

Detection of anti-mullerian hormone receptor II protein in the postnatal rat testis from birth to sexual maturity

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Summary. Anti-Mullerian hormone (AMH) produced by the immature Sertoli cells negatively regulates the postnatal Leydig cell (i.e. adult Leydig cells/ALC) differentiation, however, the mechanism is sparsely understood. AMH negatively regulates the steroidogenic function of fetal Leydig cells (FLC) and ALC. However, when this function is established in the ALC lineage and whether AMH has a function in FLC in the postnatal testis are not known. Therefore, the objectives of this study were to examine the presence of AMH receptor type II (AMHR-II) in FLC and cells in the ALC lineage in the postnatal mammalian testis using the rat model Male Sprague Dawley rats of days 1, 5, 7-21, 28, 40, 60 and 90 were used. AMHR-II in testicular interstitial cells was detected in testis tissue using immunocytochemistry. Findings showed that the mesenchymal and the progenitor cells of the ALC lineage, were negative for AMHR-II. The newly formed ALC were the first cell type of the ALC lineage to show positive labeling for AMHR-II, and the first detection was on postnatal day 13, although they were present in the testis from day 10. From days 13-28, labeling intensity for AMHR-II in the ALC was much weaker than those at days 40-90. FLC were also positive. The time lag between the first detection of the newly formed ALC in the testis and the first detection of AMHR-II in them suggests that the establishment of the negative regulatory role of AMH on ALC steroidogenesis does not take place immediately upon their differentiation; no change in cell size occurs during this period. The absence of AMHR-II in mesenchymal cells suggests that it is unlikely that the negative regulatory effect of AMH on ALC differentiation in the postnatal testis is achieved via a direct action of AMH on mesenchymal cells. The presence of AMHR-II in postnatal FLC suggests a

possible role by AMH on FLC, which warrants future investigations.

Key words: AMHR-II, Leydig cells, Mesenchymal cells, Progenitor cells, Sertoli cells, Postnatal testis, Light microscopy, Immunohistochemistry

Introduction

Anti-Mullerian hormone (AMH), a glycoprotein and a member of the transforming growth factor-beta (TGF- β) family, is an important factor in male sexual differentiation. In the male, AMH is produced by the Sertoli cells from the time of fetal sex differentiation to puberty (Josso et al., 2001). As other members of TGF- β family, AMH signals through two related but distinct receptors, both serine/threonine kinases with a single transmembrane domain, called type I and type II. Type II receptor is solely expressed in AMH target organs (Josso et al., 2001) such as the testis.

The prenatal effect of AMH in males is well documented and includes the regression of Mullerian ducts, the anlagen of the female internal reproductive tract in male fetuses (Jost, 1953). Rouiller-Fabre et al., (1998) have also showed that AMH represses aromatase activity of fetal Sertoli cells and inhibits testosterone synthesis by fetal Leydig cells (FLC). The role of AMH in the postnatal testis is not clearly identified at present. Male transgenic mice expressing very high levels of human AMH (hAMH) under the control of the mouse metallothionein-1 promoter (MT-hAMH), are incompletely masculinized externally and rapidly become infertile (Behringer et al., 1990). It is also seen that male MT-hAMH mice expressing lower levels of AMH show a reduced number of postnatally differentiated Leydig cells or adult Leydig cells (ALC, Racine et al., 1998). In contrast, AMH deficient mice exhibit marked Leydig cell hyperplasia (Racine et al., 1998). Taken together, it is suggested that AMH is a

negative regulator of ALC differentiation in the postnatal testis (Racine et al., 1998).

Theoretically, the inhibitory action of AMH on the differentiation of Leydig stem cells to progenitor cells to begin the process of ALC differentiation could be exerted either directly or indirectly. Mesenchymal cells, especially the ones in the peritubular region of the postnatal testis interstitium are shown as the primary precursors for the ALC in the adult testis (Ariyaratne et al., 2000). Therefore, if AMH action on these mesenchymal cells is direct, they should possess AMH receptors (AMHR), specifically, AMHR-II (Josso et al., 2001). However, there is no information available on the presence of AMHR type II in mesenchymal cells in the postnatal testis to determine whether AMH acts directly or indirectly to inhibit the ALC differentiation. By contrast, it is known that the mesenchymal cells around the Mullerian ducts in the fetal testis contain AMHR-II (Josso and di Clementi, 2003).

