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Secretion of Inhibin A and Inhibin B During Pregnancy and Early Postpartum Period in Japanese Monkeys

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In order to determine the profiles and sources of inhibin A and inhibin B during pregnancy in Japanese monkeys, serum samples were collected from eight monkeys for measuring concentrations of both inhibins by enzyme-linked immunosorbent assay. The term placenta was used for the localization of inhibin α-, βA-, and βB-subunits by immunohistochemistry. Serum concentrations of inhibin A showed a significant rise at the second quarter and maintained its level until term. Serum concentrations of inhibin B gradually increased until the fourth quarter. The concentration of both inhibins abruptly declined after delivery to the nonpregnant level. Positive staining of inhibin α-, βA-, and βB-subunits was observed in syncytiotrophoblast in the placenta by immunohistochemistry. These results demonstrated that large amounts of both dimeric inhibins are secreted from the placenta of Japanese monkeys.

Key Words: Inhibin A; inhibin B; placenta; Japanese monkey; pregnancy.

Introduction

Inhibin is a heterodimeric glycoprotein that has two isoforms consisting of an α-subunit with one of two β-subunits (βA or βB): inhibin A (α/βA) and inhibin B (α/βB). Although it was first identified as a gonadal factor that suppressed secretion of follicle-stimulating hormone (FSH) in the pituitary *(1,2)*, current studies have also shown its paracrine effect in ovary and utero-placental unit *(3,4)*. In female mammals, inhibin is mainly secreted from the ovary and placenta, and inhibin α, βA, and βB mRNAs have also been identified in various extragonadal tissues, including adrenal, bone marrow, pituitary, spleen, kidney, spinal cord, and brain *(5)*.

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Both the immunoreactivities and bioactivities of maternal serum inhibin during pregnancy are higher than those during the normal menstrual cycle in humans *(6,7)*. In the third trimester of human pregancy, immunoreactive (ir-) inhibin levels rise and reach the maximum level, and remain high until the end of pregnancy. After delivery, circulating ir-inhibin concentrations decrease to undetectable levels *(6)*.

Recently, a new enzyme-linked immunosorbent assay (ELISA) has been developed to measure different isoforms of inhibins in human *(8,9)* and a few results have been described about the patterns of both inhibin A and inhibin B during the menstrual cycle *(9)* and pregnancy *(10,11)*. Furthermore, it has been demonstrated that human placental cells secrete inhibin A and inhibin B during pregnancy *(12–15)*. In baboons and Japanese monkeys, serum ir-inhibin levels increased and were maximal in late pregnancy *(16,17)*. In common marmosets, serum ir-inhibin levels increased and maintained at high levels throughout pregnancy *(18)*. However, in pregnant chimpanzees, serum levels of inhibin A and inhibin B remained low throughout pregnancy, as measured by ELISA, and bioactivity also remained very low *(19)*. Thus, there seems to be different patterns in hormonal profiles of inhibin during pregnancy among different primates.

Japanese monkeys are unique macaques that live at the northernmost latitude of any other nonhuman primate, and they show distinct seasonal breeding *(20–23)*. Because of their endocrinological features, they are thought to be a suitable model for studying reproduction in human *(24)*. On the other hand, in the wild, Japanese monkeys are listed as endangered by the World Conservation Union (IUCN) *(25)*. Although it is very important to investigate the basic reproductive physiological features from views of a medical use and species conservation, there are only a few reports available on their reproductive physiology.

Nozaki et al. *(17)* reported that in pregnant Japanese monkeys, serum concentrations of ir-inhibin increased remarkably from middle pregnancy and reached its peak in late pregnancy. In addition, they demonstrated placenta obtained at d 120 of pregnancy and serum (d 60–80 of pregnancy) contained very high levels of bioactive inhibin. However,

