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Developmental Changes in Inhibin-α Gene Expression in the Mouse Testis

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Inhibin is a gonadal hormone which is composed of an α-subunit and one of two related β-subunits (β_A, β_B). Inhibin is important for pituitary FSH regulation, normal follicle development and maintenance of the estrous cycle in the female, whereas the role of inhibin in the male is less clear. Thus, we examined the expression of the inhibin-α gene in testis during sexual maturation in male mice, to try to gain insight into its functions in the male. Male mice of the ICR strain attained fertility at 6 weeks of age, and histological analysis revealed that a functional testis was formed, with seminiferous tubules which contain mature sperm and with an abundant population of Leydig cells. Parallel with this sexual maturation, inhibin-α subunit protein synthesis increased, whereas synthesis of the activin β_A and activin β_B followed with a delayed time course. Inhibin-α mRNA also increased during this critical period, and this corresponded to a change in the methylation status of the inhibin-α gene. Taken together, our data reveal that activation of inhibin-α gene during testis development correlated with the histological maturation of the testis and the acquisition of fertility in male mice.

Keywords: Fertility; Histology; Inhibin-α; Methylation; mRNA.

Introduction

Inhibin and activin are multifunctional hormones belonging to the TGF-β superfamily of proteins. Inhibin and activin were identified for their ability to inhibit and stimulate, respectively, FSH secretion and synthesis from cultured anterior pituitary cells (Ling *et al*., 1985; 1986a; 1986b; Miyamoto *et al*., 1985; Rivier *et al*., 1985; Robertson *et al*., 1985; Vale *et al*., 1986). Inhibin is a heterodimer composed of an inhibin-specific α subunit and one of two related β subunits (β_A, β_B) (Ling *et al*., 1985; Miyamoto *et al*., 1985; Rivier *et al*., 1985; Robertson *et al*., 1985), whereas activin is a dimer of the β subunits (Ling *et al*., 1986b; Vale *et al*., 1986). Inhibin α mRNA is primarily expressed in the granulosa cells of the ovary (Meunier *et al*., 1988b), the Sertoli cells of the testis (Roberts *et al*., 1989), and the adrenal cortex (Crawford *et al*., 1987).

In female mice, inhibin is important for normal follicle development, and inhibin production is tightly regulated during the estrous cycle. On the afternoon of proestrus, developing follicles produce high levels of inhibin A (Meunier *et al*., 1988a; 1988b). Following the preovulatory gonadotropin surges, ovarian inhibin production declines (Woodruff *et al*., 1989). These low inhibin levels persist through the morning of estrus, providing an environment permissive to elevated FSH, the generation of the secondary FSH surge, and recruitment of a new cohort of follicles.

In male mice, the testis is the primary source of circulating inhibin, and the α-β_B heterodimer, inhibin B is the major isoform produced (Sharpe *et al*., 1999; Woodruff *et al*., 1996). Inhibin is likely to be important for normal gonadal function, in that inhibin-α deficient mice develop testicular stromal tumors and exhibit an arrest of gametogenesis (Matzuk *et al*., 1992). In addition, transgenic mice overexpressing inhibin A (Pierson *et al*., 2000) or the inhibin-α subunit (McMullen *et al*., 2001) have small testis and reduced spermatogenesis. However, functional studies of inhibin-α expression during sexual maturation in

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Abbreviation: FSH, follicle-stimulating hormone.

the male have not been explored. It is known that the interrelationships between the hypothalamic-pituitary axis and the gonads of the male are already functional before birth. Thus, the first few weeks of postnatal life may be envisaged as a period of fine tuning rather than establishment of these interrelationships (Ojeda and Urbanski, 1988). To test the hypothesis that inhibin expression is correlated with sexual maturation in the male, expression of the inhibin-α gene and corresponding histological changes of the testis were investigated in this study.

Materials and Methods

Animals ICR strain mice were purchased from the Daehan Animal Center and maintained with 14 h light, 10 h dark illumination at 23°C, and with food and water available *ad libitum*. Male mice at 3 and 6 weeks of the age were obtained from the same litters. All experiments were performed at least four times if not otherwise noted, and representative results are shown.

Southern blot hybridization Southern blot analysis was carried out as previously described (Ko *et al*., 2003). Briefly, genomic DNA was isolated from tissues by proteinase K digestion and extraction with phenol/ chloroform. DNA was quantified in duplicate with a U.V. 2000 spectrophotometer (Pharmacia, USA). Following incubation with restriction endonucleases, 10 μg of the digested DNA was electrophoresed in a 1% agarose gel and transferred to Nytran filters (0.45 μm, Schleicher & Schuell, Germany) under vacuum. They were hybridized with ^{32}P-labeled rat inhibin-α cDNA, washed, and processed for autoradiography.

