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Immunohistochemical Expression of Inhibin-α Subunit in the Developing Rat Gonads

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ABSTRACT—In order to investigate the roles of inhibin for rat gonadal differentiation, the expression of inhibin-α subunit in the developing rat gonads was determined. Sprague-Dawley rat gonads from the gestational day (GD) 13 through the postnatal day (PD) 21 were fixed in Methacarn solution and immunohistochemically stained with a polyclonal antibody against [Tyr^{30}] porcine inhibin α-chain (1–30)NH_2 subunit raised in the goat. During fetal period, inhibin-α subunit was detected only in male gonads: slight or moderate staining was observed in Sertoli/supporting cells on GDs 14 and 15, and moderate or marked staining in Leydig cells from GD 17 to 20. Reactivity was negative in female gonads and in the Müllerian and Wolffian ducts in both sexes during fetal period. After birth, inhibin-α subunit was moderately expressed in Sertoli cells and slightly in Leydig cells on PD 21 for males, and markedly in the granulosa cells for females. These results indicate that the expression of inhibin-α subunit is stage- and cell-specific during the gonadal development and the inhibin may participate in rat testicular differentiation.

INTRODUCTION

Inhibin is a gonadal glycoprotein which regulates pituitary FSH secretion [3, 16, 24] and synthesis [27, 29]. The purification, cloning and sequencing of inhibin cDNA showed that inhibin is a heterodimer comprised of an α-subunit (18 KD) and one of two related β-subunits (14 KD, βA and βB) joined by a disulfide bond [2, 5, 8, 10, 19, 23]. After the purification of inhibin protein, it has been obvious that inhibin plays a variety of types of roles as hormone, paracrine and autocrine regulators of cellular proliferation and functions in some mammals [for reviews, 4, 7, 14, 28, 30].

Expressions of inhibin α- and β-mRNAs were seen by using *in situ* hybridization in the gonads of embryonic rats from gestational day (GD) 14 to birth [22]. Inhibin-subunits mRNAs were localized by the stage-specific and tissue-specific manners in the developing rat gonads: α-mRNA was seen in the seminiferous tubules and interstitial tissue from GD 14, βA-mRNA only in the interstitial tissue just before birth and βB-mRNA over the tubules from GD 14. Immunohistochemical localizations of α- and β-subunit proteins were exhibited in the germ, Sertoli and Leydig cells in fetal rat testes and the germ cells in the fetal ovaries [20]. These results indicate that inhibin may play roles for gonadal cell proliferation and differentiation, like the cases of immature and adult gonads.

In the present study, immunohistochemical expression of inhibin-α subunit was chronologically clarified in the fetal and prepubertal rat gonads from gestational day 13 to postnatal day 21.

MATERIALS AND METHODS

Experimental animals

Crj: CD (Sprague-Dawley) rats in 13 to 20 weeks of age were housed in constant temperature (22±2°C), relative homidity (55±10%) and light-dark cycle (lights on 7:00–19:00). The animals fed purina chow and took the tap water *ad libitum*. Cohabitation was done in the evening in the 1:1 basis of male:female. In the next morning, copulation was checked by the presence of sperm in the vaginal smear. The day when sperm-positive

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smear was found was designated as GD 0, and the day when litter was found was designated as postnatal day (PD) 0.

Preparation of tissues for immunostaining

Dams were sacrificed from GD 13 to 21 and neonates on PD 5, 11 and 21 by carbon dioxide. The gonads and genital ducts dissected from the fetuses and pups were fixed in Methacarn solution consisting of methanol, chloroform, and acetic acid, 6:3:1 in volume for a few hours to overnight. The sexes of fetuses were determined as described by Agelopoulou et al. [1]. Then, the tissues were dehydrated through a series of graded concentrations of ethanol and xylene, embedded in paraffin and sectioned in 5 μm thickness.