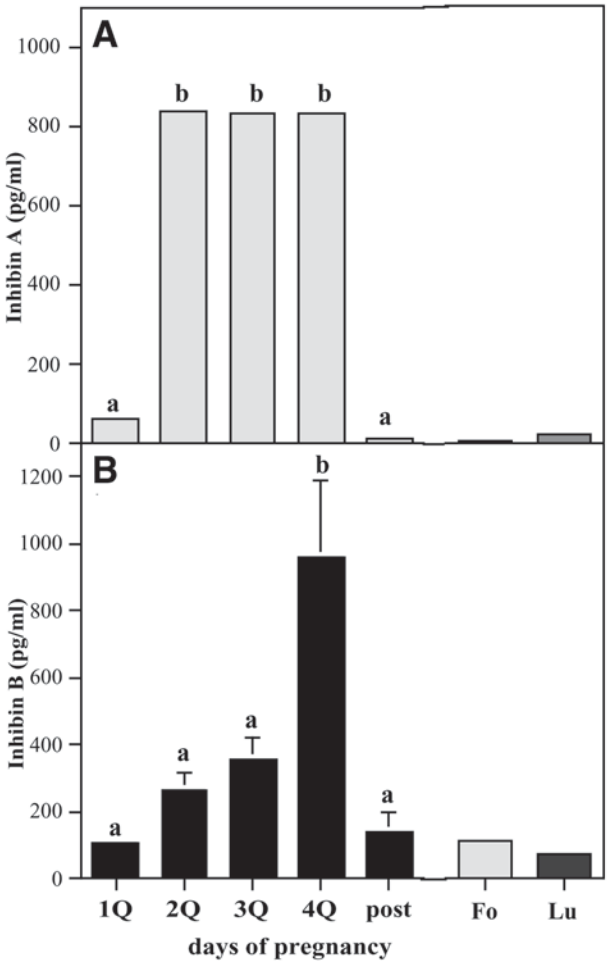

Fig. 1. Changes in maternal serum concentrations of inhibin A (**A**) and inhibin B (**B**) during pregnancy (1Q–4Q) and the early postpartum period (post) and during follicular (Fo) and luteal phase (Lu) of the menstrual cycle in the Japanese monkey. Each value represents the mean ± SEM (n = 6–8). Different letters in each bar indicate a significant difference ($p < 0.05$) between each group [Fisher's protected least significant difference (PLSD)].

there is no information available about their secretion patterns and source of inhibin A and inhibin B during pregnancy. In order to determine the profiles and the source of inhibin A and inhibin B throughout pregnancy and early postpartum period in Japanese monkeys, both serum inhibin A and inhibin B were determined using human inhibin ELISAs. In addition, the specific cellular localization of three inhibin subunits in the term placenta was also determined by immunohistochemical approach.

Results

Changes in Serum Inhibin A and Inhibin B During Normal Menstrual Cycle and Pregnancy (Fig. 1)

The mean gestation period of eight pregnancies was 165 ± 3.2 d (range: 157–180 d). The serum concentrations of inhibin A in the first quarter were above ninefold higher than those of nonpregnant female; then, the level showed a significant increase ($p < 0.05$) in the second quarter (about 14-fold higher than the first quarter), and the high levels were maintained until the end of pregnancy. After parturition, the concentrations of serum inhibin A declined significantly ($p < 0.05$) to the nonpregnant level. The concentration of inhibin A in the placental homogenate was 4509.9 pg/g placenta.

Serum concentrations of inhibin B tended to increase gradually from the second quarter, and then showed a significant increase ($p < 0.05$) in the fourth quarter. The concentrations were about ninefold higher than the first quarter. After parturition, the concentrations of serum inhibin B declined significantly ($p < 0.05$) to the nonpregnant level. The concentration of inhibin B in the placental homogenate was 3935.7 pg/g placenta.

Immunohistochemistry (Fig. 2)

Positive staining of α-, βA-, βB-subunit antisera was observed in the cytoplasm of the syncytiotrophoblast of term placenta. Normal rabbit serum did not show any immunohistochemical staining within either trophoblasts or interstitial cells.

Discussion

Only a few reports are available about the endocrinological profile of inhibin A and inhibin B during pregnancy in nonhuman primates. The present study showed the profiles of maternal serum inhibin A and inhibin B in pregnant Japanese monkeys for the first time, and it appeared that both inhibin levels were significantly high throughout pregnancy as compared to nonpregnant levels.

Although the profiles of maternal serum inhibin A and inhibin B levels have little difference, both profiles showed a remarkable increase during pregnancy and declined substantially to nonpregnant levels after parturition.

In human pregnancy, it has been reported that maternal serum inhibin A levels increased progressively to maximal concentrations in wk 36, which is 48-fold higher than nonpregnant levels. After parturition, it became substantially lower than at wk 36 of gestation *(11)*, whereas maternal serum inhibin B levels were undetectable or lower than the nonpregnant levels from early pregnancy. Therefore, inhibin A is the major type of inhibin that has more biological activities than inhibin B during human pregnancy. In Japanese monkeys, however, the present findings clearly showed that serum concentration of inhibin A indicated a remarkable rise from the second quarter of pregnancy, which is identical with the period when the luteo-placental shift completed around d 21 of the pregnancy in macaques *(26)*.

