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In Vitro Metabolism of Progesterone in the Mammary Tumor and the Normal Mammary Gland of GRS/A Strain of Mice and Dependency of Some Steroid-Metabolizing Enzyme Activities upon Ovarian Function*

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Abstract —[^{14}C] *Progesterone was incubated* in vitro *with cell-free homogenates of GRS/A mouse mammary tumor and normal mammary gland in the presence of NADPH. In the mammary tumor,* 20α-*hydroxy*-4-*pregnen*-3-*one,* 5α-*pregnane*-3,20-*dione,* 20α-*hydroxy*-5α-*pregnan*-3-*one,* 3α-*hydroxy*-5α-*pregnan*-20-*one,* 5α-*pregnane*-3α,20α-*diol and* 4-*pregnene*-3α,20α-*diol were identified as the metabolites. In the normal mammary gland,* 20α-*hydroxy*-4-*pregnen*-3-*one and* 4-*pregnene*-3α,20α-*diol were identified as the major metabolites, besides the* 5α-*reduced metabolites as minor ones. After establishment of the metabolic pathways of progesterone,* 5α-*reductase, and* 3α- *and* 20α-*hydroxysteroid dehydrogenases were suggested to be involved in the progesterone metabolism in these tissues. Activity of the* 5α-*reductase in the mammary tumor was higher than that in the normal mammary gland, but ovariectomy resulted in the reduction of this enzyme activity in the mammary tumor to the level of the normal mammary gland. By estradiol-*17β-*benzoate administration to ovariectomized tumor-bearing animals,* 5α-*reductase activity was restored to the level of the mammary tumor of intact animals.*

INTRODUCTION

THE OCCURRENCE and growth of certain types of mouse mammary tumors is controlled by endocrine factors [1]. The mammary tumor of GRS/A strain mouse, induced by force-breeding without subsequent nursing of pups, is regarded as pregnancy-dependent; the tumor grows during pregnancy but regresses after parturition [2]. The hormonal control of this type of tumor was extensively studied by *in vivo* transplantation of the tumor cell into endocrine gland-deprived and hormone-supplemented animals. Van Nie and Dux [3] showed that the growth of the tumor was dependent on ovarian hormones. Sluyser and Van Nie [4] reported that this tumor regressed after hypophysectomy but was reactivated by the administration of ovarian hormones. Yanai and Nagasawa [5] showed that *in vivo* incorporation of thymidine into the mammary tumor was stimulated by progesterone but not by pituitary hormones.

Progesterone is known to be transformed into variety of metabolites in target organs. such as uterus [6], vagina [7], hypothalamus [8], pituitary [9] and mammary gland [10]. We found that progesterone was metabolized to 5α-reduced and 20α-hydroxysteroids in the dimethylbenzanthracene (DMBA)-induced mammary tumor of rat, while the normal mammary tissue converted progesterone characteristically to 4-pregnene-3α,20α-diol [11]. The purpose of the present study is to examine the metabolism of progesterone in the GRS/A mouse mammary tumor, in comparison with that in the normal mammary gland of the same strain of mouse, and to study the

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dependency of the activities of the metabolizing enzymes upon the ovarian hormones.

MATERIALS AND METHODS

Animals and tissue preparations

Female mice of the inbred GRS/A strain, obtained from National Cancer Center Research Institute, Tokyo, were housed with males at 60–70 days of age and were subjected to force-breeding by removing pups on the day of parturition. Animals with palpable solid tumors were killed by cervical dislocation on the 18th day of the 3rd and 4th pregnancies, and the tumor was extirpated immediately after sacrifice. Macroscopically normal parts of the glands were obtained from the tumor-bearing animals and employed as normal tissue in this experiment. The isolated tissues were washed with an ice-cold 0.25 M sucrose solution, weighed, finely chopped with scissors and homogenized in 0.25 M sucrose solution (pH 7.4, 5 ml/g wet tissue). The homogenates were centrifuged at 800 ***g*** for 20 min and the supernatant fluid was employed as the cell-free homogenates.

