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Estrogen up-regulates MMP2/9 expression in endometrial epithelial cell via VEGF-ERK1/2 pathway

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ABSTRACT

Objective: To study the effect of estrogen on anovulatory dysfunctional uterine bleeding (ADUB). **Methods:** Primary endometrial epithelial cells of Hainan Lizu female was cultured and hydrolytic activity of gelatinase was determined by gelatin zymography analysis. Cellular mRNA and protein synthesis was blocked respectively to determine whether the increased expression of MMP-2/9 was induced by estrogen. The expression of VEGF was blocked by siRNA. After treatment with various factors, MMP-9, VEGF, total Erk and phosphorylated Erk expression in primary uterine epithelial cells was detected by Western blotting analysis. Cell MMP-2/9mRNA levels was measured by real-time RT-PCR. **Results:** The activity and expression of MMP2/9 was increased in the endometrium of patients with ADUB. Estrogen could up-regulate the expression of VEGF and activate Erk 1/2-Elk1 signal path. After interference by siRNA, ERK1/2 pathway was blocked in cells, and the expression of MMP-2/9 was down-regulated. ERK1/2 specific blocker U0126 blocked ERK phosphorylation, and it could down-regulate the expression of MMP-2/9. **Conclusions:** The results showed that the estrogen can increase the expression of VEGF, and thus activate ERK1/2 pathway to induce MMP-2/9 expression.

1. Introduction

Dysfunctional uterine bleeding (DUB) is an abnormal uterine bleeding which is caused by reproductive neuroendocrine disorders. It can be divided into two categories: anovulatory dysfunctional uterine bleeding (ADUB) and ovulation dysfunctional uterine bleeding. And ADUB is the most common in clinical[1,2]. Although the disease poses a indirect threat to patient's life, but it can cause anemia, infection, or even hysterectomy, and influence patient's life quality seriously[3]. However, the mechanism of bleeding has not been clarified yet. The absolute or relative lack of estrogen is considered to be a major cause of the bleeding and the irregular endometrial shedding[4], but how estrogen causes endometrial bleeding is not clear.

Matrix metalloproteinases (MMPs) are a group of zinc ion-dependent protease family, and most of the extracellular matrix and basement membrane collagen can be degradated under neutral pH. Recent studies have shown that the abnormal bleeding of ADUB may be associated with uterinethe regulation of endometrial microenvironment abnormalities[2]. And MMPs serves as an important degradation protease of extracellular matrix and collagen, weather it is involved in anovulatory uterine blood flow has attracted the attention. Recently, a large number of studies have shown that MMPs are extensive expression in normal endometrium, which is involved in the degradation and repair of endometrial[5]. Retreat of the progesterone or estrogen stimulation can increase the expression of endometrial MMP significantly, it suggested that MMPs play an important role in the process of menstruation occurred[6]. MMP-2/9 belong to gelatin enzyme, and can degrade matrix collagen and basement membrane, such as collagen type

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Ⅳ and Ⅴ, laminin protein and fiber links, *etc*. Studies have found that in the normal menstrual cycle the expression level of MMP-2/9 is significantly increased in the endometrial. In addition, the activity and expression of MMP-2/9 is also increased at endometrial stromal degradation in DUB[4]. A specific molecular mechanism remains unclear. And estrogen may be an important incentive .

One study have found that estrogen can stimulating endometrial repair and angiogenesis via stimulate the production of vascular endothelial grow factor (VEGF)[4]. VEGF can lead to uterine vascular intimal hyperplasia, vascular structural abnormalities and increase permeability in pathological situation to cause bleeding. The research reported the endometrial spiral artery contraction is inhibited and the number of venous capillaries is increased in dysfunctional uterine blood flow. While the latter was in the form of performance lumen, thin wall, twisted, forming irregular vascular access, and it increased vascular fragility.This phenomenon is particularly evident in the performance of anovulatory uterine blood flow only by the influence of estrogen[6]. This may be related to estrogen, which can stimulate the synthesis of VEGF expression. The latest research has shown that estrogen can increase the expression of endometrial VEGF, moreover the expression of VEGF and the synthesis of MMP- 2/9 have a positive correlationis closely[4,7]. However, the regulation mechanism between VEGF and MMP-2/9 is not clear. ERK1/2 signaling pathways is an important molecular pathway of VEGF downstream. It participates in the regulation of multiple gene expression[8]. But it is unclear whether VEGF can regulate the expression of MMP2/9 through ERK1/2 pathways. We can study the efffect of estrogen on the expression of MMP-2/9 by VEGF-ERK1/2 pathways based on the above researches. It is to further explore the specific molecular mechanisms of estrogen in ADUB, and the regulatory role of VEGF, then to provide a new theoretical basis for the treatment of ADUB.

