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Localization of Ovarian Inhibin/Activin Subunits in Follicular Dominance during the Estrous Cycle of Guinea Pigs

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ABSTRACT—The cellular localization of inhibin α , β_A and β_B subunits in cyclic ovaries of the guinea pig was investigated. The immunoreactivity of inhibin α , β_A and β_B subunits was localized to the granulosa cells of some large healthy follicles in each ovary throughout the estrous cycle. The number of follicles that stained was in accordance with the number of offspring typical in guinea pigs. Inhibin β_B was also localized to the granulosa cells of small antral follicles on Day 4. There were two kinds of staining patterns for inhibin α , β_A and β_B subunits on Day 12: strongly stained follicles identical to those observed on Days 8 and 16, and weakly stained follicles that showed atresia in hematoxylin and eosin (HE) stained sections. Two types of ovarian cysts were found throughout the estrous cycle in this experiment: serous cysts and follicular cysts. The incidence of serous cysts and follicular cysts were 64% and 24% of animals, respectively. There was no positive reaction for inhibin α , β_A and β_B subunits in the corpora lutea, other follicles or any kind of ovarian cyst during the estrous cycle. These results confirm that only dominant follicles stain positively for inhibin α and β_A subunits and are in agreement with the phenomenon that the follicular development of guinea pigs shows two waves of growth. This study is also the first to describe the ovarian cysts during the estrous cycle in guinea pigs systematically.

