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Original Article

The expression of marker for endometrial stem cell and fibrosis was increased in intrauterine adhesious

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Abstract: Objectives: The objective of the present study was to evaluate whether fibrotic markers and endometrial stem cell markers were abnormal expressed in endometrium of intrauterine adhesions and a female mouse model for intrauterine adhesions. Methods: We revaluated endometrial fibrosis using Masson's stain. We detected the expression of endometrium stem cell markers (CD146 and CD140b) and fibrosis markers (TGF-Beta, CTGF, collagen protein I and collagen protein III) in endometrial tissue with intrauterine adhesions using real-time PCR and S-P (Streptavidin-Peroxidase) immunohistochemistry. We create a female mouse model for intrauterine adhesions using mechanical injury, and then revalue the expression of endometrial stem cell markers and fibrosis markers in endometrial tissue of mouse model for intrauterine adhesions. Results: The ratio of the area with endometrial fibrosis to total endometrial area in intrauterine adhesious significantly increased compared with the normal endometrial tissue ($P < 0.05$); The expression levels of fibrotic markers and endometrial stem cell markers were higher in the endometrial tissue with intrauterine adhesious compared to normal endometrial tissue ($P < 0.05$). The animal experiments showed that the ratio of the area with endometrial fibrosis to total endometrial area significantly increased compared with the control group ($P < 0.05$); The expression levels of fibrotic markers and endometrial stem cell markers were higher in the endometrial tissue compared to the control group ($P < 0.05$). Conclusion: Aberrant activation of fibrosis may be involved in the pathology of intrauterine adhesious.

Keywords: Stem cells, intrauterine adhesious, fibrosis

Introduction

Intrauterine adhesion was first described by Joseph Asherman in 1948, whose name was applied to the syndrome of partial or complete obliteration of the uterine cavity by adhesions [1, 2]. It is usually known as intrauterine adhesion and Asherman's syndrome. It is characterized by recurrent abdominal pain, menstrual disturbance, amenorrhea and thin endometrium. A normal uterine cavity and adequate thickness of the endometrium is indispensable for a successful pregnancy in assisted reproductive technology (ART) cycles [3, 4]. Previous studies have reported low pregnancy rates in the presence of thin endometrium [5]. Scar tissue within the uterine cavity can partially or completely obliterate the normal cavity which involved in menstrual abnormalities, infertility, recurrent pregnancy loss and other complications later in the pregnancy [6].

Endometrial stem cells have been identified in human endometrial tissues on the basis of their functional attributes. They can reconstruct endometrium suggesting they can be used in treating disorders associated with inadequate endometrium [7]. Some specific markers of human endometrial stem cell have been identified. CD146 and platelet-derived growth factor receptor beta (PDGF-Rb) are candidate markers for endometrial stem cells [7]. Some gynecological disease was considered to be involved in endometrial stem cells, including endometriosis, endometrial cancer and endometrial polyps [8-10]. However, there is no report on the pathogenesis of IUAs with regard to endometrial stem cells/progenitor cells.

Many studies have demonstrated that stem cells derived from bone marrow contribute to the physiological remodeling and regeneration of the human uterus [11, 12]. Several growth

