Expression of *GRIM-19* in adenomyosis and its possible role in pathogenesis

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Objective: To study the expression of the gene associated with retinoid-interferon (IFN)-induced mortality 19 (*GRIM-19*) in the endometrial tissue of patients with adenomyosis and to describe the possible pathogenic mechanisms of this phenomenon.
Design: Experimental study using human samples and cell lines.
Setting: University-affiliated hospital.
Patient(s): Ectopic and eutopic endometrial tissues were obtained from 30 patients with adenomyosis, whereas normal endometrial specimens were obtained from 10 control patients without adenomyosis.
Intervention(s): Patients with rapid pathology report-confirmed adenomyosis were recruited, and eutopic and ectopic endometrial tissue samples were collected from patients who had undergone hysterectomies by either the transabdominal or laparoscopic method at Qilu Hospital. Normal endometrial tissue was collected from a group of control patients without adenomyosis.
Main Outcome Measure(s): Immunohistochemistry (IHC) was performed to evaluate the expression of *GRIM-19*, phospho-signal transducer and activator of transcription 3 (Y705) (*Y705*) (*pSTAT3*(*Y705*)), and vascular endothelial growth factor (*VEGF*) in endometrial tissue samples. The protein levels of *GRIM-19*, *pSTAT3*(*Y705*), *STAT3*, and *VEGF* were detected by Western blot. Apoptosis in endometrial specimens was assayed by TUNEL. Immunohistochemistry with an antibody directed against *CD34* was performed to detect new blood vessels in the endometrial tissue. *GRIM-19* small interfering RNA and a recombinant plasmid carrying *GRIM-19* were constructed to evaluate the effects of *GRIM-19* on the downstream factors *pSTAT3*(*Y705*), *STAT3*, and *VEGF* in Ishikawa cells.
Result(s): The expression of *GRIM-19* was down-regulated in the eutopic endometria of patients with adenomyosis compared with the endometria of patients in the control group, and it was further reduced in the endometrial glandular epithelial cells of adenomyotic lesions. Apoptosis was reduced in the eutopic endometrium compared with the control group, and it was significantly reduced in ectopic endometrial tissues. In addition, the ectopic and eutopic endometria of patients with adenomyosis displayed a much higher microvessel density. In the eutopic and ectopic endometria of patients with adenomyosis, the expression levels of *pSTAT3*(*Y705*) and *VEGF* were significantly higher than in the controls. Furthermore, down-regulation of *GRIM-19* in Ishikawa cells significantly promoted the activation of both *pSTAT3*(*Y705*) and its dependent gene *VEGF*.
Conclusion(s): Aberrant expression of *GRIM-19* may be associated with adenomyosis through the regulation of apoptosis and angiogenesis. (Fertil Steril® 2016;105:1093–101. ©2016 by American Society for Reproductive Medicine.)
Key Words: *GRIM-19*, adenomyosis, apoptosis, angiogenesis

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Adenomyosis is a commonly encountered, benign gynecologic disease that typically occurs in women of reproductive age. The characteristics of this disease include the benign invasion of the myometrium by endometrial tissue and subsequent myometrial hypertrophy. No specific studies have described the conception rates in women with and without adenomyosis. However, some studies have reported a lower pregnancy

rate (PR) in patients with adenomyosis than in women who used assisted reproductive technology (ART) (1, 2). Some studies have indicated that risk factors of adenomyosis include parity (3) and endometrial hyperplasia (4). A series of hypotheses that attempt to explain the genesis of adenomyosis has been established. Currently, the most popular hypothesis is that adenomyosis originates from the deep region (basalis) of the endometrial mucosa (5). However, the etiology and pathogenesis of the disease remain poorly understood.

The gene associated with retinoid-interferon (IFN)-induced mortality 19 (*GRIM-19*), a novel growth suppressor, encodes a 16-kDa protein that was first isolated by an antisense knock-out technique (6). Ample evidence now shows that the *GRIM-19* protein is lost or severely repressed in many cancers such as renal cell carcinomas (7) and primary prostate cancer (8). *GRIM-19* binds to signal transducer and activator of transcription 3 (*STAT3*) and inhibits its transcriptional activity (9). A conditional knockout of *STAT3* resulted in decreased apoptosis and a dramatic delay of involution in mammary tissue (10). Jennifer Rubin Grandis et al. (11) found that constitutively activated *STAT3* contributed to the loss of growth control in head and neck carcinogenesis, and her group induced apoptosis with *STAT3* antisense gene therapy in human tumors in vivo. Niu and colleagues (12) demonstrated that constitutive *STAT3* activation directly up-regulated vascular endothelial growth factor (*VEGF*) expression and stimulated tumor angiogenesis, which indicates that *STAT3* may represent a common molecular target for the prevention of the angiogenesis that occurs by different mechanisms in human cancer cell lines.

