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A quantitative and interspecific test for biological activity of anti-Müllerian hormone: the fetal ovary aromatase assay

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Summary

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance or factor, has previously been shown to sex-reverse the steroidogenic pattern of fetal mammalian ovaries through repression of aromatase biosynthesis. Study of the ontogeny of the response of cyclic AMP-stimulated aromatase activity of rat fetal ovaries to AMH has allowed us to develop a quantitative biossay for the hormone. Linear responses as a function of the logarithm of AMH concentration were observed over ranges of 0.2 - 7.5 μg/ml for the bovine protein and 0.15 - 2 μg/ml for the human protein, with a maximal decrease in aromatase activity of 90% for both proteins. Under the same *in vitro* conditions, AMH treatment did not affect cyclic AMP-stimulated fetal rat testicular aromatase activity. Partially purified chick AMH also decreased rat ovarian aromatase activity, allowing us to use this test to study AMH ontogeny in chick gonads. Analysis of the species specificity of AMH repression of ovarian aromatase activity indicated that turtle and rat fetal ovaries responded to AMH of other vertebrate classes, whereas aromatase activity of chick embryo ovaries could be repressed only by the homospecific hormone.

Key words: Müllerian-inhibiting substance, gonadal sex differentiation, cytochrome P-450 aromatase, fetal ovary

Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS) or factor (MIF) plays an important role in sex differentiation. Besides promoting regression of Müllerian derivatives in male fetuses (Jost, 1953), the hormone inhibits oogonial proliferation and induces the formation of seminiferous cord-like structures in fetal ovaries exposed to it in organ culture (Vigier et al., 1987). Both the inhibiting and virilizing effects of AMH upon the developing ovary have recently been confirmed in transgenic mice (Behringer et al., 1990). AMH has also been shown to sex-reverse fetal ovarian steroidogenesis by repressing cytochrome P_{450} aromatase enzyme biosynthesis (Vigier et al., 1989).

Up to now, the only test for anti-Müllerian activity was based upon the work of Picon (1969), showing that fetal rat Müllerian ducts in organ culture regress in the presence of AMH of other mammalian species. This labor-intensive procedure is at best semi-quantitative, and requires high concentrations of AMH to obtain unequivocal histological evidence of Müllerian regression. We have used the observation that AMH can decrease aromatase activity in the fetal rat ovary to develop a more sensitive, quantitative and interspecific bioassay.

Materials and methods

Animals

Wistar rats were used between 13 days post-coitum (the day following the night of mating was considered day 0) and 10 days post-partum. Release of fertilized eggs from the European pond turtle *Emys orbicularis* was induced by intracoelomic injection of 5 i.u. of oxytocin (Ewert and Legler, 1978). Eggs were incubated for either 55 days at 25°C or 35 days at 30°C, to obtain respectively 100% male or 100% female offspring at stage 24 of development, at which point differentiated gonads are no longer sensitive to temperature (Pieau, 1974; Pieau and Dorizzi, 1981). Gonads were removed from chick embryos (white Leghorn, *Gallus gallus* L.) which had been incubated for 8 to 17 days at 38°C, and from an adult rooster after decapitation.

Reagents

Native testicular bovine AMH was purified from incubation medium of bovine fetal testes as described (Picard and Josso, 1984). Human recombinant anti-Müllerian hormone (hAMH)

was purified from transfected CHO cell culture medium (Pepinsky et al., 1988). Purified AMH was quantified by reading optical density at 280 nm, using an extinction coefficient of 1.

Partially purified chick AMH was obtained by incubating 250 10-17-day-old chick embryo testes in organ culture for 3 days. The corresponding incubation medium (25 ml) was loaded on a 0.4 ml column containing immobilized *Lens culinaris* lectin (Sigma), equilibrated with Tris-HCl, 20 mM, pH 7.7 buffer, and eluted by addition of NaCl, 300 mM and 10% *N*-acetyl-glucosamine. The protein concentration of the pooled 3 fractions with the highest optical density at 280 nm was 0.3 mg/ml. Protein bands were visualized by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, in reducing and non-reducing conditions, as described (Picard and Josso, 1984) and biological activity was tested by the Müllerian duct assay (Picon, 1969). Incubation medium from chick heart was subjected to the same treatment and used as control.

