Healthy women and patients with endometriosis show high concentrations of inhibin A, inhibin B, and activin A in peritoneal fluid throughout the menstrual cycle

Introduction

Inhibin A, inhibin B, and activin A are dimeric proteins identified in ovarian follicular fluid that modulate ovarian steroidogenesis and pituitary follicle stimulating hormone (FSH) production (Ying, 1988). They are suggested to have local autocrine/paracrine actions in several reproductive tissues, including the ovary, placenta, and anterior pituitary (Ying, 1988; Petraglia et al., 1996; Wallace and Healy, 1996).

Using newly developed and sensitive two-site enzyme immunoassay (EIA), the changes in circulating concentrations of activin A (Muttukrishna et al., 1996), inhibin A (Muttukrishna et al., 1994), and inhibin B (Groome et al., 1996) throughout the menstrual cycle in healthy women have been described. Serum total activin A concentrations do not show major changes (Muttukrishna et al., 1996), while serum dimeric inhibin A concentrations are very high at the midluteal phase, decreasing by the onset of menses (Muttukrishna et al., 1994), and inhibin B concentrations are high during the follicular phase.

Recent investigation showed that cultured endometrial stromal and epithelial cells expressed and secreted inhibin-related subunits and protein, supporting the endometrial production of these growth factors (Petraglia et al., 1998). However, there are no data showing an endometrial contribution to peritoneal fluid.

A possible role for these proteins in reproductive function is suggested by the fact that inhibin inhibits rat oocyte maturation (O et al., 1989), while activin accelerates primate oocyte maturation (Woodruff et al., 1990). Sharing structural similarities with Mullerian inhibiting substance (MIF) and transforming growth factor-β (TGF-β), inhibin and activin are expressed in rat embryos (Voutilainen et al., 1991) and in human pre-embryos (Phocas et al., 1992).

The peritoneal fluid hormone content reflects in part ovarian secretory activities (Konicks et al., 1980; Petraglia et al., 1985; Barry-Kinsella et al., 1994), but may also have a function in the pathogenesis of infertility related to endometriosis (Viganò et al., 1994). Inhibins and activin A may also play a role in the cellular immune system, which is involved in the pathogenesis of endometriosis. These proteins have an immunomodulatory activity influencing erythroid differentiation (Eto et al., 1987), mitotic activity in thymocytes (Hedger et al., 1989), and stimulate chemotaxis and human lymphocyte interferon-γ production (Petraglia et al., 1991). Peritoneal fluid from women with endometriosis has a greater immunosuppressive effect on natural killer-mediated cytoxicity than peritoneal fluid from fertile women (Viganò et al., 1994), and TGF-β and platelet-derived growth factor (PDGF) seem to
participate in the development and maintenance of ectopic endometrial tissue (Oosterlynck et al., 1994; Whitworth et al., 1994). In particular, there are increased TGF-β concentrations in the peritoneal fluid of women with endometriosis compared to both fertile and infertile women without endometriosis (Oosterlynck et al., 1994).

The present study aimed to evaluate peritoneal fluid inhibin A and B, and activin A concentrations throughout the menstrual cycle in women with and without endometriosis. In addition, the possible expression of inhibin α-, activin βA-, and BB-subunits, and activin receptor type II and type IIB mRNAs was investigated in peritoneal tissues collected from healthy women, as well as in cultured endometriotic cells.

Materials and methods

Patients

Women (n = 72) (age range 22–33 years) were recruited at laparoscopy for infertility investigation. Informed consent was obtained from patients and the study was approved by the Human Investigation Committee. Subjects were divided in two groups:

Group 1 – control healthy women (n = 35), subdivided according to follicular (6th to 8th day) (n = 17) and luteal (22nd to 24th day) (n = 18) phase of the menstrual cycle.

Group 2 – women with endometriosis (n = 37), subdivided according to follicular (6th to 8th day) (n = 17) and luteal (22nd to 24th day) (n = 20) phase of the menstrual cycle.

Healthy women showed normal hysterosalpingography, semen analysis of the partner, postcoital test, luteal phase endometrial biopsy, normal follicular development on ultrasound at mid-cycle and a normal follicular development on ultrasound at mid-cycle and a follicular phase of the menstrual cycle from three women affected by endometriosis (Vignano et al., 1990). Diffuse cytoplasmic immunostaining for vimentin and cytokeratin was present in most (90%) cultured stromal and epithelial cells. The cytofluorimetric analysis indicated that macrophage contamination was <2%. Primary cultures were used within 3 days from cell dispersion, and cell density ranged between 6.0×10^5 – 1.0×10^6/dish (volume of culture medium was 3 ml/dish).

