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Effects of TGF-β1 on the expression of endometrial stromal cell-related protein and mRNA

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Abstract. – OBJECTIVE: To investigate the effects of transforming growth factor β1 (TGF-β1) on α-smooth muscle actin (α-SMA), insulin-like growth factor I (IGF-I), and type I collagen (Col I) expression in endometrial stromal cells as well as on fibronectin (FN) level.

PATIENTS AND METHODS: 56 patients with normal endometrial tissue obtained from surgery were selected from June 2018 to November 2019. Endometrial stromal cells were isolated from patients and then assigned to the control group and observation group (addition of TGF-β1) followed by the analysis of cellular activity by Thiazole blue staining; and α-SMA, IGF-I, Col I, and FN mRNA and protein levels by real-time fluorescent PCR and Western blot.

RESULTS: The cell proliferation rate at 12 h, 24 h, 36 h, and 72 h after culture in both groups was higher than 0 h ($p < 0.05$) with higher cell proliferation in the observation group than the control group ($p < 0.05$). Real-time fluorescence PCR results showed that the levels of α-SMA, IGF-I, Col I, and FN mRNA in endometrial stromal cells of the observation group after TGF-β1 intervention were higher than those in the control group ($p < 0.05$). Meanwhile, α-SMA, IGF-I, Col I, and FN protein level was also elevated in the observation group after TGF-β1 treatment ($p < 0.05$).

CONCLUSIONS: TGF-β1 can stimulate the proliferation of endometrial stromal cells, which may be related to regulate α-SMA, IGF-I, Col I, and FN expression.

Key Words:
Transforming growth factor β1, Endometrial stromal cells, α-smooth muscle actin, Insulin-like growth factor I, Type I collagen, Fibronectin.

Introduction

The endometrial layer refers to the layer that constitutes the inner wall of the mammalian uterus. It can respond to both estrogen and progesterone, and its level is changed with the sexual cycle[1]. Weckel et al[2] showed that the endometrium is divided into the functional and the basal layer while the endometrium is composed of the spongy layer and the dense layer. The basal layer is composed mainly of the endometrium of the myometrium. Peng et al[3] shows that endometrial fibrosis/intrauterine adhesions cause more cases; however, due to repeated abortion operations and continuous stimulation or unreasonable operation, most patients suffer from loss of uterine cavity, resulting in different adhesions on the inner wall of the uterine cavity. In clinical practice, menstruation reduction and loss are the main factors affecting the health and life of patients[4].

Transforming growth factor (TGF-β1) belongs to a newly discovered TGF-β1 superfamily that regulates cell growth and differentiation and can change the characteristics of fibroblasts[5]. TGF-β1 involves in body's inflammatory response and tissue repair, and it regulates cell growth and differentiation[6]. Di Donato et al[7] showed that the TGF-β1 has a stimulative effect. But for endometrial stromal cells, whether TGF-β1 affects α-smooth muscle actin (alpha SMA), insulin-like growth factor I (IGF-I), type I collagen (Col I), and fiber connection (FN) remains unclear[8]. This investigation selected normal endometrial tissue as the object to explore TGF-β1's effect on α-SMA, IGF-I, Col I, and FN protein and mRNA expression.

Patients and Methods

Cell Data

Fifty-six patients with normal endometrial tissue in the First Hospital of China Medical Uni-

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versity from June 2018 to November 2019 were selected as the subjects, aged from 35 to 61 years old, with an average age of 51.24 ± 4.36 years old. The course of the disease was 1-6 months, with an average of 3.15 ± 0.42 months. Inclusion criteria: (1) all patients met the diagnostic criteria for endometrial hyperplasia and were confirmed by pathological tissue examination[9]; (2) all patients planned to undergo surgical treatment and could tolerate it; (3) no hormone therapy was used in the past 6 months. Exclusion criteria: (1) patients with mental disorders, blood system diseases or autoimmune diseases; (2) combined with organic diseases, abnormal coagulation function or severe liver and kidney abnormalities; (3) patients with malignant tumors, receiving chemoradiotherapy or biological immunotherapy. This study was approved by the Ethic Committee of the First Hospital of China Medical University, and informed consent has been obtained from all participants.

