receptor complex to nuclei are correlated to the growth response of the SC 115 (Sica *et*

al., 1981). In the present study, the presence of the androgen receptor was shown in the SC 115 and also in its sublines of altered androgen dependency. Binding properties of the androgen receptor in the latter tumors were not different from those of the original SC 115 but the number of binding sites in the independent subline was significantly reduced. However, it has been reported by others that some androgen-independent sublines of the SC 115 contained androgen receptor, while in other androgen-independent sublines only trace amounts of binding component to androgen were detected (Bruchovsky *et al.*, 1975, Bruchovsky and Rennie, 1978). In this context it has been generally accepted that the presence of the cytosol steroid receptor is not a direct indication of hormone responsiveness of the tumors (Nomura *et al.*, 1977).

In the present experiments, ^3H -R 1881 was used as the ligand for determination of the androgen receptor in the cytosol of tumors. Although R 1881 binds to the androgen receptor as well as the progestin-binding component (Asselin *et al.*, 1976, Zava *et al.*, 1979), this ligand was widely used in the determination of the androgen receptor in prostates of rodents, since there were no detectable components binding to progestins in the prostates of rodents (Bonne and Raynaud, 1976, Pousette *et al.*, 1979). From the results of the inhibition experiments in the present study, the binding of R 1881 in the SC 115 also appeared to have the nature of androgen receptor. It was reported that the addition of molybdate stabilizes the binding to R 1881 in cytosol of rodent prostate (Gaubert *et al.*, 1980, Noma *et al.*, 1980). In the present study, however, the binding to R 1881 was observed without molybdate, since the preliminary experiments did not show any difference between the presence and absence of this ion in the incubation medium. Exchange of occupied binding sites with ^3H -R 1881 was not complete, and higher rates of

exchange were observed in receptors occupied with testosterone than with dihydrotestosterone. In the tissues of the SC 115, the activity of 5α -reductase is low (Bruchovsky, 1972, Bruchovsky and Meakin, 1973), and the major androgen in the tissue after the injection of testosterone is unmetabolized testosterone (Matsumoto *et al.*, 1972), so that androgen which occupies the receptor may be mainly testosterone under the experimental conditions used here. For these reasons, most of binding sites for androgens in the cytosol after testosterone injection could be measured by this method.

Depletion was equally observed in cytosols of three tumors of different androgen dependency (Fig. 3). Some autonomous sublines of the SC 115 showed nuclear androgen uptake which was similar to that observed in the androgen-dependent tumor (Bruchovsky and Rennie, 1978). However these authors noticed no specific binding in nuclear extracts of these autonomous sublines even though there was considerable nuclear androgen uptake. In the present experiments, an androgen-independent subline, CS 2, showed a depletion and replenishment of cytosolic receptor after testosterone administration, and this implied that the nuclear translocation of androgen-receptor complex took place in this tissue. Therefore, unresponsiveness to androgen in the CS 2 might be accounted for by reasons other than a defect in the nuclear translocation process. Previously, we observed that the effect of testosterone administration on an increase in RNA polymerase I was more meagre in the CS 1 than that in the SC 115 (Suzuki *et al.*, 1983).

Lefebvre *et al.* (1982) reported that the rate of nuclear uptake of dihydrotestosterone after incubation of purified nuclei with dihydrotestosterone was high in the SC 115 and one autonomous subline, but it was low in the other. This seems to indicate that androgen binding of nuclei which is not mediated by the cytosolic androgen re-

ceptor is not correlated to the hormone dependency of tumors.

The Kd of the replenished binding component in the liver after injection of glucocorticoid to rats was significantly greater than that of intact animals (Ichii, 1981). However, no significant difference in Kd's of androgen receptor before or after administration of testosterone was detected in the present study. Depletion of the R 1881-binding was observed to be dependent on the dose of testosterone administered, and similar dose-related depletion of the androgen receptor in cytosols of the prostate of rats has already been reported (Ichii, 1980). Moreover, the injection of long-acting androgen kept the cytosolic androgen receptor of rat ventral prostate in a depleted state for a long period (Ichii, 1980). Multiple injections of testosterone also delayed the replenishment of the androgen receptor. Recycling of receptors seems to be the main cause of depletion and replenishment of the androgen receptor in the tumors, since actinomycin D or cycloheximide administered simultaneously with testosterone did not influence the replenishment of the receptor in the tumors. On the other hand, there have been reports indicating the requirement of new protein synthesis in the replenishment of receptors in cytosols (Jungblut *et al.*, 1976, 1979, Korach and Ford, 1978).

Acknowledgements

The authors wish to thank Dr. K. Yamaguchi of the Shionogi Research Laboratory for donating the mouse mammary tumor (SC 115). This work was supported in part by Grants-in-Aid for Scientific Research and Cancer Research from the Ministry of Education, Science and Culture, and for Cancer Research from the Ministry of Health and Welfare, Japan.

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