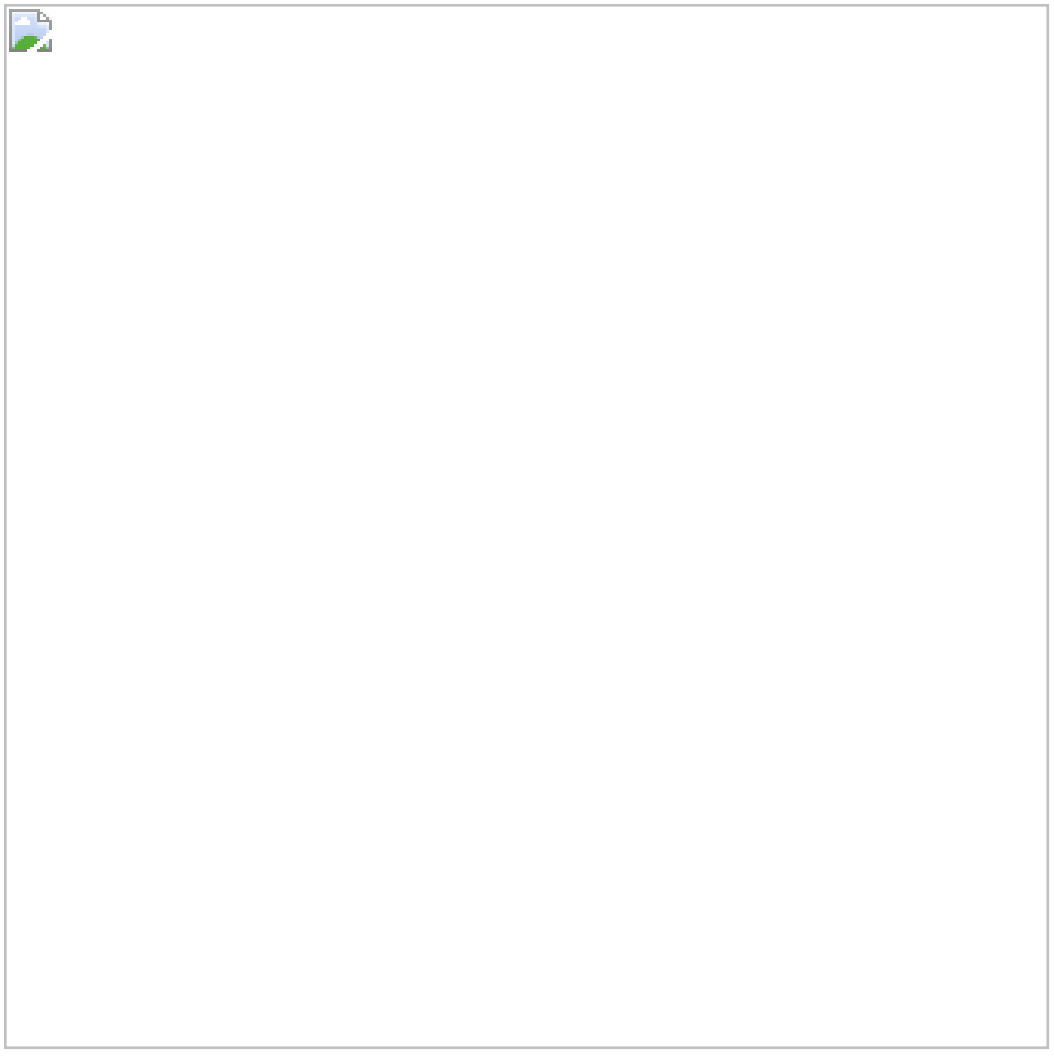

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Developmental Pattern of Androgen Receptor Immunoreactivity in the Mouse Submandibular Gland

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ABSTRACT—We studied the immunohistochemical localization of androgen receptor in the mouse submandibular gland, and developmental profiles of its expression using polyclonal human androgen receptor antibody. In the submandibular glands of both sexes, specific immunoreactivity appeared only in cell nuclei of the acini, the intercalated ducts, the granular convoluted tubules (GCT) and the excretory striated ducts. The percentage of immunoreactive cells in each region gradually declined with age during the first 90 days of postnatal development studied. The sexual difference in the percentage of immunoreactive cells was oberserved in the acini on days 20 and 30 and in the GCT on day 30. Incidence of immunoreactive cells in the female was significantly smaller than that in the male. On day 60, the percentage of immunoreactive cells of these two regions turned to increase slightly in the female but continued to decline in the male, and then it became higher in the female than in the male. In addition, one-week castration did not cause any changes in the intracellular distribution of androgen receptor and the percentage of immunoreactive cells in each region of the adult gland.