Reagents and chemicals

All reagents were of analytical grade, and organic solvents were redistilled before use. NADH and NADPH were purchased from Boehringer (Mannheim, Germany), and unlabeled steroids were obtained from Steraloids (Wilton, NH) and Ikapharm (Ramat-Gan, Israel). [4-^{14}C] Progesterone (58.5 mCi/mmole) was obtained from Radiochemical Centre (Amersham, England), and [1,2-^{3}H(N)]20α-hydroxy-4-pregnen-3-one (55.7 Ci/mmole) was purchased from New England Nuclear Corp. (Boston, MA). [^{14}C]20α-Hydroxy-4-pregnen-3-one was enzymatically synthesized from [^{14}C]progesterone by 20α-hydroxysteroid dehydrogenase in the ovarian cytosol fraction of mature rat [12]. [^{14}C]5α-Pregnane-3,20-dione was also prepared from [^{14}C]progesterone by 5α-reductase in the hepatic microsomal fraction (10,000–105,000 ***g*** precipitate) of female rat [13]. Identification of these two radioactive steroids was achieved by the same procedures employed for the identification of the radioactive metabolites. All radioactive steroids were checked for radiochemical purity by thin layer chromatography (solvent system; benzene: acetone = 4:1, v/v), before use.

Incubation

^{14}C-Labeled steroids were diluted with the corresponding radioinert steroids to the specified specific activities, and were dissolved in two drops of propylene glycol. Then, 1 ml of cell-free homogenates obtained from mammary tumors or normal mammary gland and 1 mg of NADPH were added per flask. Final volume of the incubation mixture was adjusted to 5 ml per flask with 0.25 M sucrose solution, buffered at pH 7.4 with 10 mM Tris–HCl. Incubation was performed in a Dubnoff-type incubator at 37°C under an aerobic atmosphere. The mixtures, excluding the tissue preparation, were incubated simultaneously under identical conditions for the control experiments.

Isolation and identification of the metabolites

At the end of the incubation, ^{14}C-labeled steroids were extracted with 15 ml methylene chloride. More than 90% of the initial radioactivity was recovered by repeating this extraction procedure three times. An aliquot of the extract was spotted on a thin layer of silica gel G and GF (4:1, w/w; E. Merck, Darmstadt, Germany), together with several Δ^4-3-oxosteroids (progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone and 11-desoxycortisol) as markers and developed with benzene:acetone (4:1, v/v). After development, the marker steroids were detected under ultraviolet light (wavelength, 253 nm), and the radioactive areas were autoradiographically detected. Quantitation of the metabolites was performed as previously reported [11].

For identification of the metabolites, the following criteria were employed: (1) identical mobility of the radioactive metabolite with the corresponding authentic preparation on thin layer chromatograms, developed with several solvent systems such as benzene:acetone (4:1, v/v), benzene:chloroform:methanol (6:1:1, v/v), and cyclohexane:ethylacetate:*n*-heptane (4:6:1, v/v); (2) the same chemical behavior of the radioactive metabolite with the authentic standard preparation against oxidation and acetylation [11]; (3) constant specific activities of cyrstals after repeated crystallization of radioactive metabolite with the authentic preparation from different solvent mixtures.

Chemical synthesis of 4-pregnene-3α,20α-diol

Tritiated 4-pregnene-3α,20α-diol was chemically prepared by the reduction of

[^{3}H]20α-hydroxy-4-pregnen-3-one (2.2×10^5 counts/min/mg) with $LiAlH_4$ [10]. The mixture of reduction products (yield ratio of 4-pregnene-3α,20α-diol to its 3β-isomer; 12:88) was treated with digitonin to precipitate the 3β-isomer. The final purification of 4-pregnene-3α,20α-diol to its 3β-isomer; 12:88) parative silica gel thin layer chromatography developed with benzene:acetone (4:1, v/v). Recently, chemical epimerization of 4-pregnene-3β,20α-diol to its 3α-isomer was accomplished by us, and reported elsewhere [14].

Expression of enzyme activity

After identification and quantitation of each metabolite, the enzyme activities were tentatively expressed in pmole or nmole of the relevant metabolites produced for 1 hr per mg of tissue protein. As a sufficient amount of substrate still remained at the end of incubation, saturation of the enzyme with substrate throughout the incubation period was ensured. Protein content was measured by the copper–Folin method [15].