Production of monoclonal antibody (mAb) 278 against bovine AMH (Vigier et al., 1982) and of mAb 10.6 against hAMH (Pepinsky et al., 1988) has been documented. Unlabelled chemical reagents were purchased from Sigma.

Organ culture

Gonads were explanted 3 days in organ culture, as previously described (Vigier et al., 1987). Rat gonads were explanted together with the adjacent mesonephros, which provides a 'handle' for subsequent manipulation. Aromatase activity of ovaries cultured with or without their adjacent mesonephros is comparable (results not shown). In experiments calling for association of gonads of different ages or species, rat fetal ovaries were always cultured whole, other gonads were cut into fragments of similar size. When gonads of different sex and/or age were compared for their effect upon ovarian aromatase activity, an effort was made to keep the amount of co-cultured tissue relatively constant. The mean protein content of co-cultured tissue is shown on Table 1. Culture medium was CMRL 1066 (Eurobio, France) containing 0.25 mg/ml bovine serum albumin and, for rat gonads, 0.1 mM 3-isobutyl-1-methylxanthine and 1 mM N_6,O_2-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP).

Measurement of aromatase activity

Aromatase activity was measured at the end of the culture period by the tritiated water technique (Ackerman et al., 1981) as previously described (Vigier et al., 1989). Organ culture explants were incubated for 5 hrs in groups of 4 in a Dubnoff incubator under a 95% O_2/5% CO_2 atmosphere in the presence of 0.7 μM [1β-^{3}H]androstenedione (27.4 Ci/mmol) obtained from NEN/Dupont. All experiments were carried out in triplicate unless stated otherwise. Linear regression and analysis of variance were computed using 'ABSTAT' software (Anderson-Bell, Co), the residual variance was used for comparing mean aromatase activities of control and treated ovaries.

Table 1. *Protein content of chick gonadal tissue co-cultured with 16-day-old rat fetal ovaries*

Chick tissue	Protein content (μg), mean±s.e.m.
Testis	34.41±6.72
Left ovary	46.43±8.65
Right ovary	42.41±11.3

Tissue processing and assay of protein concentration

After the aromatase assay, rat fetal ovaries and a representative sample of other cultured gonads were subjected to histological analysis as described (Vigier et al., 1989) to check for possible necrosis. All other gonads were solubilized as described (Vigier et al., 1989) and protein concentration was measured by the bicinchoninic acid assay (Redinbaugh and Turley, 1986) using the BCA protein reagent (Pierce®) with the enhanced protocol described by the manufacturer. This technique could not be used to measure protein concentration in the presence of *N*-acetyl glucosamine. Therefore the protein concentration of the eluate of the lectin column was assessed by measuring optical density at 280 nm, using an extinction coefficient of 1.4.

Results

Effect of AMH upon aromatase activity of fetal rat ovaries

Ontogeny of response of developing rat gonads to AMH

Aromatase activity of developing rat ovaries explanted 3 days in organ culture in control medium was low throughout gestation and up to 1 day after birth (Fig. 1A). The activity could be enhanced by Bt_2cAMP, particularly at 16 days p.c., at which time a tenfold stimulation in activity was obtained. AMH treatment reduced aromatase activity of Bt_2cAMP-treated explants to control levels. Results obtained later than one day post-partum, using the organ culture technique, yielded inconsistent results (not shown), probably due to defective survival of the explants. The low basal aromatase activity of fetal testes in organ culture was increased by Bt_2cAMP treatment from 16 days post-coitum onwards, however this stimulation could not be inhibited by concomittent AMH treatment (Fig. 1B).

