Evidence for local production of inhibin A and activin A in patients with ovarian endometriosis

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Objective: To evaluate the expression of inhibin A and activin A in ovarian endometriosis.

Design: Uncontrolled cross-sectional study and controlled prospective in vitro study.

Setting: Academic health centers in Siena, Udine, Sassari, and Milan, Italy.

Patient(s): A group of women (n = 19) who underwent laparoscopic excision of ovarian endometriotic cysts.

Intervention(s): Specimens of serum, peritoneal fluid, and cystic fluid, ovarian tissue for immunohistochemistry, and endometriotic cells for primary culture were collected. Cell cultures were also prepared from proliferative endometrium of women without endometriosis.

Main Outcome Measures(s): Dimeric inhibin A and activin A concentrations in biological fluids; immunostaining of α and βA subunits in ovarian endometrioma; α and βA gene expression in cultured endometriotic cells compared with normal endometrium.

Result(s): Inhibin A and activin A concentrations in the cystic fluid were slightly higher than in peritoneal fluid and significantly higher than in serum (P < .05). Immunoreactive α and βA subunits were strongly expressed both in the epithelial and stromal components of ovarian endometrioma. The relative abundance of βA mRNA was significantly decreased in endometriotic cells compared with eutopic stromal cells.

Conclusion(s): The results of the present study provide evidence for a local production and secretion of inhibin A and activin A in ovarian endometriotic cysts. (Fertil Steril 2001;75:367–73. ©2001 by American Society for Reproductive Medicine.)

Key Words: Inhibin, activin, ovary, ovarian cyst, endometriosis

Inhibins and activins are dimeric glycoproteins resulting from distinct combinations of α (18 kDa) and/or β (14 kDa) subunits. One variety of α subunit and two types of β subunit produce inhibin A (α + βA), inhibin B (α + βB), activin A (βA + βA), activin B (βB + βB), or activin AB (βA + βB). Both forms of inhibin have an inhibitory effect on pituitary FSH release, while the three forms of activin are biologically equivalent in stimulating FSH release (1). The availability of bioactive inhibin and activin is regulated by the binding protein follistatin (2). The biological effects of activins are mediated by the oligomerization of two types of transmembrane serine/threonine kinase activating receptors, while inhibins act as activin receptor antagonists and also possibly through a putative inhibin receptor (3).

Inhibin/activin subunit gene expression has been demonstrated in granulosa, theca, and lutein cells of human ovaries (4), and dimeric forms of inhibin A, inhibin B, and activin A are highly concentrated in ovarian follicular fluid (5, 6). Activins and inhibins are also produced and secreted by extragonadal tissues, such as pituitary gland, adrenals, and placenta (7–9). We have recently shown that inhibin A, inhibin B, and activin A, as well as activin receptors, are expressed in normal and neoplastic human uterine tissues (10). Two independent groups have subsequently demonstrated the presence of immunoreactive inhibin α and inhibin/activin βA but not βB subunit in human endometrium with a menstrual cycle changing pattern (11, 12). In addition, we observed that isolated cells from human endometrium and
Inhibin and activin in ovarian endometriosis

MATERIALS AND METHODS

Subjects
The study involved a group of women (n = 19) who underwent laparoscopic excision of ovarian endometriotic cysts during the follicular phase of the menstrual cycle. Informed consent was obtained from all subjects before inclusion, and the study protocol was approved by the local committees on human research. Patient age ranged between 22 and 33 years, cyst diameter measured by ultrasound was in the range of 20–110 mm, and all patients were classified as having stage III or IV endometriosis according to the revised American Fertility Society classification of endometriosis (18). Diagnosis was confirmed by laparoscopic and histological examination of the endometriotic lesions.

Fluid Sampling
Blood samples were drawn from a peripheral vein immediately before anesthesia for laparoscopy was administered, and they were allowed to clot at room temperature. Peritoneal fluid was obtained by culdocentesis during laparoscopy, while cystic fluid was collected by needle aspiration after removal of the cysts. Hemorrhagic fluids were discarded only if there was obvious contamination by peripheral blood at the puncture site. All fluid samples were centrifuged at 400 g for 10 min, and aliquots of the supernatants were stored at −20°C until inhibin A and activin A assays.

Inhibin A and Activin A Assays
Inhibin A and activin A concentrations were measured in serum, peritoneal fluid, and cystic fluid by using specific two-site enzyme immunoassays purchased from Serotec (Oxford, UK) as described elsewhere (5,19,20). The inhibin A detection limit was 20 pg/mL, with intra-assay and inter-assay coefficients of variations (CVs) for quality control samples <4.0% and <8.0%, respectively. The limit of detection for activin A was <100 pg/mL, and intra-assay and interassay CVs were 5.0% and 9.0%, respectively. Cross-reactions for each assay with the various inhibin-related proteins were <0.5%.