Healthy women showed normal hysterosalpingography, semen analysis of the partner, postcoital test, luteal phase endometrial biopsy, normal follicular development on ultrasound at mid-cycle and a completely normal diagnostic laparoscopy. Women with endometriosis had similar negative infertility investigations. Endometriosis was diagnosed at laparoscopy and was classified according to the American Fertility Society (1985) classification, and biopsy specimens were taken to confirm the diagnosis. The day of the cycle was determined by the first day of the last menstrual period during the follicular phase and by endometrial biopsy dating during the luteal phase. Peritoneal fluids were aspirated from the pouch of Douglas during the proliferative phase and by endometrial biopsy dating during the luteal phase. Peritoneal fluids were aspirated from the pouch of Douglas during the proliferative phase and by endometrial biopsy dating during the luteal phase.

Women (n = 72) (age range 22–33 years) were recruited at laparoscopy for infertility investigation. Informed consent was obtained from patients and the study was approved by the Human Investigation Committee. Subjects were divided in two groups:

Group 1 – control healthy women (n = 35), subdivided according to follicular (6th to 8th day) (n = 17) and luteal (22nd to 24th day) (n = 18) phase of the menstrual cycle.

Group 2 – women with endometriosis (n = 37), subdivided according to follicular (6th to 8th day) (n = 17) and luteal (22nd to 24th day) (n = 20) phase of the menstrual cycle.

Healthy women showed normal hysterosalpingography, semen analysis of the partner, postcoital test, luteal phase endometrial biopsy, normal follicular development on ultrasound at mid-cycle and a completely normal diagnostic laparoscopy. Women with endometriosis had similar negative infertility investigations. Endometriosis was diagnosed at laparoscopy and was classified according to the American Fertility Society (1985) classification, and biopsy specimens were taken to confirm the diagnosis. The day of the cycle was determined by the first day of the last menstrual period during the follicular phase and by endometrial biopsy dating during the luteal phase. Peritoneal fluids were aspirated from the pouch of Douglas during laparoscopy, collected in sterile tubes, centrifuged (400 g, 10 min) and the supernatants stored at −20°C until the assay. Haemorrhagic fluids were discarded only when the reason for peripheral blood contamination was obvious at the puncture site.

In the control group, a specimen of blood was collected, at the time of peritoneal fluid sampling, from the antecubital vein, allowed to clot and, after centrifugation, the serum was divided into five aliquots and stored at −20°C until hormone assay.

Table I. Oligonucleotide primers for RT-PCR detection of human inhibin α-subunit, activin βA- and BB-subunit, and activin receptor type II and type II B mRNA transcripts

<table>
<thead>
<tr>
<th>Oligonucleotide primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibin α-sense</td>
<td>5′-CAACCCACAGATGCGACAGTGC-3′</td>
</tr>
<tr>
<td>Inhibin α-antisense</td>
<td>5′-CTCCGAGGCTTCGAGCAGGCCCAG-3′</td>
</tr>
<tr>
<td>Activin βA-sense</td>
<td>5′-GGATTGGGCAAGGGGCTATGGGCCCGATC-3′</td>
</tr>
<tr>
<td>Activin βA-antisense</td>
<td>5′-GAGGAGGACAGAAGCAGGGGACAC-3′</td>
</tr>
<tr>
<td>Activin βB-sense</td>
<td>5′-TTGCCGAGAGATGATGCCT-3′</td>
</tr>
<tr>
<td>Activin βB-antisense</td>
<td>5′-TTGCCGAGAGATGATGCCT-3′</td>
</tr>
<tr>
<td>Activin receptor type II-sense</td>
<td>5′-GTGGAAGGGGAGGGGAGCCCAG-3′</td>
</tr>
<tr>
<td>Activin receptor type II-sense</td>
<td>5′-CAACTCTTCTAGAATGAGG-3′</td>
</tr>
<tr>
<td>Activin receptor type II-antisense</td>
<td>5′-CAGGATGTTTACAAATGCCCT-3′</td>
</tr>
<tr>
<td>Activin receptor type II B-sense</td>
<td>5′-CAGGATGTTTACAAATGCCCT-3′</td>
</tr>
<tr>
<td>Activin receptor type II B-antisense</td>
<td>5′-CAGGATGTTTACAAATGCCCT-3′</td>
</tr>
</tbody>
</table>

Tissue specimens

Peritoneal tissue specimens were collected from three healthy women at the time of laparoscopy, treated for extraction of total RNA using the method of Chomczynski and Sacchi (1987), and quantified by UV absorption at 260 nm. As a positive control, cultured trophoblast cells (collected from three healthy pregnant women at 40 weeks of gestation) were used within 3 days of cell preparation (Kliman et al., 1986).