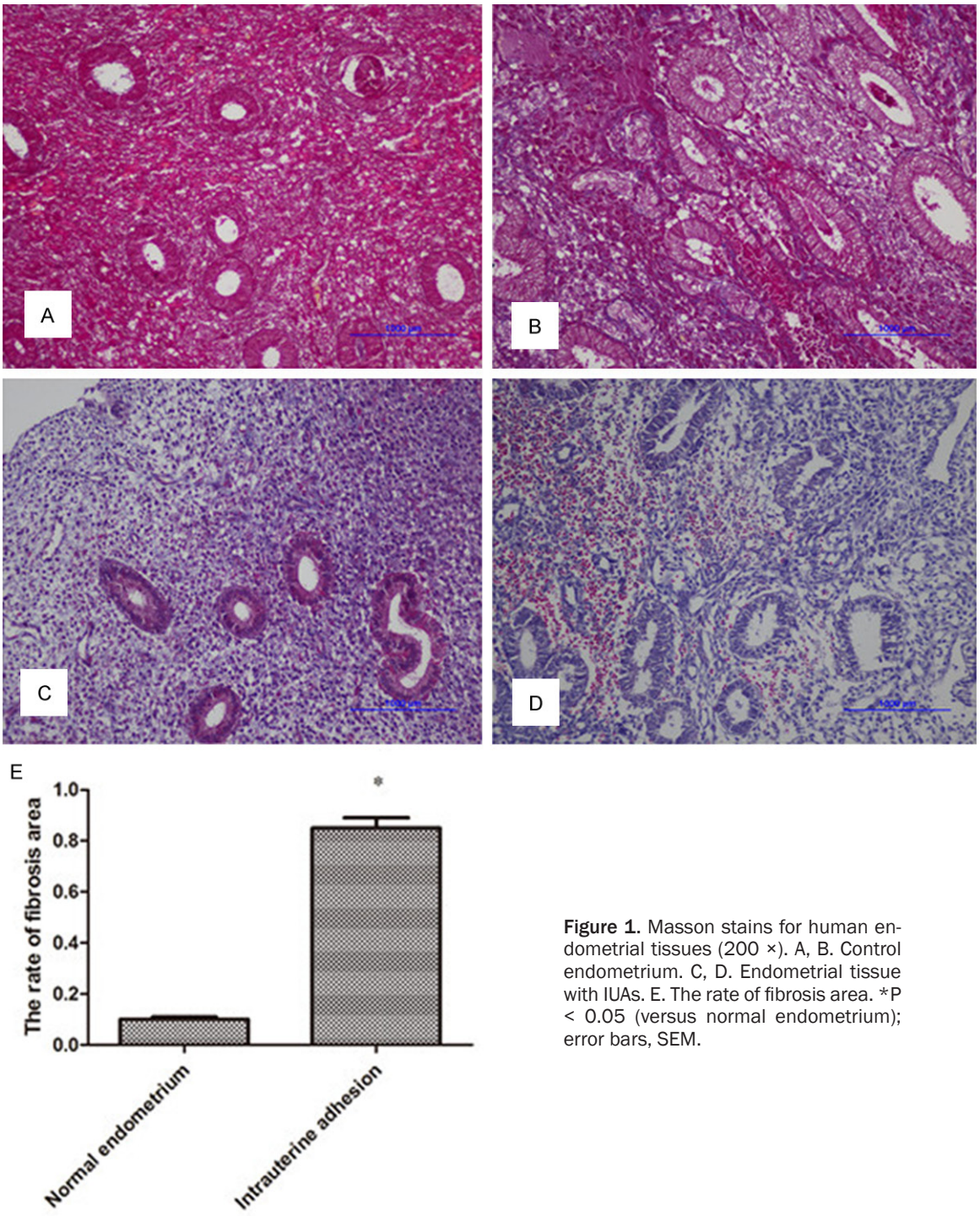


Figure 1. Masson stains for human endometrial tissues (200 ×). A, B. Control endometrium. C, D. Endometrial tissue with IUAs. E. The rate of fibrosis area. *P < 0.05 (versus normal endometrium); error bars, SEM.

factors are required for colony-forming cells/units (CFU) activity, including epidermal growth factor (EGF), transforming growth factorα (TGFα) and platelet-derived growth factor BB (PDGF-BB). Candidate endometrial stem/progenitor cells have been identified in mouse endometrial tissue as label retaining cells (LRCs). However, epithelial and most stromal LRCs do not express estrogen receptor α (Esr1). Endometrial tissue is high regenerate and they rapidly proliferate on estrogen stimulation, most likely mediated by the Esr1-expressing niche cells [13]. Normal wound healing is regulated by a complex set of interactions in a network of profibrotic and antifibrotic cytokines. These proteins include transforming growth

