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Metabolism of Androgens by the Mouse Submandibular Gland and Effects of Their Metabolites

KAZUHIKO SAWADA AND TETSUO NOUMURA

Department of Regulation Biology, Faculty of Science, Saitama University, Urawa, Japan

ABSTRACT—In the mouse submandibular gland, sexual difference becomes evident on day 30, when the granular convoluted tubules (GCT) of the gland rapidly grow in response to drastically increased levels of circulating testosterone and 5α-dihydrotestosterone (DHT) in the male. We studied the *in vitro* metabolism of both androgens by the mouse gland and the effects of their metabolites on the gland.

By the glands on days 20 and 30, ^{3}H-testosterone was not further converted to any derivatives. In contrast, ^{3}H-DHT used as a substrate was metabolized partly to ^{3}H-5α-androstane-3α,17β-diol (3α-diol) and ^{3}H-5α-androstane-3β,17β-diol (3β-diol) in both sexes (approximately 9 and 1%, respectively). On the basis of the metabolites identified, the gland contained both 3α- and 3β-hydroxysteroid dehydrogenase activities, which were not sexually different.

Neonatally-castrated mice were given daily injections of 3α-diol, 3β-diol, testosterone and DHT for 1–10 days from day 20. The relative occupied area (ROA) of GCT in the gland increased in both sexes by treatment with 3α-diol or DHT for 4 days, or by 3β-diol or testosterone for 10 days. The order of ROA gain after 4–10 days was DHT>3α-diol>testosterone>3β-diol. The mitotic activity of GCT increased in both sexes by treatment with 3α-diol or DHT for 4 days, but in only males by 3β-diol or testosterone for 10 days.

The results suggest that in the mouse gland testosterone exerts its effects without any intraglandular conversion, and that DHT primarily acts in its form, whereas its intraglandular metabolite, 3α-diol may be supplementary as a stimulant.

INTRODUCTION

In rodents, the male submandibular gland is larger than the female one and has a more complex morphology. The glandular contents of biologically active polypeptides, including nerve growth factor, epidermal growth factor, renin and proteases, are higher in the male than in the female, being responsive to androgens [3, 6, 12, 14, 16, 19, 28]. By histological, ultrastructural and morphometrical studies, both sexes experience a similar morphogenesis of the gland during perinatal development, and then the sexual difference arises at 3–4 weeks of age, when the granular convoluted tubules (GCT) grow more rapidly in the male than in the female [10, 11, 13, 21]. In a completely androgen-independent state (e.g., neonatal castration or androgen-insensitive Tfm mutation), the glands carry out the feminine development [21, 22]. The masculine development of the gland is caused by circulating androgens, testosterone and 5α-dihydrotestosterone (DHT), the latter being more effective [23]. In male mice, their circulating levels are drastically increased between days 20 and 30 [23], when sexual difference of the gland occurred morphologically.

Therefore, in order to determine how testosterone and DHT exert their effects on the mouse submandibular glands, we examined *in vitro* metabolism of these androgens by the glands, and the effects of the resulting metabolites on the glands by morphometry.

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MATERIALS AND METHODS

Animals

CD-1 mice were obtained from Charles River Japan Co. and maintained by randomly mating in

our laboratory. The animals were given a commercial diet (CRF-1: Charles River Japan Co.) and tap water *ad libitum* and were kept at 23±1°C under 12-hr artificial illumination (from 8:00 to 20:00).

