Deranged expression of follistatin and follistatin-like protein in women with ovarian endometriosis

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Abstract

Objective: To evaluate the messenger RNA (mRNA) expression and peptide localization of follistatin and follistatin-like protein (FLRG) in ovarian endometriosis, compared to healthy human endometrium.

Design: Samples of ovarian endometriotic and healthy endometrial tissues were processed by semiquantitative reverse transcriptase-polymerase chain reaction and immunohistochemistry.

Setting: Academic health centers in Siena, Italy, and Belo Horizonte, Brazil.

Patient(s): Women with endometrioma who underwent laparoscopic excision of ovarian endometriotic cysts (n = 16), and healthy, nonpregnant women (n = 18, control group).

Main Outcome Measure(s): Immunostaining and relative quantification of follistatin and FLRG mRNA in ovarian endometriosis and eutopic endometrium.

Result(s): Both ovarian endometriosis and healthy endometrium expressed and localized follistatin and FLRG. In endometriotic glands, follistatin immunostaining was homogeneously distributed throughout the cytoplasm of the epithelial cells, contrasting with normal eutopic endometrium, where follistatin expression was focal, irregular, and confined to the basal side of the glands. Follistatin-like protein was immunolocalized in the nuclei of both glandular epithelial cells and stromal cells, with less intense staining in endometriotic samples. The relative intensity of follistatin and FLRG immunostaining was significantly higher and lower, respectively, in endometriosis than in controls. The expression of follistatin mRNA was higher, while that of FLRG mRNA was lower, in ovarian endometriosis than in healthy eutopic endometrium.

Conclusion(s): Ovarian endometriotic lesions show a deranged expression of FLRG and follistatin, which are activin A-binding proteins. This may result in an altered effect of activin A on angiogenesis and/or endometrial differentiation. (Fertil Steril 2007;88:200–5. ©2007 by American Society for Reproductive Medicine.)

Key Words: Follistatin, FLRG, endometriosis, endometrium, activin A, angiogenesis

Activin A is a dimeric protein, composed of two βA subunits, that belongs to the transforming growth factor-β superfamily, a group of structurally similar but functionally diverse growth factors involved in cellular proliferation, differentiation, and apoptosis (1). Human endometrium is an important source of activin A, because stromal and epithelial cells express activin A messenger RNA (mRNA) and protein (2–9), and activin A expression and secretion increase during the menstrual cycle, reaching their highest levels in the secretory phase (4, 6, 8). Moreover, the human endometrium is a target for activin A, because the addition of activin A to cultured human endometrial stromal cells promotes the process of decidualization (10, 11).

The actions of activin A are primarily modulated by follistatin and follistatin-like protein (FLRG), which are activin-binding proteins that neutralize the biological effects of activin A (1). They are expressed by the human endometrium without significant variations throughout the menstrual cycle (8, 12), with increased expression early in pregnancy (7, 12–14). In addition, follistatin was shown to counteract activin A in stromal-cell decidualization (10, 11).

Endometriosis is a gynecological condition in women of reproductive age, which primarily produces infertility and pain, and is defined as the presence of viable endometrial glands and stroma outside the uterine cavity, mainly on the pelvic peritoneum, but also in the ovaries and rectovaginal septum, and more rarely in other sites. Endometriomas are invaginations of ovarian surface epithelium that contain endometrial tissue, and are commonly referred to as ovarian cysts lined by endometrial tissue (15).

The expression of activin A mRNA was found to be reduced in ovarian endometriotic cells compared with healthy endometrium (5), and because activin A also inhibits angiogenesis (16) and modulates immune and inflammatory responses (17, 18), it was proposed that it may have an
impact on the pathogenesis of the disease (5, 9). The present study aimed to evaluate the expression and localization of follistatin and FLRG in ovarian endometriosis.

**MATERIALS AND METHODS**

Two groups of women were included in the study: group A, women with endometrioma who underwent laparoscopic excision of ovarian endometriotic cysts (n = 16); and group B, healthy nonpregnant women (n = 18, control group).

Patients with endometrioma had an age range of 22–33 years, and a cyst diameter measured by ultrasound in the range of 20–110 mm; all were classified as having stage III or IV endometriosis according to the revised American Fertility Society classification of endometriosis (19). The diagnosis was confirmed by laparoscopic and histological examination of the endometriotic lesions.

