Effects of estrogen and progestin on expression of MMP-2 and TIMP-2 in a nude mouse model of endometriosis

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Summary

Objective: The study endeavored to observe the effects of estrogen and progestin on expression of matrixmetalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) in a nude mouse model of endometriosis (EMT) and to explore the roles of MMP-2/TIMP-2 in the pathogenesis of EMT. *Methods:* Sixty nude mice were injected in the abdominopelvic cavity with human endometrial tissue and randomly divided into four hormone treatment groups (estrogen, progestin, estrogen+progestin, and saline control; n = 15 per group). Implantation rates and gross morphological characteristics were assessed. Further, expression of MMP-2 and TIMP-2 in ectopic endometrial lesions was detected by immunohistochemistry. *Results:* The overall implantation rate of endometrial samples was 87.5% (50/60) in injected nude mice. Although there was no statistically significant difference in implantation rates between groups, the number of lesions in the progestin group was higher than that in other groups, and the size of lesions in the progestin and estrogen+progestin groups was larger than in the estrogen and control groups ($p < 0.05$). Further, expression levels of MMP-2 in the estrogen and estrogen+progestin groups were higher than in the progestin and control groups ($p < 0.05$). In contrast, expression levels of TIMP-2 in estrogen, progestin, and estrogen+progestin groups were lower than in the control group; additionally, the expression level of TIMP-2 in the progestin group was lower than in the estrogen group ($p < 0.05$). Finally, the ratios of MMP-2/TIMP-2 expression in the estrogen, progestin, and estrogen+progestin groups were higher than for the control group; in fact, this ratio was highest in the estrogen+progestin group ($p < 0.05$). *Conclusions:* The nude mouse is an appropriate model for early clinical studies of EMT, specifically in the detection of MMP-2 and TIMP-2. These proteins appear important in the pathogenesis of EMT. Specifically, estrogen can raise the expression level of MMP-2 to promote ectopic implantation of endometrial tissue. Meanwhile, progestin can inhibit the expression of TIMP-2 to raise the MMP-2/TIMP-2 ratio, which can enhance invasiveness of ectopic endometrium to promote implantation.

Key words: Endometriosis; Nude mouse; Matrixmetalloproteinase-2; Tissue inhibitor of metalloproteinase-2; Estrogen; Progestin.

Introduction

Endometriosis (EMT) occurs when functional endometrial glands and stroma exist outside the uterine cavity and affect normal physiological functions. This chronic disease is common in women of childbearing age, often leads to pelvic and abdominal pain and infertility, and affects women's quality of life [1]. Recently, studies have suggested that ectopic implantation of active endometrial histocytes, which have entered the abdominal cavity, must be achieved through adhesion, invasion, and angiogenesis [2]. Matrixmetalloproteinases (MMPs) play important roles in ectopic implantation. A key member of this family of proteins, MMP-2, is regulated by tissue inhibitor of metalloproteinase-2 (TIMP-2); the dynamic balance between MMP-2 and TIMP-2 in the body is important [3]. Additionally, some evidence indicates that the female sex hormones estrogen and progestin affect expression of MMP-2 and related proteins, and that this dysregulation may promote pathogenesis of endometriosis [4-8]. Here, we establish a nude mouse model for EMT and determine the effect of estrogen and progestin on expression of matrixmetalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) in early lesions of endometriosis (EMT).

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Materials and Methods

Human endometrium specimens. Specimens were collected from a patient who underwent a hysterectomy because of benign lesions (non-endometriosis) at the Affiliated Hospital, School of Medicine, Anhui University of Science and Technology in September 2009. The patient was 43 years old with regular menstruation, no medical complications, and no hormone use for six months preceding the procedure. The endometrium was scraped in the late secretory phase, rinsed repeatedly with cold PBS, cut into 0.5 mm^3-1 mm^3 pieces, and placed in D-Hanks solution with 200 U/ml penicillin and 200 U/ml streptomycin. Postoperative pathological biopsy confirmed that the endometrium was normal. Transfer to animals was conducted within one hour after specimen preparation.