In vitro androgen metabolism by the mouse submandibular gland

Both male and female mice were killed on days 20 and 30. Approximately 50 mg tissue of the submandibular glands were minced into about 2 mm-fragments. Minced tissues were incubated with 10 nM [1, 2, 6, 7-^{3}H]testosterone and [1, 2, 4, 5, 6, 7-^{3}H]5α-dihydrotestosterone (DHT) (specific activity, 70 and 117 Ci/mmol, respectively; Amersham) in 2 ml Hanks' solution (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 4 mM glucose-6-phosphate-2NA (Boehringer Mannheim GmbH, Germany) for 1 hr at 37°C in an atmosphere of 95% air and 5% CO_2. After 1 hr, the incubation was stopped with 3 ml ethyl acetate, containing each 100 μg of the following non-radioactive reference steroids: 4-androstene-3,17-dione (androstenedione), 5α-androstane-3,17-dione (5α-androstanedione), 5α-androstan-3α-ol-17-one (androsterone), 4-androsten-17β-ol-3-one (testosterone), 5α-androstan-17β-ol-3-one (5α-dihydrotestosterone, DHT), 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3β-diol) (Sigma Chemical Co., St. Louis, Mo, U.S.A.). After shaking, the ethyl acetate fractions were transferred to a new set of glass tubes, and the water phase was reshaken twice each time with 3 ml ethyl acetate. The resulting 9 ml ethyl acetate fraction was washed with 1 ml saturated $NaHCO_3$ and twice with 1 ml distilled water, and was evaporated. The extracted steroids were redissolved in 20 μl methanol-dichloromethane (1:1, vol/vol), and applied to TLC Silica gel plate 60 $HF_{254+366}$ (Merck, Darmstadt, Germany). The plates were developed in the solvent system, dichloromethane-diethyl ether (4:1, vol/vol), and were scanned to detect the areas of radioactivity by Chromatoscaner (Aloka, Tokyo, Japan). The radioactive areas were scraped off and steroids were extracted from the Silica gel three times with 2 ml methanol-dichloromethane (1:1, vol/vol) each time. The dried residues of radioactive steroids, except for 5α-androstanediols, were transferred to scintillation vials. After adding 4 ml scintillation fluid (6 g DPO, 0.3 g POPOP in 1,000 ml toluene), radioactivity was measured in a Packard Liquid Scintillation Counter, model 3255 with an efficiency of 50% for ^{3}H. The fraction of 5α-androstanediols was acetylated by dissolving in equal volumes of pyridine and acetic anhydride (0.2 ml, total volume) and allowing the mixture to stand overnight at room temperature. The mixture was evaporated and redissolved in 20 μl methanol-dichloromethane (1:1, vol/vol), applied to the TLC plates and developed twice in dichloromethane. The results were expressed as the percentages of total radioactivity. The protein content of each sample was determined by BCA protein assay reagent (Pierce, Rockford, IL, USA) and enzyme activities of 3α- and 3β-hydroxysteroid dehydrogenases were expressed as f mol of 3α- and 3β-diols produced from DHT per hour per mg protein, respectively. Each datum was the mean of 2–4 independent experiments. The procedure losses were less than 10% after first chromatography and less than 20% after acetylation and second chromatography (for separation of 3α- and 3β-diols), and the results were corrected for these losses.

Morphological studies

Mice of both sexes were castrated on day 0 and were subcutaneously given daily treatments with each 100 μg of 3α-diol, 3β-diol, testosterone and DHT, and the vehicle alone (0.1 ml sesame oil) for 1, 4 and 10 days starting on day 20, and they were killed on days 21, 24 and 30, respectively. All animals were subcutaneously given a single injection of colchicine (5 μg/g body weight) dissolved in 0.9% NaCl, 5 hr before they were killed to arrest cell division at metaphase. Submandibular glands were weighed and fixed in Bouin's solution, embedded in paraffin and sectioned at 8 μm. Sections were stained with Delafield's hematoxylin and eosin.

The sectional figures of submandibular glands in the microscopic enlargements (×400) were traced by the camera lucida, and the area of GCT was measured by the picture analyzer (Logitec K-510, Kantou Denshi Co., Japan) connected with a

microcomputer (NEC PC-9801DA). The area was expressed as percentages of the total area of the gland (the relative occupied area, ROA). Cell height of GCT was measured in randomly chosen five sections.

Cells in division per 500–1,000 cells were counted in the GCT of the gland. Mitotic rate was estimated by counting the cells at metaphase per 5 hr in 100 cells.

Statistical analysis

Data were statistically analyzed by Student's *t*-test or by Cochran-Cox test.