These results suggest that androgen receptor is localized primarily in cell nuclei in all four regions of the mouse submandibular gland *in situ*, and that its expression in acini and GCT is superior in the male around days 20 to 30, when sex difference of the gland becomes evident.

INTRODUCTION

In rodents, the male submandibular gland is larger than the female one and has a more complex morphology. Biologically active polypeptides, including nerve growth factor, epidermal growth factor, renin and proteases, are contained in higher concentration in the male gland, being more responsive to androgens [1, 2, 8, 12, 13, 16, 19, 26, 36]. The adult gland consists of the acini, the intercalated ducts (ID), the granular convoluted tubules (GCT) and the excretory striated ducts (SD). Among these structures, the male GCT are much more developed than the female GCT [23]. On the basis of histological, ultrastructural and morphometrical studies, both sexes in rats and mice undergo a similar morphogenesis of the gland during perinatal development, and then the sex difference appears at 3–4 weeks of age, when GCT grow more rapidly in the male than in the female [9, 11, 15, 28]. In a completely androgen-independent state (e.g., neonatal castration or androgen-insensitive testicular feminization mutation), the glands carry out the feminine development [28, 29]. The masculine development of the gland is caused by circulating androgens: 5α-dihydrotestosterone (DHT) induces cell proliferation of GCT around day 20, and then both testosterone and DHT cause morphologically masculine development and maintain GCT in fully-stimulated states [30, 32].

The mouse submandibular gland is suggested biochemically to contain both cytosolic and nuclear androgen receptors [18, 20, 32, 34]. Physico-chemical properties of androgen receptors in the mouse submandibular gland have been studied by binding assays. But the binding assays lack a detailed information of the cellular and subcellular localization of the receptor. Therefore, using a polyclonal human androgen receptor antibody, this study examined the immunohistochemical localization of androgen receptor in four regions (acini, ID, GCT and SD) of the mouse submandibular gland in the course of postnatal development.

MATERIALS AND METHODS

Animals

CD-1 mice were obtained from Charles River Japan Co. and maintained by random 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).

Immunohistochemical staining for androgen receptor

In the first group, adult male mice were castrated or subjected to sham-operation 1 week before killing. At 90 days of age, animals were killed and submandibular glands and epididymides were removed. In the second group, mice of both sexes were killed under ether anesthesia on days 0 (at birth), 5, 10, 15, 20, 30, 60 and 90, and submandibular glands were removed. Tissues were immediately embedded in O.C.T. compound (Miles Inc.), frozen and cut at 8 μm with a cryostat (Cryocut E, Reichert-Jung). Sections were mounted directly onto gelatin-coated slide, fixed in 3.7% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 min, and rinsed with 10 mM phosphate buffered saline (PBS) (pH 7.2). After preincubation with 2% normal goat serum in PBS for 30 min at room temperature, sections were incubated for 24 hr at 4°C with polyclonal human androgen receptor antibody (Affinity Bio Reagents), which was raised by immunization to rabbits with a 62 kDa fusion protein from the N-terminal domain of the human androgen receptor expressed by

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bacteria [4–6]. As a control, the primary antibody was replaced with normal rabbit serum (NRS) at a dilution of 1:16,000. No positive signals were detected in the epididymis and submandibular gland by reaction with NRS. Preabsorption test of this antibody was omitted because the antibody could specifically detect the mouse androgen receptor as described previously [33]. Sections were rinsed with PBS, and incubated with biotinylated secondary antibody. The immunoreactive products were detected by an Avidin-conjugated peroxidase kit (Vectastain ABC kit: Vector Lab., Inc.), using 0.05% 3,3'-diaminobenzidine tetrachloride (Sigma Chemical Co.) in 0.01% H_2O_2 as a chromogen. As final step, the sections were rinsed with PBS and coverslipped with 7% gelatin in 50% glycerol (vol/vol). Immunoreactivity in four regions (acini, ID, GCT and SD) of the submandibular gland was shown by the percentage of the number of immunoreactive cells per 500–1,000 cells counted.

Statistical analysis

Data were analyzed by Mann-Whitney's U-test.