Experimental animals. Sixty female nude mice 6-10 weeks old and weighing 18-24 g were purchased from SLACCAS and raised in SPF conditions. Animal protocols were approved by the university.

Endometrial transplantation. Nude mice were anesthetized via intraperitoneal injection (10% chloral hydrate 0.5 mL/20 g body weight). D-Hanks solution with endometrium samples was injected into the abdominal cavity at the puncture point under the navel on the medioventral line at a volume of 1 ml for each nude mouse (containing about 20 pieces of endometrial tissue). Mice were returned to cages for recovery. Penicillin and streptomycin were administered for three days to prevent infection. Mice were killed 18 days after operation for assessment of lesion formation in the abdominopelvic cavity after celiotomy. Ectopic lesions were removed and immediately placed in formaldehyde solution with 10% phosphate buffer for fixing, dehydration, paraffin embedding, slicing, and staining.

Table 1. — *Ectopic implantation of human endometrium in nude mice administered sex hormones or saline control.*

Group	Implantation ratio (%)	Number of lesions ($\bar{x} \pm s$)	Size ($\bar{x} \pm s$, cm)
Control	10/15 (66.7)	1.40 ± 0.52	0.26 ± 0.05
Estrogen	12/15 (80.0)	1.33 ± 0.49	0.33 ± 0.09
Progestin	15/15 (100)	2.87 ± 0.83$^{*\#}$	0.73 ± 0.20$^{*\#}$
Estrogen+Progestin	13/15 (86.7)	1.62 ± 0.51$^{\Delta}$	0.65 ± 0.16$^{*\#}$
Total	50/60 (87.5)	1.88 ± 0.90	0.52 ± 0.25
χ^2/F^{Δ}	6.240	18.358	31.796
p	0.100	0.001	0.001

$^{*}p < 0.05$, *vs* Control group; $^{\#}p < 0.05$, *vs* Estrogen group; $^{\Delta}p < 0.05$, *vs* Progestin group.

Table 2. — *MMP-2 and TIMP-2 expression in ectopic endometrium in nude mice administered sex hormones or saline control (AOD, $\bar{x} \pm s$, cm).*

Group	*n*	MMP-2	TIMP-2	MMP-2/TIMP-2
Control	10	0.225 ± 0.015	0.286 ± 0.043	0.797 ± 0.095
Estrogen	12	0.273 ± 0.032*	0.241 ± 0.019*	1.141 ± 0.190*
Progestin	15	0.237 ± 0.013$^{\#}$	0.211 ± 0.014$^{*\#}$	1.128 ± 0.086*
Estrogen+ Progestin	13	0.298 ± 0.019$^{*\#\Delta}$	0.225 ± 0.016*	1.334 ± 0.139$^{*\#\Delta}$
Total	50	0.259 ± 0.035	0.237 ± 0.036	1.119 ± 0.224
F		31.369	21.262	31.026
p		0.001	0.001	0.001

$^{*}p < 0.05$, *vs* Control group; $^{\#}p < 0.05$, *vs* Estrogen group; $^{\Delta}p < 0.05$ *vs* Progestin group.

Hormone application. Nude mice were randomly divided into four groups of 15 by a double-blind design. Mice in each group were administered the following hormones on the 7th and 14th days after operation: Group A, 0.02 mg estradiol benzoate; Group B, 5 mg progesterone; Group C, 0.02 mg estradiol benzoate + 5 mg progesterone; and Group D, 0.05 ml normal saline.