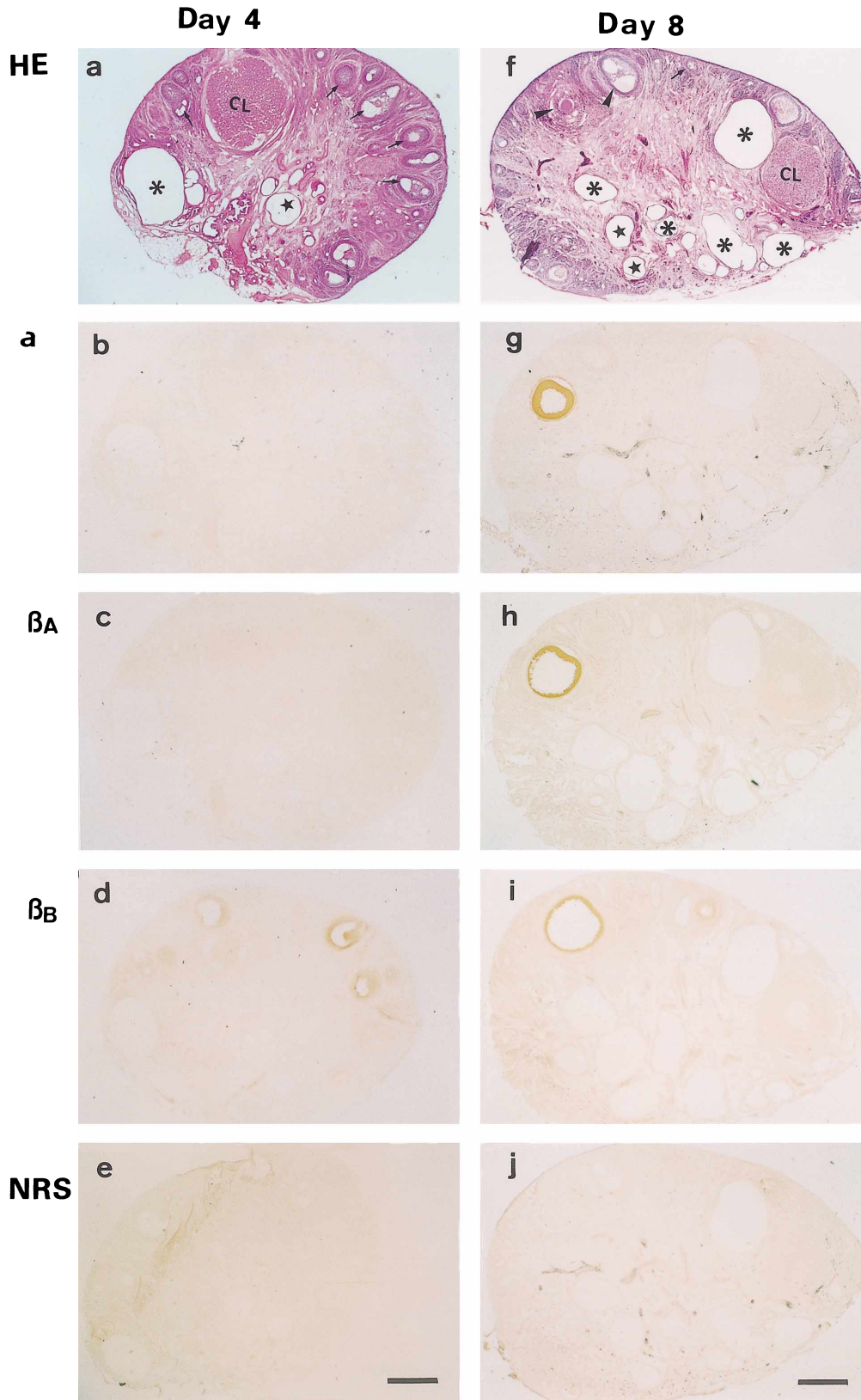
INTRODUCTION

Inhibins and activins are glycoproteins that belong to the transforming growth factor- β (TGF β) superfamily (Kingsley, 1994) and, as such, have direct and indirect effects on granulosa and theca cells that can modulate follicular development and steroidogenesis. Inhibins and activins play a very important role in determining the different fates (whether dominance or atresia) of the selected and non-selected follicles that develop in the same hormonal environment (Roche, 1996). There is evidence to suggest that the maintenance of dominance is effected by intra-ovarian paracrine signaling (Hillier, 1981) with inhibins and activins acting as important paracrine messengers (Hillier, 1991). Development of the dominant follicle is characterized by the secretion of increasingly large amounts of estradiol and inhibin A into the circulation in humans (Lockwood *et al.*, 1998). In guinea pigs, as in most mammals,

many follicles start to develop simultaneously, but most become atretic. During the luteal phase of the cycle, two waves of follicular growth are initiated, and although a few in each wave gain dominance, most follicles ultimately become atretic (Bland, 1980; Hutz *et al.*, 1990). The dominant follicle(s) in the wave produced toward the end of the luteal phase ovulate(s). The follicular dominance process is poorly understood at present. Our previous reports showed that inhibin subunits were only localized by immunohistochemistry to the granulosa cells of dominant follicles in the guinea pig ovary before ovulation (Shi *et al.*, 1999, 2000a). However, when and how the follicular dominance process in guinea pigs happen has not been documented. Therefore, further characterization of follicular growth and dynamic studies of the immunohistochemical localization of inhibin subunits during the estrous cycle in guinea pigs are required.

It is reported that cysts in the guinea pig ovaries are very easy to induce (Quattropiani, 1977; 1981; Keller *et al.*, 1987; Quandt and Hutz, 1993). Silva *et al.* (1997, 1998) have established the guinea pig as an excellent animal model to study ovarian neoplasms in humans, and some researchers have

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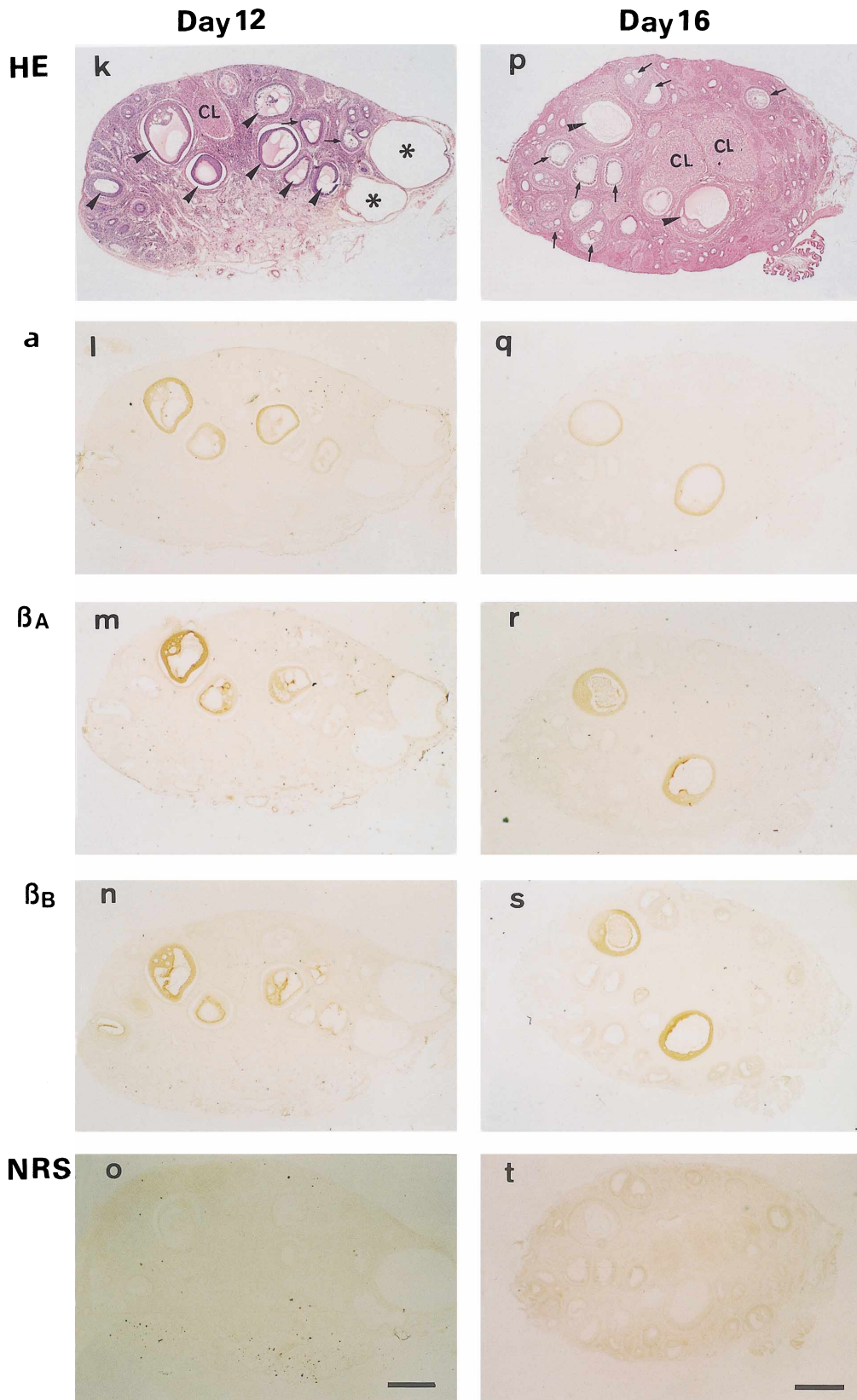


