Serum anti-Müllerian hormone dynamics during controlled ovarian hyperstimulation

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BACKGROUND: The study aim was to investigate possible changes in serum anti-Müllerian hormone (AMH) levels during controlled ovarian hyperstimulation (COH), and their possible relationship with follicular development and other ovarian hormones. METHODS: A total of 93 women undergoing COH with GnRH agonist and FSH was studied prospectively. Serum levels of AMH, inhibin B, estradiol (E₂), progesterone, testosterone and Δ⁴-androstenedione were measured when pituitary suppression was achieved (baseline), on days 6 and 8 of FSH treatment, and on the day of hCG. The number of small (<12 mm) and large (≥12 mm) antral follicles were estimated using ultrasound. RESULTS: Serum AMH levels declined progressively (baseline, 1.21 ± 0.11 ng/ml; day 6, 0.91 ± 0.09 ng/ml; day 8, 0.77 ± 0.08 ng/ml; and day of hCG, 0.53 ± 0.06 ng/ml), whereas—as expected—the other hormone levels increased during FSH treatment. Throughout COH, serum AMH levels correlated positively with the number of small but not large antral follicles, and with inhibin B serum levels. No correlation between AMH and the other hormones was observed. CONCLUSIONS: Serum AMH levels decline gradually during multiple follicular maturation, probably reflecting the dramatic reduction in the number of small antral follicles due to COH, and confirming the scarce AMH expression by larger follicles.

Key words: anti-Müllerian hormone/controlled ovarian stimulation/follicular development

Introduction

Recent clinical data have indicated that peripheral anti-Müllerian hormone (AMH) levels, as detected by a newly developed ultrasensitive assay (Long et al., 2000), during the early follicular phase of the menstrual cycle, is a useful reflector of the number of early antral follicles (de Vet et al., 2002; Fanchin et al., 2003) and of the number of oocytes retrieved in subsequent controlled ovarian hyperstimulation (COH) cycles (Seifer et al., 2002). Indeed, as with other members of the transforming growth factor β superfamily (Pepinsky et al., 1988), such as activins and inhibins, AMH is mainly expressed by the follicular granulosa cells (Vigier et al., 1984). Its secretion is probably modulated by the degree of gonadic development, as it increases from barely detectable levels at birth (Rajpert-De Meyts et al., 1999) to augmented, yet subtle, levels after puberty (Hudson et al., 1990). Also, the stage of follicular growth is likely to influence AMH expression, with predominant production during pre-antral and early antral follicular stages. In adult women, the physiological role of AMH remains astonishingly ill-established. Previous studies conducted in animals have suggested that AMH, probably via its specific type II receptors expressed in granulosa and theca cells, may reduce both aromatase activity and the amount of LH receptors in FSH-stimulated granulosa cells (di Clemente et al., 1992) and inhibit testosterone production by theca cells (Teixeira et al., 2001). In addition, growing evidence obtained in mice indicates that AMH is involved in the rate of recruitment of primordial follicles (Durlinger et al., 1999, 2002).

Little is known about the dynamics of AMH secretion when the ovarian follicles grow beyond the early antral stage and enter the final maturation process. The only report that aimed at clarifying this issue included a single AMH measurement during the pre-ovulatory phase in a limited population (Cook et al., 2000), but unfortunately this study design prevented the detection of possible fluctuations in AMH levels during the mid- to late follicular phase of the menstrual cycle. In addition, data on the plausible relationship between AMH secretion and that of other follicular hormones throughout the ultimate phase of follicular development are still lacking.

An attractive model to examine the influence of follicular development status on AMH secretion is that of COH. Indeed, COH is characterized by the thorough transformation of small antral follicles into maturing follicles as a result of exogenous FSH. This phenomenon is not reproduced during the menstrual cycle, in which the early antral follicle cohort remains nearly intact throughout the follicular phase and only a single follicle reaches maturation. Hence, the study of hormonal dynamics during COH might be particularly helpful to evaluate the
relative contribution of small and large antral follicles to peripheral AMH levels. Also, given that large antral follicles may express AMH only scarcely (Baarends et al., 1995), the multiplicity of maturing follicles could be instrumental in exacerbating overall serum AMH levels and improving their detection.