Immunohistochemistry. Rabbit anti-human MMP-2 polyclonal antibody and mouse anti-human TIMP-2 monoclonal antibody were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Three-micron serial sections were made from the removed ectopic lesions. For each sample, hemotoxylin and eosin staining was applied to one portion, and two portions were analyzed by immunohistochemistry (IHC) with the MMP-2 and TIMP-2 antibodies detected with two-step streptavidin-peroxidase (SP; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) according to the manufacturer's instructions. MMP-2 and TIMP-2 staining was analyzed with automatic image analysis software (SPSS Science, Chicago, IL), with imaging conditions for each slice as follows: 3 fields were randomly selected and input into the analysis software to determine the mean optical density value as the staining intensity of MMP-2 and TIMP-2 proteins.

Statistical methods. SPSS13.0 statistical software was applied for statistical analysis. All measured values are expressed as mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variance (ANOVA) and pairwise comparisons between the groups (SNK method) were performed to compare results from different groups, using a two-sided test, an alpha level of 0.05, and $p < 0.05$ for statistical significance.

Results

Morphology of endometrial transplants. To develop a model of endometriosis, human endometrial samples were implanted into the abdominal cavities of 60 nude mice that were divided into four hormone treatment categories. Transplants were assessed 18 days after implantation; gross assessment of mice showed adhesions in the abdominopelvic cavities and fusion of transplanted tissue with native tissues. Further, lesion texture was firm, and the surface was covered with small blood vessels (Figure 1). Implantation occurred mainly in the abdominal wall, mesentery, intestinal tubes, and liver in the abdominal cavity, and the adhesion belt was mostly in the adipose tissues of the abdominal wall, intestinal tubes, and omentum.

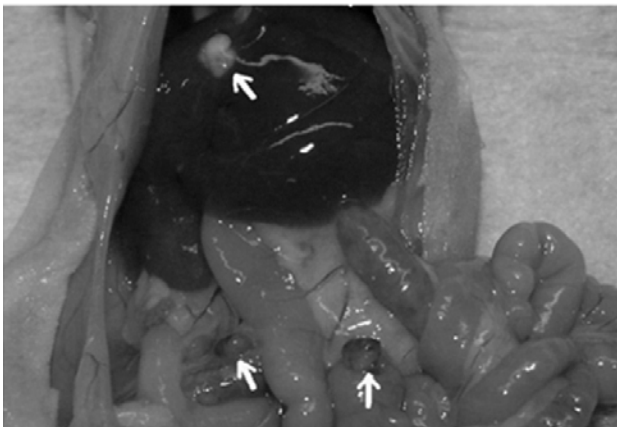

Figure 1. — Lesions of ectopic human endometrium implanted in nude mice.

Endometrial transplants survived in 50 mice (87.5%). No difference in implantation rate was detected among the four treatment groups (estrogen, progestin, estrogen+progestin, or saline control; Table 1). However, the number of ectopic lesions in mice receiving progestin was significantly greater than in other groups ($p < 0.05$). Further, the mean diameters of the lesions in the progestin group and the estrogen+progestin group were significantly greater than those in the estrogen group and the control group ($p < 0.05$).

Histological characteristics. Hematoxylin and eosin-stained excised lesions were examined under light microscopy. Single-layer columnar, cubic, or flattened epithelial cells surrounded uterine glands in samples from all groups and were infiltrated by a large number of inflammatory cells. At their edges, fiber cells proliferated, connective tissues increased, and hyaline degeneration was apparent; a large number of capillaries could also be seen. Implants from estrogen-treated mice showed proliferative changes: the number of glands were small, the glandular cavity was regular with a round or oval shape, glandular epithelial cells were single-layer columnar, glandular secretion was absent, and stroma was dense (Figure 2A). Implants from estrogen+progestin-treated mice had typical secretory changes. Further, the glandular epithelium was a single layer of cubical cells, the glandular cavity was extremely bent and expanded with secretion, stroma was edematous, cells were hypertrophic, and cytoplasm was full of lipid droplets (Figure 2B). While lesions from progestin-