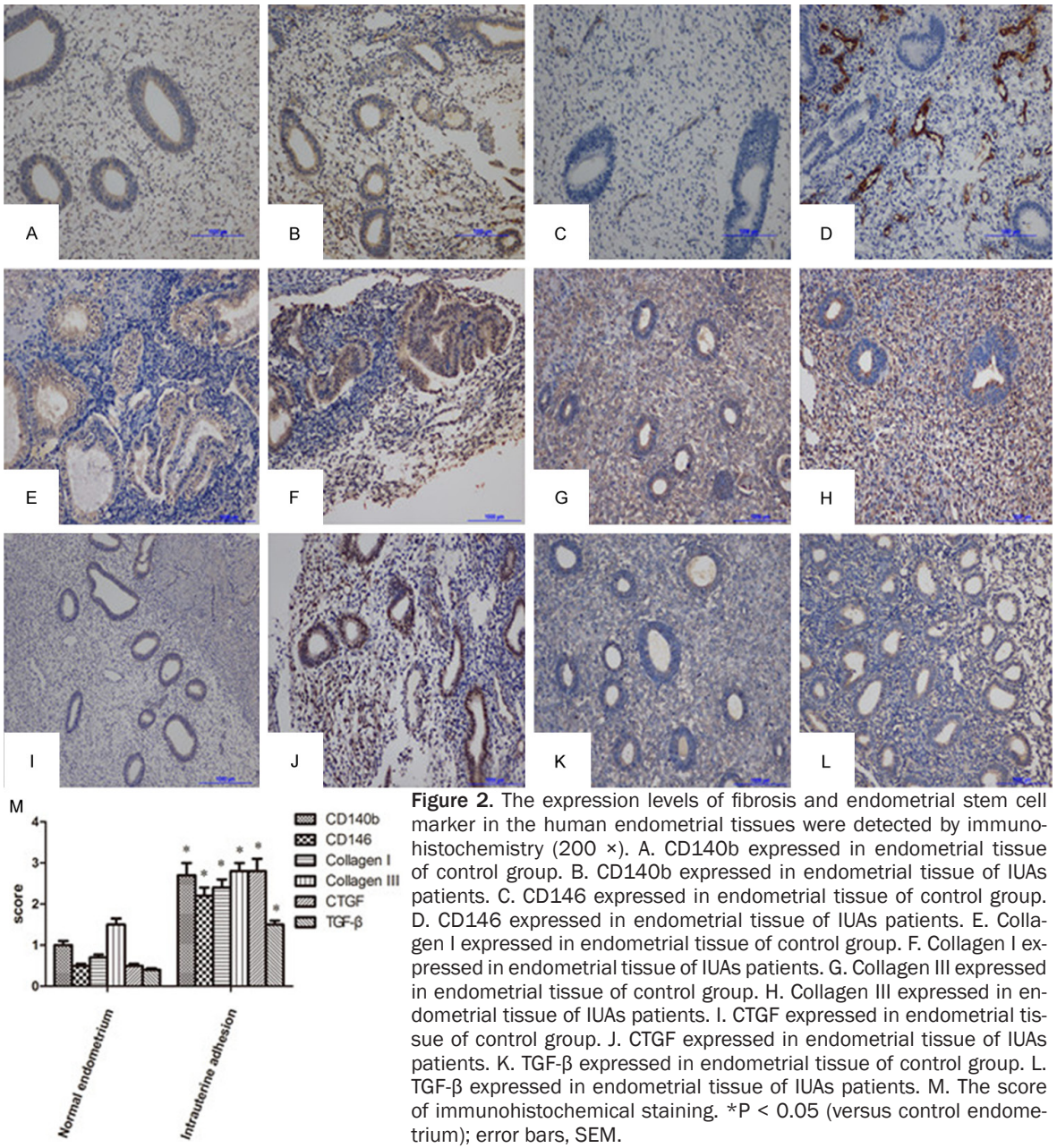


Figure 2. The expression levels of fibrosis and endometrial stem cell marker in the human endometrial tissues were detected by immunohistochemistry (200 ×). A. CD140b expressed in endometrial tissue of control group. B. CD140b expressed in endometrial tissue of IUAs patients. C. CD146 expressed in endometrial tissue of control group. D. CD146 expressed in endometrial tissue of IUAs patients. E. Collagen I expressed in endometrial tissue of control group. F. Collagen I expressed in endometrial tissue of IUAs patients. G. Collagen III expressed in endometrial tissue of control group. H. Collagen III expressed in endometrial tissue of IUAs patients. I. CTGF expressed in endometrial tissue of control group. J. CTGF expressed in endometrial tissue of IUAs patients. K. TGF-β expressed in endometrial tissue of control group. L. TGF-β expressed in endometrial tissue of IUAs patients. M. The score of immunohistochemical staining. *P < 0.05 (versus control endometrium); error bars, SEM.

factor-β (TGF-β) and connective tissue growth factor (CTGF). TGF-β has long been believed to be a central mediator of the fibrotic response, this cytokine induces fibroblasts to synthesize and contract ECM [14]. CTGF is induced by TGF-β and is considered a downstream mediator of the effects of TGF-β on fibroblasts [15]. The fibroblast is responsible for collagen deposition that is needed to repair the tissue injury [16]. The relationship between fibrosis and IUAs development remains unclear.

