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Immunoreactive inhibin in human follicular fluid in an ovarian hyperstimulation programme for in vitro fertilization: correlations and different forms

Bärbel Reichardt, Gerhard Mehltretter, Karin Lechner, Hans K Rjosk, Otto A Müller and Jochen Schopohl

Medizinische Klinik, Klinikum Innenstadt, Ludwig-Maximilians-Universität München; Gynäkologische Gemeinschaftspraxis[1]; Krankenanstalten Rotes Kreuz; München, Germany

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The inhibin concentration in 131 samples of human follicular fluid obtained from 31 women undergoing ovarian hyperstimulation for in vitro fertilization was measured using specific double antibody radioimmunoassay. We used the synthetic 1-32-α-inhibin as standard and radioiodinated 1-32-Tyr-α-inhibin as tracer. Antibodies were raised in rabbits by immunization with the synthetic peptide. Estradiol and progesterone concentrations were measured using commercial radioimmunoassays. *Results*: The inhibin concentration correlated with the estradiol ($r=0.57$, $N=88$, $p<0.0001$) and progesterone ($r=0.82$, $N=88$, $p<0.0001$) concentrations in human follicular fluid. The dosage of human menopausal gonadotropin given to individual patients correlated with the average inhibin concentration measured in their follicles ($r=0.72$, $N=23$, $p<0.0001$). Similarly, the size of follicles correlated with their inhibin content ($r=0.75$, $N=131$, $p<0.0001$). Nineteen samples of human follicular fluid originating from follicles of different size and volume were examined using gel-chromatography. In each human follicular fluid the main form of inhibin (32 kDa) was recovered. In small follicles (3 ml) we found $12.8\pm9.1\%$ (mean ± SD) of the whole immunoreactive inhibin eluating in the area of V_0 (≥80 kDa). In the larger follicles (4–7 ml), however, only $4.4\pm4.2\%$ of this large inhibin form could be found. *Conclusions*: Our data confirm that human menopausal gonadotropin stimulates ovarian inhibin production. In addition to the estradiol and progesterone concentrations, the inhibin concentration may be an index of granulosa cell function and follicular maturation. The occurrence of large molecular weight forms of inhibin in small follicles remains unclear. They may represent large precursor molecules which are proteolytically cleaved in more mature follicles.

Jochen Schopohl, Medizinische Klinik, Klinikum Innenstadt, Ziemssenstr. 1, W-8000 München 2, Germany

Inhibin is a gonadal glycoprotein which selectively suppresses FSH-mRNA production and secretion of FSH, but not of LH, from the pituitary gland (1, 2). Since inhibin was purified in 1985 (3, 4) its detection has been limited to bioassays (5, 6) and several heterologous radioimmunoassays (RIA) using antibodies against 58 kDa or 32 kDa inhibin isolated from bovine follicular fluid (7).

There are at least two biologically active inhibin molecules, named inhibin A (α-β_A) and inhibin B (α-β_B) (8), being members of a large family which includes activin, transforming growth factor β (TGFβ) and Müllerian inhibiting substance (8).

Furthermore, the amino acid sequence of both subunits of inhibin and their precursors have been analyzed and the existence of multiple molecular weight forms of inhibin in bovine follicular fluids have been demonstrated (9, 10). Dynamic changes in the different molecular form of inhibin may also play a physiologically significant role in the regulation of FSH secretion and maturation of oocytes.

In this study we describe the development of a heterologous RIA for human inhibin based on a synthetic peptide prepared from a sequence of the human inhibin α-chain. We studied human follicular fluids (hFF), derived from in vitro fertilization (IVF) patients to clarify different inhibin levels and molecular weight forms depending on follicle size, its stimulation with human menopausal gonodotropin (HMG) and the maturation of the oocyte.

Materials and methods

Patients

This study includes 131 hFF samples of 31 women (mean age 30.5 years, range 26 to 36) participating in an IVF programme. Most of the patients had regular ovulatory cycles as judged by basal body temperature charts and serum progesterone values. They were fully investigated by hysterosalpingography and the infertility was principally due to a tubal factor.