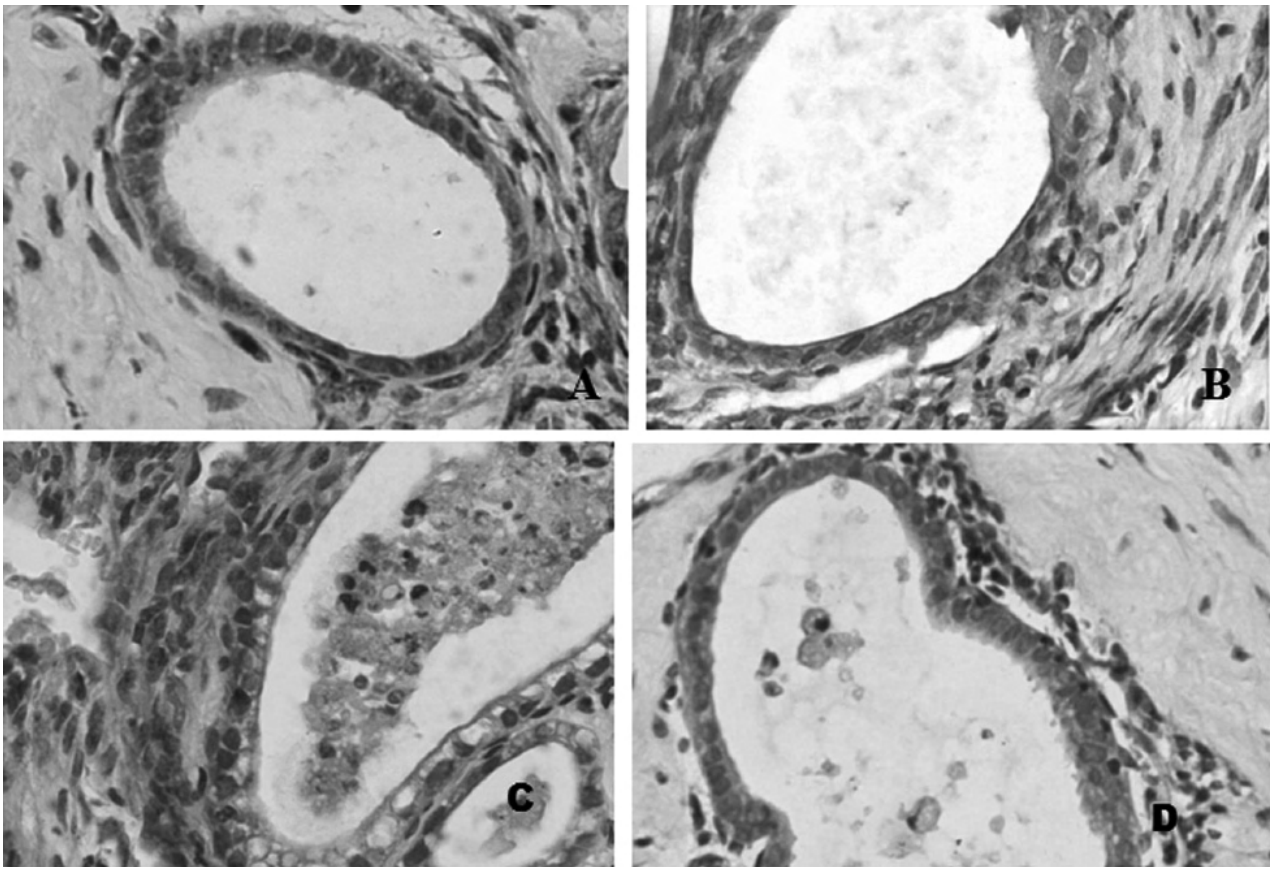

Figure 2. — Histology of ectopic human endometrial lesions in nude mice. Note: A: estrogen group; B: progestin group; C: estrogen+progestin group; D: control group.