Northern blot analysis Northern blot analysis was carried out as previously described (Cho *et al*., 1993). Briefly, testes were homogenized with denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauryl sarcosine, 0.1 M 2-mercaptoethanol). The homogenate was phenol/ chloroform extracted, and RNA was precipitated. RNA was quantified in duplicate with a U.V. 2000 spectrophotometer (Pharmacia, USA). A_{260}/A_{280} ranged from 1.8 to 2.0. Total RNA (10 μg) was electrophoesed on a 1.2% agarose gel at 70 V for 1.5 h. After RNA transfer onto Nytran filters (0.45 μm, Schleicher & Schuell, Germany) by diffusion blotting, the filters were hybridized with ^{32}P-labeled rat inhibin-α cDNA, washed, and autoradiographed with X-ray film (Konica AX film) at –70°C for 1 day.

Protein blot analysis Tissues were removed, homogenized in 400 μl of protein extraction buffer [0.1 M NaCl, 0.01 M Tris-Cl (pH7.6), 1 mM EDTA (pH 8.0), 0.1% TritonX-100, 1 μg/ml aprotinin, 100 ng/ml phenylmethylsulfonyl fluoride], and centrifuged four times. The homogenates were then mixed with an equal volume of 2× SDS-loading buffer [100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% BPB, 20% glycerol], placed in boiling water for 10 min, and centrifuged. The supernatants were transferred to fresh tubes. Samples of each extract containing 10 μg protein were heated at 70°C for 10 min, electrophoresed on a 12% acrylamide gel and subsequently transferred onto Nytran filters in 1× transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). For inhibin-α protein, the blots were incubated for overnight in blocking solution (5% nonfat dried milk, 0.02% sodium azide, 0.02% Tween) with shaking at 4°C, followed by exposure to primary inhibin-α antibodies (1:750) (Serotec, UK) overnight. For activin- β_A and activin-β_B protein analyses, primary activin-β_A and activin-β_B antibodies (1:750, 1:750) (Serotec, UK) were used respectively. They were washed in milk-TBS-Tween for 30 min and incubated with secondary anti-rabbit Ig horseradish peroxidase-linked whole donkey antibody (1:6000) (Amersham Pharmacia Biotech, USA) in azide-free blocking solution [5% nonfat dried milk, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5)] for 2 h. The secondary antibody-specific signal was detected with an ECL kit (Amersham Pharmacia Biotech, USA). Exposure time is 30 s for inhibin-α, 1 min for activin-β_A, 1 min for activin-β_B, respectively.

Histology Tissues excised from male mice at 3 and 6 weeks of age were examined for gross appearance. They were immediately fixed in fresh 4% paraformaldehyde in PBS, pH 7.4. Following overnight fixation, they were dehydrated in ethanol and embedded in paraffin. Seven-micrometer sections of tissue were prepared using a microtome (Nippon Optical Works, Japan). The sections were de-parafinized with xylene, dehydrated in absolute ethanol, and rehydrated in water. Sections were stained with hematoxylin, counterstained with eosin, and observed under a light microscope (Olympus IX70, Japan) or a steromicroscope (Leica ME Apo, Switzerland).

Results

In an attempt to understand the developmental roles of inhibin-α in the male, we initially investigated the acquisition of fertility between 3 and 6 weeks of the age in male mice. When males were mated with females with proven fertility and the number of the pregnant female mice after mating was counted, males attained 80% fertility at 6 weeks of age compared with 0% fertility at 3 weeks of age (n = 11). Next, we investigated changes in testicular morphology during this period. The testis at 6 weeks of age was observed to be larger in size than at 3 weeks of age (data not shown). Subsequent analysis of testicular histology revealed that the testis at 6 weeks of age had compacted seminiferous tubules with clearly defined lumens filled with mature sperm, whereas the seminiferous tubules were largely undifferentiated at 3 weeks of age. In addition, an abundant population of Leydig cells was observed in the testis at 6 weeks of age (Fig. 1).

To investigate inhibin subunit gene expression, we per-

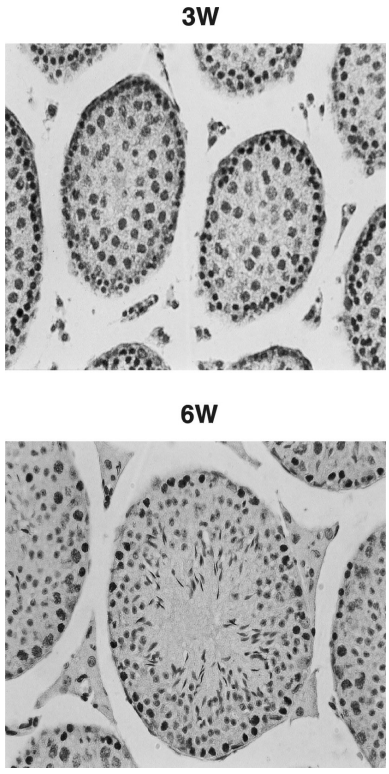

Fig. 1. Histology of the testis. Testes were obtained from mice at 3 weeks of age (3w) and 6 weeks of age (6w). Tissues were sectioned, stained, and observed at 200× magnification.