Endometriotic cell cultures

Endometrial tissue specimens were collected during the proliferative phase of the menstrual cycle from three women affected by endometriosis at the time of laparoscopy, minced into small pieces and incubated for 2 h at 37°C in a shaking water bath in 10 ml Ham’s F-10 containing 0.1% collagenase. Cells were cultured in Ham’s F-10 medium supplemented with 10% fetal calf serum and antibiotics (Vignano et al., 1993). Diffuse cytoplasmic immunostaining for vimentin and cytokeratin was present in most (90%) cultured stromal and epithelial cells. The cytofluorimetric analysis indicated that macrophage contamination was <2%. Primary cultures were used within 3 days from cell dispersion, and cell density ranged between 6.0×10^5 – 1.0×10^6/dish (volume of culture medium was 3 ml/dish).

Harvested cultured cells were treated for total RNA extraction (Chomczynski and Sacchi, 1987), and quantified by UV absorption at 260 nm.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The expression of inhibin α-, activin βA, activin B and activin receptors mRNAs was demonstrated by amplifying respective target sequences by polymerase chain reaction (PCR) according to the instructions of the GeneAmp amplification reaction kit (Perkin Elmer, Milan, Italy). Total RNA (2 µg) was reverse transcribed to prepare complementary DNA (cDNA). PCR was performed by using Taq (Thermus aquaticus) DNA polymerase.

Reaction conditions for reverse transcription were: 1 mmol/l each deoxyribonucleoside triphosphates, 1 IU of RNasin, 100 pmol of random hexamers and 200 IU of reverse transcriptase. The reaction was run at 42°C for 1 h, and then at 94°C for 5 min. The mixture was then quick chilled on ice.

The specific oligonucleotide primers designed to amplify sequences of inhibin α- (Voutilainen et al., 1991), activin βA- and activin B-subunit (Tuuri et al., 1994), and activin receptor type II (ActRII) and type II B (ActRIB) (Carcamo et al., 1990) cDNAs are shown in Table I. Computer analysis performed to study the secondary structure of the different cDNAs and to compare the synthesized oligomers to the human sequences in the MicroGenie (Beckman, Palo Alto, CA, USA) gene database bank, revealed no more than 74% homology in the former and 72% in the latter among all the other genes. Sequence
homology among the different oligomers used in the present study was avoided, excluding possible cross-reactions. For inhibin α- and activin βA subunits, cDNA amplification was performed with 35 thermal step cycles (94°C, 1 min; 60°C, 1 min; 72°C, 3 min); while for activin βB 40 thermal step cycles (94°C, 30 s; 62°C, 7 s; 72°C, 1 min) were used. For activin receptors, we followed three thermal step cycles (94°C, 30 s; 37°C, 30 s and 72°C, 1 min with 2 min ramp time), followed by 30 cycles (ActRII: 94°C, 30 s; 62°C, 7 s; 72°C, 1 min; ActRIIB: 94°C, 30 s; 60°C, 7 s; 72°C, 1 min). For each tissue analysed, the template was omitted in the amplification mixture during the RT-PCR, in order to rule out DNA contamination, and was used as a negative control.

Amplification products were visualized on a 4% agarose gel stained with ethidium bromide, and viewed on an ultraviolet light box. Size markers were purchased from Bio Ventures Inc., Los Angeles, CA, USA. The experiments were repeated at least three times and qualitatively similar results were obtained.

Inhibin A, inhibin B, and activin A assays
Inhibin A and B and activin A concentrations in the various fluids (culture medium, peritoneal fluid, and serum) was measured by using specific two-site enzyme immunoassays as previously described (purchased from Serotec, Oxford, UK) (Muttukrishna et al., 1994, 1996; Groome et al., 1996). Briefly, in each assay standard and samples were diluted as appropriate and mixed with an equal (activin A), and half volume of the sample (inhibin A and B) of distilled water containing respectively 10% SDS. After 3 min at 100°C, tubes were cooled before adding freshly prepared hydrogen peroxide solution. After an additional time of incubation at room temperature, duplicate aliquots of denatured and oxidized samples/standards were transferred to antibody-coated microtitre plates. Plates were incubated at room temperature, for 2 h (inhibin A) or overnight (inhibin B). In activin A plates, 25 µl biotinylated monoclonal antibody to the beta A subunit was added before overnight incubation. After washing with enzyme immunoassay (EIA) wash buffer [0.1 mol/l Tris–HCl, 0.15 mol/l NaCl, 10% (wt/vol) BSA, 5% (vol/vol) Triton X-100, and 0.1% (wt/vol) sodium azide, pH 7.5], 50 µl alkaline phosphate-conjugated Fab mouse anti human inhibin α subunit were used in inhibin A and inhibin B assays, and streptavidin alkaline phosphatase in activin A assay; after this, plates were incubated for 1 (inhibin A), 2 (activin A), or 3 (inhibin B) h. Plates were washed and bound alkaline phosphatase was quantitated using a commercially available enzyme immunoassay amplification system (ImmuNo Select ELISA Amplification System, Dako, Milan, Italy) which was used according to the supplier’s instructions.