RESULTS

In vitro androgen metabolism by the mouse submandibular gland

The results in Table 1 are expressed as the percentage conversion of each substrate to metabolites/hr/mg of wet tissue, and a proposed metabolic pathway of androgens in the mouse submandibular gland is shown in Figure 1. By the glands on days 20 and 30, ^{3}H-testosterone was not further converted *in vitro* to any radioactive metabolites, including DHT, and its recovery was

TABLE 1. *In vitro* androgen metabolism by the mouse submandibular gland

substrate	animals	age in days	% recovery			
			testosterone	DHT	3α-diol	3β-diol
testosterone	male	20	98.8	ND	ND	ND
		30	96.1	ND	ND	ND
	female	20	96.7	ND	ND	ND
		30	95.6	ND	ND	ND
DHT	male	20	—	79.4	9.6	1.1
		30	—	83.5	9.0	1.4
	female	20	—	78.9	8.2	1.2
		30	—	79.3	8.7	1.4

The results are expressed as the percentage conversion of each substrate to metabolites/hr/mg wet tissue. Values are the mean of 2–4 independent experiments. DHT=5α-dihydrotestosterone, 3α-diol=5α-androstane-3α,17β-diol, 3β-diol=5α-androstane-3β,17β-diol, ND=not detected.

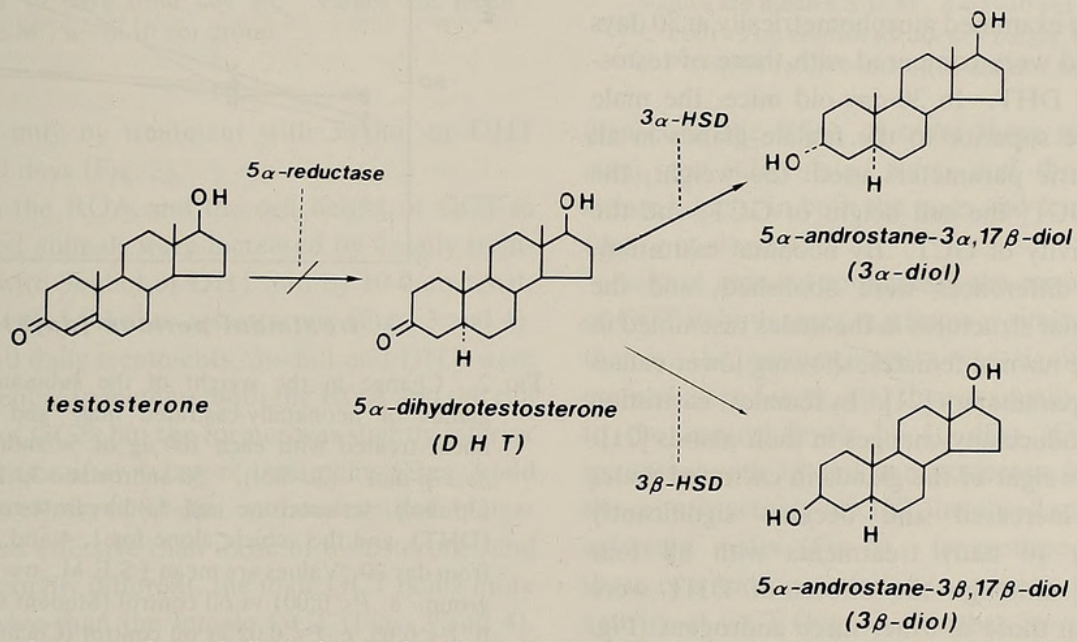

FIG. 1. A proposed metabolic pathway of androgens in the mouse submandibular gland. HSD=hydroxysteroid dehydrogenase.