On the other hand, these differences of the profiles of both inhibin A and inhibin B between Japanese monkeys and humans may be caused by anatomical differences of placenta.

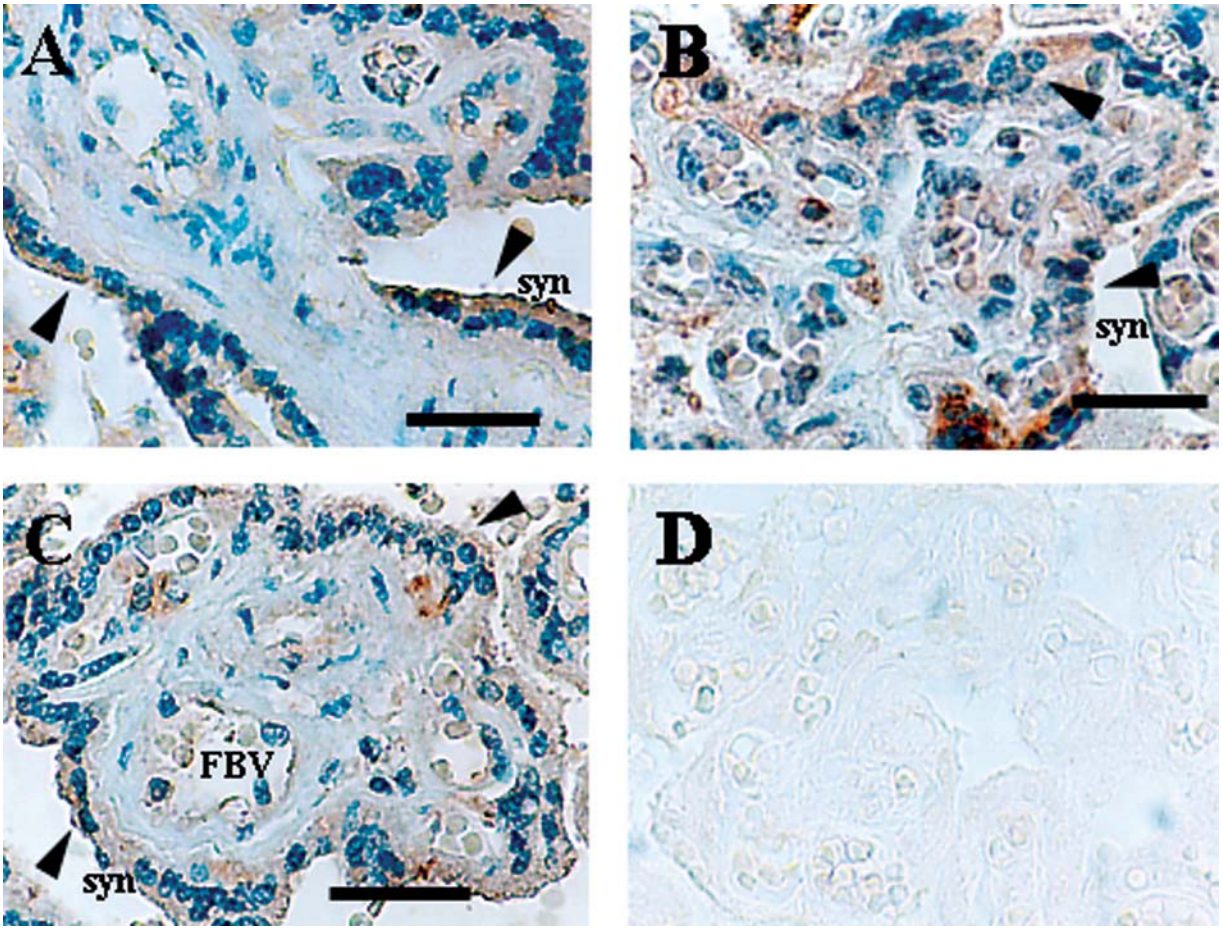

Fig. 2. Immunohistochemical staining for inhibin α-subunit (**A**), inhibin βA-subunit (**B**), and inhibin βB-subunit (**C**) in the placenta of Japanese monkey. The localization of all three subunits was observed in syncytiotrophoblast (syn). Sections incubated with normal rabbit serum (**D**) instead of primary antibody did not show immunostaining reaction. FBV = fetal blood vessel. Scale bar = 30 μm.

