Expression and Cellular Localization of Inhibin α-Subunit mRNA in Equine Fetal Gonads

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ABSTRACT. The expression of inhibin α-subunit mRNA in equine fetal gonads during pregnancy (Days 90 to 300) was examined by means of Northern blot analysis. In all samples examined, a single species of transcript was detected at the size of 1.5 kb. A digoxigenin-labeled antisense cRNA probe specific to equine inhibin α-subunit was synthesized and in situ hybridization analysis to locate the inhibin α-subunit mRNA positive cells was performed using frozen tissue sections of equine fetal ovary (day 150 of pregnancy) and equine fetal testis (day 180 of pregnancy). In the fetal ovary, positive cells were seen throughout the interstitial area but did not show any particular localization. In the fetal testis, on the other hand, the antisense cRNA hybridized almost exclusively to the interstitial cells surrounding developing seminiferous cords and Sertoli cells within the cords. Positive signals were also detected in a limited number of the interstitial cells located away from the cords. These results suggest that in equine fetal gonads, inhibin and/or inhibin α-subunit related molecules such as the monomeric form are produced and these molecules may have a paracrine/autocrine role within the gonads. — KEY WORDS: equine, fetal gonad, inhibin, Northern blot, in situ hybridization.

Inhibin was originally discovered as a gonadal polypeptide which is capable of suppressing follicle stimulating hormone (FSH) secretion from the anterior pituitary gland [23]. The polypeptide is a heterodimer composed of two subunits termed as α and β (β is further subdivided into βA and βB) [23]. Activin, an inhibin related molecule, is a homodimer of β-subunits and functions as a stimulatory factor on hypophysial FSH secretion. Thus, the β-subunit is shared either by inhibin or activin and it has been suggested that the degree of the amount of α-subunit synthesized within the cells determines the relative amount of inhibin produced [12].

Extragonadal expression of inhibin/activin subunits mRNA has been examined in adult rat tissues by means of S1 nuclease analysis by Meunier et al. [12]. They demonstrated that the inhibin α-subunit mRNA was present in a variety of tissues, such as placenta, pituitary, adrenal, spleen, kidney, brain and spinal cord, though the precise functions of inhibin in these tissues are still unknown.

In addition to its inhibitory action on pituitary FSH secretion, inhibin also plays paracrine/autocrine roles within the gonads, e.g., stimulation of in vitro androgen production from rat theca/interstitial cells [10] or Leydig cells [10], and inhibition of spermatogenesis in adult rat testes [4, 22].

The expression of inhibin α-subunit mRNA and its protein was also found in fetal gonadal tissues of the rat [18, 19], humans [3, 16] and rhesus monkeys [16], and its role during gonadal development has been predicted [3, 16, 18, 19].

Equine fetal gonads show quite unique characteristics during development. The weight of the fetal gonads increases during pregnancy and peaks around day 200 of pregnancy [2]. At this period, fetal gonads are much larger than the maternal ones due to the proliferation or hyperplasia of steroidogenic interstitial cells [6, 7] which are actively producing androgen [15, 17]. The weight of fetal gonads begins to decrease after around day 200 of pregnancy and this decrease is accompanied by a reduction in the number of interstitial cells [2]. Androgen derived from the equine fetal gonads is converted to estrogen by placental aromatase activity [14], and therefore, the pattern of serum estrogen concentrations in the pregnant mare coincides with the changes in the weight of the fetal gonads [5, 7]. However, the regulatory mechanism of androgen production from equine fetal gonads is unclear.

It may not be implausible to hypothesize that inhibin is produced in equine fetal gonads and plays a role in the regulation of androgen production, since inhibin has been reported to have a stimulatory effect on androgen production within the gonad as described above [10]. Therefore, as an initial approach to this hypothesis, we investigated the expression of inhibin α-subunit mRNA both in the testes and ovaries of equine fetuses by means of Northern blot analysis.
analysis, and also examined its localization by *in situ* hybridization.

**MATERIALS AND METHODS**

*Tissues*: Thoroughbred mares mated with stallions were purchased from Nishiya Horse Breeding Farm (Mukawa, Hokkaido), and only the mares who became pregnant were used for tissue collection. The day of mating was designated as day 0. Pregnant mares were sacrificed at each stage of pregnancy by intravenous injection with a mixture of sodium thiopental (Ravonal: Tanabe Pharmaceutical Co., Ltd., Osaka, Japan) and succinylcholine solution (Succine: Yamanouchi Pharmaceutical Co., Ltd., Osaka, Japan). Gonads were immediately collected from the equine fetuses and snap-frozen in liquid nitrogen, then stored at -70°C until used.

**Extraction of poly(A)+RNA**: Total cellular RNA was extracted from the tissue by the cesium chloride ultracentrifugation method [20]. Poly(A)+RNA was selected with Oligotex-dT30 Super (Takara, Kyoto, Japan). The quantity and quality of the extracted poly(A)+RNA were determined by spectrophotometry at 260 and 280 nm and by ultraviolet light visualization of ethidium bromide-stained agarose gels.