Steroid hormones and growth factors regulate endometrial stem/progenitor cells, they may be the microenvironment of endometrial stem cell [12]. However, there is no report on endometrial stem cell markers (CD146 and CD140b) and fibrosis markers (TGF-Beta, CFGF, collagen protein I and collagen protein III) involving in the pathology of IUAs. We assessed the expression levels of the endometrial stem cell markers CD146 and CD140b, as well as the expression of the fibrosis-related markers (TGF-Beta, CFGF, collagen protein I and collagen protein III) in IUAs, to explore the pathogenesis of IUAs.

Materials and methods

Patient samples

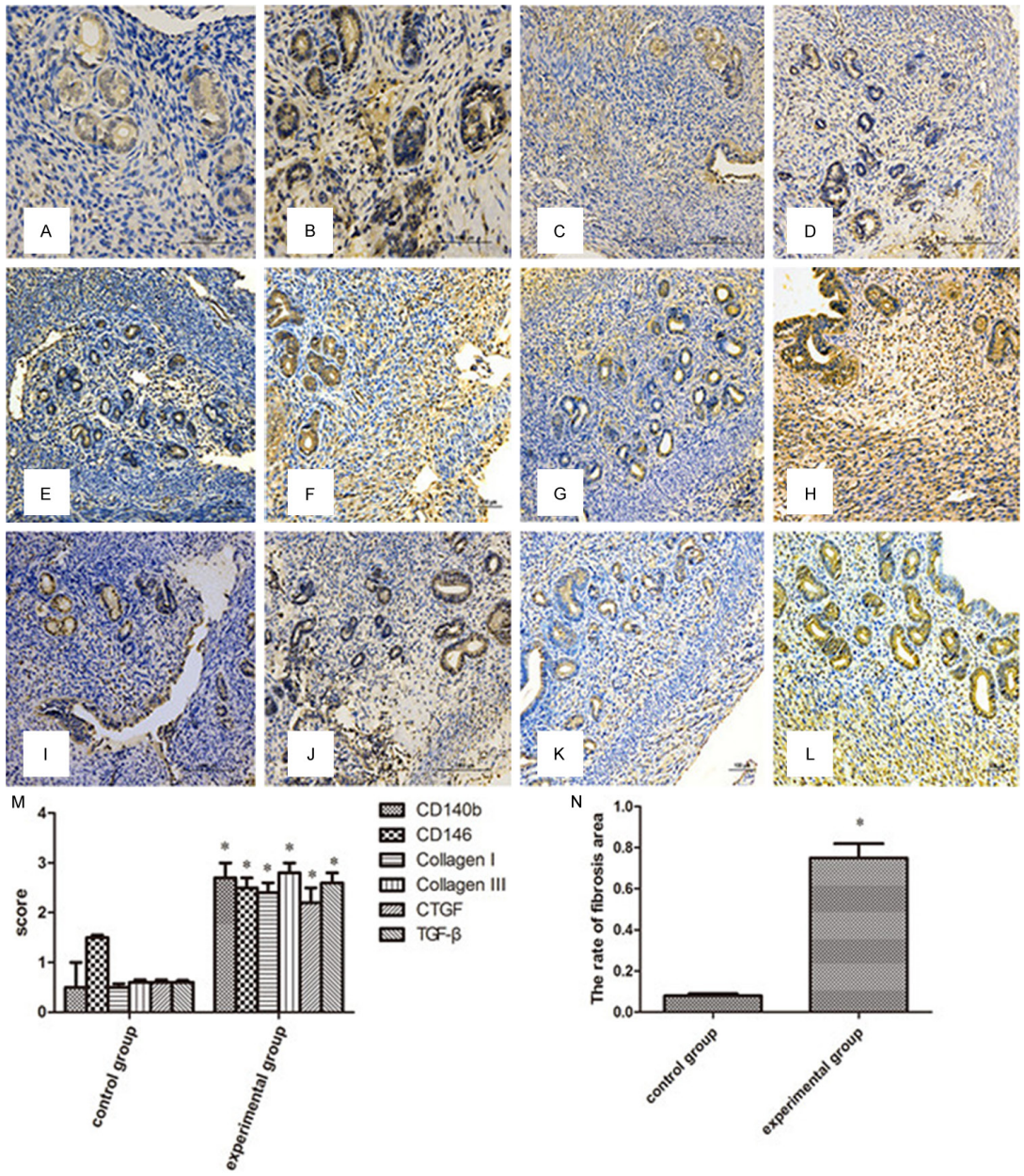
The patients were admitted to the Second Affiliated Hospital of Chongqing Medical University from January 2013 to January 2014. They were diagnosed IUAs patients by hysteroscopy. All patients were randomly selected. The study was approved by the Ethics Commission of Chongqing Medical University, and informed consents from the patients were obtained. Forty cases of normal endometrium were harvested as the control group. The patients were ages 24 to 55 years, with a mean age of 34 years. All the pathologies were confirmed by pathological examination. Patients, who had additional endometrial complications, including dysfunctional uterine bleeding, adenomyosis, polycystic ovary syndrome, and other hormone-dependent diseases, were excluded. All of the patients had regular menstrual cycles, did not receive hormone therapy during the three months before surgery, and were not pregnant or lactating during the study.