TABLE 2. 3α- and 3β-Hydroxysteroid dehydrogenase activities in the mouse submandibular gland

animals	age in days	enzyme activity (fmol·mg^{-1} protein·hr^{-1})	
		3α-HSD	3β-HSD
male	20	369.8	43.4
	30	645.4	98.1
female	20	314.3	47.6
	30	574.6	93.2

Values are the mean of 2–4 independent experiments.
HSD = hydroxysteroid dehydrogenase.

more than 95% (Table 1). On the other hand, ^{3}H-DHT was metabolized in limited amounts to tritiated 3α- and 3β-diols (approximately 9 and 1%, respectively). The amounts of metabolic products from DHT did not differ between sexes at each age (Table 1). On the basis of the metabolites identified, the gland contained both 3α- and 3β-hydroxysteroid dehydrogenase (HSD) activities. The activities of these enzymes increased between 20 and 30 days, but were not sexually different at both ages (Table 2).

Effects of 5α-androstanediols on the mouse submandibular gland

By the mouse glands, some amounts of 3α- and 3β-diols could be converted *in vitro* from DHT. Therefore, the effects of 3α- and 3β-diols on the glands were examined morphometrically at 30 days of ages, and were compared with those of testosterone and DHT. In 30-day-old mice, the male glands were superior to the female glands in all morphometric parameters used: the weight, the ROA of GCT, the cell height of GCT, and the mitotic activity of GCT. By neonatal castration, these sex differences were abolished, and the intraglandular structures in the males resembled to those in the normal females, showing lower values in all four parameters [21]. In females, castration could not induce any changes in their glands [21].

The net weight of the glands in castrated males gradually increased and became significantly heavier by 10 daily treatments with all four androgens, although the effects of DHT were greater than those of other three androgens (Fig. 2). In castrated females the gland weight gain was

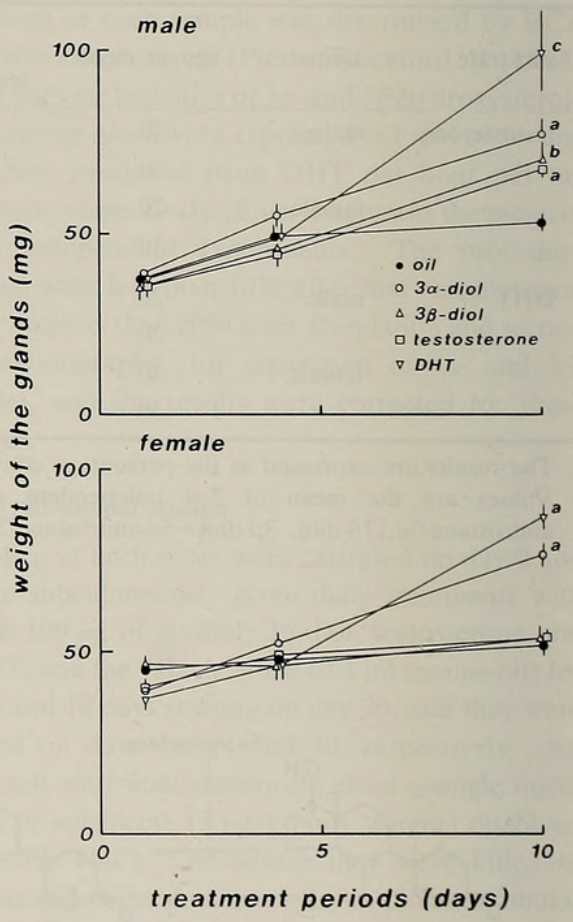

FIG. 2. Change in the weight of the submandibular glands of neonatally-castrated male and female mice, treated with each 100 μg of 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3β,17β-diol (3β-diol), testosterone and 5α-dihydrotestosterone (DHT), and the vehicle alone for 1, 4 and 10 days from day 20. Values are mean ± S.E.M., n = 4–5 per group. a: $P<0.001$ *vs* oil control (Student's *t*-test), b: $P<0.05$, c: $P<0.02$ *vs* oil control (Cochran-Cox test).