**Northern blot analysis**: The EcoRI-fragment from equine inhibin α-subunit cDNA clones (Eq-α-11, 1286 bp) [24] was used as a probe for Northern blot analysis. Rat β-actin cDNA fragment (764 bp) was amplified by means of the reverse transcription-polymerase chain reaction (RT-PCR) method. Rat β-actin Control Amplimer Set (CLONETECH, CA) was used as the primer set, and RT-PCR was performed in accordance with Takara GeneAmp RNA PCR Kit (Takara, Kyoto, Japan). The RT-PCR product was ligated to the commercial pT7Blue T-vector (Novagen, WI), and verified by sequencing the double stranded plasmid templates using an AutoRead Sequencing kit and A. L. F. DNA sequencer (Pharmacia Biotech, Sweden). These cDNA inserts were labeled with [α-32P]dCTP (3000 Ci/mm, Amersham, Tokyo, Japan) using a random primer DNA labeling kit (Boehringer Mannheim Biochemica, Germany). For Northern blot analysis, approximately 3 μg of each poly(A)+RNA extracted from equine fetal gonad during pregnancy was separated by electrophoresis in 1.0% agarose 3-[N-morpholino]propanesulfonic acid/formaldehyde gels [20], and transferred to a nylon membrane (BIOLOGY, Pall Biosupport, NY). The membrane was UV-crosslinked and hybridized using [α-32P]dCTP-labeled cDNA fragment from Eq-α-11. Hybridization was processed in the hybridization mixture (5 × SSC, 2.5 × Denhardt’s reagent, 5 mM EDTA, 0.1% SDS, 10% dextran sulfate, 100 μg/ml salmon sperm DNA) at 42°C overnight. After sequential washing with 0.5 × SSC/0.1% SDS for 15 min at room temperature, 0.1 × SSC/0.1% SDS for 15 min at room temperature, and 0.1 × SSC/0.1% SDS for 15 min twice at 50°C, the membrane was exposed to an X-ray film at -85°C. The membrane was also re-hybridized with [α-32P]dCTP-labeled rat β-actin cDNA probe as a control to evaluate the loading amount of poly(A)+RNA.

**In Situ Hybridization**: The EcoRI-fragment from equine inhibin α-subunit cDNA, Eq-α-11 [24], was subcloned into pGEM-3Z vector (Promega, WI). From appropriate linearized plasmid DNA templates, digoxigenin-UTP labeled cRNA probes (antisense riboprobes) and control probes (sense riboprobes) were synthesized using T7 RNA polymerase (DIG RNA Labeling Kit, Boehringer Mannheim Biochemica, Germany). Equine fetal gonads from days 150 (ovary) and 180 (testis) of pregnancy were embedded in O. C. T. Compound (Miles Laboratories, IN) and cut into 5-μm thick sections by cryostat, then mounted on slides coated with 2.3% VECTABOND REAGENT (VECTOR LABORATORY INC., CA) in acetone. Sections were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 60 min at room temperature, then washed for 10 min in PBS. The sections were then processed according to the method of Hirota et al. [9]. Digoxigenin-labeled cRNA in the tissue sections was detected after overnight incubation with an anti-digoxigenin alkaline phosphatase-conjugated antibody (Nucleic Acid Detection Kit, Boehringer Mannheim Biochemica, Germany) at a dilution of 1:500. After being counterstained with hematoxylin, the slides were mounted in glycerol gel and examined under bright-field microscopy.

**RESULTS**

*Expression of inhibin α-subunit mRNA in equine fetal gonads detected by Northern blot analysis*: To examine the expression of inhibin α-subunit mRNA in equine fetal gonads, Northern blot analysis was performed on poly(A)+RNA extracted from samples from days 90 to 300 of pregnancy. As shown in Fig. 1, a single species of inhibin α-subunit mRNA was expressed in all samples and the size of each transcript was 1.5 kb.

*In situ hybridization analysis of inhibin α-subunit mRNA in equine fetal gonads*: In the equine fetal ovary of day 150, some but not all the interstitial cells were positive for the inhibin α-subunit mRNA (Fig. 2A). These cells did not show any particular localization, and were scattered throughout the interstitial tissue of the fetal ovary. On the other hand, in the fetal testis of day 180, the positive cells were localized in the interstitial area, surrounding the cord-like structure which is the forerunner of seminiferous tubules (Fig. 2B) [5]. Some, but not all seminiferous cords, contained inhibin α-subunit mRNA positive cells (Fig. 2B).

The specificity of the staining was verified using digoxigenin-labeled sense cRNA as a probe which resulted in no staining (Fig. 2C and D).

**DISCUSSION**

In the present study, the expression of inhibin α-subunit mRNA and its localization in equine fetal gonads were...
examined by means of Northern blot and in situ hybridization analyses.