The inhibin A and B, and activin A plates were read at 490 nm on an automated EIA plate reader (BROi: Basic Radim Immunoassay Operator, Radim spa, Pomezia, Italy). The inhibin A detection limit was 20 pg/ml serum, with intra- and interassay coefficients of variations (CVs) for quality control samples <4.0 and 8.0% respectively. The assay detection limit for inhibin B was <30 pg/ml in serum, and <15 pg/ml in peritoneal fluid and culture medium. Within- and between-plate CVs in this case were less than 5.0 and 9.0% respectively. The limit of detection for activin A was <100 pg/ml, and intra- and interassay CVs were 5.0 and 9.0% respectively. Cross-reactions for each assay with the various inhibin-related proteins were <0.5%.

Statistical analysis
The data were analysed by using the Wilcoxon and the Mann–Whitney U-tests.

Results
Expression of inhibin and activin subunit mRNAs
RT-PCR analysis performed on total RNA showed that peritoneal tissue and cultured endometriotic cells express specific mRNAs for inhibin α-, activin βA- and βB-subunits (Figure 1). The DNA size corresponds to those detected in trophoblast cells used as positive controls (Figure 1).

Using total RNA isolated from the same tissues, the RT-PCR also generated two DNA fragments corresponding to activin receptor type II (ActRII) and type IIB (ActRIIB) respectively (Figure 1).

![Figure 1. Expression of inhibin α-, activin βA- and βB-subunit, and activin receptor type II (ActRII) and activin receptor type IIB (ActRIIB) mRNAs by RT-PCR in endometriosis and peritoneal tissue of healthy women. Size markers from BioMarker™ LOW, Bio Ventures, Inc. Left to right: marker (M); endometriosis (end); peritoneum (per); trophoblast as positive control (troph); and negative control without template (nc).](image1)

![Figure 2. Mean ± SEM inhibin A, inhibin B, and activin A concentrations in media collected from cultured endometriotic cells.](image2)
Peritoneal fluid growth factors

Figure 3. Mean ± SEM inhibin A, inhibin B, and activin A concentrations in serum (black bars) and peritoneal fluid (white bars) collected from healthy women according to follicular and luteal phases of the menstrual cycle. *P < 0.05.

Concentrations of inhibin A, inhibin B, and activin A in culture medium

Medium collected from cultured endometriotic cells contained discrete amounts of inhibin A (56.1 ± 4.7 pg/ml mean ± SEM), inhibin B (27.13 ± 4.1 pg/ml), and activin A (1.51 ± 0.12 ng/ml) (Figure 2).

Concentrations of inhibin A, inhibin B, and activin A in peritoneal fluid and serum

In healthy women, inhibin A concentrations in peritoneal fluid collected in the follicular phase (209.65 ± 60.95 pg/ml) were significantly lower than those collected in the luteal phase (740.13 ± 143.31 pg/ml; P < 0.05) of the menstrual cycle (Figure 3). In the same subjects, serum inhibin A concentrations were constantly and significantly lower than peritoneal concentrations both in the follicular (25.59 ± 1.7 pg/ml) and in the luteal phase (21.82 ± 0.95 pg/ml) of the menstrual cycle (P < 0.001) (Figure 3).

Inhibin B peritoneal concentrations did not show any significant difference between follicular (174.98 ± 24.35 pg/ml) and luteal (125.51 ± 21.90 pg/ml) phases of the menstrual cycle, but were constantly and significantly higher than in serum, in both phases of the menstrual cycle (follicular: 15.06 ± 2.41 pg/ml; luteal: 50.81 ± 3.4 pg/ml) (P < 0.001 respectively) (Figure 3).