Fig. 1. Immunohistochemical staining of inhibin subunits in guinea pig ovary during the estrous cycle. Sections were stained with hematoxylin and eosin (HE) (a,f,k,p). Various sections were stained with anti-inhibin α -subunit serum (b,g,i,q), anti-inhibin β_A subunit serum (c,h,m,r), anti-inhibin β_B subunit serum (d,i,n,s), and normal rabbit serum (NRS) (e,j,o,t). Sections were stained from ovaries on Days 4 (a,b,c,d,e), 8 (f,g,h,i,j), 12 (k,l,m,n,o) and 16 (p,q,r,s,t). Arrowheads represent large antral follicles; arrows represent small antral follicles; *, serous cyst; , follicular cyst; CL, corpora lutea. Bar=1 mm.

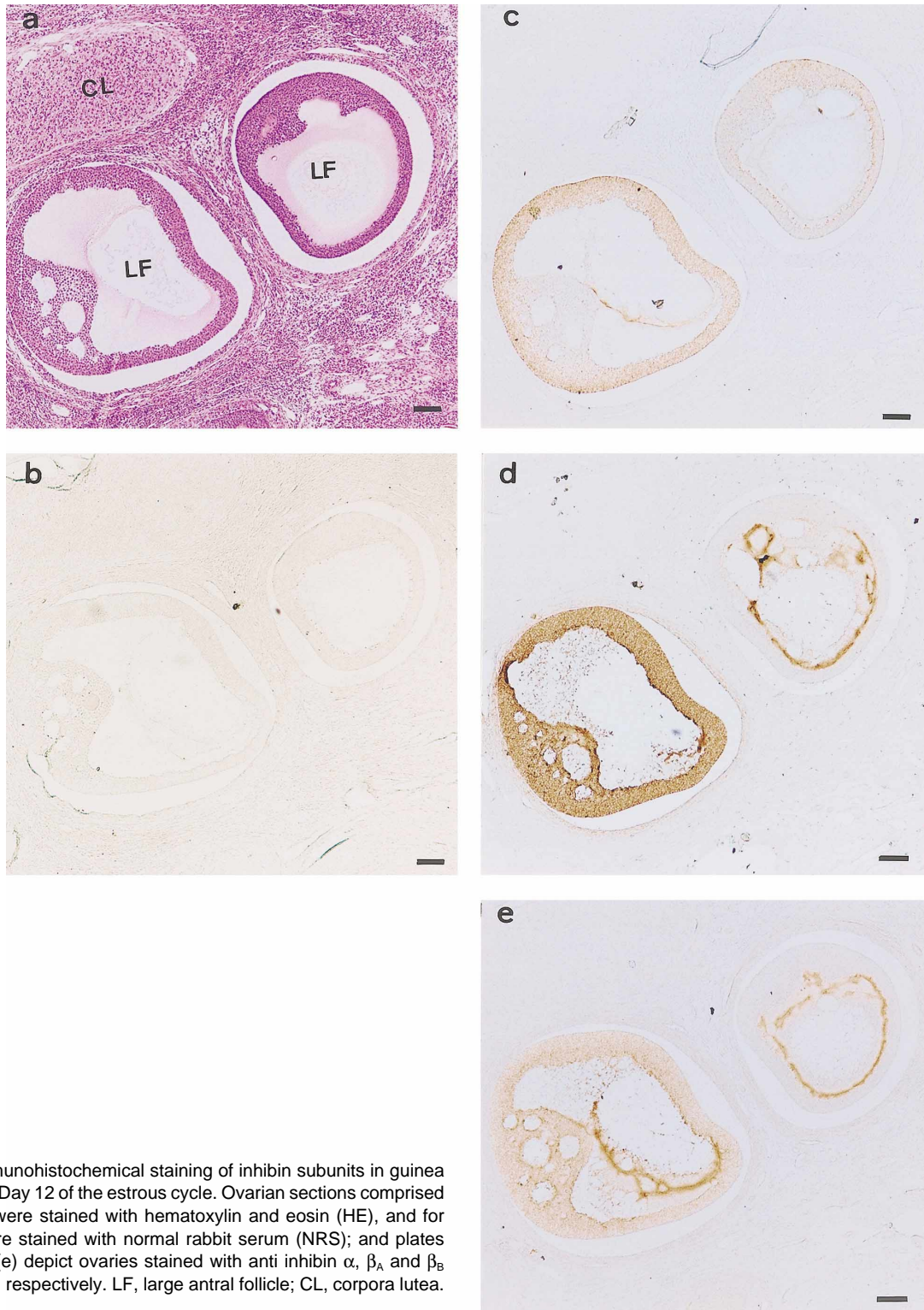


Fig. 2. Immunohistochemical staining of inhibin subunits in guinea pig ovary on Day 12 of the estrous cycle. Ovarian sections comprised in plate (a) were stained with hematoxylin and eosin (HE), and for plate (b) were stained with normal rabbit serum (NRS); and plates (c), (d) and (e) depict ovaries stained with anti inhibin α , β_A and β_B subunit sera, respectively. LF, large antral follicle; CL, corpora lutea. Bar=100 μm .

suggested that the cystic ovary in guinea pigs is a good model for studying polycystic ovarian syndrome (PCOS) in humans, although cyst incidence and induction by estradiol may be strain specific (Quandt and Hutz, 1993; Campion *et al.*, 1996). However, it is still lack of study in ovarian cysts throughout the estrous cycle in guinea pigs until now.