In order to clarify the evolvement of AMH secretion from earlier to later stages of follicular maturation, the dynamics of AMH levels was investigated during pituitary-desensitized COH cycles, together with its possible relationship with follicular development status and hormonal secretion.

Materials and methods

Subjects

A total of 93 infertile women, aged from 24 to 41 years, was studied prospectively. In order to be included, the women had to meet the following criteria: (i) both ovaries present; (ii) no current or past diseases affecting ovaries or gonadotrophin or sex steroid secretion, clearance or excretion; (iii) body mass index (BMI) ranging from 18–27 kg/m²; (iv) no current hormone therapy; and (v) adequate visualization of ovaries on transvaginal ultrasound scanning. Indications for IVF–embryo transfer were sperm abnormalities (51%), tubal abnormalities (27%), unexplained infertility (18%) and endometriosis (4%). Informed consent was obtained from all women, and the investigation was approved by the authors’ internal Institutional Review Board.

COH protocol

All women received a timed-release GnRH agonist, leuprolide acetate (1 mg/day, s.c.) (Lucrin; Abott-France Pharmaceuticals, Rungis, France) from cycle day 21 onwards. On day 3 of the subsequent cycle, complete pituitary desensitization was confirmed by the detection of low serum levels of estradiol (E₂) and gonadotrophin. Patients also underwent a conventional ultrasound examination to exclude the presence of ovarian cysts and to verify that the endometrial thickness was <5 mm. Recombinant FSH therapy (Gonal-F; Serono Pharmaceuticals, Boulogne, France) was then initiated at a dosage of 225 IU/day, whereas daily GnRH agonist administration was continued until the day of hCG administration (Gonadotrophine Chorionique “Endo”; Organon Pharmaceuticals, Saint-Denis, France; 10 000 IU, i.m.). Daily FSH doses and timing of hCG administration were adjusted according to the usual criteria of follicular maturation. Administration of hCG was performed when at least three follicles exceeded 17 mm in diameter and E₂ levels per mature follicle (>17 mm diameter) were >300 pg/ml. Oocytes were retrieved 36 h after hCG administration using transvaginal ultrasound-guided aspiration. All embryo transfers were performed 2 days after oocyte retrieval using Frydman catheters (CCD Laboratories, Paris, France). The luteal phase was supported with micronized progesterone (Estima; Effik Pharmaceuticals, Bièvres, France; 400 mg/day) administered by the vaginal route and starting on the evening of embryo transfer.

Hormone and follicle measurements

On the day in which pituitary desensitization was confirmed (baseline), on days 6 (d6) and 8 (d8) of FSH treatment, and on the day of hCG administration (dhCG), women underwent measurement of serum levels of AMH, inhibin B, E₂, progesterone, testosterone and Δ⁴-androstenedione (AD) at approximately 09:00 h. All blood samples were obtained by venipuncture; the serum was then separated and frozen in aliquots at −20°C until used for subsequent centralized analysis. Serum AMH levels were determined using an ultrasensitive enzyme-linked immunosorbent assay (ELISA) (Beckman-Coulter, Villepinte, France) as described previously (Long et al., 2000). For AMH, functional sensitivity was 0.24 ng/ml and intra-assay and inter-assay coefficients of variation (CV) were <5 and <8% respectively. Serum inhibin B levels were determined using a double antibody ELISA (Serotec, Varilhes, France) as described previously (Groome et al., 1996). For inhibin B, functional sensitivity was 15 pg/ml and intra-assay and inter-assay CV were <6 and <9% respectively. Serum E₂ and progesterone levels were determined using an automated multi-analyser system with chemiluminescence detection (ACS-180; Bayer Diagnostics, Puteaux, France). For E₂, functional sensitivity was 15 pg/ml and intra-assay and inter-assay CV were 8 and 9% respectively. For progesterone, functional sensitivity was 0.1 ng/ml and intra-assay and inter-assay CV were 8 and 9% respectively. Serum testosterone and AD levels were measured using a radioimmunoassay technique (Beckman-Coulter, Villepinte, France); functional sensitivity was 0.1 ng/ml and intra-assay and inter-assay CV were 8 and 9% respectively.