Creation of a female mouse model for intrauterine adhesions using mechanical injury

All animal experiments were approved by the Ethical Committees of Chong Qing Medical University. Six-month-old virgin female Kun Ming mouse were maintained in collective cages in an appropriate room with controlled temperature and with a 12-h light cycle and fed with standard mouse chow and water. 8-week-old Kun Ming female mice were used to create Asherman's syndrome mouse model [17]. Briefly, after exposure to the inhaled anesthetics, a vertical incision was made in the abdominal wall and the uterus exposed. A small incision was made in the each uterine horn at the utero-tubal junction and the horn traumatized in a standardized method using 27 Gauge needle inserted two-thirds of the way through the lumen, rotated and withdrawn four times. A total of 10 mice were randomized to uterine damage or abdominal surgical incision alone (control group). Two estrous cycles after uterus damage, the uterine horn were collected. The hematoxylin and eosin (H&E) as well as Masson stain for histological evidence of endometrial fibrosis.

Immunohistochemistry

The tissues were embedded in paraffin and cut into 5-μm sections. The sections showing typical endometrial structures by hematoxylin and eosin (H&E) staining were included in this study. Immunohistochemistry was performed according to the SP kit instructions (SP-9000, ZSGB-BIO, Beijing, China). After dewaxing and hydration, the sections were heated in citrate buffer (pH 6.0, Sigma-Aldrich, USA) in a microwave oven for 20 min for antigen retrieval. The sections were then cooled naturally to room temperature. The sections were washed for three min × 3 cycles. The sections were then incubated in 3% aquae hydrogenii dioxidi for 15 min at room temperature and washed with PBS for 3 min × 3 cycles. The sections were blocked in 5% donkey serum (ab7475 Abcam Company) for 30 min at 37°C. Anti-CD146 rabbit monoclonal antibody (1:100, ZA-0539, ZSGB-BIO, Beijing, China), Anti-CD146 rabbit polyclonal antibody (1:100, bs-1618R, Bioss, Beijing, China), anti-CD140b rabbit polyclonal antibody (1:100, bs-0231R, Bioss, Beijing, China), anti-CTGF goat polyclonal antibody (1:100, sc-14939, Santa Cruz Biotechnology, Inc., USA), anti-TGF-β rabbit polyclonal antibody (1:100, bs-0086R, Bioss, Beijing, China), anti- Collagen I (COI) rabbit polyclonal antibody (1:100, bs-10423R, Bioss, Beijing, China), and anti-Collagen III (COIII) rabbit polyclonal antibody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The rate of fibrosis area
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Figure 3. The expression levels of fibrosis and endometrial stem cell marker in the mouse endometrial tissues were detected by immunohistochemistry (200 ×). A. CD140b expressed in endometrial tissue of control group. B. CD140b expressed in endometrial tissue with IUAs. C. CD146 expressed in endometrial tissue of control group. D. CD146 expressed in endometrial tissue with IUAs. E. Collagen I expressed in endometrial tissue of control group. F. Collagen I expressed in endometrial tissue with IUAs. G. Collagen III expressed in endometrial tissue of control group. H. Collagen III expressed in endometrial tissue with IUAs. I. CTGF expressed in endometrial tissue of control group. J. CTGF expressed in endometrial tissue with IUAs. K. TGF-β expressed in endometrial tissue of control group. L. TGF-β expressed in endometrial tissue with IUAs. M. The score of immunohistochemical staining. N. The rate of fibrosis area. *P < B0.05 (versus control group); error bars, SEM.