Immunohistochemistry

Sections were deparaffinized with xylene and hydrated in decreasing concentrations of ethanol, and incubated in 6 M urea (ICN Biomedicals Inc.) at room temperature for 30 min and to block endogenous peroxidases with 0.5% periodic acid (Sigma Chemical Co.) for 15 min. Sections were subsequently rinsed with 10 mM phosphate buffered saline (PBS, pH 7.4, Sigma Co.) for 20 min, blocked non-specific staining with 1.5% normal rabbit serum in 10 mM PBS including 0.5% casein (Wako Pure Chemical Industries Ltd.) for 20 min and then incubated with avidin and biotin blocking solution (Vector Laboratory Inc.) for 15 min, each at room temperature. After that, sections incubated overnight at 4°C with the polyclonal antibody against [Tyr^{30}] porcine inhibin α-chain (1–30)NH_2 raised in the goat (gifted from Prof. Sasamoto, Tokyo University of Agriculture and Technology) at a dilution of 1:40,000 in 10 mM PBS including 0.5% casein. Dose-response study indicated that this dilution of the antibody gave optimal labelling results. Following this incubation the sections were rinsed with PBS and then treated with 0.5% biotinylated rabbit anti-goat secondary antibody (Vector Lab. Inc. ABC-peroxidase staining kit Elite) diluted in 10 mM PBS containing 0.5% casein for 30 min at room temperature. Sections were again washed in PBS and subsequently incubated with 2% avidin-biotin complex (Vector Lab. Inc. ABC kit Elite) in 10 mM PBS for 60 min at room temperature. Avidin and biotin were prepared at least 30 min before applied to the sections to allow the complex to form. The sections were again washed in PBS, and the bound antibody was visualized with 0.05% 3,3′-diaminobenzidine tetrachloride (Sigma Chemical Co.) in 10 mM Tris-buffered saline (Sigma Chemical Co.) and 0.01% H_2O_2 for 4 min.

Controls included (a) replacing the primary antibody with normal goat serum, (b) using the primary antibody that had been pre-incubated overnight at 4°C with 1 μg/ml porcine inhibin-α subunit (1–32) (Peninsula Lab. Inc.) before this mixture was applied to the section in order to check the specificity of the primary antibody and (c) omitting the primary antibody to check the specificity of the secondary antibody.

RESULTS

Specificity of antibody

Preparations which were stained with the antibody to inhibin, with the immunoneutralized antibody, and with normal goat serum were shown in Fig. 1. Inhibin antibody stained the immature rat Sertoli cells on PD 21, but the neutralized antibody or normal goat serum did not stain any cells. Therefore, these results showed that this polyclonal antibody specifically stained inhibin-containing cells, because Sertoli cells might be regarded as the major source of inhibin in the immature male rat [see reviews].

Immunohistochemical localization

The immunohistochemical localizations of inhibin-α subunit in developing gonads were summarized in Table 1. The first positive staining was gained in most of the Sertoli cells on GD 14 (Fig. 2. A) and this sign was also seen in a few Sertoli cells on GD 15. At the second, the Leydig cells were positively stained from GD 17 to PD 5. The intensity of staining was marked on GDs 17 (Fig. 2. B) and 18 and gradually decreased with development. The number of the Leydig cells with positive reaction was also decreased after GD 20. However, female gonads and mesonephric tubules, Müllerian and Wolffian ducts in both