Response to mammalian AMH

Since 16-day-old rat fetal ovaries showed the greatest degree of inhibition of Bt_2cAMP-stimulated aromatase activity by AMH, this developmental stage was chosen to study dose-response relationship (Fig. 2). A linear log/dose response to AMH treatment was demonstrated between 0.2 and 7.5 μg/ml of bovine AMH (r=0.964, P<0.01) and between 0.15 and 2 μg/ml for hAMH (r=0.918, P<0.01). Intra-assay and interassay variations were respectively 12.3% (n=3) and 14.6% (n=3) for bovine AMH and 12.2% (n=3) and 19.2% (n=4) for human AMH. Recombinant human AMH was more active than bovine AMH in this system, with an ED_{50} equal to 0.42 μg/ml compared to 1.12 μg/ml for bAMH. Monoclonal antibodies to bAMH and hAMH, added to the culture medium at a concentration equal to five times that of the hormone, decreased the bioactivity of their antigens by 90% and 70% respectively (Fig. 2).

Response to chick AMH

Chicken AMH obtained by lectin chromatography of testicular tissue-incubation media exhibited the

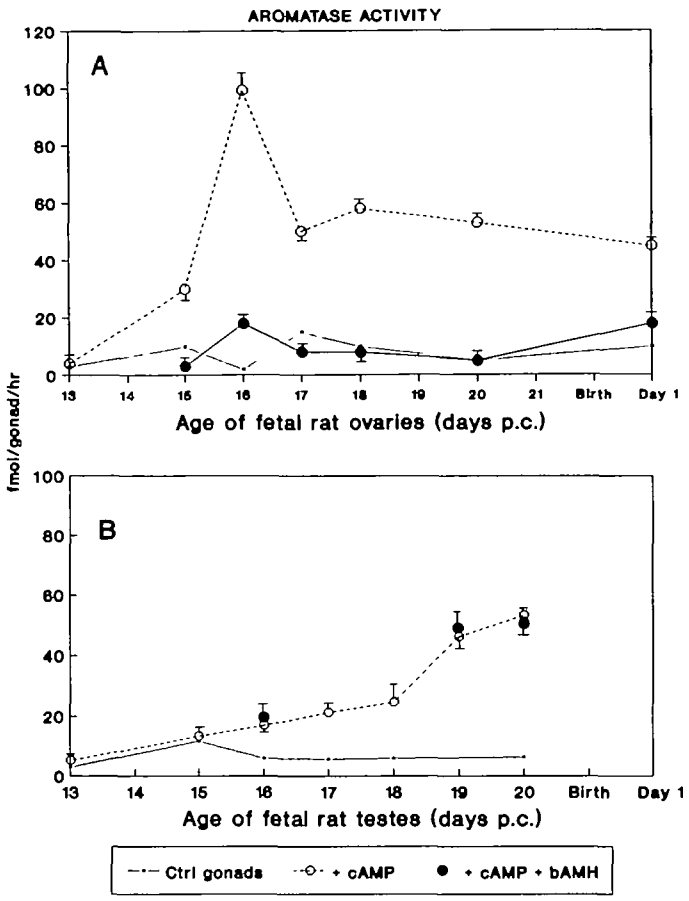


Fig. 1. (A) Rat ovarian aromatase response to AMH was studied in whole rat ovaries maintained in organ culture from 13 days p.c. to 1 day after birth. Basal aromatase activity was low, at all stages studied. Stimulation by 0.1 mM Bt_2cAMP is maximal at 16 days and can be inhibited by 10 μg/ml bAMH from 15 days p.c. to 1 day after birth. Values shown represent mean and s.e.m. of experiments usually performed in triplicate. At 16 days p.c., $n=6$ for controls and Bt_2cAMP+ AMH-treated ovaries, and $n=15$ for Bt_2cAMP treated ones. (B) Aromatase activity in rat fetal testes was studied from 13 to 20 days p.c. Stimulation of aromatase activity by 0.1 mM Bt_2cAMP increased with fetal age whether or not 10 μg/ml bAMH was added to the culture medium. Mean and s.e.m. of triplicate experiments are shown. p.c.: post-coitum.