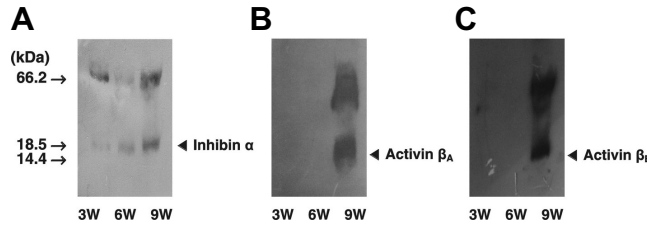

Fig. 2. Western blot protein analysis. Western blot analysis was performed as described in the **Materials and Methods**. Proteins (10 μg) from testes at 3 weeks (3w), 6 weeks (6w), and 9 weeks (9w) of age were loaded and detected using primary antibodies to the inhibin-α subinit (A), or activin β_A (B), or activin β_B subunits (C). The Western blot shown is representative of four independent experiments.

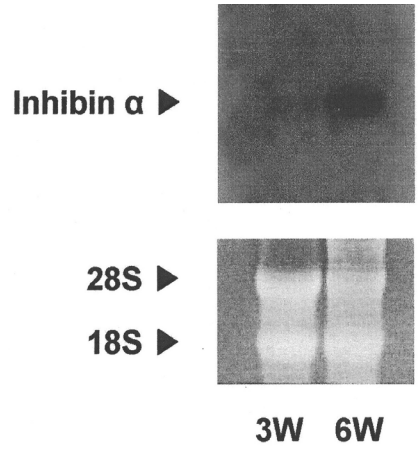

Fig. 3. Inhibin-α subunit mRNA expression. RNAs were purified from 3 or 6 week testes, quantified, and electrophoresed on 1.2% agarose gels. After transfer to Nytran, the filters were hybridized with ^{32}P-labled inhibin-α cDNA. The amount of RNA was normalized to the 28S and 18S ribosomal RNA content.

formed Western blot analysis for inhibin-α protein, Northern blot analysis for inhibin-α mRNA levels, and Southern blot analysis for to investigate the status of the genomic inhibin-α DNA, respectively. We found that inhibin-α protein, which has a molecular size of 18 kDa, is first detected at 3 weeks of the age and is increased in amount at 6 and 9 weeks of age (Fig. 2A). Smiliar patterns of syntheses of the inhibin and activin β_A (14 kDa) and β_B (14 kDa) subunits were observed, although expression was delayed compared to a subunit and the proteins were readily detectable only at 9 weeks of age (Figs. 2B and 2C). When we directly investigated gene expression, inhibin-α subunit mRNA levels increased dramatically between 3 and 6 weeks of age (Fig. 3), preceding the increase observed in inhibin-α protein levels (Fig. 2A). Finally, to test for alterations in the genomic DNA that might be associated with expression of the inhibin-α subunit gene, we examined the methylation status of the gene. Genomic DNA from the testes of 3 or 6 week old male mice was digested with the restriction enzyme *Sac*I and hybridized with inhibin-α cDNA. This analysis revealed an additional band in the DNA from the 6 week animal compared to the 3 week animal (Fig. 4A, left). When we digest with both *Sac*I and *Eco*RI, two additional bands were observed in the 6 week DNA (Fig. 4A, right). *Sac*I does not cut GAG^{m5}CTC on one strand, while *Eco*RI shows a reducd rate of cleavage in hemi-methylated DNA and does not cut DNA that contains GAATT^{m5}C on both strands. These studies suggest that genomic DNA surrounding the inhibin-α subunit gene in the testis is more heavily methylated at 3 weeks of age than at 6 weeks of age, and that de-methylation of the gene is associated with its transcriptional activation.