The aim, then, of this project is to determine the cellular localization of inhibin α , β_A and β_B subunits in cyclic and cystic ovaries of guinea pigs.

MATERIALS AND METHODS

Animals and the sample collection

Twenty-five adult female guinea pigs (*Cavia porcellus*) of the Hartley strain were used at 3-6 months of age, the same as in our previous report (Shi *et al.*, 1999). They were housed under controlled lighting (lights on 05:00–19:00hr), and provided with commercial food pellets and tap water *ad libitum*. Estrous cycles were recorded by daily examination of the vaginal membrane and smears were taken by lavage whenever the vagina was open. The day of ovulation was estimated as the day when maximal cornification was seen in the smear before the ovulatory influx of leukocytes, and was designated as Day 0 of the cycle (Norris and Adams, 1979; Lilly *et al.*, 1997). Days of the estrous cycle followed thereafter consecutively. The normal estrous cycle in the guinea pig has a mean length of 16.1±0.2 days with a range of 13–22 days (n=25). We used only animals showing at least 2 consecutive 15–17 day cycles just prior to the experiment. Groups of animals were necropsied at 4 day interval (5 animals per day). For characterization of cysts in ovaries during the estrous cycle, one ovary of each animal was analyzed histologically. For determination of the cellular localization of inhibin α , β_A and β_B subunits in the ovaries, another one ovary of each animal was analyzed immunohistochemically. Bloods were collected into heparinized centrifuge tubes after intracardiac method sampling under ether anesthesia at 10:00–12:00 hr of the various days after estrus.

Immunohistochemistry

Ovaries were removed immediately after necropsy and fixed in 4.0% paraformaldehyde solution at room temperature overnight. After fixation, the ovaries were sectioned as in our previous reports (Shi *et al.*, 1999). Cystic ovaries were sectioned through the cyst wall to the thickest part of the parenchyma in an attempt to identify functional ovarian tissue. Sections were used for immunohistochemical localization of inhibin subunits as in our previous reports (Otsuka *et al.*, 1997; Nagata *et al.*, 1998; Shi *et al.*, 1999). Briefly, sections (6 μ M) were cut and mounted on slides coated with poly-L-lysine. The slides were dried, then deparaffinized with xylene and rehydrated in graded ethanol before washing in water. To expose epitopes sections were autoclaved for 15 min at 121°C in sodium citrate buffer (0.01 M, pH 6.0). The sections were incubated in 3% H₂O₂ in methanol at 32°C for 30 min to reduce endogenous peroxidase activity and then followed by 0.5% casein-tris saline (CTS: 0.05 M Tris-HCl with 0.15 M NaCl, pH 7.6) at 37°C for 1 hr to quench nonspecific staining. Then the sections were incubated for 24–36 hr at 4°C with polyclonal antibodies against inhibin subunits. The antibodies against each inhibin subunit were: anti-[Tyr 30] porcine-inhibin α -chain (1–30)-NH₂ conjugated to rabbit serum albumin, anti-inhibin β_A (81–113)-NH₂ (#305–24-D) and anti- β_B (80–112)-NH₂ (#305-25-D) (kindly provided by Dr. W. Vale, The Salk Institute for Biological Studies, La Jolla, CA, U.S.A.). Binding sites of antibodies were visualized by ABC Kit Elite, and 0.05% 3, 3'-diaminobenzidine tetrachloride (Sigma Chemical Co. St. Louis, MO, U.S.A.) in 10 mM tris-buffered saline containing 0.01% H₂O₂ for 1 min. Specificity of the antibodies was examined using normal rabbit serum instead of primary antibody. In order to identify the cell types within the ovary, the adjacent serial section was stained with hematoxylin and eosin (HE).

Evaluation of immunostaining sites

The percentages of area in the granulosa cells and other cells that stained positive for the inhibin subunit were determined by analyzing sections of ovary during the estrous cycle. The images were captured in a personal computer (NEC, Tokyo, Japan) using a Nikon photomicroscope with a \times 40 objective (Nikon, Tokyo, Japan), and the areas were determined using an image analysis system with a Graphic Digitize (PIAS, Osaka, Japan). The repeatability of measurements, expressed as the coefficient of variation for ten measurements of a positive area, was 1.94% at 10 mm² and 1.62% at 100 mm², respec-

tively. The percentages of area of granulosa cells that stained positive for inhibin subunits more than 50% are considered strongly positive (++), between 10–50% are considered as positive (+), and less than 10% are considered as negative (–).