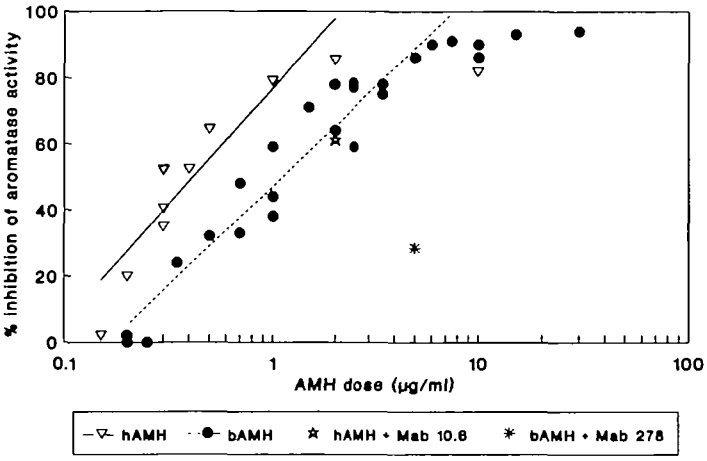


Fig. 2. Quantitative bioassay for AMH based upon inhibition of aromatase activity of Bt_2cAMP-stimulated 16-day-old rat fetal ovaries. Inhibition of aromatase activity of hormone-treated ovaries was determined in comparison with the aromatase activity of contralateral ovaries cultured without bAMH or hAMH. Each point represents the mean of triplicate experiments. Inhibition of aromatase activity was proportional to the logarithm of the concentration of either bAMH or hAMH in the culture medium. Half-maximal inhibition (ED_{50}) was obtained with 1.12 μg/ml bAMH and 0.42 μg/ml hAMH. Monoclonal antibodies to either bAMH or hAMH decreased hormone bioactivity at a hormone/mAb ratio of 1/5: the concentration of mAb-treated bAMH (5 μg/ml) and hAMH (2 μg/ml) required to elicit the same biological effect as untreated AMH of the same species (respectively 0.5 and 0.6 μg/ml) was increased by 90 and 70% respectively.

expected reduction-sensitive pattern on polyacrylamide gels (Fig. 3). Its apparent relative molecular mass, respectively 160×10^3 for the dimer and 76×10^3 for the monomer, was slightly higher than that of human recombinant AMH. A contaminant, of approximately 72×10^3 M_r in non-reducing conditions is resolved into 2 smaller subunits in reducing conditions. The biological activity of the preparation was tested by the aromatase (Table 2) and Müllerian duct assay (Fig. 4). Strongly positive results were obtained with both techniques. Assuming that the hormone preparation is approximately 30% pure, chick AMH at an estimated concentration of 12 ng/ml produced a 93% decrease of aromatase activity and induced complete regression of rat fetal Müllerian ducts at 20 μg/ml. A precise dose/response curve cannot be constructed, in the absence of an accurate assay for chick AMH concentration.

AMH production by chick gonads studied by the aromatase technique

The ontogeny of AMH production by chick gonads was studied by exposing 16-day-old fetal rat ovaries to gonadal tissue of developing chickens, and measuring the aromatase activity of the rat ovaries at the end of the culture period. Results are shown in Fig. 5. The ability of chick testes to decrease rat fetal aromatase activity is high between 8 and 17 days of incubation, and is lost in the adult. The left fetal ovary exhibits increasing anti-aromatase activity after ten days, while the stunted right ovary and fetal chick cardiac tissue have no significant effect.