AMH has also been suggested as a negative regulator of ALC steroidogenesis. Addition of AMH to purified adult mouse Leydig cells (Racine et al., 1998) and R2C and MA-10 adult Leydig cell lines (Teixeira et al., 1999; Fynn-Thompson et al., 2003) decreases steroid hormone production (Racine et al., 1998; Rouiller-Fabre et al., 1998) by down regulating steady-state mRNA levels of enzymes in the steroidogenic pathway (Racine et al., 1998) and interfering with cAMP signaling (Fynn-Thompson et al., 2003). However, it is also reported that higher levels of intra-testicular testosterone is present in ethane dimethane sulphonate-treated adult rats after 35 days who were treated with AMH/MIS from day 11-14 after EDS treatment (Salva et al., 2004).

AMHR-II mRNAs, but not the protein AMHR-II, have been detected in developing and adult rat testis (Barrends et al., 1995) adult Leydig cells (Lee et al., 1999). Yet, no precise information is available on when and/or which cell type of the ALC lineage first gain AMHR-II. In addition to the cells of ALC lineage, the postnatal testis interstitium contains FLC (Mendis-Handagama et al., 1987, 1998; Kerr and Knell, 1988; Ariyaratne and Mendis-Handagama, 2000; Baker et al., 2003) which also have the steroidogenic potential. However, to our knowledge, there is no documentation on the presence of AMHR-II in FLC. In this study, we have used the first polyclonal antibody against AMHR-II to address this important issue which is crucial to understand the establishment of the regulatory role of AMH in ALC and FLC in the postnatal testis interstitium, and on ALC differentiation.

Materials and methods

Animals

Pregnant female Sprague Dawley rats were purchased from Harlan Industries (Madison, WI). They were housed one rat per cage, under conditions of

controlled temperature (25°C) and lighting (14L:10D) in the animal facility of The University of Tennessee College of Veterinary Medicine. Rats were provided food (Agway Prolab rat formula, Syracuse, NY) and water ad libitum. These rats were examined for litters twice daily (morning and evening) and the day of birth of pups was considered as Day 1 of postnatal life.

AMHR-II Antibody

A complete description of the antibody used to immunolocalize AMHR-II is published elsewhere (18). Briefly, a bacterially produced protein constituted by the extracellular domain of the human AMHR-II fused to a His6 tag was used to immunize a rabbit, and the resulting serum after 3 months was purified by affinity with the fusion protein as indicated in the O'Quick Pure System kit (Sterogene, Isnes, Belgium). As previously described (Gouedard et al., 2000; di Clemente et al., 2003), This antibody recognizes both the immature and mature forms of AMHR-II in CHO cells permanently transfected with AMHR-II and not in parental CHO cells, by western-blotting.

Testicular tissue preparation

Twenty one groups of male rats of the following ages were used (n=5 per group); 1, 5, 7-21, 28, 40, 60 and 90 days of age. They were killed by CO₂ inhalation and both testicles of each rat were harvested and fixed in Bouin's fluid by immersion fixation for 5-6 hours, processed and prepared for immunocytochemistry (Mendis-Handagama et al., 1998; Ariyaratne and Mendis-Handagama, 2000). Sections of 5 μ m in thickness were cut from these testis blocks and adhered on ProbeOn Plus glass slides (Fisher Scientific, Pittsburgh, PA). The animal protocol used (protocol#731) was approved by the Institutional Animal Care and Use Committee.

Immunocytochemistry

To immunolocalize AMHR-II, testis tissue sections were deparaffinized, incubated in 3% hydrogen peroxide for 20 minutes to quench endogenous peroxidase activity, and then incubated in goat serum (Biogenex, San Ramon, CA) overnight at 4°C to eliminate non-specific binding. AMHR-II antibody was used at an optimum dilution of 1:100, and the incubations were carried out at 4°C overnight. In control incubations, tissue sections were incubated with preimmune serum. The antigen-antibody complex was visualized using a super sensitive biotin-streptavidin-peroxidase method (Biogenex, San Ramon, CA). Finally, the sections were counter-stained with Mayer's hematoxylin (Sigma, St. Louis, MO), dehydrated in a series of increasing concentrations of ethanol and cover-slipped under Permunt (Fisher Scientific, Fair Lawn, NJ).