Criterion for cysts

Ovarian morphology provides diagnostic criteria in cystic ovaries in this experiment (Quattropani, 1977; 1981; Keller *et al.*, 1987). Follicular cysts are large with a thin wall made up of one or several layers of granulosa cells, have an increased amount of fluid, and do not contain an oocyte. Serous cysts are lined with a simple epithelium that varies from low cuboidal to columnar, usually characterized by ciliated epithelium, solitary cilium or a tuft of cilia.

Radioimmunoassays for inhibin, estradiol, progesterone, testosterone, FSH and LH

Plasma concentrations of inhibin were measured using a rabbit antiserum against bovine inhibin (TNDH-1) and ¹²⁵I-labeled 32 kDa bovine inhibin (Shi *et al.*, 1999). The inhibin antiserum (TNDH-1) displayed no significant cross-reaction with LH, FSH and prolactin of rats, cattle and sheep, GnRH, transforming growth factor β or activin, whereas the antiserum cross reacts with inhibin Pro α C and free inhibin α -subunit (Kaneko *et al.*, 1995). Results were expressed in terms of 32 kDa bovine inhibin. The intra- and inter-assay coefficients of variation were 10.8 and 12.2%, respectively. Plasma concentrations of estradiol, progesterone and testosterone were determined by double-antibody RIA system using ¹²⁵I-labeled radioligands (Taya *et al.*, 1985). Antisera against estradiol (GDN 244), progesterone (GDN 377) and testosterone (GDN 250) were kindly provided by Dr. G. D. Niswender (Animal Reproduction and Biotechnology, Colorado State University, Fort Collins, CO, U.S.A.). The intra- and inter-assay coefficients of variation were 5.8 and 11.4% for estradiol, 3.5 and 13.4% for progesterone and 6.5 and 8.2% for testosterone, respectively.

Concentrations of plasma FSH were measured by heterologous

Table 1. Immunohistochemical staining of inhibin subunits in guinea pig ovaries

	Inhibin subunits											
	α				β_A				β_B			
Days of estrous cycle	4	8	12	16	4	8	12	16	4	8	12	16
Preantral follicle	–	–	–	–	–	–	–	–	–	–	–	–
Small antral follicle	–	–	–	–	–	–	–	–	+	+	–	–
A few large antral follicles	–	++	+	++	–	++	+	++	–	++	+	+
Other large antral follicles	–	–	–	–	–	–	–	–	–	–	–	–
Corpus luteum	–	–	–	–	–	–	–	–	–	–	–	–
Cysts	–	–	–	–	–	–	–	–	–	–	–	–

The immunohistochemical staining was determined as positive (+), strongly positive (++) or negative (–).

double-antibody RIA using NIDDK RIA kit for rat FSH as described previously (Shi *et al.*, 1999). Iodinated preparation was FSH-I-5, and the antiserum used was anti-rat FSH-S-11. Results were expressed in terms of NIDDK rat FSH-RP-2. The intra- and inter-assay coefficients of variation were 4.8% and 6.8%, respectively.

Concentrations of plasma LH were measured by heterologous double-antibody RIA using an anti-bovine LH monoclonal antibody (#5; kindly provided by Dr. J. F. Roser, University of California, Davis, USA), rat LH-I-5 (provided by NIDDK RIA kit) for radiiodination and highly purified guinea pig LH (kindly provided by the Wisconsin Re-

Table 2. Numbers of follicles stained positively for inhibin α and β_A subunits in the guinea pig ovary during the estrous cycle

Days of estrous cycle	4	8	12	16	
Staining character ^{a)}	+,++	++	+	++	++
Number ^{b)}	0	2.3±0.4	2.5±0.3	2.2±0.5	2.2±0.5

^{a)}: The immunohistochemical staining was determined as positive (+), strongly positive (++) or negative (-) ^{b)}: Each value represents the mean \pm SEM of five animals.