(1:100, bs-0948R, Bioss, Beijing, China) were incubated with the sections overnight at 4°C. Negative controls included omission of primary antibody and use of irrelevant primary antibodies. The corresponding secondary antibodies, which were conjugated to horseradish peroxidase (Bioss Biotechnology), were incubated with the sections for 1 h at room temperature. The sections were washed in PBS for 3 min × 3 cycles. The sections were incubated in horseradish enzyme labeled chain avidin solution (Bioss Biotechnology) for 30 min at 37°C and washed in PBS for 3 min × 3 cycles. The proteins were visualized by diaminobenzidine (DAB). All of the sections were observed by three independent pathologists using a light microscope. A total of 22 representative high power fields (40 ×) were chosen, and the positively-stained cells were counted for each sample. Slides were evaluated independently by 3 pathologists for distribution and intensity of signal as described by De Falco et al [17]. Intensity was scored from 0 to 3:0 (absent immunopositivity); 1 (low immunopositivity); 2 (moderate immunopositivity); 3 (intense immunopositivity). An average of 22 fields was observed for each tissue. All values were represented as the mean ± standard error (mean ± SEM).

qPCR

Total RNA was isolated using a RNA pure high-purity total RNA rapid extraction kit (BioTeke, RP1201, China) in strict accordance with the instructions provided with the kit. cDNA was synthesized using the iSCRIPT cDNA synthesis kit(Bio-Rad). The primers used for amplifying a-SMA, TGF-β, CTGF, Collagen III and Collagen I, and GAPDH were synthesized by Guangzhou Funeng Co., Ltd. The real-time PCR kit was purchased from Guangzhou Funeng Co., Ltd. PCR conditions were 95°C for 10 s, 60°C for 20 s, 72°C for 10 s. The experiments were performed in triplicate for every sample.

Western bolt

Tissues were lysed with a RIPA buffer containing protease inhibitors. Aliquots of the lysates containing 25 μg of total protein were run on a SDS-polyacrylamide gel. The proteins were transferred to a PVDF membrane. The membrane was incubated overnight at 4°C in TTBS containing 5% non-fat milk powder (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Anti-CTGF goat polyclonal antibody (1:500) or anti- Collagen I rabbit polyclonal antibody (1:400) was incubated with the membrane for 2 h at 37°C. Secondary antibodies that were conjugated to horseradish peroxidase were incubated with the membrane for 1 h at 37°C. The proteins that were revealed by western blotting were visualized by chemiluminescence (Biyuntian Company). The densities of bands were analyzed by a gel imaging system and calculated compared to the internal control.

Statistical analysis

SPSS 17 software was used to perform the statistical analysis. One-way analysis of variance was used for comparison the rate of fibrosis area. The difference between two groups was compared using the independent t-test. *P* values less than .05 were considered statistically significant.

Results

The degree of endometrial fibrosis in endometrium

The ratios of the area with endometrial fibrosis to total endometrial area were calculated. The IUAs group had a higher ratio compared with control group respectively, (P < 0.01) (**Figures 1** and **4**).

Immunohistochemistry

TGF-β, CTGF, Collagen III, Collagen I and CD140b were expressed both in the cytoplasm and nucleus of the epithelial and stromal cells.