The stimulation of multiple follicles was initiated on the third cycle day by giving HMG ("Humegon",

Organon, Germany) 150–450 IU im daily. From the eighth cycle day onwards follicular growth was assessed by daily measurement of plasma estradiol and progesterone and by vaginal ultrasonography using a 5.0 MHz transducer (Kretz, Austria). Human chorionic gonadotropin (HCG, "Pregnyl", Organon, Germany) 10 000 IU im was given to the patients to induce ovulation when the mean diameter of the largest follicle reached 15–17 mm. Oocyte pick-up was performed approximately 35 h after HCG injection by means of ultrasonically guided transvaginal follicular puncture. Oocytes were classified as degenerated, pre-mature and mature according to appearance of the cumulus-oocyte complex as described by Krebs and Al-Hasani (11). All oocytes were inseminated with husband sperm 2–6 h after aspiration. Sperm preparation was performed with a swim-up technique or Percoll density-gradient centrifugation. The oocytes were cultured in growth media and up to three embryos were transferred to the patients at the 2–6 cell stage approximately 40 h after insemination.

We used only hFFs without the addition of follicular rinsing fluid. All hFFs were centrifuged at 1400 × *g* for 10 min to remove cellular fragments and blood cells and kept frozen at −18°C until further investigations. The experiments were in accordance with the ethical methods of the responsible committee on human experimentation and with the Helsinki Declaration.

Inhibin-RIA

The inhibin immunoreactivity was measured by specific double antibody RIA using an antibody directed against human inhibin-α-1-32, a synthetic fragment derived from the NH_2-terminal sequence of the human α-subunit (Peninsula Lab., UK). Antibodies were raised in two male rabbits by monthly immunization with the synthetic peptide (1–32) coupled to bovine thyroglobulin according to the carbodiimide method (12). The resulting antiserum was used in a final dilution of 1:2000.

This synthetic fragment of human α-chain was also applied as standard. The radiotracer used in the RIA was human 1-32-Tyr-α-inhibin which had been labelled with ^{125}I on the terminal tyrosine by the iodogen method (13).

The incubation volume consisted of 0.1 ml antibody and 0.1 ml standard or unknown. Preincubation was carried out at 4°C over 24 h. After one day tracer incubation bound-free separation was performed using the double antibody PEG method.

The non-specific binding was less than 5% and tracer binding 15 to 20%. The ED_{50} of the standard curve was 0.05 nmol/l and the minimum detectable dose of inhibin found in the assay was 0.011 nmol/l.

Duplicate aliquots of each sample were assayed and the inter- and intra-assay coefficients of variation were 11.5% and 7.5% respectively. There was no cross-reaction with LH (500 U/l), HCG (200 U/l), FSH (500 U/l), porcine (7.8 nmol/l) and bovine inhibin (15.6 nmol/l), inhibin-like peptide (20 nmol/l) or with activin (50 nmol/l) and TGF-β (100 nmol/l). Displacement curves of several pools of unpurified hFF and recombinant human inhibin A in this RIA were parallel to that of the 1-32-α-inhibin standard (Fig. 1). Recombinant human inhibin A was a gift from Dr Schwall, Genentech, CA.

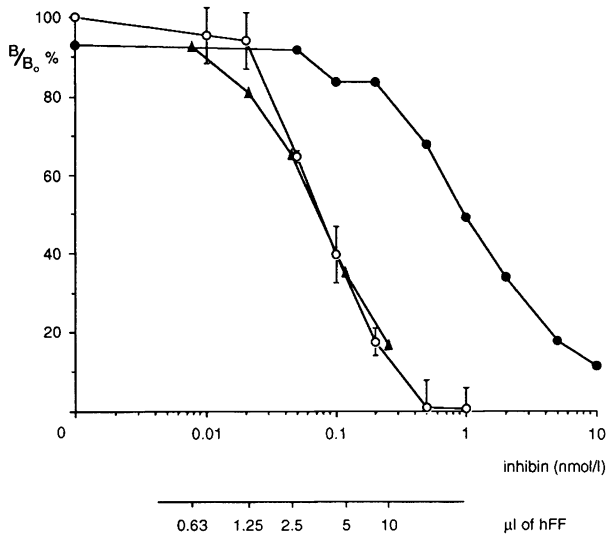

Fig. 1. Displacement curve of recombinant human inhibin A (—●—) in comparison to the standard curve with α-inhibin 1-32 (—○—, x ± SD) and unpurified hFF (—▲—).

Estradiol and progesterone assay

In addition to inhibin, estradiol (E_2) and progesterone (P) concentrations were measured in the same samples by RIA using commercially available kits (Baxter). Because of the high concentration of steroids in hFF the samples were diluted 1:1000 in E_2-free and P-free serum before measurement.

Gel chromatography by Sephadex G-75

Gel chromatography was performed using a column of 1 × 60 cm with Sephadex G-75 (Pharmacia), prepared and evaluated as described in the users' manual of Pharmacia. The elution buffer contained 0.1 mol/l acetic acid with 0.1% BSA.