2. Materials and methods

2.1. Culture of endometrial epithelium primary cells

After total hysterectomy, the intima was promptly scraped down from tissue, endometrial epithelium cells were cultured[9]. Related agreement was signed by patients, and it was agreed by ethics committee. Culture condition was as follows: 15% fetal bovine serum, 37 ℃, 5% CO_2. When cell confluence reached 80%, treatments were added. But in RNAi process, cell confluence was just 50%. When Cell confluence reached 80%, cells were washed 3 times with PBS, then the cells were cultured in steroid-free medium (1640), and was added with 10 nM 17 β -estradiol (E_2). They were cultured for 24 h.

2.2. Interference of VEGF RNA

Cells were cultured in medium without antibiotics for 12 h, at 50% convergence degree. Different concentration of RNA interference reagent (A), RNA transfection reagent (B) was diluted by RNA transfection medium. Before transfection, A was mixed with B, incubated for 30 min. Then cells were washed with PBS for 6 h later. After that, cells were cultivated in 30% serum DMEM.

2.3. Blocking phosphorylation of ERK1/2 with U0126

When cells convergence degree reached 80%, cells were then cultured in steroid-free medium for 12 h. After that, U0126 and E_2 were respectively added. After 24 h, medium and cell were harvested for detection of MMP-2/9 and other proteins.

2.4. Zymography in situ

The gelatinase activity of endometrial tissue was test according to the EnzCheck Collagenase Kit instructions[10]. The samples in experimental group were from ADUB patients, samples in control group from usual leiomyoma patients. Endometrial histological section was washed 3 times and placed on the cover glass for incubation at 37 ℃ for 2 h, then incubated in reaction liquid with fluorescently-labeled DQ-gelatin for 2 h. After that, endometrial histological section was washed with PBS and prolong antifade reagents in order. Finally, endometrial histological section was observed under fluorescence microscope. The specificity was confirmed by MMP inhibitor 1, 10-phenanthroline.

2.5. Gelatin zymography

Sample was extracted from culture medium according to Gelatin zymography instruction. Experiment condition was as follows: 0.1% Gelatin SDS PAGE (8%), 160 v, 70 min. It was rinsed in 0.25% Triton-×100 rinse for 30 min, incubated at 37 ℃ for 48 h [Tris 50, pH 7.6, 5, $CaCl_2$ 5 mmol/L, NaCl 0.2 mmol/L, and 0.02% (w/v) Brij-35]. PAGE was discolorated after Coomassie brilliant blue staining for 2 h, then it was observed under molecular imaging scanner (human MMP-2/9 mixture as standard product).

2.6. Western blotting

Protein was extracted from endometrial epithelium cells treated with various factor, according to the manufacturer's protocol. For detection of MMP-9,VEGF, the total ERK and phosphorylated ERK1/2, 20 μg protein was separated on a 10% SDS-PAGE gel and transferred to PVDF. Membranes were incubated overnight with primary antibodies, and proteins were visualized using appropriate secondary

antibodies conjugated to horse radish peroxidaseand scanned using ECL Plus.

2.7. Real-time RT-PCR

Total RNA was extracted using the TRIzol reagent following the manufacturer's instructions (Invitrogen). One microgram of total RNA was reverse transcribed using a *Taq*Man Reverse Transcription Reagents Kit (Applied Biosystems), and then 2 μg of cDNA was added to the real-time quantitative PCR (RT-PCR) system (Applied Biosystems) for evaluation of the relative mRNA levels of MMP2 and MMP9. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primers used to amplify specific gene products were as follows: GAPDH sense,5′-TGCCATCAACGACCCCTTCA-3′; GAPDH antisense, 5′-TGACCTTGCCCACAG-3′; MMP-2 sense, 5′-ATAACCTGGATGCCGTCGT-3′; MMP-2 antisense, 5′-AGGCACCCTTGAAGAAGTAGC-3′; MMP-9 sense, 5′-GAACCAATCTCACCGACAGG-3′; MMP-9 antisense, 5′-GCCACCCGAGTGTAACCATA-3′; The Ct (threshold cycle) value of each sample was calculated from the threshold cycles using the software embedded in the RT-PCR machine (SDS 2.3), and the relative expression of MMP2 and MMP9 mRNA was normalized to the GAPDH level. Relative expression level was determined by normalizing the expression of each gene to that of the GAPDH gene following the $2-\triangle\triangle C_t$ method after amplification using the Power SYBR green Master Mix (Applied Biosystems).