The control group had an age range of 27–37 years, and consisted of women with regular menstrual cycles who underwent hysteroscopy for the evaluation of uterine-cavity morphology or laparoscopic tubal sterilization. Specimens were collected during the proliferative phase, initially determined from the number of days since the last menstrual period, and confirmed by ultrasound (20) and by the historical criteria of Noyes et al. (21). Subjects who had received steroid treatment during the past 6 months, and with pituitary, thyroid, or adrenal disorders, were excluded from the study. Informed consent was obtained from the women before their inclusion in the study, for which local institutional review board approval was granted.

Representative samples of all ovarian endometriomas were cut from the cyst wall lined by endometriotic tissue. These endometriosis samples and endometrial specimens from controls were in part fixed by immersion in 10% buffered formalin for immunohistochemistry, and in part were immediately submerged in an RNA stabilization reagent (RNALater; Qiagen, Milan, Italy) for extraction of total RNA and subsequent qualitative and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

**Semiquantitative RT-PCR**

Differences in mRNA expression between the control and study groups were estimated by semiquantitative RT-PCR, with the use of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. Samples were disrupted and homogenized with the use of Mixer Mill MM 300 (Qiagen), and total RNA was extracted with the RNeasy Protect Mini Kit and treated with RNase-free DNase according to the manufacturer’s instructions (Qiagen). Total RNA was quantified by ultraviolet absorption at 260 nm.

The reverse-transcription reaction was performed with a Thermoscript RT-PCR system (Invitrogen, Milan, Italy). First-strand cDNA was synthesized with the use of 1 μg total RNA. After denaturation, template RNA, oligo (dT) primers, and 10 mmol/L of dNTP were mixed for 5 minutes at 65°C. Fifteen units of reverse transcriptase were added in the presence of complementary DNA (cDNA) synthesis buffer (250 mmol/L Tris acetate, pH 8.4, 375 mmol/L potassium acetate, and 40 mmol/L stabilizer), 40 U RNase inhibitor, 0.1 mmol/L dithiothreitol, and diethyl pyrocarbonate-treated water to make a volume of 20 μL. The mixture was incubated at 50°C for 45 minutes, heated to 85°C for 5 minutes to stop the reaction, and stored at −20°C. Two microliters of the product were used for the PCR reaction.

The PCR was performed under the following conditions: 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 25 mmol/L of each dNTP, 1 U recombinant Taq DNA polymerase, and 0.4 μmol/L (final concentration) primers (Invitrogen) in a 50-μL total volume. The specific primers used to amplify cDNA fragments corresponding to follistatin (Genebank accession no. J03771), common to both splice variants, were: 5’GCCACCTGAGAAAGGCC-TAC’3 (sense) and 5’ACAGACAGGCTATCCGGC-TACT’3 (antisense) (included intron size, 904 base pairs [bp]; expected size, 201 bp). The FLRG was amplified using primers of the sequence 5’ACCTGAGGCTATGTTACC’3 (sense) and 5’TGTGGCAGGAGATGTAG’3 (antisense) (included intron size, 792 base pairs [bp]; expected size, 198 bp). The GAPDH primers were 5’GAAGGTGAAGGTGATGGTGAGCTCA’3 (sense) and 5’CTGAGAACGGGAAGCTTGC’3 (antisense) (expected size, 300 bp).

Computer analysis, performed to compare the synthesized oligomers with the human sequences in the gene database of the National Center for Biotechnology, using the BLAST program (22), revealed no significant homology to other genes or pseudogenes.

The PCR for follistatin consisted of 30 thermal cycles of 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 20 seconds. The PCR for FLRG consisted of 32 thermal cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by a final step of 10 minutes at 72°C. The GAPDH amplification was carried out for 30 seconds at 94°C, 30 seconds at 52°C, and 30 seconds at 72°C for 28 cycles, followed by a final step of 10 minutes at 72°C.

The number of PCR cycles was established after testing a range of 20–40 cycles to ensure that the amount of DNA product remained in the logarithmic range of the amplification curve, so that reliable semiquantified comparisons could be made. Each sample had a negative control in which the reverse transcriptase was omitted in the reaction mixture so as to rule out genomic DNA contamination. A negative control without RNA was also used.