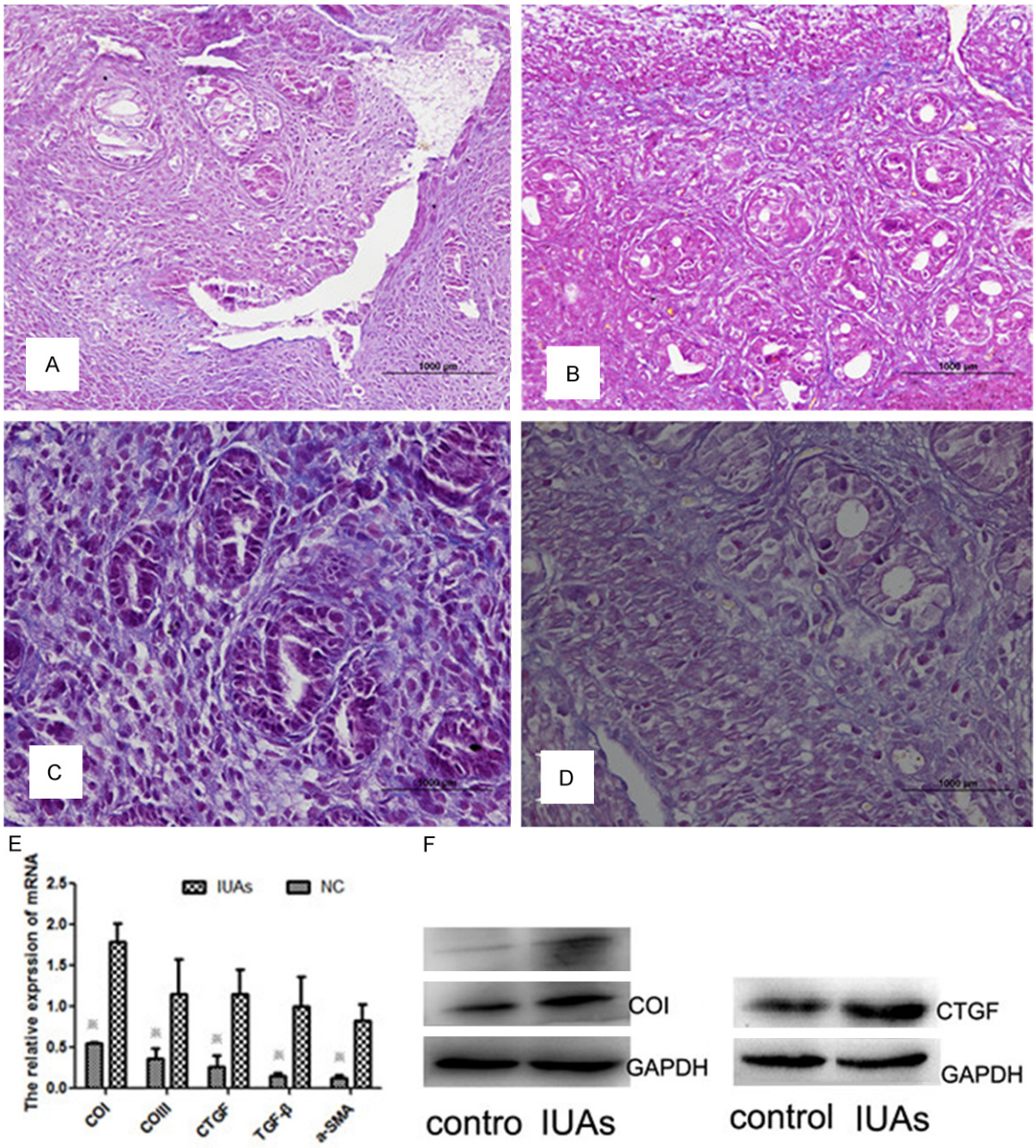

Figure 4. A, B. Masson stains for mouse endometrial tissues of control group (200 ×). C, D. Masson stains for mouse endometrial tissues with IUAs (200 ×). E. The relative expression of mRNA detected by qPCR. F. The protein expression levels of Collagen I and CTGF detected by Western blotting. *P < 0.05 (versus control endometrium); error bars, SEM.

CD146 was mainly expressed in the cytoplasm of the stromal vascular endothelial cells (**Figures 2** and **3**). CD140b was strongly expressed in the endometrium of the IUAs patients. However, it was moderate expressed in the control group (**Figure 2**). CD146 was strongly expressed in the endometrium of the IUAS patients; it was weakly expressed in the control group. CO I and CO III showed very strong expression in the endometrium of the IUAS patients; they were weakly expressed in the control group. TGF-β and CTGF showed strong expression in the endometrium of the IUAS patients; however, they were weakly or could not be detected in the control group (**Figure 2**).