Results

Immunocytochemistry

All elongated spindle-shaped cell types in the testis interstitium, which included the mesenchymal cells (peritubular and centrally located), Leydig progenitor cells, endothelial cells and pericytes were negative for AMHR-II at all ages studied (few selected ages are shown in Figs. 1, 2). Newly formed ALC were present in the testis interstitium from the latter part of the postnatal day 10, however, they did not express AMHR-II immediately upon their appearance. On postnatal day 13, a weak positive cytoplasmic labeling for AMHR-II was first observed in some of these newly formed ALC (Fig. 1A) and other such cells, which were still negative for AMHR-II were also present in the testis interstitium (Fig. 1A). Although the number of the ALC in the testis interstitium positive for AMHR-II was increased from postnatal days 13 through 28, the intensity of the staining for AMHR-II in these cells during this developmental period appeared to be the same (Fig. 1A-D). By contrast, the intensity of the label in ALC together with the number of AMHR-II positive ALC in the testis interstitium appeared to be markedly increased at postnatal day 40 (Fig. 2A), 60 (Fig. 2B) and 90 (Fig. 2C) above younger levels (i.e. from days 13-28).

From birth up to postnatal day 21 (few selected ages

are shown in Fig. 1A-C), no cell type in the seminiferous tubules (i.e. Sertoli cells and germ cells), demonstrated positive labeling for AMHR-II. However, beginning at days 28, an extremely weak cytoplasmic label, which appeared to be above the background label was seen in the seminiferous tubules, confined to few areas of Sertoli cell cytoplasm (Figs. 1D, 2A,B). As we were particularly focusing on the cells of Leydig cell lineage we did not pursue further studies on cells in the seminiferous tubules, and therefore, we are unable to further discuss this point.

Fetal Leydig cells showed a positive cytoplasmic label for AMHR-II and they were the only cells in the testis that were labeled positive for AMHR-II at postnatal days 1, 5, and 7-12. Positive labeling for AMHR-II in fetal Leydig cells in a 10 day old postnatal rat testis is shown in Figure 2D.

Discussion

AMH is well known for its role during Müllerian duct regression. Recently, several *in vivo* and *in vitro* studies have shown that AMH also regulates Leydig cell steroidogenic function (Rouiller-Fabre et al., 1998; Racine et al., 1998; Fynn-Thompson et al., 2003; Salva et al., 2004) but the mechanism of action of AMH upon these cells is poorly understood. The first component of AMH signaling pathway, AMHR-II mRNA is expressed