Statistical analysis

Correlations between the measured variables were tested by *t*-distribution. Correlation between the maturity of the oocytes and inhibin content of the hFFs was performed using the Mann-Whitney U-test.

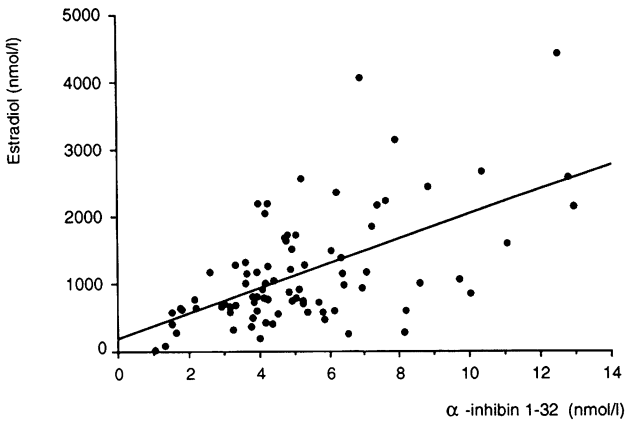

Fig. 2. Correlation between concentration of α-inhibin 1-32 and estradiol measured in human follicles (r = 0.57, N = 88, p < 0.0001).

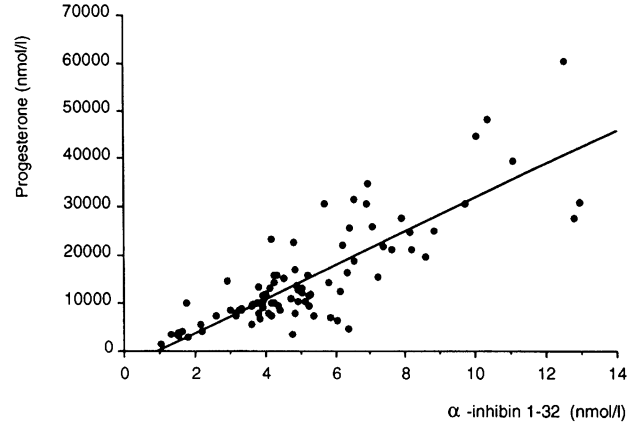

Fig. 3. Correlation between concentration of α-inhibin 1-32 and progesterone measured in human follicles (r = 0.82, N = 88, p < 0.0001).

Results

A positive correlation was found between the size of 131 human follicles and their inhibin content, obtained from 31 IVF patients (r = 0.75, N = 131, p < 0.0001). The follicles varied in size from 1 to 7 ml, according to the content of hFF.

The dosage of exogenous FSH, given as daily HMG injections to individual patients to stimulate follicular growth, correlated with the average inhibin concentration measured in their follicles (r = 0.72, N = 23, p < 0.0001).

The inhibin concentration correlated with the E_2 concentration (r = 0.57, N = 88, p < 0.0001) in 88 samples of hFF derived from 26 women undergoing IVF treatment (Fig. 2). A positive correlation was also found between the inhibin and P concentration (r = 0.82, N = 88, p < 0.0001) in the same 88 hFFs obtained from the same 26 IVF patients (Fig. 3).

The inhibin content of 88 hFF of 26 IVF patients was analysed in comparison to the maturity of the according oocyte (11). In follicles with a mature oocyte we found a lower inhibin content (0.013 nmol) than in follicles having a degenerated or immature oocyte (0.023 nmol, p < 0.005).

We examined 19 samples of hFF of 6 IVF patients by gel chromatography using a Sephadex G-75 column. These hFFs originated from follicles of different size and volume (3–7 ml). Recovery after chromatography was about 90%. In each hFF we found a peak with a distribution coefficient (Kd) of 0.34, representing the main inhibin form of 32 kDa. In small follicles with a volume of 3 ml we found a second peak in the area of V_0 (Kd 0–0.08), where 12.8 ± 9.1% of the whole immunoreactive inhibin was recovered (N = 6). In contrast, in the larger follicles (4–7 ml) we found only 4.4 ± 4.2% of inhibin eluating with the void volume (N = 13), shown in Figs. 4, 5 and Table 1. Dilutions of both peaks were parallel to the slope of the standard curve of α-inhibin 1–32. The molecular mass of both peaks exceeded 22 kDa, indicating that there are no isolated α-chains (18 kDa) found in hFF.