Discussion

Our studies revealed that in the ICR strain of mice, sexual maturation was attained between 3 and 6 weeks of age. This correlated with the histologic observation of clearly defined seminifierous tubules with lumens and the presence of both mature sperms and abundant Leydig cells, responsible for the synthesis of androgens. Thus, this

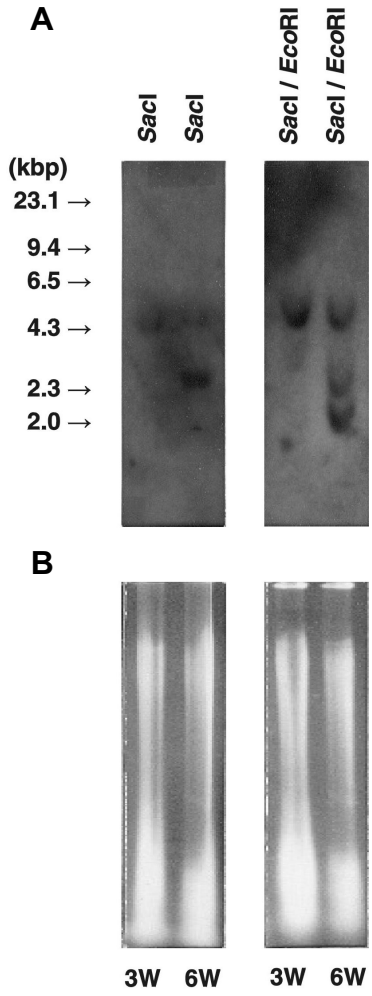

Fig. 4. Southern blot DNA methylation analysis. **A.** Genomic DNAs were purified from testis at 3 or 6 weeks of age, digested with the indicated restriction enzymes, electrophoresed on 1.0% agarose gels. After transfer to Nytran, the filters were hybridized with ^{32}P-labled inhibin-α cDNA. **B.** Gels were stained with ethidium bromide after digestion with restriction enzymes and electrophoresis.

suggests that spermatogenesis and steroidogenesis, two major functions of the testis, are initiated by 6 weeks of age in the male ICR mice.

During this same period, expression of the inhibin-α subunit gene was also dramatically changed. There is little mRNA or protein expression at 3 weeks of age, but both are readily detectable and up-regulated by 6 weeks of age. In mice, it was reported that level of inhibin-α mRNA level peaked at birth and then decreased using dot-blot hybridization (Tone *et al*., 1990). A gradual increase in inhibin-α subunit gene expression after birth, however, is also expected since GATA-1 declined and disappeared between 2 weeks to 5 weeks after birth in the mouse testis (Ito *et al*., 1993) and an inverse relationship was shown between GATA-1 and inhibin-α (Zhang *et al*., 2003). It is known that the activity of the inhibin-α gene promoter is regulated by GATA-1 (Feng *et al*., 1998). In addition, as explained below, activity of DNA methyltransferase which can influence inhibin-α gene expression reveals two peaks after birth in mice. Thus, there is a possibility that inhibin-α gene expression reveals a biphasic pattern in mice. In rats, different results regarding inhibin-α gene expression have been reported. High levels of inhibin-α mRNA from 8 days to 25 days after birth were reported in one study (Meunier *et al*., 1988b), while high levels at 20 days to 25 days of age and low thereafter were reported in another study (Keinan *et al*., 1989). In addition, the appearance of activin β_A and activin β_B at 9 weeks of age was unexpected, since mRNAs for these subunits were undetectable during this period in the rat (Meunier *et al*., 1988b).

The regulation of gene expression in eukaryotes is carried out by a large number of independent mechanisms operating at different levels. One important epigenetic factor which plays a role in this process is DNA methylation at the 5′ position of cytosine resides (5-Methylcytosine) (Schmitt *et al*., 2002). Our studies suggest that activation of expression of the inhibin-α subunit gene in the testis is associated with de-methylation of the gene. When genomic DNAs from the testis were digested with the restriction enzymes *Sac*I or *Eco*RI, which can or cannot digest DNA depending on the status of methylation, and hybridized with ^{32}P-inhibin-α cDNA, additional bands were observed at 6 weeks of age, indicating a decrease in the methylation of these specific restriction recognition sites. It is known that the inhibin-α promoter contains no *Sac*I and *Eco*RI sites within the 700 bp upstream of the transcriptional start site, and that the nearest downstream site is a *Sac*I site at +156 bp (Su and Hsueh, 1992). Thus, it is likely that the additional *Sac*I and *Eco*RI sites accessible after demethylation are located within intron I, the size of which is estimated 1.7 kb (Su and Hsueh, 1992). Interestingly, others have reported a gradual decrease in DNA methyltransferase activity after birth in mice, with the exception of two peaks, at 6 days and 18 days after birth (Benoit and Trasler, 1994), consistent with undermethylation and activation of the gene during this time interval. That methylation is an important mechanism of regulating this gene is supported by studies in human prostate cancer, where the gene is down-regulated (Mellor *et al*., 1998) and is also reported to be hypermethylated (Schmitt *et al*., 2002).

Collectively, our studies reveal that the inhibin-α gene is transcriptionally activated in male mice coincident with the onset of histological changes that define sexual maturation of the testis and the animal. Whether inhibin is a maker for these developmental events or plays any direct role in bringing about maturation of the testis through its well-reported autocrine regulatory functions remains to be established.

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