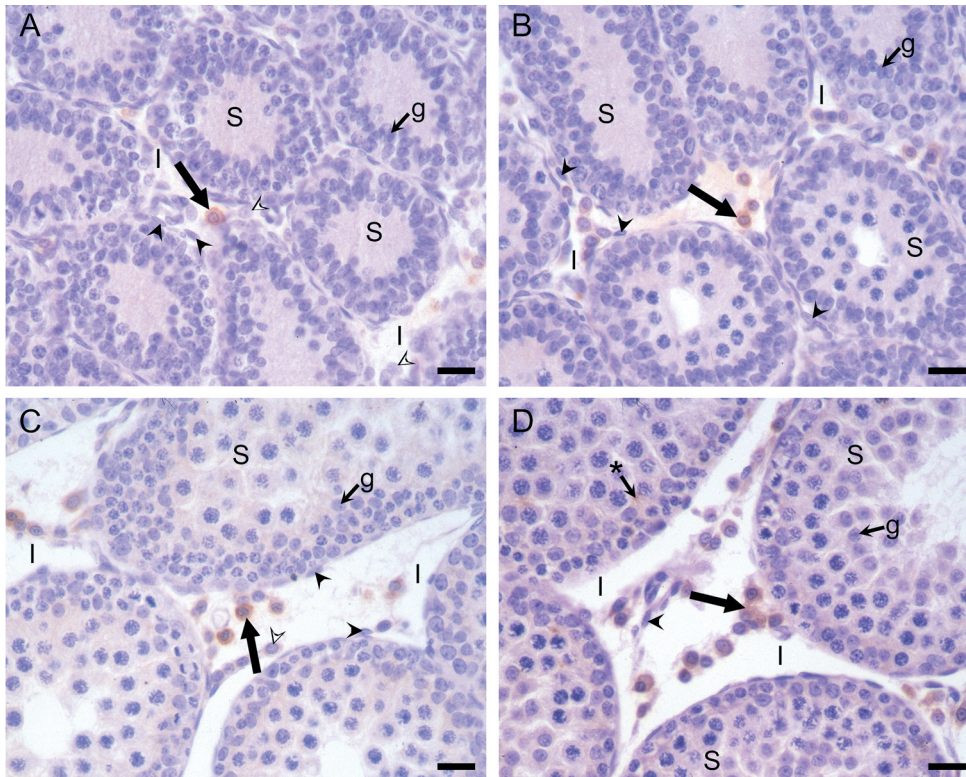


Fig. 1. Representative photomicrographs of rat testes at postnatal days 13 (A), 15 (B), 21 (C), 28 (D) immunolabeled for AMHR-II. Arrow depicts positive labeling in Leydig cells. Solid arrow heads (black) depict elongated spindle-shaped cells in the testis interstitium which show negative labeling for AMHR-II. Open arrow heads (white) show newly formed Leydig cells which are still negative for AMHR-II. No immunolabeling is visible in the seminiferous tubules (S) at postnatal days 13 (A), 15 (B), and 21 (C), however, at postnatal day 28 (D) sparse labeling is visible in few areas of the seminiferous tubules confined to the cytoplasm of few Sertoli cells (asterisks). I: testis interstitium; g: germ cells. Bar: 13.5 μ m.

by 21 day-old rat Leydig cells (Barrends et al., 1995; Lee et al., 1999) and by adult mouse and rat Leydig cells or cell lines (Fynn-Thompson et al., 2003). However, up to now, no immunohistological localisation of AMHR-II protein in the testis during the postnatal period had been performed on tissue sections of prepubertal testes in any mammalian species. Using a polyclonal antibody against the extracellular part of human AMHR-II, we show that surprisingly, AMHR-II protein is strongly expressed by Leydig cells compared to Sertoli cells, from birth to adulthood. These results are similar to those obtained on adult testis using a monoclonal anti-AMHR-II antibody directed against a specific epitope of human AMHR-II (Salhi et al., 2004), or using mice expressing beta galactosidase under the control of Amh2 promoter (Jeyasuria et al., 2004).

Establishment of the population of ALC in the postnatal testis is important for the attainment of puberty and regulation of testicular functions in the adult male mammal. This population of ALC differentiates primarily, if not exclusively, from the mesenchymal cells of the postnatal testis interstitium that are located around the seminiferous tubules (i.e. peritubular mesenchymal cells; Ariyaratne et al., 2000). Among the factors that regulate the postnatal ALC differentiation, it is suggested that AMH is a negative regulator of this process (Racine et al., 1998). This suggestion was originally based on the observations of Leydig cell

hyperplasia and absence of postnatally differentiated Leydig cells in the testes of AMH deficient and transgenic mice, respectively (Racine et al., 1998). Later, it has been reported that 21 day-old rat Leydig cells respond to AMH by decreasing DNA synthesis (Salva et al., 2004) and that AMH inhibits regeneration of at Leydig cells after EDS treatment (Salva et al., 2004).

If the negative regulatory effect of AMH on mesenchymal cell differentiation into Leydig cells in the postnatal testis is direct, it is logical to expect receptors for AMH, i.e. AMHR-II, in these mesenchymal cells. Immunocytochemical findings of the present investigation demonstrated clearly, the absence of AMHR-II in mesenchymal cells and progenitor cells at any age tested. Additionally, AMHR-II were absent in newly formed ALC prior to day 13. This trend seen in the ALC lineage for positive labeling for AMHR-II suggests that function of AMH in ALC is established with the maturation of the newly formed ALC following their differentiation. Additionally, these observations suggest that the negative regulatory role of AMH on the process of ALC differentiation is unlikely to be direct. This concept is not in agreement with two previously published reports (Lee et al., 1999; Salva et al., 2004), which concluded that AMH has a direct role in the regulation of postnatal testicular development. However, the validity of this conclusion of these studies (Lee et al., 1999; Salva et al., 2004) is in question due to the reasons