RESULTS

Identification of metabolites

[^{14}C]Progesterone (8.5×10^4 counts/min, 4.1 nmole) was incubated with the cell-free homogenates of mammary tumor (7–18 mg protein) and normal mammary gland (7–9 mg protein) in the presence of NADPH (1.34 μmole). After 1 hr of incubation of it with the mammary tumor homogenates, 20α-hydroxy-4-pregnen-3-one, 5α-pregnane-3,20-dione, 20α-hydroxy-5α-pregnan-3-one, 3α-hydroxy-5α-pregnan-20-one, 5α-pregnane-3α,20α-diol and 4-pregnene-3α,20α-diol were obtained as the metabolites, while in the normal mammary gland, 20α-hydroxy-4-pregnen-3-one and 4-pregnene-3α,20α-diol were obtained. These metabolites were identified according to the procedures described in Materials and Methods, and final identification was achieved by the constant specific activities of the crystals after repeated crystallization of the radioactive metabolites with their authentic preparations (Table 1).

Metabolic pathway of progesterone

According to the time course study of progesterone metabolism in the cell-free homogenates of the mammary tumor, there was a rapid decrease in the amount of substrate concomitant with the immediate rise in 20α-hydroxy-4-pregnen-3-one. 3α-Hydroxy-5α-pregnan-20-one increased during the first 45 min of the incubation and then decreased, while 5α-pregnane-3α,20α-diol slowly increased up to the end of the incubation (75 min). Among the three minor metabolites, 5α-pregnane-3,20-dione showed a peak at 10 min of incubation and then fell to an undetectable level (data not shown).

The cell-free homogenates of the mammary tumor or the normal mammary gland were incubated with the two immediate metabolites, [^{14}C]5α-pregnane-3,20-dione and [^{14}C]20α-hydroxy-4-pregnen-3-one, respectively, and the yield of metabolites was compared with that obtained from the incubation with [^{14}C]progesterone. The major metabolite of 5α-pregnane-3,20-dione was 3α-hydroxy-5α-pregnan-20-one in both the mammary tumor and the normal mammary gland. 20α-Hydroxy-5α-pregnan-3-one and 5α-pregnane-3α, 20α-diol were also detected in smaller yields. The major metabolite of 20α-hydroxy-4-pregnen-3-one in the mammary tumor was 5α-pregnane-3α,20α-diol, whereas, in the normal mammary gland, 4-pregnene-3α,20α-diol was found predominant (Table 2).

In order to establish the pathway furthermore, cell-free homogenates of the mammary tumor and the normal mammary gland were incubated with [^{3}H]progesterone together with tracer amount of [^{14}C]5α-pregnane-3,20-dione. After 1 hr of incubation, the radioactivities of ^{14}C and ^{3}H in each metabolite were measured and the ratio of ^{14}C to ^{3}H was calculated. As shown in Table 3, most of 5α-pregnane-3,20-dione was metabolized to 5α-pregnane-3α,20α-diol via 3α-hydroxy-5α-pregnan-20-one, but another pathway through 20α-hydroxy-5α-pregnan-3-one was also present as shown by the fact that the ratio of ^{14}C to ^{3}H in 5α-pregnane-3α,20α-diol was lower than that in 5α-pregnane-3,20-dione and that 20α-hydroxy-5α-pregnan-3-one contained a significant amount of ^{14}C radioactivity.

Cofactor preferences

The cell-free homogenates of the mammary tumor or the normal mammary gland were incubated with [^{14}C]progesterone, [^{14}C]5α-pregnane-3,20-dione, and [^{14}C]20α-hydroxy-4-pregnen-3-one in the presence of NADH or NADPH. After 1 hr of incubation, the metabolites were quantitated. The results indicated that the 5α-reductase, and 3α- and 20α-hydroxysteroid dehydrogenases prefered NADPH to NADH (data not shown).

Table 1. Recrystallization analysis of the metabolites

Tissue	Metabolite identified as*	Solvent system	Specific activity (counts/min/mg)
Tumor	5α-Pregnane-3,20-dione (0.77)		449†
		Chloroform: *n*-heptane	442
		Dioxane: water	449
		Ethylacetate: *n*-heptane	439
Tumor	3α-Hydroxy-5α-pregnan-20-one (0.58)		1024†
		Ethanol: water	1026
		Ethylacetate: *n*-heptane	1039
		Methylene chloride: *n*-heptane	1011
Tumor	20α-Hydroxy-5α-pregnan-3-one (0.53)		449†
		Ethanol: water	450
		Tetrahydrofuran: water	437
		Ethylacetate: *n*-heptane	444
Tumor	20α-Hydroxy-4-pregen-3-one (0.43)		566†
		Ethanol: water	565
		Methylene chloride: *n*-heptane	562
		Chloroform: *n*-heptane	565
Tumor	5α-Pregnane-3α,20α-diol (0.38)		392†
		Tetrahydrofuran: water	376
		Methylene chloride: *n*-heptane	389
		Methanol: water	374
Tumor	4-Pregnene-3α,20α-diol (0.28)		5409†
		Ethanol: water	5245
		Methanol: water	5331
		Acetone: water	5151
Normal gland	20α-Hydroxy-4-pregnen-3-one (0.43)		672†
		Methylene chloride: *n*-heptane	671
		Chloroform: *n*-heptane	675
		Acetone: water	677
Normal gland	4-Pregnene-3α,20α-diol (0.28)		6664†
		Ethanol: water	6445
		Methanol: water	6562
		Acetone: water	6744