RESULTS

Effect of castration on immunohistochemical expression of androgen receptor in adult epididymis and submandibular gland

In the epididymis in sham-operated male mice at 90 days of age, the immunopositive products were detected in the cell nuclei, not in the perikaryal cytoplasm of both the epithelial and stromal cells (Fig. 1a). Castration did not induce any distinct changes in the intracellular distribution of the immunoreactive products (Fig. 1b).

As observed in the epididymysis, the immunopositive materials were also found only in the cell nuclei, not in the perikaryal cytoplasm, of all four regions (acini, ID,GCT and SD) in the submandibular gland of sham-operated males (Fig. 1c). The percentage of immunoreactive cells in acini, ID, GCT and SD was $64.4\pm1.32\%$, $78.8\pm1.57\%$, $68.3\pm0.75\%$ and $77.0\pm0.89\%$ (n=3), respectively. In the submandibular gland, castration had no influence on the intracellular distribution of the immunopositive products (Fig. 1d) and the occurrence of immunoreactive cells (acini=$65.1\pm1.03\%$, ID=$79.1\pm1.00\%$, GCT=$72.8\pm2.26\%$. SD=$77.2\pm0.80\%$ (n=3)).

Ontogeny of immunohistochemical expression of androgen receptor in submandibular gland

In neonatal mice, the submandibular glands of both sexes were composed of the developing acini and ID. In newborn acini, signals showing androgen receptor were found in almost all the cells (Fig. 2a, b). The percentage of immunoreactive cells in acini decreased gradually with age and

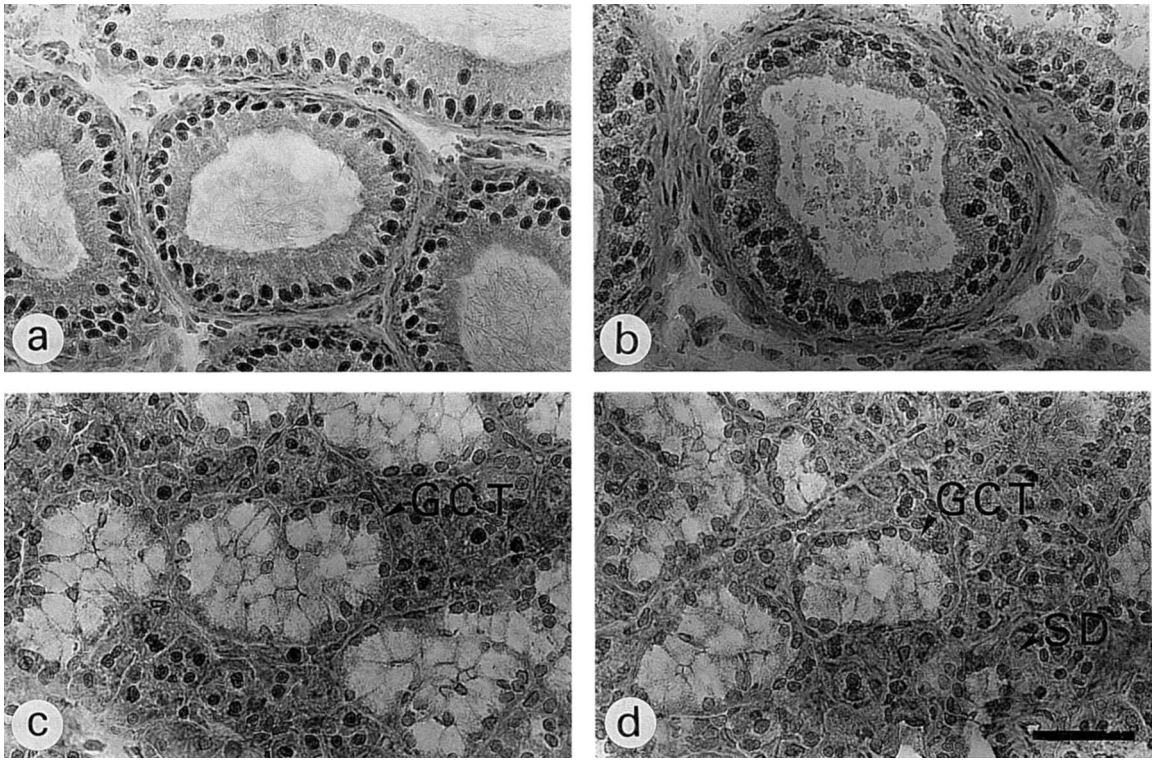

FIG. 1. Immunohistochemical localization of androgen receptor in the epididymis and the submandibular gland in castrated or sham-operated mice. Mice were castrated or subjected to the sham-operation one week before killing, and were killed at 90 days of age. Nuclei in the epididymal epithelial and stromal cells reacted with the polyclonal human androgen receptor antibody. In the submandibular gland, cell nuclei in acini, intercalated ducts (ID), granular convoluted tubules (GCT) and excretory striated ducts (SD) also reacted. (a) Epididymis in sham-operated mouse. (b) Epididymis in castrated mouse. (c) Submandibular gland in sham-operated mouse. (d) Submandibular gland in castrated mouse. Bar=50 μm.