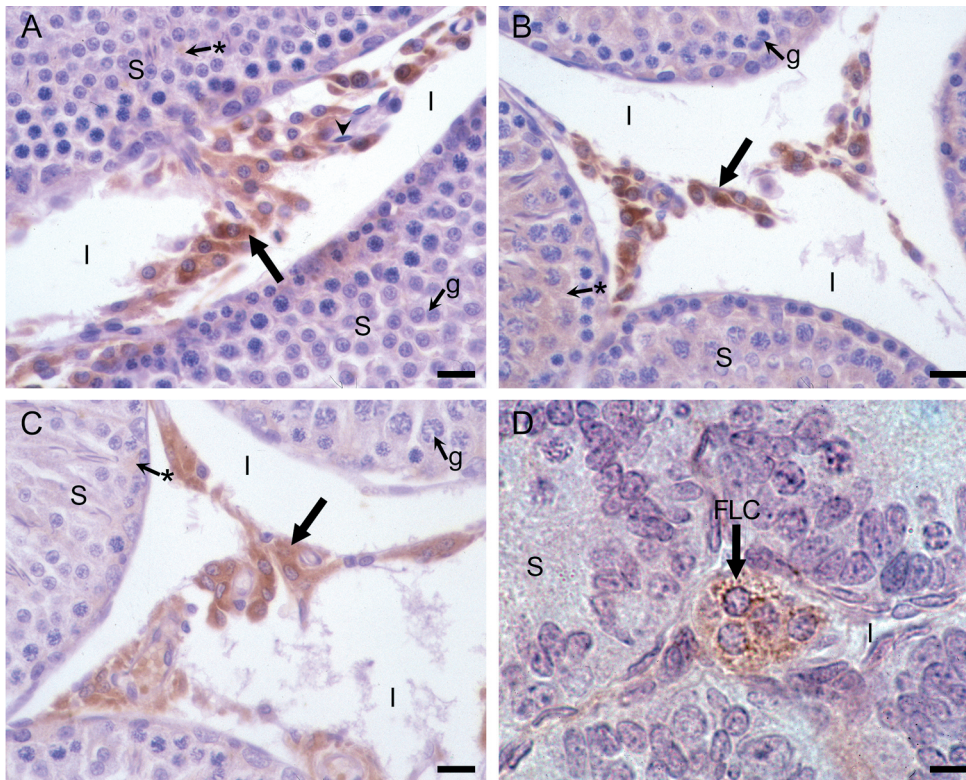


Fig. 2. Representative photomicrographs of rat testes at postnatal days 40 (A), 60 (B) and 90 (C) immunolabeled for AMHR-II. Arrow depicts positive labeling in Leydig cells. Note that the intensity of the immunolabel for AMHR-II in Leydig cells at these postnatal ages are much stronger than those present at younger ages shown in Figure 1. Solid arrow heads (black) depict elongated spindle-shaped cells in the testis interstitium which show negative labeling for AMHR-II. At postnatal day 60 (B) some labeling was visible in the seminiferous tubules (S) confined to the cytoplasm of several Sertoli cells (asterisks). Figure 2D demonstrates a cluster of FLC immunolabeled for AMHR-II in a 10 day old rat testis. I: testis interstitium; g: germ cells. Bars: A-C, 13.5 μ m; D, 34 μ m.

AMHR-II in testicular interstitial cells

discussed below.

First, the study of Lee et al. (1999) did not show the presence of AMHR-II protein expression in mesenchymal cells. Instead, they (Lee et al., 1999) based their conclusion on the presence of AMHR-II mRNA expression in progenitor cells, which were in suspension; latter cell type is the second cell stage in the ALC lineage (Mendis-Handagama and Ariyaratne, 2001), which is beyond the onset of the process of precursor cell differentiation. Moreover, these cells were obtained from 21 day old rats and at this age, not only the progenitor cells but the newly formed ALC are present in the testis interstitium. Therefore, the isolated cells in suspension used in their study should include a mixture of progenitors and newly formed ALC. In contrast, the present study convincingly demonstrates using testis tissues *in situ*, but not cell suspensions, that any spindle-shaped cell in the testis interstitium, which includes mesenchymal precursor cells and the progenitor cells of the ALC lineage, do not show positive immunolabeling for AMHR-II at any stage of development. Instead, the newly formed ALC from postnatal day 13 onwards, were positively labeled for AMHR-II; these cells are observed in tissue sections as circular or polygonal profiles. Therefore, it appears that the newly formed ALC at postnatal days 21, which are positive for AMHR-II, are mistakenly identified as Leydig progenitors in the cell suspensions of the latter study (Lee et al., 1999).