*R_f value on thin layer choromatogram developed with benzene:acetone (4:1, v/v) is indicated in parenthesis.
†Specific activity of the mixture before crystallization.

Table 2. Yield of metabolites obtained from incubations of progesrerone, 20α-hydroxy-4-pregnen-3-one and 5α-pregnane-3,20-dione with cell-free homogenates of mammary tumor or normal mammary gland

		Yield of metabolite (%)						
Tissue*	Substrate†	Progesterone	20α-Hydroxy-4-pregnen-3-one	5α-Pregnane-3,20-dione	20α-Hydroxy-5α-pregnan-3-one	3α-Hydroxy-5α-pregnan-20-one	5α-Pregnane-3α,20α-diol	4-Pregnene-3α,20α-diol
GRS/A Tumor	Progesterone	(58.7)‡	27.1	2.0	1.6	4.8	1.1	0.6
	20α-Hydroxy-4-pregnen-3-one	0.5	(65.7)	N.D.§	1.3	0.3	11.6	2.3
	5α-Pregnane-3,20-dione	N.D.	N. D.	(61.0)	2.2	18.3	1.1	N.D.
Normal gland	Progesterone	(39.3)	51.2	N. D.	1.6	1.0	0.5	2.0
	20α-Hydroxy-4-pregnen-3-one	1.0	(75.2)	N.D.	N.D.	N.D.	4.2	6.3
	5α-Pregnane-3,20-dione	N.D.	N.D.	(46.6)	1.8	37.2	3.0	N.D.

*Protein contents of the cell-free homogenates of mammary tumor, 10.5 mg: normal mammary gland, 4.8 mg.

†[^{14}C]Progesterone, 4.3×10^4 counts/min, 3.7 nmole; [^{14}C]5α-pregnane-3,20-dione, 3.8×10^4 counts/min 63.6 nmole; [^{14}C]20α-hydroxy-4-pregnen-3-one, 4.1×10^4 counts/min, 450 pmole.

‡Percentage of recovered substrate in paranthesis.

§Not detected.

Table 3. *Metabolic patterns of progesterone in cell-free homogenates of mammary tumor or normal mammary gland. The cell-free homogenates of the mammary gland* (4.8 mg *protein*) *and the mammary tumor* (10.5 mg *protein*) *were incubated with* [^{3}H]*progesterone* (4.1×10^5 counts/min, 3.2 nmole) *in the presence of* [^{14}C]5α-*pregnane-3,20-dione* (9.8×10^3 counts/min, 440 pmole). *After* 1 hr *of incubation, radioactivities due to* ^{14}C *and* ^{3}H *in each metabolite were counted*

Tissue	Radionuclides	Metabolite (counts/min)						
		Progesterone	20α-Hydroxy-4-pregnen-3-one	5α-Pregnane-3,20-dione	20α-Hydroxy-5α-pregnan-3-one	3α-Hydroxy-5α-pregnan-20-one	5α-Pregnane-3α,20α-diol	4-Pregnene-3α,20α-diol
GRS/A Tumor	^{3}H	202,330	117,520	4150	4370	19,900	5890	3840
	^{14}C	20	20	1290	240	5220	1300	10
	$^{14}C/^3H \times 100$	0.01	0.02	31.02	5.46	26.23	22.02	0.37
Normal gland	^{3}H	158,820	209,310	1300	5070	6430	5060	10,430
	^{14}C	20	20	1250	210	4960	3120	10
	$^{14}C/^3H \times 100$	0.01	0.00	95.90	4.12	77.13	61.58	0.12

Influence of ovariectomy and supplementary administration of estrogen upon enzyme activities