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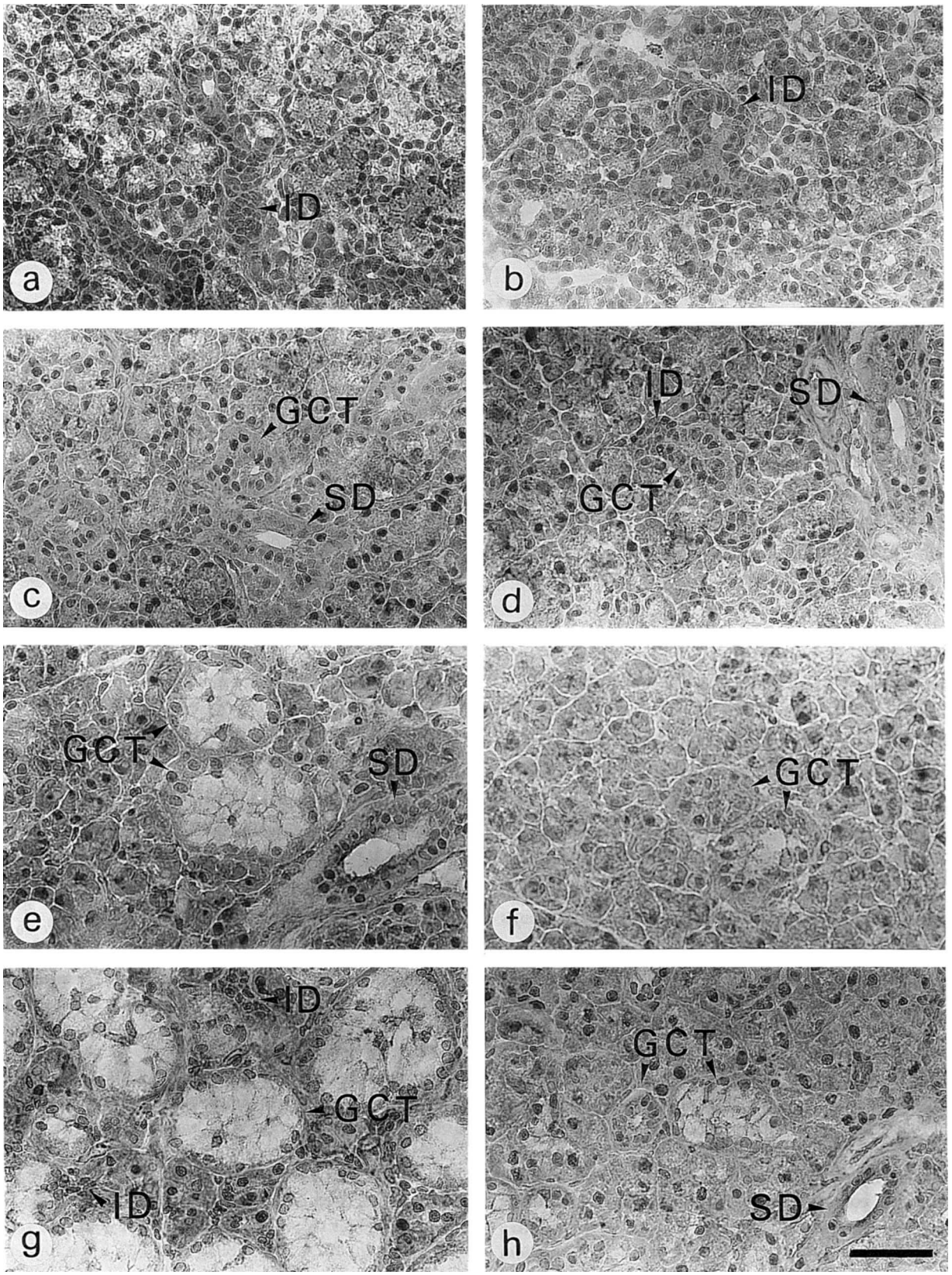

FIG. 2. Immunohistochemical localization of androgen receptor in the mouse submandibular gland. Cell nuclei in the acini, the intercalated ducts (ID), the granular convoluted tubules (GCT) and the excretory striated ducts (SD) positively reacted with the polyclonal human androgen receptor antibody. (a) 0-day-old male. (b) 0-day-old female. (c) 20-day-old male. (d) 20-day-old female. (e) 30-day-old-male. (f) 30-day-old female. (g) 90-day-old male. (h) 90-day-old female. Bar=50 μm.