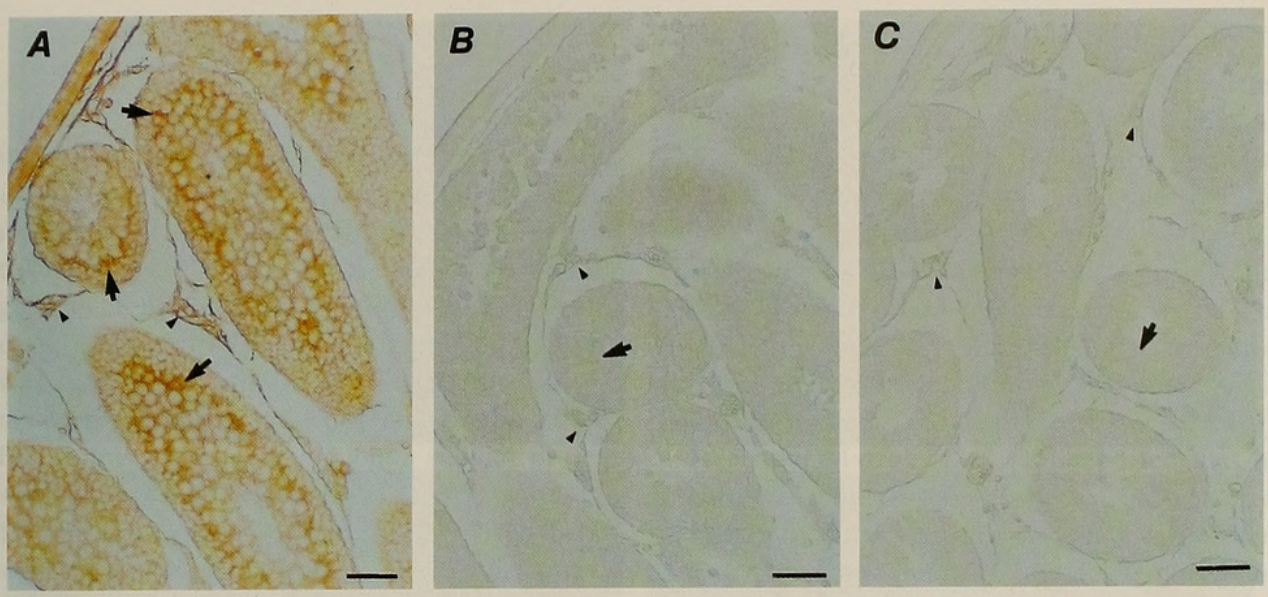

FIG. 1. Demonstration of staining specificity. Male gonads on PD 21 incubated with the inhibin-α antibody (A), with the neutralized antibody (B) or with normal goat serum (C). Staining is seen in the Sertoli cells (arrow) and the Leydig cells (arrow head) of A, but not those of B or C. Bars: 50 μm.

TABLE 1. Immunohistochemical localization of inhibin-α in the developing rat gonads

	Male							Female						
	Gonad				Duct		MT	Gonad				Duct		MT
	G	S	P	L	W	M		G	Gr	T	I	W	M	
GD 13	-	-			-		-	-				-		-
14	-	++			-	-	-	-	-		-	-	-	-
15	-	+		-	-	-	-	-	-		-	-	-	-
16	-	-		-	-	-	-	-	-		-	-	-	-
17	-	-		+++	-		-	-	-		-	-	-	-
18	-	-	-	+++	-		-	-	-		-	-	-	-
19	-	-	-	++/+	-			-	-		-		-	
20	-	-	-	++	-			-	-		-		-	
21	-	-	-	+/-	-			-	-		-		-	
PD 5	-	-	-	+/-	-			-	-	-	-		-	
11	-	-	-	-	-			-	-	-	-			
21	-	++	-	+/-	-			-	+++	-	-			

GD: Gestational day, PD: Postnatal day, G: Germ cell, S: Sertoli/supporting cell, P: Peritubular cell, L: Leyding cell, W: Wolffian duct, M: Müllerian duct, MT: Mesonephric tubule, Gr: Granulosa/supporting cell, T: Theca cell, I: Interstitial/stromal cell, Grade, −: No detectable, +: Slight but above background levels, ++: Moderate, +++: Marked staining, Shade box was shown that the cells or tissues were not found in that day.