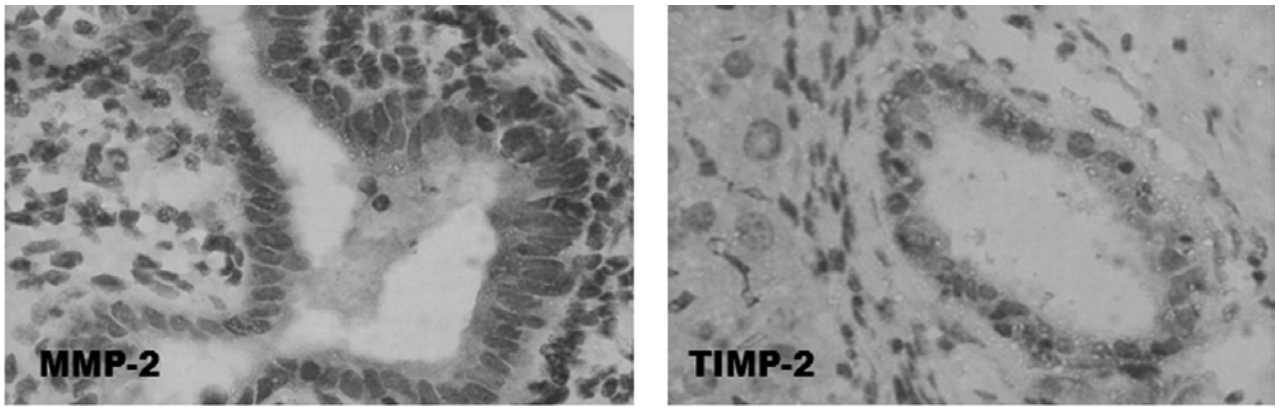

Figure 3. — Positive expression of MMP-2 and TIMP-2 (400 ×).

treated mice also expressed secretory features, compared with the estrogen+progestin group these changes were less apparent and atypical (Figure 2C). Finally, lesions from the control group had proliferative changes, but the glands were fewer, thin, and short, glandular epithelium was mostly single-layer flattened, some glands were incomplete, stroma was less dense, and connective tissue proliferation significantly infiltrated the implants (Figure 2D).

Immunohistochemistry of ectopic endometrium. MMP-2 and TIMP-2 are typically detected in cytoplasm of normal and ectopic endometrial glands and stroma; the glands are generally highly positive [8]. MMP-2 and TIMP-2 were both detected in our ectopic endometrial samples by IHC with anti-MMP-2 and anti-TIMP-2 antibodies, visualized as brownish-yellow staining (Figure 3).

MMP-2 and TIMP-2 staining was compared between groups using a software that analyzes staining intensity. The difference in MMP-2 and TIMP-2 protein expression between treatment groups was statistically significant ($p < 0.05$). Pairwise comparisons between groups indicated that MMP-2 expression in ectopic endometrium from

mice in the estrogen and estrogen+progestin groups was significantly higher than in the control and progestin groups ($p < 0.05$). The difference in MMP-2 expression between lesions from mice in the progestin and control groups was not statistically significant. Conversely, TIMP-2 expression in ectopic endometrium from mice in the estrogen, progestin, and estrogen+progestin groups was significantly lower than in the control group. Further, TIMP-2 expression was significantly lower in lesions from the progestin group than in the estrogen group ($p < 0.05$). The differences in TIMP-2 expression in ectopic endometrium between the estrogen+progestin, estrogen, and progestin groups were not statistically significant. Additionally, the ratio of MMP-2 expression to TIMP-2 expression also differed. Pairwise comparisons between groups indicated a significantly higher MMP-2/TIMP-2 ratio in ectopic endometrium from mice in the estrogen, progestin, and estrogen+progestin groups compared to controls; further, the MMP-2/TIMP-2 ratio of lesions from the estrogen+progestin group was significantly higher than for the estrogen and progestin groups ($p < 0.05$). In contrast, there was no significant difference in the MMP-2/TIMP-2 ratios between the estrogen group and the progestin group.