The results of Northern blot analysis revealed that as early as day 90 of pregnancy, a single species of inhibin α-subunit mRNA was expressed in the equine fetal gonad. The size of the transcript was 1.5 kb and this is in accordance with the size observed in adult equine gonads as reported previously [24], suggesting the same splicing mechanism in the transcription of equine inhibin α-subunit in fetal gonads.

In the ovaries of mid-gestational fetal human [16] and late-gestational fetal rhesus monkey [16], the expression of inhibin α-subunit was barely detected by immunohistochemistry, but instead, inhibin/activin βA- or βB-subunits were expressed abundantly, suggesting that in these primate tissues, activin rather than inhibin was produced predominantly. On the other hand, in the equine fetus, the expression of inhibin α-subunit mRNA was detected by Northern blot analysis both in the ovarian and testicular samples examined. Furthermore, our preliminary experiment confirmed that inhibin/activin β-subunit (βA and βB) mRNA was also expressed within these tissues but its expression level was lower than that of inhibin α-subunit mRNA (data not shown). These results suggest that inhibin and/or inhibin α-subunit related molecules, rather than activin are produced in these tissues.

In the equine fetal ovary on day 150 of pregnancy, in situ hybridization analysis showed that inhibin α-subunit mRNA positive cells were localized in the interstitial area of the tissue section, though no particular localization was observed as was in the equine fetal testis. In the equine fetal ovary, due to the great enlargement caused by hyperplasia of the interstitial cells, only about a half of the surface remains covered with cortical tissue [6], and primordial follicles are confined only to the thin germinal epithelium on the surface [1]. The areas of fetal ovaries observed for in situ hybridization did not contain any primordial follicles, therefore, additional study is required to clarify whether inhibin α-subunit mRNA is expressed in primordial follicles in the equine fetal ovary.

In the fetal human testis, Sertoli cells adjacent to the basement membrane of the seminiferous tubules and Leydig cells were immunopositive for inhibin α-subunit [16]. In addition, in the fetal ovine testis, the expression of inhibin α-subunit mRNA was localized within the seminiferous cords of the developing fetal testis, and immunoreactive inhibin α-subunit was detected in Sertoli cells within the seminiferous cords and in a small proportion of Leydig cells [21]. The localization of inhibin α-subunit mRNA in the equine fetal testis was similar to that of fetal human inhibin α-subunit protein [16] and fetal ovine testicular inhibin α-subunit mRNA and protein [21]. It is of interest to note that in the equine fetal testis, inhibin α-subunit mRNA positive cells in the interstitial area were mostly localized at the surroundings of the seminiferous cords. Although we have no available data to identify these positive cells at present, based on the analogy obtained from the humans [16] and sheep [21], the cells surrounding seminiferous cords correspond to (immature) Leydig cells.

Recently, Nambo et al. [13] reported the presence of high concentrations of immunoreactive (ir)-inhibin in the plasma of pregnant mares and demonstrated that ir-inhibin was present in the equine fetal ovary, though its homogenate showed no suppressive activity on pituitary FSH secretion. This does not, however, exclude the possibility that inhibin and/or inhibin α-subunit related molecules probably

![Fig. 1. Northern blot analysis of inhibin α-subunit mRNA in equine fetal gonads during pregnancy. Three micrograms of poly(A)RNA extracted from each tissue was electrophoresed and analyzed as described in Materials and Methods. A single species of inhibin α-subunit transcript was detected in all of the samples at the size of 1.5 kb. Numbers and symbols above the autoradiogram represent the day of pregnancy and the sex of the fetus, respectively.](image-url)
produced in equine fetal gonads might be functioning as paracrine/autocrine factors. The functions of inhibin within gonads has been shown by some investigators: inhibin produced from Sertoli cells during the spermatogonial development in the rat was reported to be acting as an inhibitory paracrine factor [4, 22], while activin stimulated spermatogonial proliferation [11]. In addition, inhibin stimulated androgen secretion from theca-interstitial cells of the rat [10] and human [8] in vitro, and inhibin also enhanced LH/hCG-induced androgen synthesis when added to a newborn rat Leydig cell culture [10], while activin suppressed it [10]. These reports suggest that in both the equine fetal ovary and testis, inhibin produced from interstitial cells might play a paracrine/autocrine role in the regulation of androgen production, and inhibin produced from Sertoli cells might be suppressing spermatogonial development as reported in the rat [4, 22]. Further studies to elucidate the precise roles of inhibin and/or inhibin α-subunit related molecules in equine fetal gonads are awaited.

Fig. 2. In situ hybridization of inhibin α-subunit mRNA in equine fetal ovary (A and C, day 150 of pregnancy) and testis (B and D, day 180 of pregnancy). Digoxigenin-labeled antisense cRNA probe (A and B) specific for equine inhibin α-subunit mRNA and control probe (sense probe, C and D) were used. Cells indicated by arrows are positive for inhibin α-subunit mRNA. ST, seminiferous tubule. Magnification. × 200 (A-D).
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