Instruments and Equipment

DMEM/F12 medium (Gibco co., Grand Island, NY, USA); SP immunohistochemical staining kits (Fuzhou Maixin Biological Technology co., Ltd, Fuzhou, China); DAB kit (Fuzhou Maixin Biological Technology co., Ltd, Fuzhou, China); TGF-β1 (Beijing Baiao Lebock Technology co. Ltd, Beijing, China); α-SMA, IGF-I, Col I, and FN antibody (Shanghai Yuanmu Biotechnology co., Ltd, Shanghai, China); Cyclin-streptomycin (Gibco co., Grand Island, NY, USA); fluorescence quantifier (Bioneer, Daejeon, Korea); protein electrophoresis apparatus, membrane transfer tank, protein electrophoresis tank (Bio-Rad, Hercules, CA, USA); PCR instrument (Agilent Technology co., Ltd, Santa Clara, CA, USA); PH meter, type 920 (Thermo Fisher Scientific, Waltham, MA, USA).

Isolation and Culture of Endometrial Stromal Cells

Endometrial stromal tissue was collected under aseptic operation and was washed with PBS to remove excess fat and mucus, followed by being cut into paste as much as possible and subsequent transferring to a centrifuge tube. Trypsin concentration of 0.125% was added to digest cells for 5-10 minutes and centrifuged for 3 min at 1000 rpm to remove supernatant, and 0.8 mg/mL type I collagenase was then added to the tissue precipitation (the control volume ratio was 1:5) for 90 min digestion. The cell suspension was prepared by repeated blowing and left standing. Cells were filtered by 200 and 400 mesh screen and centrifuged for 8 min at 1000 rpm; then, the precipitate was used to obtain stromal cells and red blood cells. The resuspended cells were cultured in medium FBS-DMEM/F12 (fetal bovine serum- Dulbecco's Modified Eagle's Medium/F12) with a concentration of 10.0% and inoculated with trypsin at a concentration of 0.25% for passage and digestion. The third generation of cells was assigned to the control group and the observation group. Control group: the cells were cultured only with the medium. Observation group: medium and TGF-β1 at different concentrations were added to the cells. Both groups were cultured continuously for 24 h, and reserved after culture[5,10].

Cell Activity Test

Cell activity was detected by thiazole blue staining. After treatment, cell density was adjusted to 5×10^4 cells/mL and added into a 96-well plate with 200 mL for each well and cultured for 72 hours. 5 mg/mL thiazole blue was added to each well (20 mL/well) for 4 h followed by the addition of dimethyl sulfoxide (DMSO). After shaking gently for 10 min, the absorbance value was measured at the wavelength of 490 nm. Cell proliferation rate = (1-OD value of experimental group/control group) ×100.00%[11]1.

α-SMA, IGF-I, Col I, and FN mRNA Level

(1) Extraction of total RNA. After treatment, RZ lysate was added to cells, mixed and kept resting for 5 min at room temperature, followed by the addition of 200 mL of chloroform and vortex for 15 s. Centrifugation was performed for 10 min at 12000 rpm. The water phase was transferred to a new test tube by a pipettor, and anhydrous ethanol was added. After fully mixing and homogenizing, the obtained solution and precipitation were transferred to a CR3 adsorption column. 30 s centrifugation was performed at 1200 rpm at 4°C to remove waste liquid. 500 mL protein solution RD was then added to the CR3 adsorption column and centrifuged at 30 s at 1200 rpm to remove supernatant. The bleach solution RW500 mL was added to the CR3 adsorption column for 2 min standing followed by 30s centrifugation at 1200 rpm at 4°C. After that, idling centrifugation was performed for 2 minutes at 12000 rpm to remove the residual liquid in CR3. The CR3 adsorption column was transferred to a new centrifuge tube and centrifuged for 2 minutes after elution. The

Table I. Dosage of reverse transcription RNA samples.

Sample ID	Sample on sample (uL)	ddH_2O does (uL)
Control group	4.0	6.5
Observation group	2.5	8.0

final liquid was total RNA. (2) Reverse transcription. Nuclease-free centrifuge tubes were used for the reaction in an ice bath, and RNA was added according to the concentration of RNA samples (Table I)[12].