Discussion

We describe the development of an inhibin RIA, based on an available 1–32 peptide of the human α-chain of inhibin, demonstrating that this assay measures inhibin in native follicular fluid and recombinant human inhibin A, as shown in the displacement curves (Fig. 1).

Sinosich et al. (14) developed a similar homologous RIA based on the cyclic synthetic peptide 6-30-α-inhibin, whereas Franchimont et al. (15) used 5-24-α-inhibin to measure inhibin.

In comparison to heterologous assay designs (7), using polyclonal antisera raised in rabbits against purified bovine inhibin, our RIA recognizes only the α-chain of human inhibin and does not show any cross-reaction with porcine or bovine inhibin or other members of the inhibin family, e.g. activin or TGFβ.

Our assay probably recognizes not only the most bioactive 32 kDa form of human inhibin, but also a range of higher molecular weight forms (120, 108, 88, 65 and 58 kDa) that have been identified in bovine follicular fluid using monoclonal antibodies specific for each subunit (20 and 13 kDa) of bovine follicular fluid 32 kDa inhibin (9).

The occurrence of larger molecular weight forms (≥ 80 kDa) of inhibin in small follicles (3 ml) is unclear. Many polypeptide hormones are generated by the cleavage of larger precursor molecules and most precursors are unable to exert their full activity until they are processed to mature forms. It may be speculated that these larger forms of inhibin represent large precursor molecules (16, 17, 9) which are proteolytically cleaved in the more mature follicles (> 4 ml). Miyamoto et al. (9)

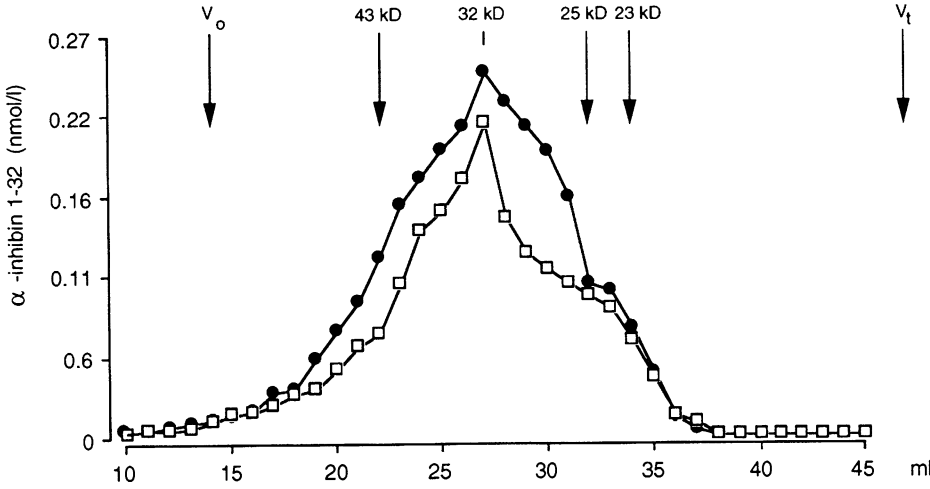

Fig. 4. Gel chromatography of two hFFs obtained from follicles of 6 ml (—□—) and 7 ml (—●—) using a Sephadex G-75 (1 × 60 cm) column. For each chromatography 1.5 ml of hFF was applied on the column; the fraction size was 1.25 ml.

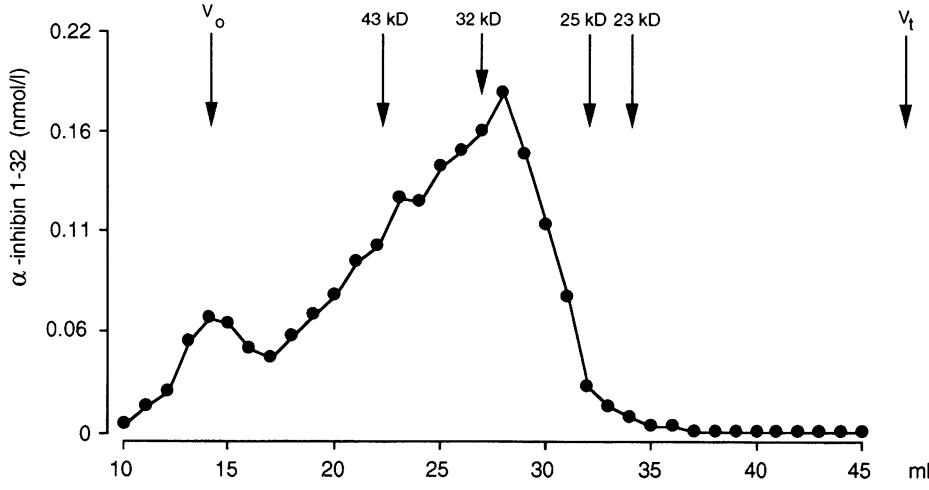

Fig. 5. Gel chromatography of an hFF obtained from a follicle of 3 ml (—●—) using a Sephadex G-75 (1 × 60 cm) column.