At baseline, on day 8, and on the day of hCG, ovarian ultrasound scans were performed using a 4.5–7.2 MHz multi-frequency transvaginal probe (Siemens Elegra; Siemens S.A.S., Saint-Denis, France). The objective of ultrasound examinations was to evaluate the number and sizes of ovarian antral follicles. For the purposes of the present study, the antral follicles were sorted into small (3–11 mm diameter) and large (12–22 mm diameter) sizes. The choice of this threshold for defining small and large follicles was arbitrary and based on the fact that, in the menstrual cycle, the sizes of non-dominant follicles remain <12 mm whereas only the dominant follicle develops further and matures (Pache et al., 1990).

Statistical analysis

The measure of central tendency used was the mean, while the measure of variability was the standard error of the mean (SEM). Longitudinal changes in hormone levels during COH were assessed using ANOVA with repeated measures. Relationships between two different continuous variables was assessed by correlation. The Fisher r to z-test was used to determine if the coefficient of correlation (r) was significantly different from zero. A P-value < 0.05 was considered statistically significant.

Results

Hormone and follicular profiles during COH

Hormone and follicular dynamics during COH are depicted in Figure 1. Serum AMH levels displayed a remarkable, gradual decrease during COH (baseline, 1.21 ± 0.11 ng/ml; d6, 0.91 ± 0.09 ng/ml; d8, 0.77 ± 0.08 ng/ml; and dhCG, 0.53 ± 0.06 ng/ml) (P < 0.001). It is noteworthy that this phenomenon was paralleled by a decrease in the number of small antral follicles (16.6 ± 0.6, 10.8 ± 0.6 and 4.0 ± 0.4 follicles <12 mm in diameter on baseline, d8 and dhCG respectively). Furthermore, a positive and steady correlation between serum AMH levels and number of early antral follicles was noted throughout COH (r = 0.73, P < 0.0001; r = 0.65, P < 0.0001 and r = 0.73, P < 0.0001 on baseline, d8 and dhCG respectively). In contrast, no correlation was observed between serum AMH levels and the number of growing follicles (>12 mm diameter) at any observation day. As expected, the remaining ovarian hormone levels (inhibin B, E₂, progesterone, testosterone and AD) increased progressively and significantly in response to exogenous FSH treatment (P < 0.001), which corresponded to

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the concomitant increase in the number of large antral follicles (≥12 mm diameter).

Serum AMH levels showed a positive correlation with serum inhibin B levels. Incidentally, the strength of this relationship tended to increase gradually throughout COH ($r = 0.30$, $P < 0.02$ on baseline; $r = 0.33$, $P < 0.005$ on d6; $r = 0.42$, $P < 0.0002$ on d8; and $r = 0.55$, $P < 0.0001$ on dhCG). In contrast, no correlation between serum AMH levels and the remaining follicular hormones was observed except for serum progesterone levels on the day of hCG ($r = -0.31$, $P < 0.008$).

**Overall population characteristics and COH results**

The women’s mean age and menstrual cycle length were 32.8 ± 0.4 years and 29.1 ± 0.3 days respectively. COH was continued for 11.1 ± 0.2 days and required 2382 ± 73 IU of FSH. No patient received hCG before the 9th day of FSH treatment. Mean numbers of total oocytes, mature oocytes, available embryos and transferred embryos were 9.1 ± 0.4, 7.6 ± 0.4, 5.1 ± 0.3 and 2.6 ± 0.9 respectively. A total of 89 women underwent embryo transfer. Rates of clinical pregnancy (presence of intra-uterine gestational sac with cardiac activity) and ongoing pregnancy (≥12 weeks of amenorrhoea) per embryo transfer were 27 and 21% respectively.