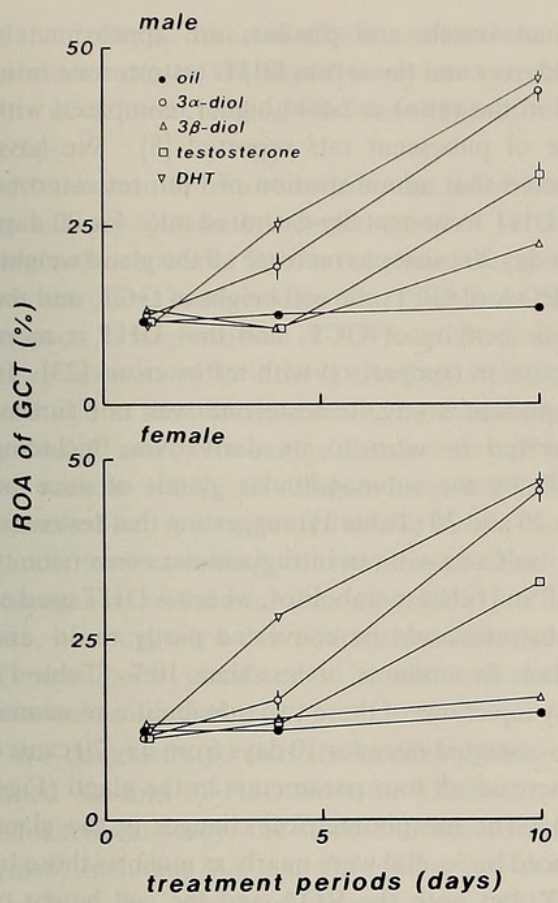

FIG. 3. Change in the relative occupied area (ROA) of the granular convoluted tubules (GCT) of the submandibular glands of neonatally-castrated male and female mice, treated with each 100 μg of 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3β,17β-diol (3β-diol), testosterone and 5α-dihydrotestosterone (DHT), and the vehicle alone for 1, 4 and 10 days from day 20. Values are mean ± S.E.M., n=8–10 per group.

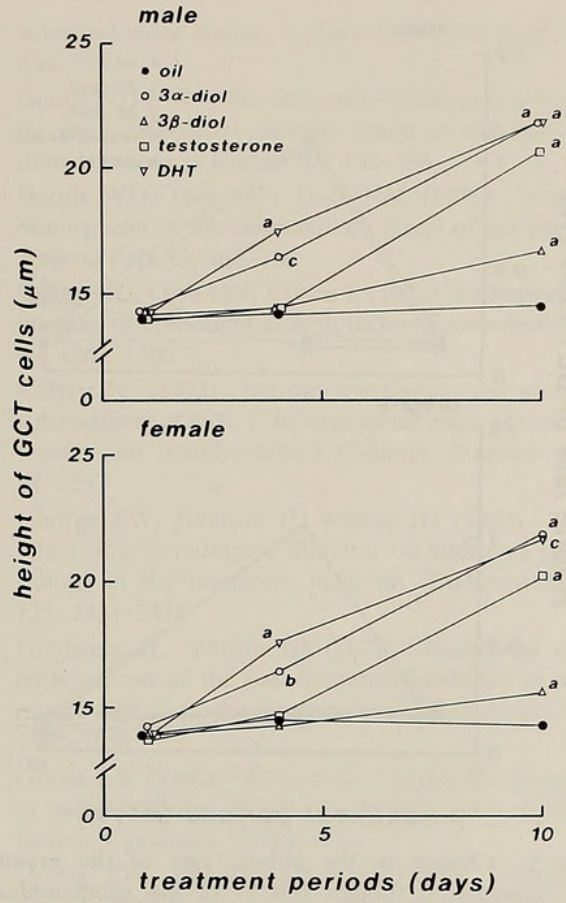

FIG. 4. Change in the cell height of the granular convoluted tubules (GCT) of the submandibular glands of neonatally-castrated male and female mice, treated with each 100 μg of 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3β,17β-diol (3β-diol), testosterone and 5α-dihydrotestosterone (DHT), and the vehicle alone for 1, 4 and 10 days from day 20. Values are mean ± S.E.M., n=8–10 per group. a: $P < 0.001$ *vs* oil control (Student's *t*-test), b: $P < 0.005$, c: $P < 0.001$ *vs* oil control (Cochran-Cox test).

caused only by treatment with 3α-diol or DHT after 10 days (Fig. 2).