Table 1. Percentage of the whole immunoactive inhibin eluating in the area of V_0 depending on the size of the follicles (Figs 4, 5). Most of the immunoreactive inhibin eluates at a Kd of 0.34 corresponding to a molecular weight of 32 kDa.

Follicular size	6–7 ml	5 ml	4 ml	3 ml
	1.3%	7.3%	1.2%	7.3%
	2.5%	5.8%	0.0%	13.9%
	0.0%	2.9%	11.5%	9.6%
		12.6%	2.9%	24.1%
		7.0%	2.0%	0.0%
				21.7%
No. of samples	3	5	5	6
Mean (%)	1.3%	7.1%	3.5%	12.8%
SD±	1.2	3.5	4.6	9.1

suggested that at least the intact 32 kDa inhibin composed of 20 kDa and 13 kDa inhibin subunit linked by disulphide bridges is essential for inhibin activity and—as shown in our experiments—probably necessary for the maturing processes of the oocyte.

The hFF of the preovulatory follicle contains steroid and peptide factors that exert paracrine and autocrine control on maturation of the antral follicle (18), specifically of the oocyte-corona-cumulus complex (19). In normal and hyperstimulated ovulatory cycles the hFF content of E_2 and P as markers of granulosa cell function and follicular maturity is well established (19–21).

This study demonstrates that in the late follicular phase there is a positive correlation between inhibin, E_2 and P concentration in hFF (Figs. 2, 3). A positive

correlation has been found between serum inhibin and E_2 concentration during the follicular phase after hyperstimulation (21) and in the normal human menstrual cycle (22). We found that larger follicles contain more inhibin than smaller ones, probably because the inhibin secretory potential increases with the number of granulosa cells in larger follicles. Tsuchiya et al. (23) also expected the amount of circulating basal inhibin being secreted to be in correlation with the number of granulosa cells. We confirm that inhibin—besides E_2 and P—may be a valid index of granulosa cell function and follicular development during the hyperstimulation cycle.

This study shows that in response to ovarian hyperstimulation by exogenous HMG there is an increase in production of human ovarian inhibin. This stimulation of inhibin by HMG, which contains equal amounts of FSH and LH, is probably mediated by LH and FSH together, because it has recently been shown that LH stimulates inhibin production in human granulosa cells as well as FSH (24, 25). According to McLachlan et al. (26) and Bicsak et al. (27) our finding confirms that inhibin is a follicular product under FSH control, with granulosa cells playing the main role of production (16, 28).

Our findings of significantly lower inhibin levels in follicular fluids with mature oocytes in comparison with those with degenerated oocytes are not yet understood. Rising inhibin levels in serum (21) and follicular fluid during hyperstimulation have raised the possibility of using inhibin responses to ovarian hyperstimulation as an index of outcome in IVF and embryo transfer (26). Franchimont et al. (15) investigated inhibin levels in follicular fluid and concluded that inhibin appears to be an index of follicular maturation, since the inhibin content of follicles characterized as immature by their E_2/P ratios and E_2 is lower than that of mature follicles. These divergent statements may seem surprising, but in contrast to Franchimont et al., who classified the maturity of the oocyte by measuring E_2 and P in follicular fluid, we used the microscopic analysis of Al-Hasani (11) to characterize the oocyte and its grade of maturation. Because of these different kinds of classification a comparison of the opposing results is not appropriate.

No significant positive correlation could be found between follicular size and maturation grade of the oocyte. Larger follicles containing more inhibin do not necessarily indicate that the oocyte is mature. In larger follicles we found mainly the high bioactive 32 kDa inhibin (Fig. 4)—follicular fluid with mature oocytes may contain less higher molecular weight forms of inhibin but more 32 kDa inhibin.

A reduced inhibin production in very mature follicles indicates the luteinization of granulosa cells induced by HCG injection (29). We propose that a follicle with high inhibin levels might be too much hyperstimulated or does not luteinize by HCG injection.

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