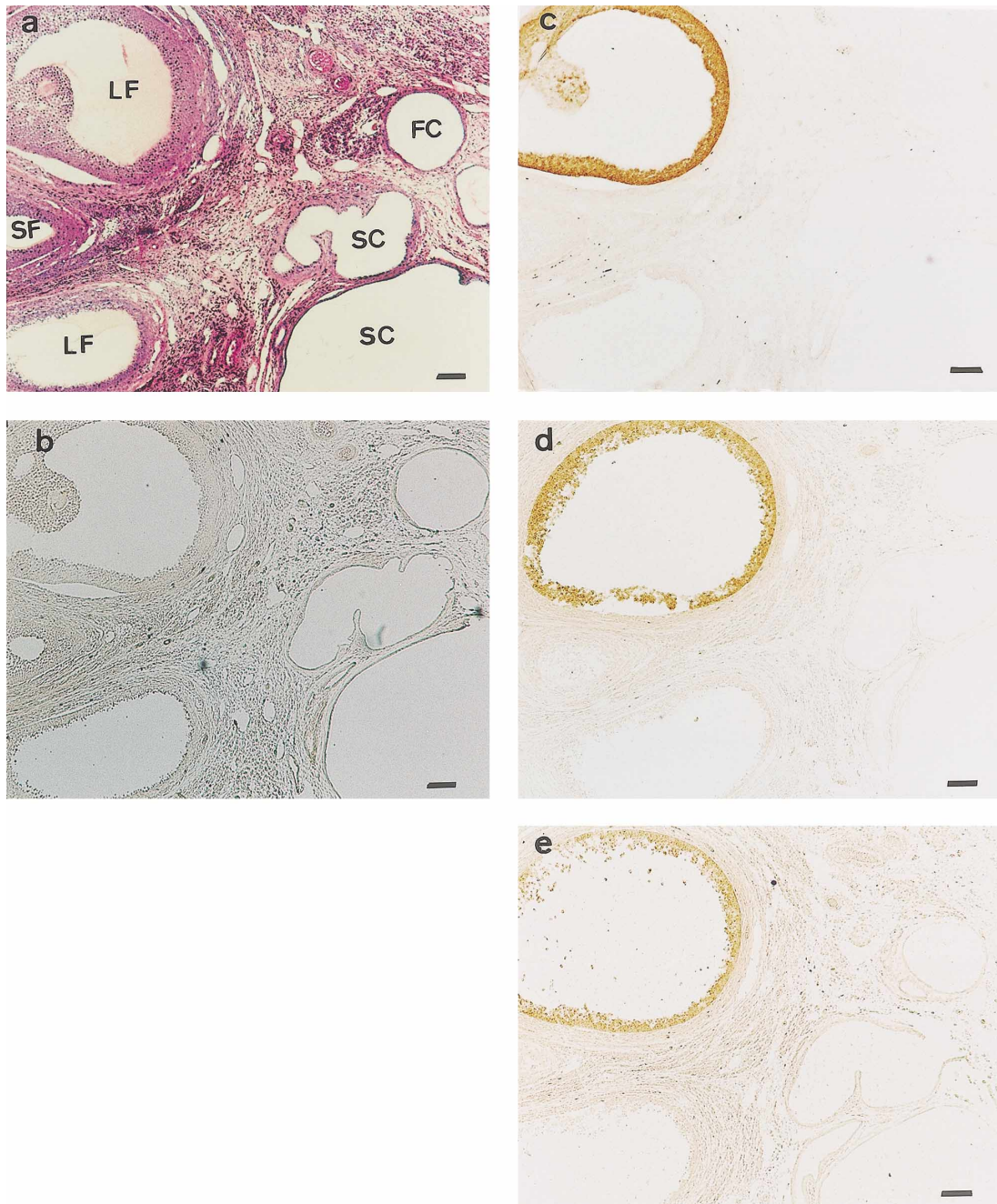


Fig. 3. Immunohistochemical staining of inhibin subunits in cystic ovaries of the guinea pig on Day 0 of the estrous cycle. Ovarian sections comprised in plate (a) were stained with hematoxylin and eosin (HE), and plate (b) were stained with normal rabbit serum (NRS); and plates (c), (d) and (e) depict ovaries stained with anti-inhibin α , β_A and β_B subunit sera, respectively. LF, large antral follicle; SF, small antral follicle; SC, serous cyst; FC, follicular cyst. Bar=100 μ m.

gional Research Center, Madison, WI, USA) as reference standard (Matteri *et al.*, 1987). The assay sensitivity was 150 ng/ml. The intra- and inter-assay coefficients of variation were 7.8 and 10.2%, respectively.

Statistics

All data were expressed as mean±SEM. When a significant effect was obtained with one-way ANOVA, the significance of the difference between two means was analyzed by using Duncan's multiple-range test (Steel and Tottie, 1960). A value of P<0.05 was considered to be statistically significant.

RESULTS

Immunohistochemistry of the cyclic ovaries (Fig. 1, 2, Table 1, 2)

On day 4 after ovulation, small antral follicles were posi-

tively stained for inhibin β_B subunits, but not for inhibin α or β_A subunits. On Day 8, the granulosa cells of some large healthy follicles in each ovary were clearly stained for inhibin

Table 3. Incidence of ovarian cysts in guinea pig ovaries during the estrous cycle

Ovary	Normal	Cyst		
		serous	follicular	serous and follicular
Number	8/25	11/25	1/25	5/25
incidence (%)	32	44	4	20