Peritoneal fluid activin A concentrations in healthy women did not show any significant difference between follicular (1.52 ± 0.24 ng/ml) and luteal phase (1.74 ± 0.29 ng/ml) (Figure 3). In the same subjects, peritoneal fluid activin A concentrations were significantly higher than in serum
Patients with endometriosis showed peritoneal fluid inhibin A concentrations in the same range as healthy women, and did not show any significant difference in comparison to healthy women at the different phases of the menstrual cycle, becoming significantly higher during the luteal phase ($P < 0.05$) (Figure 4). Similarly, peritoneal fluid inhibin B and activin A concentrations in patients with endometriosis did not significantly differ from healthy women, and were independent of the stage of the disease and/or of the phase of the menstrual cycle (Figure 4).

**Discussion**

The present study confirming the presence of inhibin A, inhibin B, and activin A in the peritoneal fluid of healthy women (Hemmings et al., 1992; Billiar et al., 1995), firstly demonstrated that: (i) dimeric inhibin A concentrations in peritoneal fluid change throughout the menstrual cycle, in women without or with endometriosis; (ii) peritoneal fluid inhibin A, inhibin B, and activin A concentrations are higher than in serum; (iii) human peritoneum collected from healthy women and endometriotic cells express mRNA for inhibin α- and activin βA- and βB-subunits, and for activin type II and type IIB receptors.

Peritoneal fluid originates from several sources: ovary, peritoneum, and endometrium (Konicks et al., 1980; Petraglia et al., 1985; Barry-Kinsella et al., 1994). An ovarian origin of peritoneal hormones is suggested by the observation that ovarian hormones, such as 17-β-oestradiol and progesterone (Barry-Kinsella, 1994), β-endorphin and met-enkephalin (Petraglia et al., 1985), show peritoneal concentrations higher than in peripheral circulation. Since human granulosa cells and the corpus luteum express inhibin α-, and activin βA- and βB-subunit mRNAs (Roberts et al., 1993; Eramaa and Ritvos, 1996; Mirò and Hillier, 1996), and ovarian follicular fluid contains large amounts of activin A, inhibin A and inhibin B (Muttukrishna et al., 1996; Magoffin and Jakimiuk, 1997), an ovarian contribution of inhibin A, inhibin B and activin A to the peritoneal fluid may be suggested. Increased inhibin A concentrations during the luteal phase in peritoneal fluid may result from the corpus luteum, probably mediated in autocrine/paracrine mode by increased PGE2 luteal production (Eramaa and Ritvos, 1996).

Further sources of inhibin and activin in peritoneal fluid may also be the peritoneum and/or endometrium. In fact, an endometrial secretion of inhibin related proteins in culture media and in the circulation has been very recently reported (Petraglia et al., 1998). In addition, we report that peritoneal and endometriotic cells express inhibin–activin subunit mRNAs and endometriotic cells also secrete the dimeric proteins in culture medium.

However, the lack of difference in peritoneal fluid inhibin and activin A concentrations between controls and patients with endometriosis suggests that ectopic endometrial tissue does not contribute significantly to the protein content in the peritoneal fluid.

The possible role of peritoneal fluid inhibin-related protein remains to be elucidated. Several possible target organs or functions may be postulated. The finding of peritoneal fluid inhibin A, inhibin B, and activin A concentrations several-fold higher than in serum suggests that oocytes are exposed to high concentrations of the proteins in the peritoneal cavity and in the Fallopian tube, and an effect of dimeric inhibin on oocyte maturation and/or fertilization may be suggested (Billiar et al., 1995; Seifer et al., 1997). Thus, inhibins and activin A in follicular fluid could be released into the peritoneal cavity during ovulation and may be important in stimulating the oviduct and the uterus in connection with ovulation, pre-embryo development and implantation (Petraglia et al., 1996).

These proteins may also modulate the cellular immune system (Eto et al., 1987; Hedger et al., 1989; Petraglia et al., 1991), which is involved in the pathogenesis of endometriosis. Our finding of no difference in peritoneal inhibin A, inhibin B and activin A concentrations between controls and endometriosis patients suggests that these proteins are not involved in the immunological pathogenesis of endometriosis.

In conclusion, the high concentration of inhibin A, inhibin B, and activin A in peritoneal fluid and the presence of inhibin subunits and activin receptor mRNAs in peritoneum in healthy women and in patients with endometriosis suggest a possible local action.

**Acknowledgements**

The present study was conducted in the ambit of the activities of the Associazione Sviluppo Ormoni e Donna Atenei Toscani (ASSODAT), Italy.

**References**


Peritoneal fluid growth factors


Received on February 2, 1998; accepted on June 5, 1998