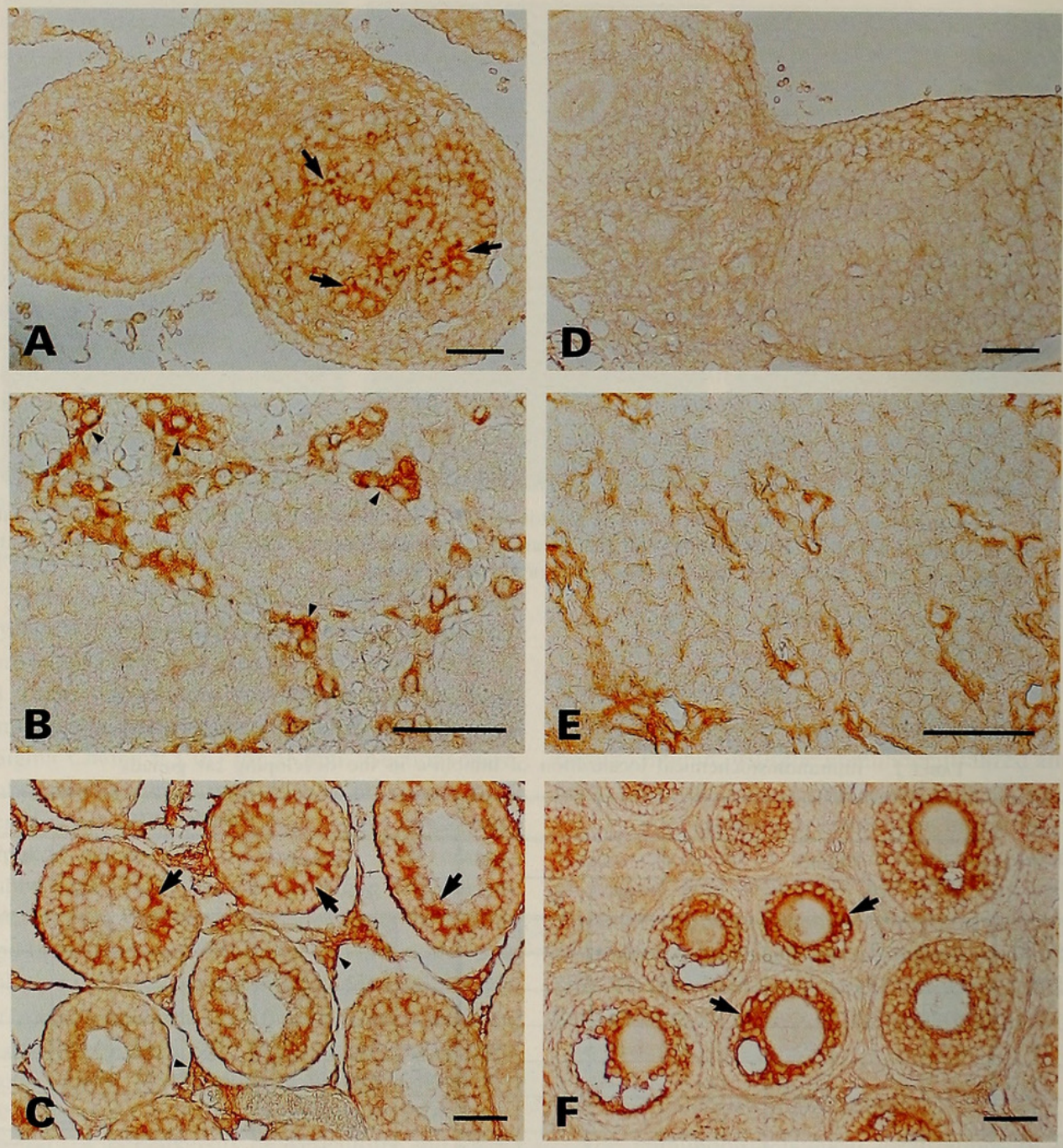

FIG. 2. Immunohistochemical localizations of inhibin-α in the perinatal gonads. (A) Male gonad on GD 14 shows the moderate staining in the Sertoli cells (arrow). (B) Male gonad on GD 17 expresses the marked staining in the Leydig cells (arrow head). (C) Male gonad on PD 21 is stained moderately in the Sertoli cells (arrow) and weakly in the Leydig cells (arrow head). (D) and (E) Female gonads on GDs 14 and 17, respectively, do not express any positive sign. (F) Female gonad on PD 21 expresses the marked reactivity in the granulosa cells (arrow). Bars: 50 μm.

sexes were negative during prenatal period (Fig. 2. D and E).