The activities of 5α-reductase and 20α-hydroxysteroid dehydrogenase were examined in the mammary tumors and the normal mammary glands of intact, ovariectomized and ovariectomized–estrogen treated animals. After bilateral ovariectomy on day 12 of pregnancy, one group of mice received subcutaneously 0.5 μg estradiol-17β-benzoate dissolved in 0.1 ml of soy bean oil from days 2 to 5 after the operation and the other group was treated with the vehicle alone. These animals were sacrificed on the 6th day after the operation. The tissue from intact animals was obtained on day 18 of pregnancy as the control.

mammary gland was not significantly altered by these treatments. Activity of the 20α-hydroxysteroid dehydrogenase in the mammary tumor was lower than that in the normal mammary gland (Fig. 1B). In the mammary tumor, this enzyme activity was not affected by the ovariectomy–estrogen treatment. However, in the normal mammary gland, the activity was significantly increased by ovariectomy ($P<0.05$) and remained at the elevated level even after the estrogen treatment.

DISCUSSION

The present studies demonstrate that 5α-reductase, and 3α- and 20α-hydroxysteroid dehydrogenases contribute to progesterone

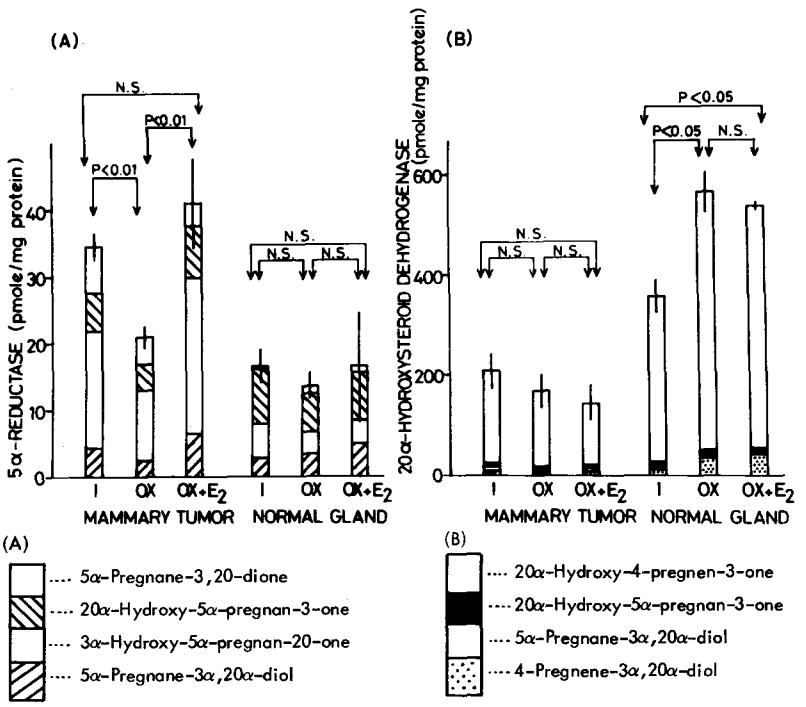

Fig. 1. Activities of 5α-reductase (A) and 20α-hydroxysteroid dehydrogenase (B) in mammary tumor and normal mammary gland. The tissues were obtained from intact animals (I), ovariectomized animals (OX), and ovariectomized and estradiol-17β-benzoate treated animals ($OX+E_2$). The cell-free homogenates of the tissues were incubated with [^{14}C]progesterone (8.5×10^4 counts/min, 4.2 nmole) in the presence of NADPH (1.34 μmole) for 1 hr at 37°C. Activities of the 5α-reductase and the 20α-hydroxysteroid dehydrogenase were obtained from the amounts of total 5α-reduced and 20α-hydroxy metabolites, respectively, per mg of tissue protein.

Activity of the 5α-reductase in the mammary tumor of the intact animals was higher than that in the normal mammary gland (Fig.1A). The activity of this enzyme in the mammary tumor was significantly reduced by ovariectomy ($P<0.01$) but was restored by treatment with estradiol-17β-benzoate. On the other hand, the enzyme activity in the normal

metabolism in GRS/A mouse mammary tumor. Metabolic pathways of progesterone in mammary tumor and normal mammary gland are diagrammed in Fig. 2, on the basis of the results from the time course study, metabolism of progesterone and related steroids and double tracer experiment. Marked difference in the metabolic pattern of pro-