Effect of AMH upon aromatase activity of non-mammalian fetal ovaries

Chick ovaries

Spontaneous aromatase activity of 17-day-old chick ovaries was extremely high, insensitive to Bt_2cAMP treatment (not shown), and could not be significantly decreased by hAMH, 10 μg/ml (Fig. 6A). The effect of homospecific AMH was tested by co-culturing chick ovaries with chick testes of the same age. The

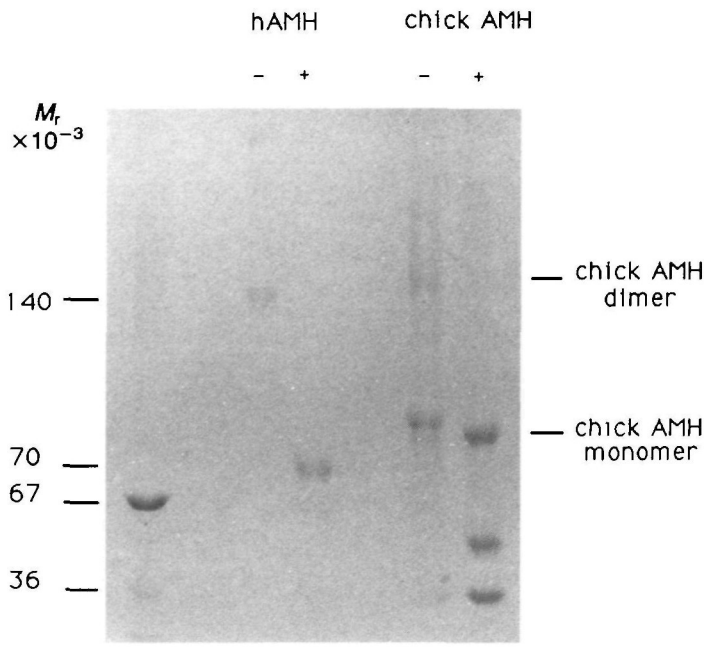


Fig. 3. Polyacrylamide gel electrophoresis of partially purified chick AMH. Lane 1: Size markers (bovine serum albumin and lactate dehydrogenase); lanes 2, 3: Recombinant hAMH; 4, 5: fraction of secreted proteins from chick embryonic testicular tissue eluted from a *Lens culinaris* lectin affinity column. +, reducing conditions; −, non reducing conditions. Under non-reducing conditions, hAMH migrates as a 140×10^3 M_r homodimer, in the lectin eluate two protein bands are visible, a high M_r one slightly retarded compared to the human AMH dimer, and a low M_r contaminant. Under reducing conditions, hAMH migrates as a single 70×10^3 M_r subunit, the chick subunit is slightly larger, and the contaminant is split into two low M_r bands. The calculated M_r for chick AMH in this experiment is 160×10^3 M_r for the dimer and 76×10^3 M_r for the monomer. The human AMH dimer (140×10^3 M_r) and monomer (70×10^3 M_r) were used as relative molecular mass markers in addition to those loaded on lane 1.

Table 2. *Anti-aromatase activity of partially purified chick AMH*

	Protein concentration* (μg/ml)	Approximate AMH concentration† (μg/ml)	Anti-aromatase activity‡ (%)
Partially purified chick AMH§	35	12	93
Control ‖	35	0	0

* Protein concentration was estimated by measuring optical density at 280 nm (see Materials and methods).
† Assuming a 30% purity of partially purified chick AMH.
‡ Decrease of aromatase activity of 16-day-old fetal rat ovaries cultured for 3 days in presence of Bt_2cAMP.
§ Lectin column eluate of secreted proteins from chick testicular tissue.
‖ Lectin column eluate of secreted proteins from cardiac tissue.