At the same time, we found that the expression pattern of fibrosis and endometrial stem cell marker in mouse model of IUAs was in consistence with human IUAS. CD146, CD140b, TGF-β, CTGF, Collagen III and Collagen I all showed strong expression in the mouse endometrium of the IUAS; however, they were weakly or could not be detected in the control group (**Figure 3**).

Results of real-time PCR

Collagen I mRNA expression was significantly increased in the mouse endometrium of the IUAs group compared to that in the control group ($P < 0.05$). The Collagen III mRNA expression was increased about 2-fold in the mouse endometrium of the IUAs group compared to that in the control group ($P < 0.05$). The CTGF mRNA expression was increased about 3-fold in the mouse endometrium of the IUAs group compared to that in the control group ($P < 0.05$). The TGF-β mRNA expression was increased about 5-fold in the mouse endometrium of the IUAs group compared to that in the control group ($P < 0.05$). The a-SMA mRNA expression was increased about 4-fold in the mouse endometrium of the IUAs group compared to that in the control group ($P < 0.05$).

The protein expression levels of Collagen I and CTGF detected by Western blotting

Collagen I protein expression was higher in the mouse endometrium of the IUAs group compared to that in the control group ($P < 0.05$). CTGF protein expression was higher in the mouse endometrium of the IUAs group compared to that in the control group ($P < 0.05$).

Discussion

Many studies have reported that endometrial stem cells contribute to endometrial repair physiologically; however, the deregulated proliferation and differentiation of these stem cells leads to endometrial diseases such as endometrial polyps, endometriosis and endometrial cancer [8, 18, 19]. The role the endometrial stem cell has not demonstrated in IUAs. CD146 and CD140b is endometrial stem marker of endometrial stem cell [7]. We detected the expression of CD146 and CD140b in the endometrial tissue of IUAs. We found that, the expression of CD146 and CD140b was increased in the endometrial tissue of IUAs compared with that of control group (**Figure 2**). We created a mouse model of IUAs in order to verify whether CD146 and CD140b expressed higher in IUAs. We found the expression of CD146 and CD140b was increased in endometrial tissue of IUAs than that of control group (**Figures 3** and **4**). It may suggest that endometrial stem cells increase in IUAs.

In order to investigate the role of fibrosis in IUAs, we detected fibrosis using Masson stains. Then we detected the marker fibrosis including a-SMA, TGF-β, CTGF, Collagen III and Collagen I. Transforming growth factor β (TGF-β) is a central mediator of fibrogenesis. We found the ratios of the area with endometrial fibrosis to total endometrial area were increased in IUAs compared to that of control group. The expression of TGF-β, CTGF, Collagen III and Collagen I was increased in endometrial tissues with IUAs than that of control group. Furthermore, we found that, the ratio of the area with endometrial fibrosis to total endometrial area was increased in mouse endometrial tissues with IUAs compared to control group. The expression of a-SMA, TGF-β, CTGF, Collagen III and Collagen I was increased in mouse endometrial tissues with IUAs than that of control group.

TGF-β is activated and upregulated in fibrotic diseases, modulating fibroblast phenotype and function, inducing myofibroblast transdifferentiation [20]. Endogenous TGF-β plays an important role in the pathogenesis of cardiac fibrotic and hypertrophic remodeling [21]. We suspected TGF-β involved in the remodeling and fibrosis of endometrial tissues after injured. CTGF is induced by TGF-β and is considered a downstream mediator of the effects of TGF-β on fibroblasts [15]. In fibrotic liver, connective tissue growth factor (CTGF) is constantly expressed in activated hepatic stellate cells (HSCs) and acts downstream of TGF-β to modulate extracellular matrix production [22]. In our study, the increased of fibrosis marker suggested that fibrosis play an important role in the pathogenesis of IUAs.

This is the first report to describe the role of the marker of endometrial stem cell and fibrosis in IUAs. Endometrial stem cells and microenvironment (niche) are responsible for regenerating endometrium [23-25]. We hypothesized that the increased expression of fibrosis, including a-SMA, TGF-β, CTGF, Collagen III and Collagen I

altered the endometrial stem cell differentiation niche. Endometrial stem cells were wrong induced to fibroblast, resulting in IUAs. However, the specific mechanism and pathway of fibrosis in IUAs need to be further studied.

Disclosure of conflict of interest

None.

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