2.8. Statistical analysis

The experimental data was expressed as the mean±standard error form and was analyzed by *t*-test. The confidence interval of 95% was calculated and $P < 0.05$ was considered as significant difference.

3. Results

3.1. Expression and activitiy of MMP-2/9 in endometrial tissues of ADUB patients

MMP2/9 expression and activitiy was compared between ADUB and control by *in situ* zymography. Figure 1 showed the staining for MMP2/9 in endometrial biopsy samples obtained from normal fertile women during menstrual cycle. MMP2/9 staining intensity was greatest in the ADUB (Figure 1). But some weak staining was seen in normal normal fertile women during menstrual cycle. This indicated that the expression and activitiy of MMP-2/9 in endometrial tissues of ADUB were increased.

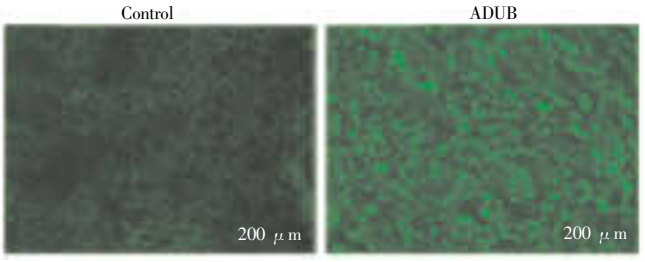

Figure 1. Expression and gelatinolytic activitiy of MMP-2/9 increased in endometrial tissues of ADUB.
In situ zymography showed increased gelatinolytic activity of MMP-2/9 in the endometrial tissues of ADUB patients (bright green fluorescence). The length of the bar is 200 μm.

3.2. Effect of estrogen on expression of MMP2/9 in endometrial epithelial cell

The results of RT-PCR indicated that estrogen induced a marked decrease in MMP2/9 mRNA transcripts. Western blotting results were in agreement with this result of RT-PCR. MMP2/9 mRNA and protein expression were significantly up-regulated as compared with the control group (Figure 2A-C).

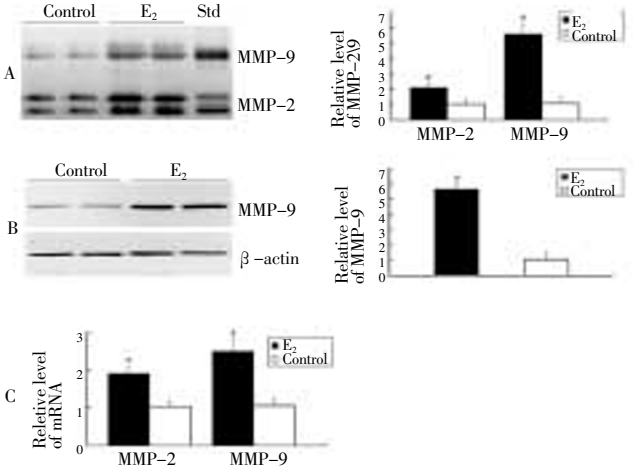

Figure 2. Expression of MMP-2/9 increased in EECs stimulated with estrogen.
A. Expression of MMP-2/9 in medium of primary EECs detected by gelatin zymography.
Std, Standards of human MMP-2/9. The moleculear weight of them are 92 kD (above) and 72 kD (bottom), respectively. * $P<0.05$ compared with control.
B. Expression of MMP-2/9 in EECs detected by Western blotting.
The molecular weight of MMP-9 and β-actin were 92 kD and 42 kD, respectively. * $P<0.05$ compared with control.
C. Level of MMP-2/9 mRNA detected by Real-time RT-PCR.
* $P<0.05$ compared with control. Experiments were repeated 4 times with similar results.

3.3. Expression of ERK1/2 and VEGF stimulated by estrogen

Extracellular signal-regulated coyness (ERK1/2) is conservative mitogen-activated protein kinase in

mammalian. It participates in the development of many different disease. This result showed expression of phosphorylation of ERK (p-ERK)1/2 was significantly increased in endometrial epithelial primary cells after stimulation by estrogen (P<0.05, Figure 3A), and VEGF was also significantly increased (P<0.05, Figure 3B).