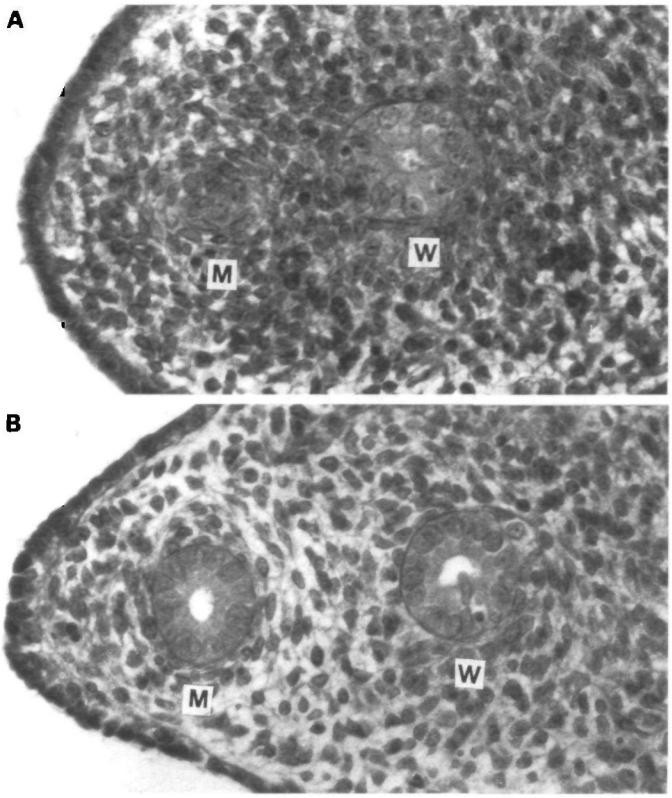


Fig. 4. Anti-Müllerian activity of partially purified chick AMH, studied by the Müllerian duct assay. (A) The rat Müllerian duct, cultured in the presence of partially purified chick AMH, is either undetectable, or reduced to a fibrous whorl, as shown here. Similar results were obtained in all 6 fetal rat reproductive tracts cultured in the presence of the hormone. Hematoxylin and Eosin, ×187. (B) Müllerian ducts cultured in control buffer show no sign of regression. M: Müllerian duct; W: Wolffian duct.

aromatase activity of chick embryonic ovaries was significantly inhibited by co-culture with chick testes ($P < 0.01$), whether expressed per gonad (Fig. 6A) or per μg of ovarian protein (not shown).

Turtle ovaries

In the turtle *Emys orbicularis* at the only developmental stage tested, the ovaries exhibited spontaneous ovarian aromatase activity (Fig. 6B), which was not modified by Bt_2cAMP treatment (results not shown). Bovine AMH at the concentration of 10 μg/ml reduced aromatase activity by 79.2% ($P < 0.01$), nearly down to the level observed in control testes of the same age.

Discussion

The data presented indicate that the aromatase activity of 16-day-old rat fetal ovaries can be used as an end-point for a bioassay of AMH bioactivity. This compares favorably with the Müllerian duct bioassay (Picon, 1969), because it is quantitative and because approximately 10 times less hormone is required to achieve