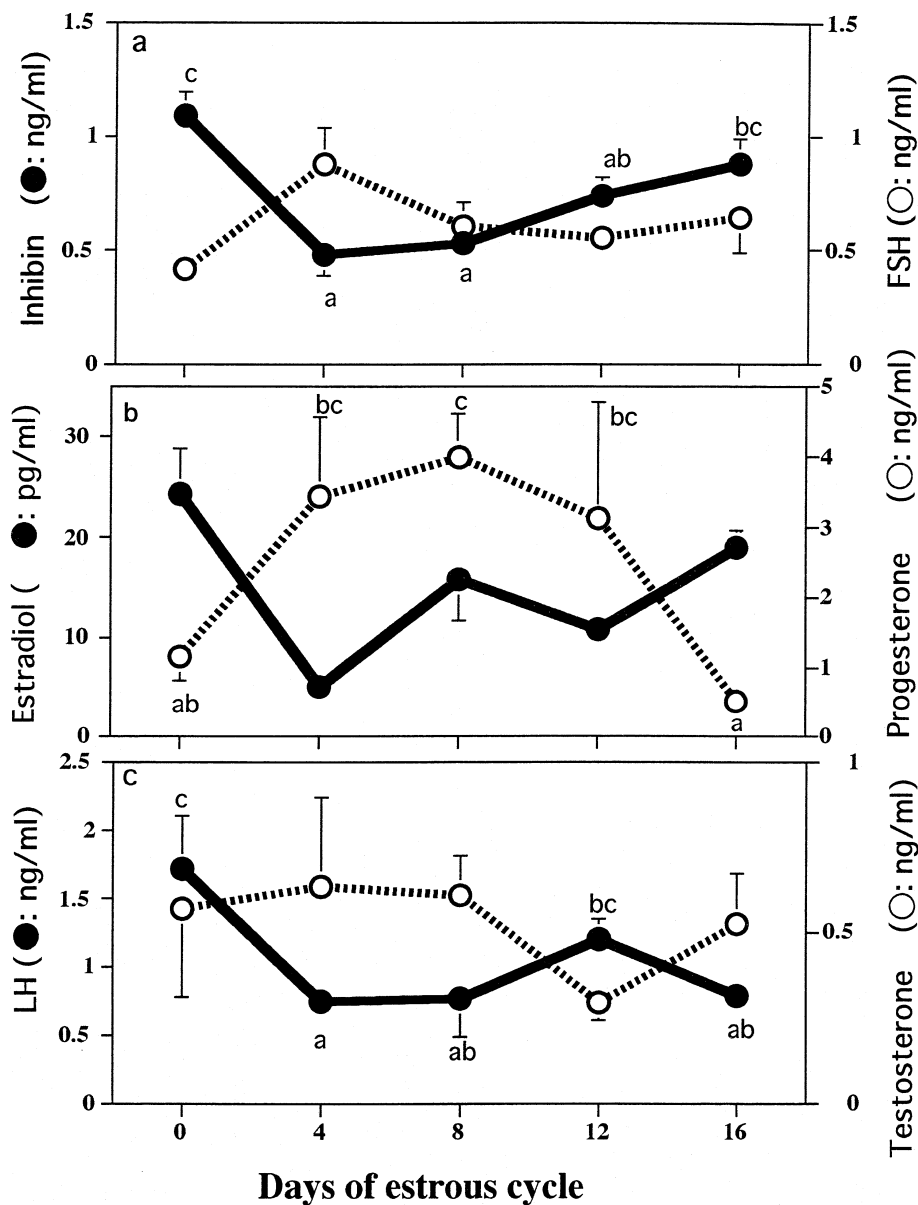


Fig. 4. Changes in concentrations of inhibin, FSH (a), estradiol, progesterone (b), LH and testosterone (c) during the estrous cycle in guinea pigs. Each value represents the mean±SEM of five animals. Error-bars within the circle are not shown. Points with no common letters indicate a significantly different values (P<0.05) (Duncan's multiple-range test).

α , β_A and β_B subunits. Similar results were observed on Day 16 (Fig. 1, Table 1) and Day 0 (data not shown). On Day 12, there were two kinds of staining characteristics for inhibin α , β_A and β_B subunits: strongly stained follicles identical to those observed on Days 8 and 16, and weakly stained follicles which appeared atretic in histology sections in which the rate of pyknotic granulosa cell nuclei was more than 5% evaluated (Shi *et al.* 2000b, Fig. 2). The number of follicles strongly stained was in accordance with the numbers of offspring in guinea pigs (Table 2). There were no positive reactions for inhibin α , β_A and β_B subunits in corpora lutea or in other follicles on any days of the estrous cycle.

Incidence of cystic ovaries and immunohistochemistry of cystic ovaries

Two types of cystic ovaries were observed. Serous cysts arise from the rete ovarii and are characterized by ciliated epithelium (Quattropani, 1977, 1981). Serous cysts were present in 64% (16/25) of our normally cycling animals. Follicular cysts were characterized by several thin layers of granulosa cells (Keller *et al.*, 1987). Follicular cysts were present in 24% (6/25) of our normally cycling animals. Thus, serous cysts were more common than follicular cysts (Table 3). The follicular cysts always coexisted with serous cysts and located at the area closed to serous cysts (Fig. 1, Table 3). Only a few follicles stained positively for inhibin subunits in each of the cystic ovaries (Fig. 3). This is in agreement with what we observed in normal ovaries. There were no positive reactions for inhibin α , β_A and β_B subunits in serous cysts or follicular cysts (Table 1, Fig. 3).

Changes in the plasma concentrations of FSH, LH, inhibin, estradiol, progesterone and testosterone during the estrous cycle

At ovulation (Day 0), plasma FSH was low but the inhibin and estradiol concentrations were high (Fig. 4). After ovulation, plasma FSH increased but inhibin and estradiol decreased. On Day 4, plasma inhibin and estradiol were at their nadirs. Plasma progesterone was low on Days 16 and 0, and high on Days 4, 8 and 12. Plasma LH was high on Day 0, decreased after ovulation and increased again on Day 12.

DISCUSSION

This is the first paper to show immunohistochemical localization of inhibin α , β_A and β_B subunits to cyclic ovaries of the guinea pig throughout the estrous cycle. We show that only a few large healthy follicles were positively stained for inhibin α and β_A subunits throughout the estrous cycle, and the number of follicles stained was in accordance with the numbers of offspring in guinea pigs. This certainly suggests that these follicles may be dominant follicles.