The placenta of macaques are characterized as bidiscoid, implanted in a superficial, straight connection between maternal and fetus tissues, whereas the human placenta is characterized as a single disk, implanted in an interstitial, irregular connection between maternal and fetus tissues *(27)*.

Using the immunohistochemical technique in the present study, the localization of inhibin α-, βA-, and βB-subunits was observed in the syncytiotrophoblast of Japanese monkey placenta. Because of a sudden decline in both inhibin A and inhibin B after parturition, the large amount of both inhibins contained in the placental homogenate, and the result of immunohistochemistry, it was suggested that the major source of both inhibins are syncytiotrophoblast, the same as in human placenta *(12–15)*.

Because bioactive inhibin was present in both placental homogenate and peripheral blood *(17)*, it is likely that inhibin plays an important role in regulating FSH secretion during pregnancy in Japanese monkey. In human, it has been reported that an inverse relationship between FSH and inhibin in maternal serum existed during pregnancy, and bioactive inhibin increased progressively with the development of pregnancy. Moreover, serum FSH levels remain at nadir during pregnancy, whereas serum inhibin A levels are higher than those of the normal menstrual cycle and rise progressively in late pregnancy. These results suggest that inhibin A plays an important role in suppressing FSH secretion and resulted in regulating ovarian folliculogenesis throughout pregnancy *(7,11,28)*, although inhibin B is also important during late pregnancy. Because inhibin has a high degree of homology with transforming growth factor (TGF)-β *(29)*, the suggestion has been made that it may have a local effect as growth and differentiation factors *(5)* and inhibin could be an important factor in the development of fetus and placenta. Recent studies report its applicability as an indicator of gestational diseases such as Down's syndrome, intrauterine growth restriction, pre-eclampsia, and so forth *(10, 30)*. However, the role of inhibin during pregnancy is still uncertain and further investigations are required. Because of the similarity to humans and high levels of both inhibin A and inhibin B during pregnancy, Japanese monkeys could be suitable models to understand the roles of both inhibins during pregnancy.

On the other hand, in pregnant chimpanzees, Kondo et al. *(19)* reported that both maternal serum inhibin A and inhibin B remained at low levels throughout pregnancy, and bioactive inhibin in placental homogenates was at a very low level. They suggested that in chimpanzees, steroids, rather than inhibin, are important in regulating FSH secretion in the late pregnancy, which is similar to sheep *(31)*. Although humans, Japanese monkeys, and chimpanzees are in the same order as primates, the profiles of inhibin secretion suggested that local factors within the feto-placenta unit and regulating FSH secretion during pregnancy are different among species.

In conclusion, we demonstrated that both circulating inhibin A and inhibin B in the pregnant Japanese monkey are at high levels during the second and fourth quarters of pregnancy. We also observed the localization of all inhibin α-, βA-, and βB-subunits in the syncytiotrophoblast of placenta by immunohistochemistry. These results suggest that large amount of inhibin A and inhibin B secrete from the placenta during Japanese monkey pregnancy. Therefore, it appears that the relative importance of both inhibins in the regulation of FSH secretion and/or fetal and placental development during pregnancy might be different than in humans.

Materials and Methods

Animals

Six to eight pregnant Japanese monkeys (*Macaca fuscata fuscata*) at least 8 yr old and weighing 7–9 kg were used. They were housed individually in an air-conditioned room with controlled temperature (20 ± 5ºC) and lighting (lights on: 0600 to 1800 h) at the Primate Research Institute, Kyoto University, Japan. They were fed standard monkey pellet food with sweet potatoes or fruit daily and allowed free access to water. All animal husbandry except blood collection was performed between 0900 and 1100 h, and the occurrence of menses were also checked at those times. Females were mated from d 11 to 15 d of the menstrual cycles by introducing a male into each female's cage.