Second, it is an established fact that the primary source of androgens in the adult mammalian male is Leydig cells. Salva et al. (2004) reported that AMH inhibits regeneration of Leydig cells in EDS-treated rats. However, the mean values for both intratesticular androgen levels (i.e. both testosterone and 3 α -diol) and serum testosterone levels reported in this study (Salva et al., 2004) were two fold higher in MIS/AMH-treated rats compared to vehicle-treated rats. These observations are sufficient evidence to prove that Leydig cell differentiation has taken place in these MIS/AMH-treated rats, as efficiently as or more efficiently than in vehicle-treated rats although the authors have concluded that MIS inhibits regeneration of Leydig cells in EDS-treated rats. Clearly, these values on mean serum and intratesticular testosterone levels do not support the fact that reduced numbers of Leydig cells have differentiated in MIS-treated rats compared to vehicle-treated controls. Additionally, it is difficult to comprehend how the steroidogenic capacity of Leydig cells in MIS-treated rats could be greater than vehicle-treated rats reported in this paper, because MIS/AMH has shown to be a negative regulator of Leydig cell steroidogenic function in previous studies (Racine et al., 1998; Rouiller-Fabre et al., 1998), including a study published by the same group of investigators (Teixeira et al., 1999).

Several previous studies have suggested that AMHR-II is restricted to Sertoli cells using *in situ* hybridization techniques, (di Clementi et al., 1994;

Barrends et al., 1995; Teixeira et al., 1996) but at this juncture, focus of our study was only on cells in the testis interstitium and therefore, we are unable to comment or discuss this point any further. The present investigation demonstrated clearly for the first time that AMHR-II protein is detected in cells of ALC lineage beginning from the newly formed ALC at postnatal day 13 through mature ALC. Also, we show for the first time that there is a time lag between the first appearance of the newly formed ALC (i.e. on postnatal day 10) and gaining of AMHR-II activity in them. Therefore, it is apparent that the regulatory role of AMH on ALC function in the postnatal testis, i.e. inhibitory effect on steroidogenesis (Racine et al., 1998; Rouiller-Fabre et al., 1998; Teixeira et al., 1999; Fynn-Thompson et al., 2003) is not established immediately upon their differentiation from mesenchymal precursors. Gaining the AMHR-II in the ALC population is suggestive of activation of the functional changes required to respond to AMH action. It is also important to note that at these early ages (i.e. from younger than 21 days), the ALC size and morphology do not change (Mendis-Handagama et al., 1987, 1998; Ariyaratne and Mendis-Handagama, 2000), even though they were functionally advancing as demonstrated by the present study. Therefore, it is possible that the gaining of the AMHR-II in postnatally differentiated ALC marks the initiation of the functional maturational process of these cells towards the next cell stage of the ALC lineage. Additionally, it is logical to interpret that the observed differences in the intensity of the positive labeling in ALC between postnatal days 13-28 and 40-90 may be a reflection of the amount of AMHR-II in these Leydig cells. However, it is important that these hypotheses should be tested prior to making any firm conclusions.

To date, it is accepted that sex differentiation is driven by two independent testicular hormones (Jost, 1953); testosterone is produced by the FLC and acting through the androgen receptor, promotes the virilization of the Wolffian ducts, masculinization of the external genitalia and urogenital sinus (Wilson, 1992), whereas AMH signalling through a plasma membrane receptor (Imbeaud et al., 1995) induces the regression of the Mullerian ducts (Josso et al., 1993; Lee and Donohoe, 1993). However, the observation of the positive AMHR-II labeling of FLC is intriguing because, this finding suggests that AMH may also have a regulatory role on the postnatal FLC that needs to be identified in future investigations. Thus, "a gonadal hormone with multiple functions" (Lee and Donohoe, 1993) appears to be a perfect description for AMH.

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