We also observed that there were inverse relationships between plasma FSH and inhibin (Fig. 4). FSH is the key hormone stimulating the emergence of waves of follicles and its decline is associated with the selection of dominant follicles.

As the dominant follicles emerge, the increasing production of inhibin and estradiol could decrease FSH secretion and so restrict the growth of non-dominant antral follicles. The dominant follicles could survive this decrease in FSH by the local action of factors such as inhibin, activin and IGF-1; these substances might sensitize the follicular cells to gonadotropin stimulation, thereby compensating for the decrease in FSH. On the other hand, non-dominant follicles would not have these survival mechanisms and would therefore be destined to become atretic (Findlay, 1993; Kaneko *et al.*, 1995).

In the present study, on day 4 after ovulation, small antral follicles were positively stained for inhibin β_B subunits. However, there was no positive staining for inhibin α or β_A subunits in the ovary on Day 4. This might suggest that activin B has some function on FSH secretion (Sugino *et al.*, 1988) and was in accordance with the low concentrations of plasma inhibin.

It has been reported that Day 12 represents a transitional stage when animals varied in their ability for compensatory ovulation (Hermreck and Greenwald, 1964). The follicles that will ovulate during a specific estrous cycle are usually set aside by day 12 in guinea pigs. Also at that time, regression of the corpora lutea occurs, follicular atresia has already begun, and follicles exceeding 700 μm in diameter begin to develop. Day 12, thus, seems to be a critical period in the development of the dominant follicle during the guinea pig estrous cycle (Hermreck and Greenwald, 1964; Bland, 1980; Hutz *et al.*, 1990). Our morphological (Hutz *et al.*, 1990) and endocrinological (Shi *et al.*, 1999) studies have confirmed that the follicular development of guinea pigs shows two waves of growth (Bland *et al.*, 1980). The report of two different staining characteristics for inhibin α , β_A and β_B subunits in different follicles on Day 12 of this experiment is in agreement with the phenomenon of follicular development in guinea pigs manifesting two waves previously reported (Bland, 1980; Hutz *et al.*, 1990). Additionally, atretic follicles usually showed no reaction with inhibin antisera. Only dominant follicles at the early stage of atresia, on Day 12 of the estrus cycle, were positively stained for inhibin α , β_A and β_B subunits. This is different from other rodents in which even the granulosa cells of atretic follicles are positively stained (Meunier *et al.*, 1988).

An important obstacle in the investigation of the development of ovarian epithelial neoplasms has been the lack of an animal model in nonhuman species (Moulton, 1978). The characteristics of serous cysts in guinea pigs is very similar to epithelial ovarian neoplasms, as epithelial cells are cylindrical, and many of the cells have cilia in the apical border. The results of this experiment support the supposition that guinea pigs can be used as animal models for epithelial tumors of the human ovary (Silva *et al.*, 1997; 1998). But many ovarian cysts in guinea pigs evidently develop spontaneously rather than as the result of artificial stimulations. This is similar to some reports (Quattropani, 1977; 1981; Keller *et al.*, 1987), but different from others (Moulton, 1978; Silva *et al.*, 1997; 1998). This difference may be due to strain specificity.

Our results also show that there exist large antral follicles

that stain for inhibin α , β_A and β_B subunits within cystic ovaries in the guinea pig. Polycystic ovarian syndrome (PCOS) is a human disorder that causes infertility due to chronic anovulation or oligo-ovulation. It affects 2.5–7.5% of the female population (Dahlgren and Janson, 1994). PCOS is a disorder in which the dominant follicle does not develop normally, but rather becomes cystic. These cysts prevent the normal development and ovulation of other follicles. In this experiment, we found that cystic ovaries are common in guinea pigs. In addition, guinea pigs with cystic ovaries still cycled normally and had large antral follicles that stained positively for inhibin α , β_A and β_B subunits. In this manner, then, it is likely that the guinea pig is more similar to the cow than human. In the cow, cysts are dynamic structures, most of them regress over time and are replaced during subsequent follicular waves (Takagi *et al.*, 1998). This suggests that the cystic follicles in guinea pigs may serve as a good model for the study of cystic follicles in cows.

The lack of staining for inhibin α , β_A and β_B subunits in serous cysts and follicular cysts in ovaries of guinea pigs were distinct from granulosa cell tumors and ovarian cancers. It is reported that the immunohistochemical staining for the inhibin subunits is predominantly in granulosa cell tumors and in the majority of mucinous cancers (Burger *et al.*, 1996; 1998). In the present study, the follicular cysts always coexisted with serous cysts and located at the area closed to serous cysts. It may suggest that the serous cysts have influence on follicular developments in guinea pigs. This is in accordance with a report in which the serous cysts only affected the reproductive capacity in old guinea pigs (Keller *et al.*, 1987).

In conclusion, the results show that only large antral follicles are positively stained with inhibin α and β_A subunits throughout the estrous cycle in guinea pigs. These positively staining follicles are presumably dominant follicles. The results of this experiment support the supposition that guinea pigs can be used as animal models for epithelial tumors of the human ovary. The results also suggest that there exist dominant follicles even in cystic ovaries, which distinguishes this paradigm from PCOS in humans, and allows for apparent ovulation. The results of the lack of staining for inhibin α , β_A and β_B subunits in ovarian cysts of guinea pigs were distinct from granulosa cell tumors and ovarian cancers.

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