The reaction system was heated for 5 min at 70°C, and then, cooled on ice for 2 min. 4 mL 5×Buffer, RAasin 0.5 mL, and M-MLV 1uL were continuously added to the solution, and then, placed in the PCR solution after fully mixing, and the bath was completed at 25°C for 10 min, at 42°C for 50 min, and at 95°C for 5 min heating and termination of the reaction. The final product was placed on ice for freezing and preservation at -20°C[13]. (3) Detection method. The real-time fluorescence PCR method assessed the α-SMA, IGF-I, Col I, and FN mRNA expression, and primer sequences were shown in Table II. According to the requirements of this study, amplification reaction parameters were set: 95°C for 10 min; 40 cycles of 95°C 10 s; 60°C 20 s; 72°C 30 s. Finally, it was saved for 5 min at 4°C[3].

α-SMA, IGF-I, Col I, and FN Protein

For protein extraction we followed the following steps. (1) Two groups of treated cells were taken and put into a 6-well plate. After the growth density reached 80.0%, the medium was removed and washed with PBS for 3 times. (2) Radioimmunoprecipitation assay (RIPA) lysate 150-200 mL was added to cells. After repeated blowing, the lysate was transferred to EP tube 1.5 ml, and placed on ice for 30 min to ensure full cell lysis. If necessary, cells could have oscillated. (3) Centrifugation was performed for 15 min at 14000 rpm at 4°C. (4) A certain volume of protein solution was taken, and its concentration was measured on an ultraviolet spectrophotometer. The protein concentration was determined by the BCA method. (5) 5× sample loading buffer was added to protein, and denaturation was performed for 10 min at 99°C.

For protein sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis we followed the following steps. (1) Enough electrophoretic fluid was added into the gel, the comb was pulled out, and the concentration of protein samples of the same quality was added into the gel according to the concentration. (2) The electrophoresis clips were placed in the electrophoresis tank to complete the installation and debugging of the equipment. Electrophoresis was performed at a constant voltage of 80 V to ensure that all protein samples were electrophoretic to the gel's bottom, and electrophoresis was completed.

For protein transfer we followed the following steps. (1) Gel was removed and protein's molecular weight was determined. The corresponding position of the gel was cut and placed on the filter paper in the film clip. (2) The polyvinylidene difluoride (PVDF) membrane soaked in methanol was taken out, and the membrane was washed with transfer liquid routinely, and the membrane was covered on the corresponding gel. (3) After determining the position of PVDF film and gel,

Table II. Primer design sequence of real-time fluorescence PCR.

Genetic types	Primer design	Length
α-SMA	Forward: 5′-TGGTGGGCCGCAGAACATGTGC-3′ Reverse: 5′-GCGAGCACAGAATTAATACGAC-3′	25
IGF-I	Forward: 5′-CTTAGTTGCGTTACTTTCTTG-3′ Reverse: 5′-CTGTCACCTTTTCCAGTTT-3	20
Col I	Forward: 5'-TTGGAAGGATGGCATACAC-3' Reverse: 5'-CAATGAAGTTGACTGGACTC-3'	31
Fn	Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3' Reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3	30
β-actin	Forward: 5′-CGCTTCGGCAGCACATATACTA-3′ Reverse: 5′-CGCTTCACGAATTTGCGTGTCA-3	32

Table III. Comparison of cell activity between the two groups ($\bar{x} \pm s$).

Group	0h	12h	24h	36h	72h
Observation group	0.00 ± 0.00	0.16 ± 0.05	0.47 ± 0.14	0.64 ± 0.17	0.86 ± 0.19
Control group	0.00 ± 0.00	0.09 ± 0.03	0.32 ± 0.11	0.50 ± 0.14	0.71 ± 0.17
Between groups	F = 4.089	$p = 0.036$			
Between time points	F = 5.291	$p < 0.021$			
Between groups · be-tween time points	F = 6.131	$p = 0.035$			

the film clip was placed in the film slot followed by the addition of transfer liquid into the transfer groove for film transfer.

Finally, PVDF membrane was then placed in 5% skim milk (prepared by Tris-Buffered Saline and Tween – TBST) and sealed for 1.5 h. The PVDF membrane was placed on a shaker and shaken for 3 times.

We then did incubation with primary and secondary antibodies and development. The PVDF membrane was taken and incubated with primary and subsequent secondary antibody solution. TBST was used for 3 times of continuous cleaning followed by the addition of a luminescent liquid to the PVDF membrane after final treatment for membrane exposure and development.