Both the ROA and the cell height of GCT in castrated animals were increased by 4 daily treatments with 3α-diol or DHT and by 10 daily treatments with 3β-diol or testosterone (Figs. 3 and 4). After 10 daily treatments, 3α-diol and DHT were nearly equal to increase both the ROA and the cell height of GCT, but the former was slightly inferior to the latter after 4 days of treatments (Figs. 3 and 4). The effects of 3β-diol on these two parameters were less effective than those of testosterone, and were sexually different, the male GCT being more responsive than the female GCT (Figs. 3 and 4). Both 3α- and 3β-diols could not cause any increases in the ROA of other three regions, the acini, the intercalated ducts and the excretory striated ducts, in both the male and female glands (data not shown).

3α-Diol induced to increase the mitotic activity of GCT in both sexes in a manner similar to DHT, that is, the mitotic activity was significantly increased after 4 days, and then gradually decreased to the control levels by 10 days (Fig. 5). By treatment with 3β-diol or testosterone for 10 days, the mitotic activity of GCT increased somewhat in castrated males (Fig. 5). In castrated females, these two androgens failed to increase the mitotic activity of GCT (Fig. 5). Neither 3α- nor 3β-diol had effects on the mitotic activities of other three

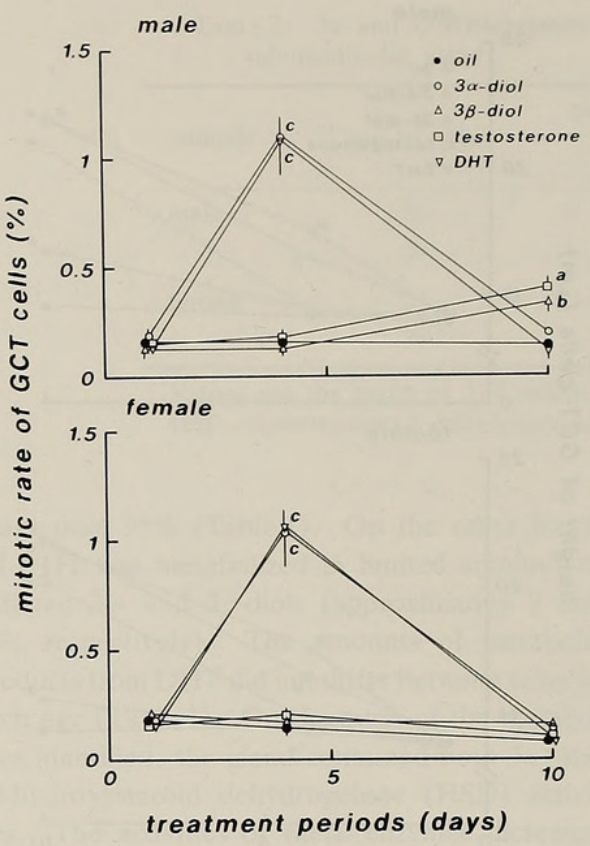

FIG. 5. Change in the mitotic rate of the granular convoluted tubules (GCT) of the submandibular glands of neonatally-castrated male and female mice, treated with each 100 μg of 5α-androstane-3α,17β-diol (3α-diol), 5α-androstane-3β,17β-diol (3β-diol), testosterone and 5α-dihydrotestosterone (DHT), and the vehicle alone for 1, 4 and 10 days from day 20. Values are mean$\pm$S.E.M., n=8–10 per group. a: $P<0.001$, b: $P<0.002$ *vs* oil control (Student's *t*-test), c: $P<0.001$ *vs* oil control (Cochran-Cox test).

regions in the gland of either sex (data not shown).