No influence of clinical indications for IVF–embryo transfer on serum AMH levels was observed. Similarly, the duration and dose of FSH treatment failed to correlate with AMH levels. In contrast, serum AMH levels were positively correlated with the total number of oocytes retrieved, in particular at baseline.
(r = 0.43, P < 0.0001). Afterwards, the strength of such a relationship declined progressively (r = 0.42, P < 0.0002 on d6; r = 0.27, P < 0.03 on d8, and r = 0.22, P = NS on dhCG). In keeping with this, there was a positive, yet weaker, relationship between AMH levels and the number of mature oocytes. The numbers of available and transferred embryos were not associated with serum AMH levels. Consistently, baseline AMH levels correlated positively with the number of pre-ovulatory follicles (r = 0.36, P < 0.002) and serum E2 levels (r = 0.25, P < 0.04) on dhCG.

As the present study was not designed to evaluate the predictability of serum AMH levels on IVF-embryo transfer outcome, data presented hereafter are merely descriptive. Patients who became pregnant (n = 22) tended to show higher serum AMH levels than those who did not (n = 69) (1.40 ± 0.14 versus 1.19 ± 0.23 ng/ml at baseline; 1.07 ± 0.25 versus 0.70 ± 0.25 ng/ml on d6; 0.71 ± 0.08 versus 0.45 ± 0.21 ng/ml on d8; and 0.42 ± 0.16 versus 0.26 ± 0.06 ng/ml on dhCG). These differences did not reach statistical significance on any observation day. No significant differences were observed in serum AMH levels in patients who either had, or had not, achieved an ongoing pregnancy.

Discussion
The aim of the present study was to investigate the dynamics of AMH secretion and its possible relationship with follicular development status (correlations with the number of follicles measuring <12 mm or ≥12 mm in diameter) during pituitary-desensitized COH cycles. The possible link between AMH secretion and the secretion of other ovarian glycoprotein and steroid hormones was also evaluated. The use of COH as a model to study the evolvement of AMH secretion during the later stages of follicular growth was motivated by the total conversion of small antral follicles into large maturing follicles in response to FSH stimulation—a phenomenon which is virtually absent in spontaneous cycles. Indeed, the non-physiological, extensive switch of small to large antral follicles that characterizes COH would be helpful in monitoring possible variations in serum AMH levels resulting from follicular growth and maturation. The results of the present study indicated that, in parallel with the progressive reduction in the small antral follicle cohort and the increase in the number of larger follicles, a remarkable decline in peripheral AMH levels occurs. Consistently, serum AMH levels correlated with the number of <12 mm but not ≥12 mm follicles throughout COH. Taken together, these data suggest that AMH is preferentially and constantly secreted by small antral follicles during COH, and provide support to the hypothesis that differentiation of granulosa cells during follicular growth is likely to alter their ability of expressing AMH (Baarends et al., 1995).

The present results are in agreement with those of previous experiments conducted in rats, which demonstrated a decreased AMH expression in large antral follicles and corpora lutea as compared with small antral follicles in vitro (Baarends et al., 1995). Nonetheless, the present results diverge from preliminary data reported by others (Cook et al., 2000), who showed an increase in serum AMH levels from the early to the late follicular phase of spontaneous cycles. The relative steadiness in the amount of small antral follicles throughout the follicular phase in the menstrual cycle, which contrasts with the dramatic attrition of the small antral follicle pool due to the multiple follicular maturation triggered by COH, may constitute a plausible explanation for these conflicting data. Furthermore, the physiological mechanisms implicated in the scarce expression of AMH by larger follicles and its possible consequences on the growth and differentiation of the follicle-oocyte complex during the late follicular phase remain unknown.

Yet, other factors may have contributed to the observed decrease in serum AMH levels during COH. One such factor is follicular atresia, which may impair AMH secretion (Baarends et al., 1995). Unfortunately, the design of the present study did not permit the singling out of healthy from atretic follicles to further address this issue. Other factors that might have played a role in the reduction in AMH levels are the estrogens. These hormones have been implicated in the down-regulation of AMH and AMH type II receptor mRNA in the ovary (Baarends et al., 1995). Although the present data failed to show a direct relationship between serum AMH and E2 levels during COH, the hypothesis of an inhibiting effect of supraphysiological E2 levels on AMH secretion could not be ruled out by the present study and deserves further investigation. Also, androgens have been reported to hinder AMH production by theca cells (Teixeira et al., 2001). The opposite serum profiles of both hormones during FSH treatment observed in the present study are consistent with this hypothesis, although a negative correlation between serum AMH and testosterone or AD levels has not been identified.