Immunohistochemistry
Tissue samples were obtained from ovarian (n = 3) and ileal (n = 1) endometriotic cysts, fixed in 10% buffered formalin, embedded in paraffin, and routinely processed. To improve antigen retrieval, paraffin sections were rehydrated and heated in a microwave oven (800 W for 5 min and 300 W for 10 min) using 0.01 M of citrate buffer, pH 7.3. All sections were stained by the peroxidase-streptavidin-biotin technique. Briefly, after blockade of endogenous peroxidase, the sections were incubated overnight with the primary antibody, followed by biotinylated link antibodies and peroxidase streptavidin (LSABTM; Dako, Glostrup, Denmark) for 30 min each. After the peroxidase development with diaminobenzidine, the slides were counterstained in Mayer hematoxylin, dehydrated, and mounted in Eukitt.

The following highly specific antisera were used: rabbit anti-α (1-26)-Gly-Tyr diluted 1:100 and mouse anti-BA (81-113)-NH2, diluted 1:25. These were affinity-purified polyclonal antisera raised against synthetic peptide fragments and kindly donated by Dr. W. Vale (Salk Institute, San Diego, CA). Their specificity has been evaluated elsewhere (21), and no cross-reaction between the three antisera has been observed. Negative controls consisted of sections where the primary antibody was replaced by nonimmune rabbit or mouse serum. The immunostaining was evaluated by estimating the distribution and the intensity of positive cells. A positive reaction was characterized by the presence of granular brown staining in the cytoplasm.

Culture of Endometriotic Cells and Eutopic Endometrial Cells
Primary cultures of endometriotic cells were prepared from four ovarian endometrioma cyst linings. Uterine endometrium was also collected from six women without endometriosis undergoing laparoscopy for infertility during the proliferative phase of menstrual cycle. Tissue handling, transport, and cell isolation were performed as described elsewhere (22). Briefly, the tissues were digested with collagenase, and single stromal cells were separated from large clumps of epithelium by differential sedimentation at single gravity. Endometriosis stromal cells and endometrial epithelial and stromal cells were plated at the same density and allowed to proliferate in Ham’s F-10 medium supplemented with 10% FCS and antibiotics. RNA extraction was performed when the cultures became subconfluent, and this was generally achieved within 7 days of cell dispersion.
Analysis of Inhibin/Activin Gene Expression by Reverse Transcriptase Polymerase Chain Reaction

The cultured cells were harvested in phenol-guanidine isothiocyanate (23). Total RNA was extracted with chloroform and precipitated with isopropanol by 12,000 g centrifugation at 4°C. In order to digest any contaminant genomic DNA, RNA samples were treated with 5 U DNAse (Promega RQ1) at 37°C for 10 min and precipitated with 0.1 M of sodium acetate pH 5.2 and 80% ethanol by centrifugation at 12,000 g. The RNA pellet was washed twice with 75% ethanol, resuspended in diethylpyrocarbonate-treated water, and quantified by light absorbance at 260 nm. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the GeneAmp RNA PCR kit purchased from Perkin Elmer (Roche Molecular Systems, Branchburg, NJ). First-strand cDNA was synthesized from 2 μg of total RNA. After denaturing the template RNA and primers at 70°C for 10 min, 50 U RT was added in the presence of buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 mM MgCl₂, 0.5 mM dNTP mix, and 20 U RNAse inhibitor. The mixture (20 μL) was incubated at 42°C for 55 min, then heated at 70°C to stop the reaction and stored at −20°C.

RT-PCR was performed in a final volume of 50 μL. Two microliters of the first-strand synthesis reaction were incubated with buffer II, 1.5 mM MgCl₂, 0.2 μM sense and antisense primers, 0.2 mM dNTP mix, and 1.25 U Taq DNA polymerase. The specific primers used to amplify cDNA fragments corresponding to inhibin α subunit, inhibin/activin βA subunit, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1. Because the inhibin α subunit gene possesses a 1.7-kb intron in the region encoding the precursor αN (24, 25), the upstream primer was designed to span this intron and thereby rule out amplification of genomic DNA. The primers for inhibin/activin βA subunit were designed to encompass part of the cDNA sequence corresponding to the pro-β precursor (common to βA and βB) and part of the specific βA sequence (24). The primers for GAPDH were designed to span an intron along 104 bases (26).