Amplification products (15 μL) were visualized on a 2% agarose gel stained with 4% ethidium bromide, and photographed under ultraviolet light. The expected bands were
analyzed by densitometry analysis performed with Image J software (National Institutes of Health, Bethesda, MD), and the relative amounts of follistatin and FLRG mRNAs were calculated as follistatin:GAPDH and FLRG:GAPDH mRNA ratios.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded specimens were cut into 4-μm sections and stained by immunohistochemistry with the use of the avidin-biotin-peroxidase method. All samples and controls were processed together. After exposure to 1% H₂O₂ in methanol to block endogenous peroxidase, sections were treated with normal goat serum (Oncogene Research Products, San Diego, CA) for 30 minutes to suppress nonspecific binding.

For follistatin localization, rabbit anti-follistatin antiserum was diluted 1:500 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and applied on the slides for 12 hours at 4°C, as previously described (23). Rabbit anti-follistatin antiserum is a specific polyclonal antibody raised against recombinant human follistatin of 288 amino acids (FS-288). The specific polyclonal rabbit anti-FLRG antibody was used as previously described (12,13,23).

Sections were then treated with biotinylated goat anti-rabbit IgG (Oncogene Research Products) for 30 minutes at room temperature, washed in PBS, and incubated with the avidin-biotin-peroxidase complex (Oncogene Research Products) for 30 minutes. Peroxidase activity was visualized by exposing the sections for 3 minutes to 1 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in PBS containing 0.3% H₂O₂. Sections were then counterstained with hematoxylin. In the negative controls, the primary antibody was replaced by normal rabbit IgG (Oncogene Research Products) or normal rabbit serum at an equivalent dilution.

**Assessment of Staining**

The individual intensity of immunostaining of tissue sections was scored under light microscopy as follows: 0 for no staining, 1 for weak staining, 2 for moderate staining, 3 for strong staining, and 4 for strong and widespread staining. Epithelial and stromal tissues were analyzed and scored by two independent observers, blind to each patient’s group. Interobserver agreement was >80%, and discordant cases were resolved by discussion with a third examiner.

**Statistical Analysis**

After assessment by the Kolmogorov-Smirnov test, normally distributed data were expressed as means ± SE, and differences between groups were evaluated by unpaired t-test. Otherwise, results were expressed as medians and analyzed by the Mann-Whitney U test. Significance was assumed when \( P < .05 \).

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**FIGURE 1**

Representative RT-PCR bands of GAPDH (300 bp), follistatin (201 bp), and FLRG (198 bp) in human endometrium (Control) and ovarian endometriosis. m, size marker; nc, negative control. Charts represent mean ± SE of ratios between follistatin and GAPDH mRNAs, and FLRG and GAPDH mRNAs. * \( P < .01 \), **\( P < .001 \), versus control endometrium (t-test).
RESULTS

**Follistatin mRNA Expression**

The RT-PCR generated a single fragment 201 bp long, corresponding in size to the predicted follistatin mRNA fragment (201 bp) (Fig. 1). No amplified fragments caused by DNA contamination were detected in any of the experiments.

When evaluated by semiquantitative RT-PCR, the expression of follistatin (expressed as the follistatin:GAPDH mRNA ratio) was significantly higher ($P<.001$) in ovarian endometriosis than in eutopic endometrium (Fig. 1).

**Follistatin Peptide Localization**

Ovarian endometriosis expressed follistatin immunostaining in both the glandular epithelium and the stroma (Fig. 2B,C). In endometriotic glands, follistatin was homogeneously distributed throughout the cytoplasm of epithelial cells, in contrast with normal eutopic endometrium, where follistatin expression was focal, irregular, and confined to the basal side of the glands (Fig. 2A). In the stroma of endometriotic specimens, similar to normal eutopic endometrium, follistatin immunostaining was scattered and less intense compared to the epithelial compartment. The relative intensity of glandular follistatin immunostaining was significantly higher ($P<.01$) in endometriosis than in controls (Fig. 3A).

**FLRG mRNA Expression**

As shown in Figure 1, the predicted band corresponding in size to the FLRG (198 bp) product was obtained. No amplified fragments caused by DNA contamination were detected in any of the experiments.