Discussion

EMT mouse model. Although EMT is a benign disease, some characteristics resemble those of malignant tumors, such as invasion ability, which is capable of local or remote metastasis and invasion and damage to other tissues [9]. To study the pathogenesis of EMT and its influencing factors, an animal model must be established. Similar to a previously reported study in rats [10], we injected human late secretory endometrial samples into the abdominal cavity of nude mice. Implanted mice were treated with estrogen and/or progestin to determine the effects of female sex hormones on implantation/EMT pathogenesis and MMP-2 and TIMP-2 expression. We obtained surviving ectopic endometrial implants in 87.5% of injected mice. These implants displayed typical endometrial glands and stroma, with proliferative and secretory morphological characteristics. However, 12.5% of the ectopic endometrial implantation failed, perhaps because endometrial samples were inadequate.

While there was no difference in implantation success between different hormone treatment groups, the mean size of implanted lesions in the progestin group was significantly larger than that of any other group, and the mean lesion size in the progestin group and the estrogen+progestin group was significantly larger than that in the estrogen group and the control group. Thus, progestin can promote the growth of ectopic endometrium. Studies have shown that progestin can induce secretion of ectopic endometrium, resulting in glandular cavity expansion and thus increasing the number of ectopic implants and implantation area [11]. Therefore, progestin stimulation may aide in the development of appropriate EMT animal models.

Estrogen and progestin influence MMP-2 and TIMP-2 expression. MMPs are important for the degradation of extracellular matrix (ECM), with MMP-2 playing a significant role in many current studies. MMP-2 is secreted by a variety of cells, such as fibroblasts, macrophages, endothelial cells, and malignant cells [12]. Through the degradation of ECM and basement membrane, MMP-2 provides space for new blood vessels and promotes tumor cell invasion and metastasis [13]. The studies on human EMT [14] and mouse EMT models [15] find that, compared with normal endometrium, ectopic endometrium has a stronger ECM degradation activity. Indeed, studies have shown that MMP-2 is highly expressed in stroma and epithelial cells in ectopic endometrium of patients with endometriosis, suggesting that MMP-2 may promote EMT occurrence and development through the degradation of ECM and basement membrane [16]. Additionally, ectopic endometrium has enhanced MMP-2 production, which increases the hydrolytic ability of this tissue. In turn, increased hydrolysis degrades the surrounding basement membrane and damages the connection of peritoneal stromal cells, causing the ectopic endometrium to grow and expand [17]. We found that MMP-2 protein expression in human endometrium ectopically implanted to nude mice was higher in animals treated with estrogen than in the control group; concurrently, TIMP-2 protein expression decreased, resulting in the increase of the ratio of MMP-2/TIMP-2 in the lesions. Thus, estrogen can promote the expression of ectopic endometrial MMPs and inhibit TIMPs, promoting ectopic endometrial invasion and growth. This effect on MMP-2/TIMP-2 may be an important factor for estrogen to promote EMT occurrence and development [18]. We also found that MMP-2 protein expression in ectopic endometrium in the progestin group was not significantly different from that in the control group. However, in these lesions TIMP-2 expression significantly decreased, resulting in a higher MMP-2/TIMP-2 ratio and increased ectopic endometrial invasion. Progestin is generally considered an inhibitor of MMPs; this hormone reduces estrogen production and directly or indirectly inhibits MMP gene expression and protein secretion [19, 20]. These results indicate that the increase in the MMP-2/TIMP-2 expression ratio regulates endometrium growth and expansion, not the activity of a specific hormone.

In summary, estrogen and progestin can regulate MMP-2 and TIMP-2 expression in human endometrium ectopically implanted in nude mice. An increase in the MMP-2/TIMP-2 ratio may lead to extracellular matrix degradation and angiogenesis, promote implantation and growth, and facilitate remote implantation and metastasis. Therefore, MMP-2 and TIMP-2 expression in ectopic endometrium may be used clinically to determine EMT invasion and metastasis and evaluate prognosis. In addition, estrogen and progestin can regulate EMT expression, which has important significance for exploring new methods of clinical diagnosis and treatment.

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