PCRs for α- and βA consisted of 30 thermal cycles of 94°C for 30 s, 56.5°C for 30 s, and 72°C for 60 s. For GAPDH, nine thermal cycles (94°C for 20 s, 59°C for 5 s, and 72°C for 70 s) were followed by 16 cycles of 94°C for 20 s, 58°C for 10 s, and 72°C for 70 s. The first cycle was preceded by denaturation at 94°C for 3 min, and the last cycle was followed by extension at 72°C for 5 min. The number of PCR cycles was established after testing a range of 20–40 to ensure that they would not reach the plateau phase.

The product of a first-strand reaction performed without RT was also submitted to the PCR protocol to serve as negative control. A sample of the PCR mixture (15 μL) was resolved on a 2.5% agarose gel stained with ethidium bromide and photographed under UV light. The expected bands were analyzed by densitometry, and data were expressed as the ratio between α- and βA cDNAs and the corresponding GAPDH cDNA.

Statistical Analysis

Data were tested for normality (symmetry and kurtosis) and for homogeneity of variances. Since the underlying assumptions of conventional analysis of variance were not met in all instances, the nonparametric Kruskal-Wallis analysis of variance was applied to test differences between groups, followed by Dunn’s test for multiple comparisons. The results are described as means and standard errors (SE), and those affected by considerable skewness are reported also as medians (27). P<.05 was considered significant.

RESULTS

Endometriotic cysts contained dimeric inhibin A and activin A in concentrations higher than those found in periph-

<table>
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<tr>
<th>Target</th>
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<th>Oligonucleotide sequence</th>
<th>Position, bases</th>
<th>Size, base pairs</th>
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<td>5’CAGCCACAGATGCCAAGCTGT 3’</td>
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<td></td>
<td></td>
<td>5’CTCCGAGGCCTGACGAGCGAG 3’</td>
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<tr>
<td>βA</td>
<td>M13436</td>
<td>5’GTGGTTCCGAGTCAAGACAG 3’</td>
<td>611–630</td>
<td>787</td>
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<td>5’GAGGTGGCAAAAGGGCTATGGCAGCCAT 3’</td>
<td>1368–1397</td>
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<tr>
<td>GAPDH</td>
<td>J04038</td>
<td>5’TCTCACTACAGAAGGTGGTTGAG 3’</td>
<td>4445–4469</td>
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<td></td>
<td></td>
<td>5’TCCCTGAGGCCATGTGGGCCCAT 3’</td>
<td>4766–4788</td>
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*Excluding the intron “H” equivalent to bases 4619–4722. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Immunoreactive inhibin $\alpha$ and inhibin/activin $\beta$A subunits were strongly expressed in the epithelial and stromal components of ovarian endometriomata (Fig. 2). Specifically stained cells were observed homogeneously along the glands and stroma of the endometriotic implants. A weaker but also specific immunostaining for both $\alpha$ and $\beta$A subunits was observed in ileal endometriosis (Fig. 2). Immunostaining was more concentrated in the endometrioid-like glandular epithelium and was focally distributed in the stroma, while the surrounding intestinal wall did not stain at all.

As shown in Figure 3, the genes encoding inhibin/activin $\alpha$ and $\beta$A subunits were expressed in endometriotic cells maintained in culture. The expression of $\alpha$ subunit was undetectable in 5 of 10 cultures but was evident in 2 of 4 samples of endometriotic stromal cells, as well as in 2 of 3 cultures of stromal and 1 of 3 cultures of epithelial cells from eutopic endometrium. The gene expression of $\beta$A subunit was observed in all 10 RNA samples analyzed. This allowed us to perform a densitometric analysis of the PCR bands in order to assess semiquantitatively the differences in $\beta$A gene expression between different cell types. The relative abundance of $\beta$A mRNA with respect to GAPDH was significantly decreased in endometriotic stromal cells ($0.5 \pm 0.1$ arbitrary densitometric units) compared with stromal cells from eutopic endometrium ($2.7 \pm 0.3$ densitometric units, $P<.05$).

**DISCUSSION**

In the present study, three complementary approaches were used to demonstrate the local production of inhibin A and activin A by ovarian endometriosis. First, we found significant concentrations of dimeric inhibin A and activin A in the cystic fluid of ovarian endometriomas. The use of specific two-site enzyme immunoassays assure us that this finding was not due to cross-reaction with other inhibin-related proteins or with inactive precursors (5, 19). The fact that inhibin A and activin A concentrations in cystic fluid were slightly higher than in peritoneal fluid and significantly higher than in peripheral blood is further evidence supporting a local secretion of these proteins, since transudation from external sources would not explain the 5-fold gradient in favor of the cystic environment.