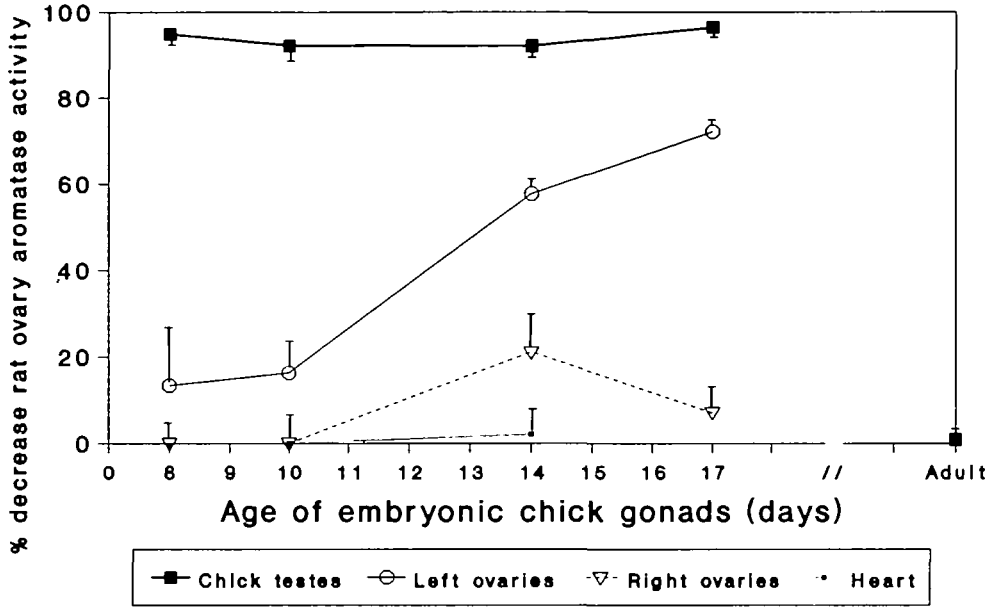
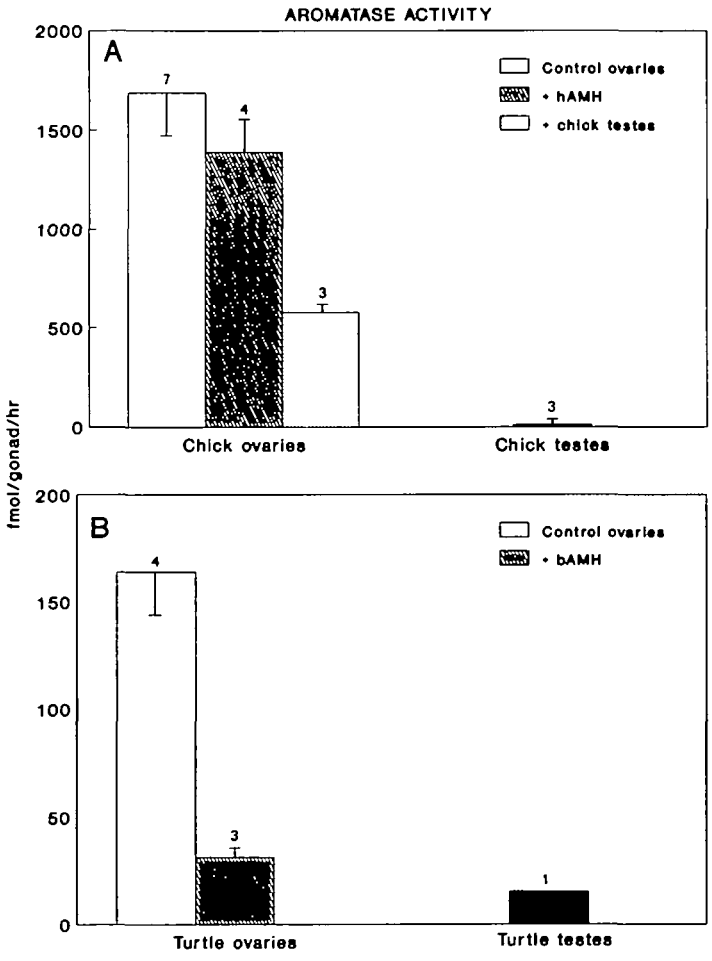


Fig. 5. Ontogeny of AMH production by chick gonads at various ages. 16-day-old rat fetal ovaries were cultured in groups of 4 either in control medium, supplemented with 0.1 mM Bt_2cAMP, or in the same medium in association with developing chick fetal gonads or with chick heart. The mean protein content of chick gonads co-cultured with one rat fetal ovary is shown on Table 1. Rat fetal ovarian aromatase activity is dramatically decreased by association with embryonic but not adult testicular tissue. Rat ovarian aromatase is also decreased by co-culture with the left chick ovary at 14 and 17 days. The stunted right ovary and fetal chick heart had no demonstrable effect. Results shown are the mean and s.e.m. of quadruplicate experiments, except for experiments with 10-day-old chick ovaries (n=8).



half-maximal inhibition of aromatase activity. Differences in bioactivity between mammalian hormone preparations is probably due to the purification method used: bAMH is purified from tissues obtained at autopsy, while the recombinant molecule is purified from cell culture medium. The bioassay also responds to chick AMH and can be used for testing AMH secretion by gonadal tissue: chick embryonic testes, between 8- and 17-days-old, and chick fetal ovaries aged 14 or 17 days, which had been previously reported to exhibit anti-Müllerian activity by the Picon bioassay (Hutson et al., 1981), were bioactive in the aromatase assay as well. Cyclic AMP is required to accelerate maturation of the aromatase enzymatic activity of the fetal rat ovaries, which do not normally synthesize aromatase in significant amounts prior to birth (Picon et