Another factor that might be involved in the ontogenesis of AMH is FSH. Data available in the literature with respect to this issue are, however, contradictory. Some investigators considered that FSH might inhibit AMH mRNA expression, as was shown in neonatal rat testis (Kuroda et al., 1990), whereas others thought that AMH might either impair (Durlinger et al., 2001) or foster (McGee et al., 2001) FSH-induced follicular growth. The present data did not contribute much to the elucidation of this issue; nonetheless, no influence was seen on serum AMH dynamics in relation to either the duration of administration or the total dose of exogenous FSH administered. In addition, mean serum AMH levels at baseline (achievement of pituitary suppression, 1.21 ± 0.11 ng/ml) were similar to those reported during the early follicular phase of the menstrual cycle (Cook et al., 2000; de Vet et al., 2002; Fanchin et al., 2003), thereby indicating that endogenous FSH suppression has little or no effect on AMH secretion by early antral follicles.

It is noteworthy that serum AMH and inhibin B levels correlated positively throughout COH. Although notable differences in serum profiles of both glycoproteins were seen during FSH treatment, this positive relationship may be attributed to the fact that inhibin B is also secreted by small antral follicles. In addition, previous studies have shown that, after a transient increase from the early to the mid-follicular phase, inhibin B production declines when the follicle nears the pre-ovulatory phase (Groome et al., 1996)—a phenomenon that may signal follicular maturation. The present observation of a progressive strengthening of AMH and inhibin B
correlation during follicular development is consistent with this. Incidentally, late follicular-phase progesterone levels showed a negative correlation with AMH. This phenomenon might be explained by the reported association between serum progesterone levels on the day of hCG and follicular maturity (Fanchin et al., 1995, 1996).

Baseline serum AMH levels were predictive of ovarian response to COH. Indeed, a positive correlation was observed between baseline serum AMH levels and peak E2 levels, the number of large antral follicles, and the number of oocytes obtained on the day of hCG. These results expand the data produced by other investigators (Seifer et al., 2002), who reported a positive correlation between day 3 AMH levels and the number of oocytes obtained in a successive IVF–embryo transfer cycle. The results are also in keeping with the recent demonstration in a separate group of patients that day 3 serum AMH levels reflect the size of early antral follicle cohort more accurately than day 3 FSH and inhibin B (Fanchin et al., 2003). Hence, the present observation that AMH levels measured after pituitary desensitization by GnRH agonists remain a useful predictor of ovarian response to COH opens new perspectives into the assessment of ovarian status. Incidentally, the present study was not powered to address the plausible relationship between AMH levels and IVF–embryo transfer outcome, and this is an issue that warrants further investigation.

In conclusion, the present results provide the first detailed information on the evolution of AMH levels through the ultimate phases of follicular development in women. During multiple follicular maturation, serum AMH levels decline progressively along with the reduction in the number of small antral follicles. Hence, it is conceivable that AMH is barely expressed in granulosa cells of larger pre-ovulatory follicles. Although the physiological mechanisms implicated in such a process remain undetermined, these results are in keeping with basic research data indicating that AMH is preferentially secreted by pre-antral and early antral follicles (Baarends et al., 1995). However, further studies to elucidate both the regulation and physiological pertinence of this hormone in adult women are needed. An involvement of AMH in the inhibition of primordial follicle recruitment has been observed in the mouse ovary (Durlinger et al., 1999, 2002), and this opens new insights into the mechanisms that control the initiation of folliculogenesis. From a clinical standpoint, the present results showing that AMH levels measured after pituitary suppression by GnRH agonists remain predictive of COH outcome may encourage further investigation on the value of AMH measurements during COH in the evaluation of ovarian responsiveness to exogenous gonadotrophins, follicular quality, and perhaps also embryo implantation outcome.

References


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