A previous study (13) indicated that decreased apoptosis and increased proliferation and angiogenesis exert a vital effect on the occurrence and development of adenomyosis. Another study (14) demonstrated that the level of *pSTAT3* was significantly higher in the eutopic endometria of women with endometriosis. An increase in angiogenesis was also found by three-dimensional power Doppler sonography in adenomyotic tissues (15). As mentioned previously, *GRIM-19*, a novel apoptotic regulatory protein, plays an important role in apoptosis and angiogenesis by inhibiting *STAT3* activity. However, no investigator has reported a correlation between *GRIM-19* expression and *STAT3-VEGF* signaling in the context of adenomyosis. Thus, the purpose of this study was to investigate the potential roles of *GRIM-19* in the development of adenomyosis.

MATERIALS AND METHODS

Subjects and Sample Collection

This study was reviewed and approved by the Institutional Review Board of Qilu Hospital of Shandong University. Written informed consent was obtained from all human subjects. Thirty patients who had undergone hysterectomies by either the trans-abdominal or laparoscopic method and who displayed rapid pathology report-confirmed adenomyosis were recruited (age range, 31–41 years). Eutopic and ectopic (diffuse adenomyotic) samples were then collected from these patients (n = 13, secretory phase; n = 17, proliferative phase). The body mass index (BMI) of the recruited patients ranged from 17.66–29.40 (mean ± SD, 23.22 ± 3.09). The indications for surgery were progressive dysmenorrhea only (n = 11), menorrhagia only (n = 5), or progressive dysmenorrhea and menorrhagia (n = 14).

After informed consent was obtained, healthy endometrial biopsy tissues were collected from 10 patients using a single-type endometrial biopsy-mistogy tube (Ningbo). These 10 patients exhibited normal hormonal profiles and showed no uterine abnormalities, as confirmed by laparoscopic exploration during tubal recanalization (n = 4, secretory phase; n = 6, proliferative phase; age range, 33–39 years). The BMI of the recruited control patients ranged from 18.82–26.81 (mean ± SD, 22.89 ± 2.29).

None of the patients had received steroid hormone therapy (HT) within the past 3 months, and none exhibited any obvious internal medicine or surgical comorbidities. Women with endometrial abnormalities, pelvic endometriosis, fibroids, ovarian cysts, or lesions were excluded. Each tissue specimen was divided into two parts, as follows: a portion was frozen in liquid nitrogen for protein analysis, and the remainder was fixed in 4% formalin for cell death determination and immunohistochemistry (IHC).

In Situ TUNEL

DNA fragmentation in apoptotic cell nuclei was assessed with an in situ cell death detection kit according to the manufacturer's protocol (In Situ Cell Death Detection Kit, Roche). Briefly, after deparaffinization and rehydration, protease K (20 μg/mL; Roche) was applied to tissue sections to increase the permeability of the cells. Next, the sections were incubated with the TUNEL reaction mixture (the treatment group was given a 1:9 mixture of Solution 2 to Solution 1, whereas the negative control group was given Solution 2 only) in a humidified chamber for 60 minutes at 37°C. Converter-POD solution was then added, and the slides were incubated for an additional 30 minutes at 37°C. The sections were then stained with a commercial 3,3′-diaminobenzidine kit before sample analysis by light microscopy. The TUNEL positive cells were counted in five randomly chosen fields under a microscope (×400).

Immunohistochemistry

Representative paraffin-embedded soft tissues were cut into 4-mm-thick sections. Tissue sections were deparaffinized and rehydrated in graded alcohol solutions. Antigen retrieval was then performed in citrate buffer (pH 6.0, 15 minutes), and endogenous peroxidase activity was eliminated by incubation in 3% hydrogen peroxide. The sections were incubated with an anti-*GRIM-19* antibody (Abcam, monoclonal antibody [mAb], 1:500), an anti-*pSTAT3* antibody (Tyr705, Abcam, mAb, 1:100), an anti-*VEGF* antibody (Abcam, polyclonal antibody [pAb], 1:150), or an anti-*CD34* antibody (Zhongshan, mAb) overnight at 4°C. The primary antibody was replaced with phosphate-buffered saline (PBS) in the negative controls. On the following day, the sections were incubated with a biotin-labeled secondary antibody. To visualize protein expression, a chromogenic reaction was performed with a 3,3′-diaminobenzidine color reagent kit according to the

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