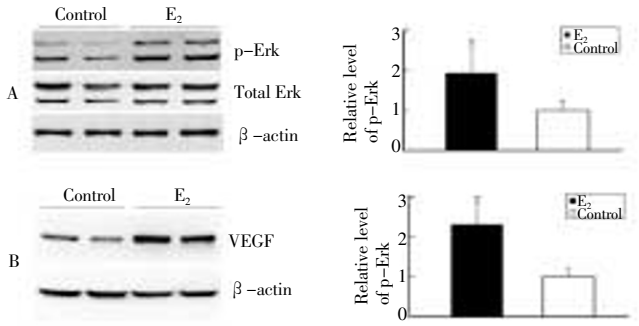

Figure 3. Estrogen activated ERK1/2 signaling pathway and induced VEGF expression.
A. The level of p-ERK1/2 expression in EECs stimulated with 10 nM E_2.
p-ERK, total ERK (sum of p-ERK and no-p-ERK), molecular weight of p-ERK and total ERK were 44 kD (above) and 42 kD (bottom), respectively. The molecular weight of β -actin was 42 kD. * P<0.05 compared with control.
B. The level of VEGF expression in EECs dealed with 10 nM E_2.
The molecular weight of VEGF was 147 kD.* P<0.05 compared with control. Experiments were repeated 4 times with similar results.

3.4. ERK1/2 inhibitors attenuate estrogen mediated MMP-2/9 expression in in endometrial epithelial cell

To further investigate the signaling pathways involved in estrogen mediated MMP-2/9 expression, endometrial epithelial cell were treated with specific inhibitors of MAP kinases. U0126, inhibitors of the MEK/ERK1/2 pathway, strongly inhibited MMP-2/9 mRNA expression. Pretreatment with U0126 (0.5 U/mL) attenuated the effect of estrogen (10 nM) on MMP-2/9 expression in endometrial epithelial cell (Figure 4A-B). This results demonstrate that MEK/ERK1/2 inhibitors could block estrogen mediated MMP-2/9 expression. Additionally, we also found that the expression of VEGF induced by estrogen don't denpen on ERK1/2 pathway (Figure 4C).

3.5. Estrogen mediated MMP-2/9 expression via VEGF-ERK1/2 pathway

Analysis of RT-PCR and Western blotting showed that pretreatment with VEGF siRNA inhibited the effect of estrogen (10 nM) on ERK1/2 and MMP-2/9 expression in endometrial epithelial cell. These findings demonstrated that VEGF deficiency in endometrial epithelial cell would block MMP-2/9 by inhibiting ERK1/2 transcriptional activity (Figure 5).

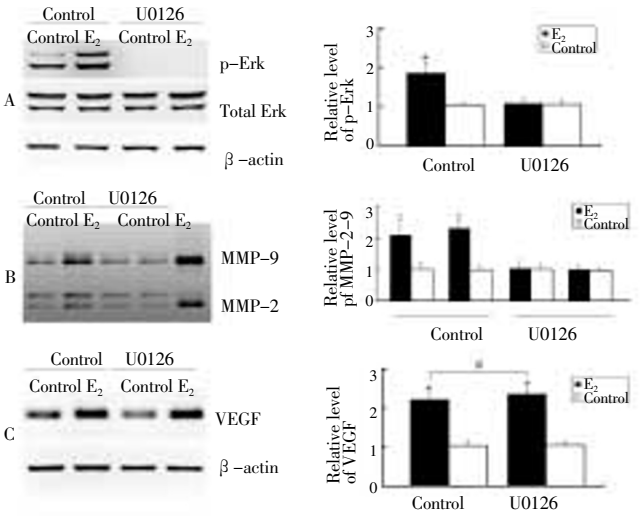

Figure 4. Estrogen-induced MMP-2/9 expression was inhibited after activation of ERK signal pathway was blocked with U0126.
A. U0126 (0.5 U/mL) effectively blocked phosphorylation of ERK.
The molecular weight of p-ERK and total ERK were 44 kD (above) and 42 kD (bottom), respectively. The molecular weight of β -actin was 42 kD. * P<0.05 compared with control.
B. The estrogen-induced MMP-2/9 expression in EECs stimulated with 10 nM E2 was blocked by U0126 (0.5 U/mL).
Std, Standard of human MMP-2/9. The molecular weight of them were 92 kD (above) and 72 kD (bottom), respectively. * P<0.05 compared with control.
C. Blocking of p-ERK did not affect estrogen-induced VEGF expression in EECs treated with 10 nM E_2.
*P<0.05 compared with control. # P>0.05 compared with control after p-ERK blocked by U0126. Experiments were repeated 3 times with similar results.