The day of luteinizing hormone (LH) surge was designated d 0 of the pregnancy, and the pregnancy was divided into five stages as follows: the first quarter (0–39 d); the second quarter (40–9 d); the third quarter (80–119 d); the fourth quarter (120 d until parturition) and postpregnancy (1 wk after parturition). All of the blood samples were drawn at 1500–1700 h from cubital vein without anesthesia once in a quarter and a week after parturition. Monkeys were well acclimated to blood collections. After vaginal delivery, the placenta was immediately fixed in 4% paraformaldehyde (Sigma Chemical Co., St Louis, MO, USA) in 0.05 *M* phosphate-buffered saline (PBS), pH 7.4, embedded in paraffin or homogenized with normal saline (1 g placenta/mL) using homogenizer (Physcotoron; Nichion Ltd, Tokyo, Japan), and centrifuged at 25,000*g* for 30 min at 4ºC. The serum samples and placental homogenate were stored at –80ºC until the assay. All of the animal care and the experimental protocol were approved by the Guidelines for the Care and Use of Laboratory Primates prepared by the Primate Research Institute, Kyoto University, Japan (1986).

Enzyme-Linked Immunosorbent Assay

Concentrations of inhibin A and inhibin B in peripheral serum and placental homogenate were measured using commercial ELISA kits (Serotec Ltd, Oxford, Oxon, UK) developed for human inhibin A and inhibin B *(8,9)*. Recombinant human inhibin A and inhibin B were used as the standard, respectively. The detection limit of the assay for inhibin A was 3.9 pg/mL, and for inhibin B, it was 15.6 pg/mL. As a pilot study, dose-response curves of serially diluted placental and testicular homogenates (3.125–50 mg for assay of inhibin A, 25–50 mg for assay of inhibin B) and serum samples (25–100 µL) were parallel to the standard curves of inhibin A and inhibin B, indicating that it was possible to measure the concentrations of inhibin A and inhibin B in peripheral serum and placental homogenate of the Japanese monkey. Specificity of inhibin A and inhibin B ELISAs was described in a previous article *(32)*. As controls, five samples of female monkeys' sera of follicular phase (d –3 to –14; d 0 = LH surge) or luteal phase (d 3–14) respectively were pooled to make the follicular-phase sample and the luteal-phase sample.

Immunohistochemistry

The paraffin-embedded placenta were cut into 6-µm-thick serial sections and placed on slide glasses coated with 0.01% poly-L-lysine (Sigma Chemical Co., St Louis, MO, USA).

After tissue sections, they were deparrafinized with xylene and treated with autoclave in 0.01 *M* sodium citrate buffer (pH 6.0) at 121ºC for 15 min to retrieve the antigen. The sections were then incubated with 3.0% H_2O_2 in methanol at room temperature for 1 h and 0.5% casein-PBS at 37ºC for 80 min to protect them from nonspecific staining. Following treatment, the sections were incubated with primary antiserum against each inhibin subunit, diluted with 1:10 Block Ace (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan), for 16 h at 37ºC. The primary antisera against α-, βA-, and βB-subunits were used at dilutions of 1:6000,1:10000, and 1:4000, respectively. The primary antiserum against each inhibin subunit was anti-[Tyr30]-porcine inhibin α-chain (1–30)-NH2 conjugated to rabbit serum albumin ([Tyr30]-porcine inhibin α-chain (1–30)-NH2, kindly provided by Dr. N. Ling (Neuroendocrine Inc., San Diego, CA, USA); anticyclic inhibin βA (81–113)-NH2 (#305-24-D) and anticyclic inhibin βB (80–112)-NH2 (#305-25-D) were kindly provided by Dr. W. Vale (The Salk Institute for Biological Studies, La Jolla, CA, USA). For negative control, normal rabbit serum was used instead of primary antiserum. After the incubation, the sections were treated with 0.25% (v/v) biotinylated goat anti-rabbit secondary antiserum (Vecta-

stain ABC kit; Vector Laboratories, Burlingame, CA, USA) in 1:10 Block Ace for 1 h at 37ºC and were then incubated with 2% avidin–biotin complex (Vectastain ABC kit) in 1:10 Block Ace for 30 min at 37ºC. Peroxidase activity was shown by incubation in 0.025% 3,3'-diaminobenzidine tetrachloride (DAB; Sigma Chemical Co., St Louis, MO, USA) in 0.01 *M* PBS, pH 7.4, containing 0.01% H_2O_2 for 1–30 min. The sections were counterstained with Lily-Mayer's hematoxylin and observed under the light microscope.

Statistical Analysis

All data were presented as mean ± SEM. When a significant effect was obtained with one-way analysis of variance (ANOVA) and the significance of the difference between two means was analyzed using Fisher's PLSD method. A value of $p < 0.05$ was considered to be statistically significant.

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