When evaluated by semiquantitative RT-PCR, the expression of FLRG mRNA (expressed as FLRG:GAPDH mRNA ratio) was significantly lower in ovarian endometriotic tissue than in healthy endometrium ($P<.01$, Fig. 1).

**FLRG Peptide Localization**

In normal eutopic endometrium, FLRG was localized in the nuclei of both glandular epithelial cells and stromal cells (Fig. 2E,F), with a similar intensity and regularity of expression in both tissue compartments. On the other hand, in ovarian endometriotic tissue, FLRG expression was markedly less intense than in healthy eutopic endometrium, and showed weaker staining in the glands and stroma (Fig. 2G). The relative intensity of FLRG immunostaining was significantly lower ($P<.01$) in endometriosis than in controls (Fig. 3B).

**DISCUSSION**

The present study showed that ovarian endometriotic implants express and localize follistatin and FLRG, supporting the concept that endometriotic cells, more than healthy human endometrium (6, 8, 12, 13, 23), have the capacity to synthesize activin A-related proteins.

Although these proteins are defined as activin-binding (1), they showed opposite changes in endometriosis: the mRNA expression and the intensity of staining of follistatin were significantly higher, while those of FLRG were lower, in endometriosis than in healthy endometrium. This is not surprising, considering that follistatin and FLRG also show different tissue-expression profiles: FLRG is highly expressed in the placenta, testis, skin, and cardiovascular tissue, while follistatin expression is considerably higher in the pituitary and ovary (24). Even in the same biological process (e.g., wound healing), follistatin and FLRG distribution...
within the wound differs, because distinct factors regulate their respective expressions (25).

The differences that we observed in follistatin expression between endometriosis and normal endometrium appear to be of greater magnitude at the mRNA rather than the protein level, although only semiquantitative methods were employed. Actually, part of the follistatin produced in the cell is secreted, and is not kept bound to the membrane or in the cytoplasm. Thus, immunohistochemistry may not detect the whole amount of protein being produced in the tissue, while RT-PCR seems to indicate that the follistatin gene has been transcribed at a higher level in endometriosis.

Previous findings of reduced expression of activin A mRNA in cells isolated from endometriotic cysts (5), together with the present data on the different expression of activin A-binding proteins, point to a derangement of the activin A pathway in endometriosis. Indeed, follistatin and FLRG regulate the interaction of activin A with its receptors, and hence its biological availability (1). Thus, activin A induces endometrial decidualization, while the addition of follistatin prevents such an effect (10, 11).

The findings that [1] activin A inhibits, while follistatin induces, angiogenesis (16); [2] activin receptors are expressed by endothelial cells (26) and endometrial blood vessels (6); and [3] FLRG is expressed by vessel walls of the human endometrium (12, 13, 23) together underscore the putative role played by activin A in endometrial angiogenesis. Indeed, endometriotic cells require a viable blood supply to develop, and increased angiogenic activity is involved in the pathogenesis of the disease (15). The increased expression of follistatin in endometriotic implants may reduce the tissue availability of free activin A, thereby favoring local angiogenesis.

The reduced expression of FLRG in endometriotic cells does not fit in with this hypothesis, but other mechanisms of action may be suggested. Indeed, FLRG is able to modulate fibronectin-mediated cell-cell or cell-matrix adhesions in hematopoietic cells (27), and because fibronectin is a potential regulator of endometriosis implant attachment in the peritoneal cavity (28–30), a synergistic effect of FLRG and fibronectin in the pathogenesis of endometriosis may be suggested.

In conclusion, we showed that follistatin and FLRG are differently expressed in ovarian endometriosis and normal endometrium in the proliferative phase. These differences may account for a local distinct pattern of activin A modulation, which in turn may have important effects in the pathophysiology of endometriosis. The possible roles of follistatin and FLRG in the pathogenesis of endometriosis warrant further investigation, to determine whether their effects are mediated by the local activin pathway or through distinct actions upon the attachment, persistence, and progression of ectopic tissue.

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REFERENCES

FIGURE 3
Scores of follistatin (A) and FLRG (B) immunostaining in human endometrium and ovarian endometriosis, expressed as medians and quartiles. *P < .01 versus controls (Mann-Whitney U test).