Second, we found intense immunostaining corresponding to the $\alpha$ and $\beta$A subunits in the epithelial and stromal components of the ovarian endometriomas. The ovary itself is a source of inhibins and activins, which could reach the endometriotic cyst and account for the presence of immunoreactive subunits in the endometriotic cells as well as for the dimeric proteins recovered in cystic fluid. However, we observed specific inhibin/activin immunostaining also in ileal endometriosis, with a confinement of the antigens to the endometrial island within the intestinal wall. In addition, our third experimental approach confirmed previous observa-
tions that endometriotic cells express the mRNAs encoding α and βA subunits (13). Combined, these data provide evidence for a local production and secretion of inhibin A and activin A in ovarian endometriotic cysts.

We also asked the question of whether the gene expression of inhibin and activin subunits in endometriosis cells would differ from normal endometrial cells cultured in similar environmental conditions and for the same time. Indeed, we observed a lower expression of βA gene in stromal cells derived from endometriotic cysts compared with stromal cells derived from eutopic endometrium. This finding must be interpreted cautiously since the semiquantitative assessment of gene expression does not determine the actual dimension of the difference observed. Besides, the functional characteristics of cultured cells do not necessarily represent their biological behavior in situ. Nevertheless, differences in the expression of growth factors may be a marker of activity of endometriosis cells, particularly those prone to form larger implants. In consonance with this hypothesis, we have observed elsewhere that cells obtained from endometriotic lesions express the basic fibroblast growth factor gene to a lesser extent than eutopic endometrial cells (22).

FIGURE 2
Representative examples of ovarian endometriotic cysts (a, c, e) and ileal endometriosis (b, d, f) immunostained for inhibin/activin subunits α (a, b) or βA (c, d). Bottom panels show negative controls of ovarian (e) and ileal endometriosis (f). Endometrial glands are lined by endometrial surface epithelium and surrounded by a thin rim of endometrial stroma. Specific immunostaining is indicated by the presence of granular brown staining in the cytoplasm. Original magnification was ×400.

The possible role of inhibins and activins in the pathogenesis of endometriosis deserves investigation. Based on the present results, it cannot be suggested that endometriotic cells have the intrinsic property of secreting higher amounts of inhibin A or activin A than normal endometrial cells. The presence of inhibin A and activin A in peritoneal fluid is not a specific finding in endometriosis either. Our previous observations suggested that inhibins and activins released by the endometriotic implants are unlikely to affect the systemic levels of these proteins, because their levels in peritoneal fluid do not differ from those found in healthy women across the menstrual cycle (13).

At present, we have no knowledge of a controlled study on the circulating levels of dimeric inhibins and activins in women affected by endometriosis, but the serum levels of inhibin A and activin A observed in the present study of patients with advanced ovarian endometriosis are quite similar to those reported for healthy women (19, 28). This observation should be confirmed by a controlled study because it frustrates the promise of using inhibins and activins as serum markers for endometriosis, but the likelihood of negative results prevented us (and perhaps other investigators) from pursuing this objective. We did not attempt to determine serum inhibin B levels in women with endometriosis because, like uterine endometrium (11, 12), ovarian endometriotic cells fail to express the inhibin/activin \( \beta B \) subunit (data not shown). It is possible, however, that some alteration in follicular phase inhibin B and/or luteal phase inhibins occurs in patients with endometriosis as a nonspecific indicator of abnormal follicular development, ovulation, or luteal function.

In contrast to the lack of evidence for a systemic role of inhibins and activins produced by endometriosis, there is evidence suggesting a local role of such proteins. Endometri-
otic cells express two types of activin receptor (13), making them a potential target for autocrine effects of inhibins and activins. These effects are still to be elucidated in endometrial cells, but it is conceivable that activin A may regulate their proliferation and/or differentiation, as occurs in other cell types belonging to steroid hormone-responsive tissues, such as prostate (29), breast (30), and trophoblast (16). It is also noteworthy that low concentrations of TGFβ, a growth factor to which activins and inhibins keep considerable homology, can stimulate mitosis in cultured endometrial stromal cells (31).

The present demonstration of inhibin A and activin A production by ovarian endometrioma suggests that this family of regulatory proteins may play a role in local modulation of endometriosis cell growth and differentiation. The future development of agonists and antagonists for these molecules should provide new tools of investigation to assess the importance of inhibins and activins in the pathogenesis of endometriosis.

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References