Fig. 6. (A) Species specificity of AMH effect upon aromatase activity of chick embryonic ovaries. One, quartered, 17-day-old chick embryo ovary was cultured either in control medium, or in the presence of 10 μg/ml hAMH, or in association with 2 halved chick embryonic testes of the same age. Chick testes, in groups of 2, each cut in two, were cultured in control medium. Similar results were observed if results were expressed per μg of ovarian protein (not shown). The number of replicates performed is shown. Chick ovary aromatase activity is significantly ($P < 0.01$) inhibited by co-culture with chick testes, but not by treatment with hAMH. (B) Aromatase activity of embryonic turtle gonads. Four gonads were used per replicate, the number of replicates is shown. Spontaneous aromatase activity of the ovary is relatively high, and can be inhibited by bAMH, 10 μg/ml. Results represent mean and s.e.m. of gonadal aromatase activity, expressed per gonad.

al., 1985). The nucleotide has been shown to block the effect of AMH upon rat fetal Müllerian ducts (Ikawa et al., 1984), but does not block the effect of AMH upon the fetal ovary.

Like the Müllerian duct assay, the bioassay based upon aromatase activity of fetal ovaries is interspecific only within certain limits. Rat and turtle ovaries respond to heterospecific AMH while the chick ovarian aromatase activity is affected only by the homospecific hormone, whether partially purified from testicular incubation media or released by embryonic gonads. These findings are in keeping with those reported by Tran and Josso (1977), who observed regression of rat Müllerian ducts co-cultured with chick testes, but no effect of mammalian fetal testicular tissue upon chick Müllerian ducts. Purification of chicken AMH to homogeneity from secretory proteins of 8-week-old male chickens has been reported, using a complex sequence of purification steps (Teng et al., 1987); in our hands significant purification, yielding a highly bioactive protein was achieved by a single passage through a lectin column.

Estrogen is thought to play a major role in the gonadal differentiation of many vertebrate species (Dorizzi et al., 1991). Repression of constitutive estrogen production and diversion to testosterone production may be needed for testicular differentiation to occur. Certainly, for AMH to inhibit Müllerian duct development, estrogen must not be present (Hutson et al., 1982; Newbold et al., 1984). Could AMH play a role in testicular differentiation by blocking estrogen synthesis in the primitive gonad? AMH inhibited aromatase biosynthesis in fetal ovaries in all three vertebrate classes investigated, regardless of their genotypic or environmental mode of sex determination, but its effect was not demonstrable in rat testicular tissue (Fig. 1B). One possible explanation could be the absence of AMH receptors in the testicular cells producing aromatase, or their saturation by endogenous AMH.

Bioactivity of peptide hormones is mediated by receptor-ligand interaction at the cell membrane level. Why rat ovarian and Müllerian receptors should be less stringent than chick receptors in their binding specificity requirements is difficult to understand and an answer to this question would necessitate isolation of AMH receptors in these species. An alternative possibility deserves consideration. The AMH molecule undergoes proteolytic cleavage at a monobasic site (Pepinsky et al., 1988); studies are in progress to determine whether this cleavage is required for its activation. If AMH proves to be similar in this respect to other TGF-β-like proteins, insensitivity of chick ovaries and Müllerian ducts to mammalian AMH might be caused by failure of chick tissues to cleave the mammalian AMH molecule. For instance, it has been suggested that mouse cells cannot be infected by human HIV even when the human CD4 receptor is present because they lack a suitable proteolytic enzyme (Stephens et al., 1990). Further investigation into structure/activity relationship for the AMH molecule is necessary to address this problem and will be facilitated by the availability of the quantitative bioassay we describe.

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