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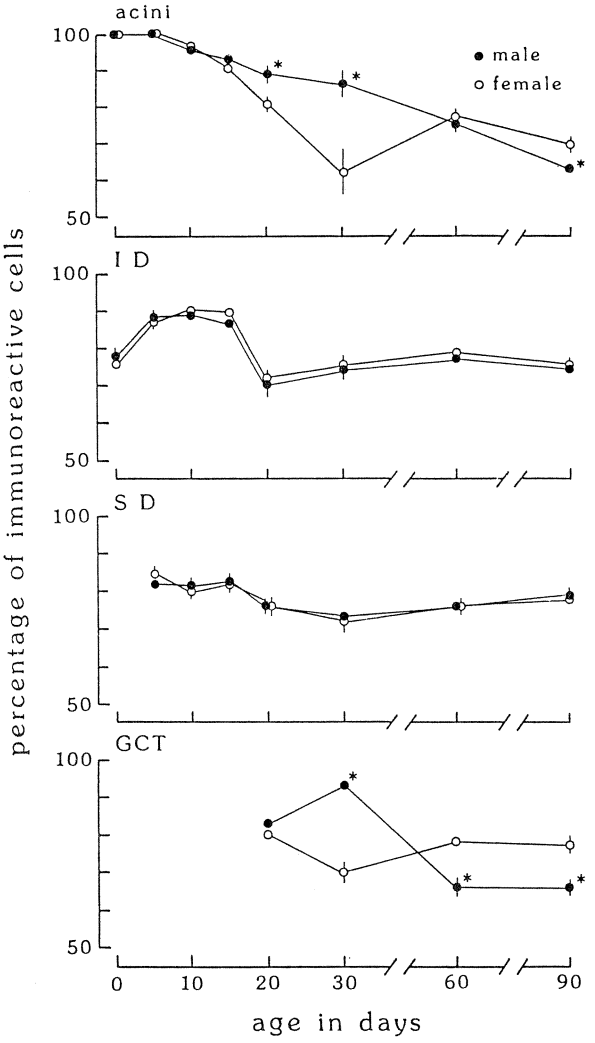

FIG. 3. Developmental changes in the percentage of androgen receptor-immunoreactive cells per 500–1,000 cells counted in the acini, the intercalated ducts (ID), the excretory striated ducts (SD) and the granular convoluted tubules (GCT) of the mouse submandibular gland. Values are mean±S.E.M., n=3 per group. *: $P<0.05$ vs. age-matched female (Mann-Whitney's U-test).

fell to approximately 63% in the male and 69% in the female at 90 days of age although decreased significantly in the female at 20 and 30 days of age compared to that in the male (Fig. 3).

At birth, 75–78% of the ID cells in both sexes showed positive signals in cell nuclei (Fig. 3). The percentage of immunoreactive ID cells slightly increased until 10 days, then gradually declined through 20 days. No sexual difference was observed at any ages examined (Fig. 3).

SD cells, composed of a layer of cuboidal and low columnar cells, began to appear at 5 days, when positive nuclear immunostaining was observed in 81–84% of them (Fig. 3). The percentage of immunoreactive cells was constant and showed no sexual difference at any ages examined (Fig. 3).

GCT cells, consisting of a layer of low columnar cells began to appear at 20 days and 80–83% of them were positively reacted (Figs. 2c, d and 3). Then GCT cells developed to tall columnar or pyramidal cells by 30 days (Fig. 2e, f). In the male, the percentage of immunoreactive cells in GCT increased to approximately 93% at 30 days, but then decreased to 66% by 60 days and remained at the level until 90 days. In contrast, the percentage of immunoreactive cells in the female decreased to 72% at 30 days and the level maintained until 90 days. Therefore, it became significantly lower in the male than in the female on days 60 and 90 (Fig. 2g, h and 3).