DISCUSSION

The mouse submandibular glands contain both cytosolic and nuclear androgen receptors [15, 17, 20, 26, 27]. The cytosolic receptor in the male glands increases during postnatal development and attains adult levels by day 20 [17, 26], while circulating levels of androgens (testosterone and DHT) begin to rise on day 20 [17, 23]. In our male mice on day 30 [23], circulating DHT levels (1.0$\pm$0.21 ng/ml), which is produced by testes [24] and metabolized from testosterone in the peripheral tissues [1, 9] including liver, prostate, epididymis, seminal vesicle and phallus, are approximately 4-fold over and the serum DHT/testosterone ratio (1:3 in the ratio) is 2-fold higher, compared with those of pubescent rats reported [8]. We have reported that administration of both testosterone and DHT to neonatally-castrated mice for 20 days from day 20 causes to increase all the gland weight, the ROA of GCT, the cell height of GCT, and the mitotic activity of GCT, and that DHT is more effective in comparison with testosterone [23]. In the present study, testosterone was not further converted *in vitro* to its derivatives, including DHT, by the submandibular glands of mice on days 20 and 30 (Table 1), suggesting that testosterone itself acts without intraglandular conversion to DHT and other metabolites, whereas DHT used as a substrate could be converted partly to 3α- and 3β-diols in amounts of less than 10% (Table 1). Daily injections of these two metabolites to neonatally-castrated mice for 10 days from day 20 caused to increase all four parameters in the gland (Figs. 2–5). The morphometrical changes in the gland induced by 3α-diol were nearly as much as those by DHT, but both the ROA and the cell height of GCT were slightly lower by treatment with 3α-diol than by DHT after 4 days (Figs. 3 and 4). On the other hand, 3β-diol was less effective than testosterone (Figs. 3 and 4). Thus these DHT metabolites produced by the gland have also androgenic effects on the gland growth, and particularly 3α-diol has a potency similar to DHT. These results indicate that, *in vivo,* DHT itself acts primarily, and that its metabolites, 3α-diol may act secondarily on the gland.

As described before [23], by treatment with DHT, the ROA and cell height of GCT in the glands of both sexes are increased for 7–20 days of treatments, whereas the mitotic activity is increased for 2–7 days only. Similar results were obtained by treatment with 3α-diol (Figs. 3–5). These androgens primarily affect the cell nucleus to initiate the mitosis, followed by the sequence of cellular events in GCT. In contrast, the mitotic activity was increased slightly and only in males by treatment with 3β-diol or testosterone for 10 days (Fig. 5). Although ample time may be allowed for peripheral conversion of the latter androgens to active mitogenic androgens and for accumulation

of their effects, different effects among these four hormones can be explained solely as a consequence of different binding affinities to the androgen receptor.

Metabolism of testosterone by the submandibular salivary glands has been reported in some mammalian species. By the domestic boar glands, testosterone is converted *in vitro* to its 5α-reduced products, a large amount of DHT and 3α-diol, and lesser amounts of 3β-diol [4]. Androstenedione is produced as the main metabolite by the rat glands *in vitro* [2] and by the canine glands *in vivo* [29]. In the present study, testosterone was not further converted *in vitro* to its derivatives by the mouse glands, corresponding to the results from the homogenate of the adult murine gland [7]. Therefore, it is suggested that androgen pathway in the submandibular glands may be different among mammalian species.

We demonstrated that the murine glands contained 3α- and 3β-HSD activities (Table 2). By histochemical study, the steroid metabolizing enzymes, including 3α- and 3β-HSDs, are mainly found in the excretory striated ducts (SD) in human [25] and pig [5]. In these two species, the GCT are absent in their glands [18]. The SD of the rodent gland are transformed to the GCT during puberty [10, 11, 13, 18, 21]. Therefore, the SD in human and pig have been considered to be identical with both the GCT and SD in rodents. In our mice, the ROA of GCT plus SD are superior in the males to those in the females on days 30–90 [21], but the activities of 3α- and 3β-HSDs were not sexually different on day 30 (Table 2). In order to solve these inconsistent results, further research is required to examine the histochemical localization of these enzymes in the glands of our mice.

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