During postnatal period, positive staining was observed in most of the Sertoli cells for male gonads and the granulosa cells for female gonads on PD 21 (Fig. 2. C and F), and the intensity of staining was moderate or marked. The slight and/or faint staining was found in the Leydig cells on PDs 5 and 21. However, there were no detectable staining to the antibody in any other cells.

DISCUSSION

Pattern of immunohistochemical expression of inhibin-α subunit was partially consistent with the *in situ* identification of its mRNA [22]. Inhibin-α mRNA was localized in the tubular and interstitial tissues of male gonads during the third trimester gestational period from GD 14 to GD 21. Ogawa et al. [20] reported that inhibin-immunoreactivity was seen in the tubular and interstitial cells for males and the germ cells for females on GD 18.5. But in this experiment, inhibin-α subunit protein was only expressed in Sertoli cells on GDs 14 and 15, and in Leydig cells from GD 17. This inconsistency might be depend on the applicaiton of different types of antibodies, because antigens used were [Tyr^{20}] inhibin-α subunit (1–20) by Ogawa *et al.* [20] but [Tyr^{30}] porcine inhibin α-chain (1–30)NH_2 in our experiment.

Positive reactions in Sertoli cells on GDs 14 and 15 suggest that inhibin might be related to the testicular differentiation of the gonad, especially to the formation of seminiferous tubules and aggregation of the Sertoli cells around the germ cells like activin-A and -B, the homodimers of inhibin-βA and -βB subunits, in immature rat testis [13] because the sexual differentiation of gonad begins from the late GD 13 [11, 12, 17]. However an elucidation of the exact role of inhibin and/or inhibin-α subunit for the seminiferous tubule formation or other developmental events will require further investigation.

This is the first report of the positive staining in the fetal Leydig cells from GD 17. Immunoreactivity in purified Leydig cells which prepared from adult rats had been shown by Sharpe *et al.* [25] and Risbridger *et al.* [21]. Sharpe et al. [25] reported that Leydig cells in adult rats made little contribution to the intratesticular and blood levels of inhibin. However, inhibin modulates LH-induced androgen biosynthesis by testicular cells [9]. Leydig cells have synthetic ability of steroid hormones during the late gestation period. Therefore, inhibin expression in the fetal Leydig cells after GD 17 indicates that inhibin might modulate to the steroidogenesis in the fetal Leydig cells. Further study is needed to elucidate the role of inhibin on steroidogenesis and/or other physiological roles in the fetal Leydig cells.

Postnatal expression of inhibin in the Sertoli and granulosa cells was consistent with many previous works [see reviews and 18]. Inhibin contributes to negative feedback loop of FSH secretion from the pituitary in the immature rats. And also inhibin is concerned with the paracrine roles for testicular and ovarian functions: steroidogenesis in the Leydig and thecal cells, increase in the number of follicles, and germ cell-Sertoli cell interaction in immature and mature rats [see reviews and 6, 26].

Recently, Matzuk *et al.* [15] reported that inhibin-α was a tumor-suppressor gene of the gonadal stromal tumors by using inhibin-α-deficient mice. Mice homozygous for the deleted α-subunit gene were susceptible to the development of differentiated gonadal stromal tumors as early as 4 weeks of age, but were normal for the development of the gonads and external genitalia before tumor development by histological examination. Therefore, it is suggested that other factors may be participated in the sexual differentiation of the gonads.

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