DISCUSSION

In the present study, the androgen receptor-immunoreactivity was observed only in cell nuclei in murine epididymis and submandibular gland (Fig. 1). This result showed that androgen receptor was localized primarily in cell nuclei, not in the perikaryal cytoplasm in the androgen target cells *in situ*. This finding is in agreement with previous studies in liver, kidney, brain, reproductive organs, and other tissues in rats [3, 14, 24, 25, 27, 35], mice [33] and human [27], and demonstrated that the androgen receptor, as well as the estrogen receptor [37], was a nuclear protein. In the rat prostate and epididymis, castration caused to decline a staining intensity of immunoreactable androgen receptor in cell nuclei of the epithelial cells within 7 days [24]. In the present study, however, castration induced no changes in androgen receptor-immunoreactivity in cell nuclei of both epithelial and stromal cells of epididymis for at least 7 days after operation (Fig. 1a, b), suggesting that a withdrawal pattern of immunoreactive androgen receptor by castration might be species specific.

The present study also demonstrated that in the submandibular gland, the most frequent appearance of nuclear androgen receptor-immunoreactivity was observed in the acini (Fig. 3). Moreover, the cell nuclei in ID, GCT and SD also contained positive immunoreactivity in adult male and female mice (Figs. 2 and 3). These results were in agreement with that of autoradiographic study of tritiated androgen binding by Morrel *et al.* [21]. The present study further examined developmental profiles and sex difference in the proportion of androgen receptor-immunoreactive cells among the four regions of the gland. In acini and ID, the proportion of the androgen receptor-immunoreactive cells was at high levels during early postnatal period of 0–15 days of age, and then decreased with age (Fig. 3). In these early ages, the binding activity of androgen receptor in the mouse gland was, however, lower than that in the adult gland [20, 34], and DHT could not influence on the neonatal gland [10, 17]. Whereas the binding affinity of the androgen receptors was constant after 20 days of age [20, 32], it was higher in the gland within 7 days of age than in the gland after puberty [20]. Thus, characteristics of the androgen receptors in the neonatal mouse gland are different from those in the postpubertal gland. Therefore, although the immunoreactive androgen receptor molecules are rich in the neonatal gland, they may

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not fully exert their abilities due to functional immaturity.

The percentage of androgen receptor-immunoreactive acinar cells was higher in the male gland than in the female gland at 20 and 30 days, but became rather lower in the male than in the female at 90 days (Fig. 3). The acinar cells contain amylase activity, which is increased by androgen [9, 11]. Therefore, it suggests that the androgen-dependent amylase activity in acini may be sexually different: more in the male at 20 and 30 days of age, and more in the female at 90 days.

In male mice, circulating levels of androgens (testosterone and DHT) drastically increased between days 20 and 30 [30], and sexual difference in the mouse gland was evident on day 30 by increasing in the relative portion of GCT to total glandular area and cell height of GCT [28]. The cytosolic androgen receptor in the gland increases during postnatal development [20, 34] and its maximum binding (Bmax) is significantly higher in the male than in the female on days 20 and 30 [32]. In the present study, GCT contained androgen receptor-immunoreactivity in cell nuclei, and percentage of the immunoreactive cells was higher in the male than in the female on day 30 (Fig. 3). These results suggest that around 30 days of age, male GCT can respond timely to a drastic increase in circulating androgen level to cause their masculine development by an increase of androgen-reactive cell population. In addition, the mitosis occurred more easily by androgen in male GCT than in female GCT around this age [30], when Bmax of the cytosolic androgen receptor was higher in the male gland [32]. On the other hand, Bmax of the cytosolic androgen receptor in the adult mouse gland on day 90 was rather higher in the female than in the male [32], and androgen-induced DNA synthesis was inferior in the male gland to that in the female gland [22]. As described before [31], circulating androgens, testosterone and DHT, act in their intact forms to increase the mitotic activity of GCT in the mouse gland. The sex difference in androgen responsiveness of GCT was demonstrated by this androgen receptor-immunoreactivity study (Fig. 3) as well as by the previous biochemical androgen binding study [32]. These results suggest that the occurrence of sexual dimorphism of the mouse gland may be correlated to sex difference in both levels of androgen receptors in GCT cells and of circulating androgens around 20 to 30 days of age.

The present study demonstrated that both ID and SD cells in the mouse gland had androgen receptor-immunoreactivity in their cell nuclei and no sex difference in the androgen-reactive population during observation period (Fig. 3). Roles of androgens on these duct regions are not yet clear, because their relative portions to total glandular area and their mitotic activities are neither sexually different nor affected by androgens [28–30].

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