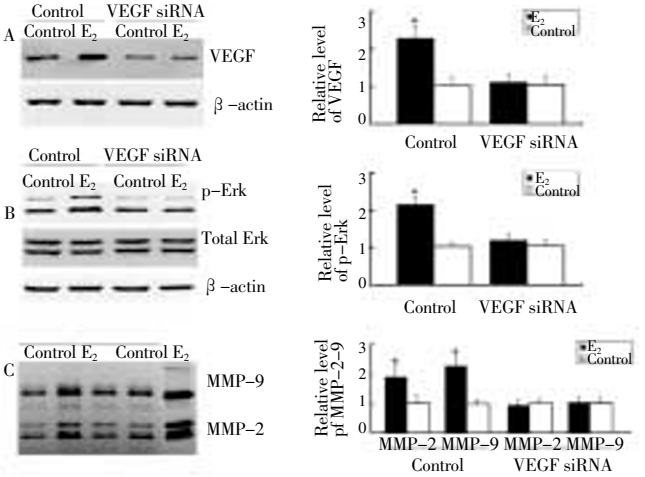

Figure 5. Knockdown of VEGF expression with VEGF siRNA desensitized estrogen-induced phosphorylation of ERK and MMP-2/9 increasing.
A. The expression of VEGF in EECs was effectively blocked with VEGF siRNA. The molecular weight of VEGF and β -actin were 147 kD and 42 kD, respectively. * P<0.05 compared with control. B. Knockdown of VEGF with VEGF siRNA blocked estrogen-induced activation of ERK signal pathway in EECs stimulated with 10 nM E_2. The molecular weight of p-ERK and total ERK are 44 kD (above) and 42 kD (bottom), respectively. The molecular weight of β -actin is 42 kD. * P<0.05 compared with control. C. Knockdown of VEGF with VEGF siRNA inhibited estrogen-induced MMP-2/9 expression in EECs dealed with 10 nM E_2. Std, standard of human MMP-2/9. The molecular weight of them are 92 kD (above) and 72 kD (bottom), respectively.* P<0.05 compared with control. Experiments were repeated three times with similar results.

4. Discussion

Proteins of MMP family are involved in the breakdown of extracellular matrix in normal physiological processes, such as tissue remodeling, cancer metastasis and invasion, angiogenesis, autoimmune diseases and so on. Most MMPs are secreted as inactive proproteins which are activated when they are cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type Ⅳ and Ⅴ collagens[11-14]. MMP-2/9 also known as gelatinase A and B, belonging to type Ⅳ collagenase can be encoded by this gene degrades type Ⅳ and Ⅴ collagens. MMP-2/9 hydrolytic activity were enhanced in ADUB patients. This study indicates that the expression and activitiy of MMP-2/9 in endometrial tissues of ADUB were increased and estrogen plays an important role in ADUB. To further investigate the relation of MMP-2/9 and estrogen, we stimulate endometrial epithelial cell with estrogen and found that MMP2/9 mRNA and protein expression were significantly up-regulated as compared with the control group. Therefore, We suspect that, MMP2/9 were increased in ADUB patients, because estrogen stimulates endometrial tissues in long time. For this reason, extracellular matrix is broken down significantly which can lead to the destruction of the extracellular environment, then fragile and bleeding of endometrium.

VEGF mediated angiogenesis palys an important role in development and repair of endometrium. Numerous studies have shown that VEGF involved in angiogenesis is associated with activation of ERK1/2[15]. This study also demonstrated that the expression of VEGF could be induced by estrogen, and the ERK1/2 was also up-regulated and activated[15]. To further investigate the signaling pathways involved in estrogen mediated MMP-2/9 expression, endometrial epithelial cell were treated with specific inhibitors of MAP kinases and VEGF siRNA. And then it shows that ERK1/2 inhibitors could block estrogen mediated MMP-2/9 and VEGF expression. Moreover, ERK1/2 activation is down-regulated when VEGF was declined with fresh serum-free medium containing estrogen. The results demonstrate that estrogen significantly elevates MMP-2/9 espression in endometrial epithelial cell via VEGF- ERK1/2 pathway. Future studies will hopefully contribute to the identification of drug targets for ADUB. Our findings may provide a new direction for ADUB treatment, and VEGF could be a protencial targets for ADUB prevention.

Conflict of interest statement

We declare that we have no conflict of interest.

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