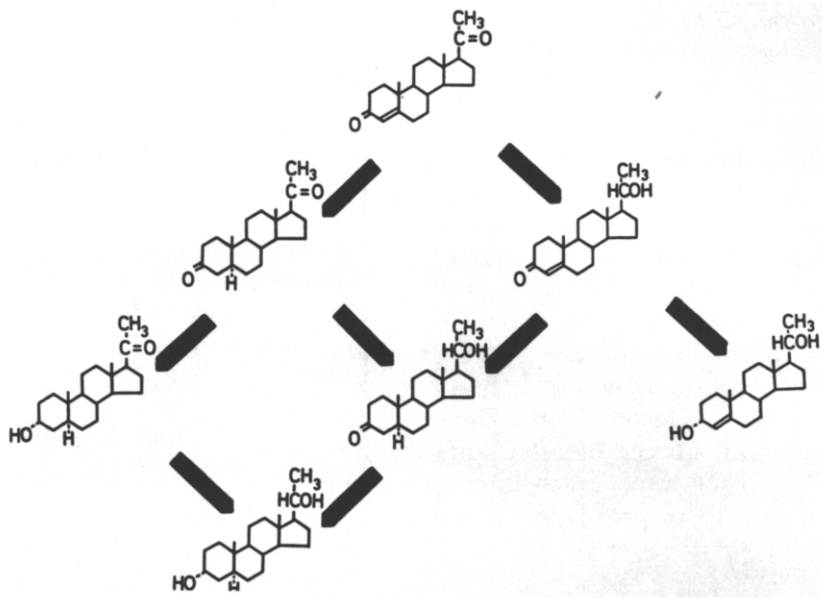

Fig. 2. Proposed metabolic pathway of progesterone in mammary tumor and normal mammary gland of GRS/A strain mice.

gesterone in this tumor from that in the DMBA-induced mammary tumor of rat was the formation of a steroidal allylic alcohol, 4-pregnene-3α,20α-diol, which was characteristic of the normal mammary gland but has never been observed in the mammary tumor of rat [11]. The occurrence of this unusual metabolite has been explained as resulting from the absence or limited activity of 5α-reductase, so that the 3α- and 3β-hydroxysteroid dehydrogenases directly attacked Δ^4-3-oxosteroids. This hypothesis has been verified, since 5α-reductase activity was either undetectable or very low in the normal mammary glands of rat [11] and mouse [10]. However, in GRS/A mouse mammary tumor, 4-pregnene-3α,20α-diol was obtained as a progesterone metabolite in spite of the presence of appreciable 5α-reductase activity.

From this result, it was suggested that there would be a different species of 3α-hydroxysteroid dehydrogenase capable of synthesizing steroidal allylic alcohol that is different from the enzyme which reduces 3-oxo group of ring A-saturated substrates. This is supported by the heterogeneity of this enzyme in the normal mammary gland and in the DMBA-induced mammary tumor of rat [11].

The relative activities of 5α-reductase and 20α-hydroxysteroid dehydrogenase in GRS/A mouse mammary tumor compared to the normal mammary gland were in good agreement with the results of the DMBA-induced mammary tumor to the normal mammary gland of the rat. The mammary tumor has higher 5α-reductase activity and lower 20α-hydroxysteroid dehydrogenase activity than those of the normal mammary gland.

These two characteristics of progesterone metabolism in GRS/A mouse mammary tumor, i.e., an allylic alcohol formation like the normal mammary gland and an enchanced 5α-reductase activity like the DMBA-induced mammary tumor, are reflected in the results of the following transplantation studies of this tumor. When the tumor was transplanted to the gland-free fat pad of virgin hosts, it grew as a normal mammary gland, but if the host animals were pregnant, it was transformed into tumor [16].

Progesterone metabolism in its target tissues were widely reported. Among the metabolites of progesterone, 5α-pregnane-3,20-dione which is formed in chick oviduct was more potent in stimulating avidine synthesis than progesterone itself [17]. 5α-Reduced metabolites of progesterone were also important for gonadotrophin secretion from rat pituitary [18].

The existence of a progesterone receptor was demonstrated in GRS/A mouse mammary tumor [19]. Since progesterone receptor was induced by estrogens [20], estrogen priming was considered as being necessary for progesterone action. In other words, the existence of a progesterone receptor is regarded as an evidence for estrogen action. Our findings in this study indicate that 5α-reductase would be an indicator for estrogen action in mammary tumor and also suggested the importance of

this enzyme for the growth of the mammary tumor.

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