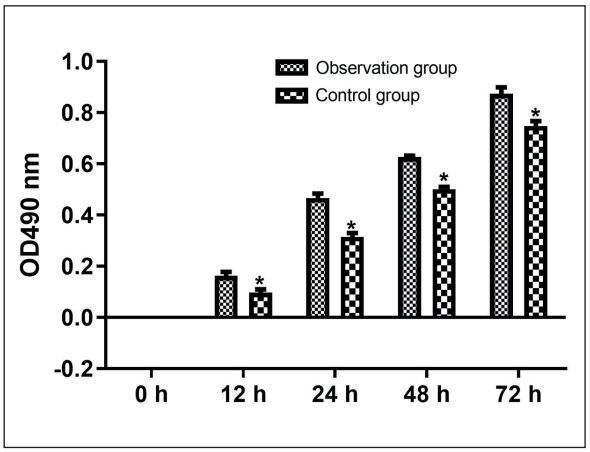

Figure 1. Comparison of cell activity between the two groups. Compared with observation group, *$p < 0.05$.

Statistical Analysis

SPSS 18.0 software (SPSS Inc. Released 2009, PASW Statistics for Windows, Chicago, IL, USA) was utilized for processing. The counting data was tested by the Chi-square test, represented by n (%), and the measurement data was tested by the Student's *t*-test, represented by (mean ± SD), and the difference was statistically significant at $p < 0.05$.

Results

Comparison of Cell Activity Between Two Groups

The proliferation rate of 0 h cells in two groups did not show differences ($p < 0.05$) but significantly increased at 12 h, 24 h, 36 h, and 72 h after culture ($p < 0.05$) with a higher proliferation rate in the observation group than the control group ($p < 0.05$) (Table III, Figure 1).

Comparison of α-SMA, IGF-I, Col I, and FN mRNA Level Between Two Groups

Real-time fluorescence PCR results show that the observation group of endometrial stromal cells after TGF-β1 intervention showed higher α-SMA, IGF-I, Col I, and FN mRNA levels than the control group ($p < 0.05$) (Table IV, Figure 2).

Comparison of Alpha SMA, IGF-I, Col I, and FN Protein Levels

Western blot showed that after TGF-β1 intervention, the observation group presented higher

Table IV. Comparison of α-SMA, IGF -I, Col I and FN mRNA level between the two groups ($\bar{x} \pm s$).

Group	α-SMA mRNA	IGF-I mRNA	Co1 I mRNA	FN mRNA
Observation group	0.71 ± 0.14	0.67 ± 0.11	0.69 ± 0.12	0.72 ± 0.15
Control group	0.32 ± 0.07	0.41 ± 0.09	0.37 ± 0.08	0.40 ± 0.08
t	6.392	7.431	5.464	6.224
p	0.000	0.000	0.000	0.000

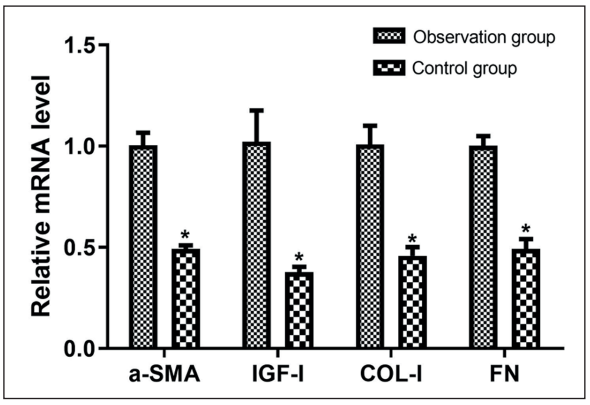

Figure 2. Two groups of α-SMA, IGF-I, Col I, and FN mRNA level. Compared with observation group, $*p < 0.05$.

α-SMA, IGF-I, Col I, and FN protein levels than the control group ($p < 0.05$) (Figure 3).

Discussion

TGF-β1 involves in inflammation, tissue repair, and embryonic development and promotes cell growth and differentiation. Kitson et al[14] has shown that TGF-β1 can directly participate in interstitial tissue fibrosis and promote cell division and proliferation, promote the transformation of epithelial cells into myofibroblasts, and help improve cell activity. In this study, the cell proliferation rate of the observation group was higher than the control group at 12 h, 24 h, 36 h, and 72 h after culture ($p < 0.05$), indicating that TGF-β1 could promote the activity of endometrial stromal cells. De Boer et al[15] have shown that endometrial tissue with normal structure and function is conducive to embryo breeding, and endometrial thickness, on the other hand, it can undergo periodic changes under the combined action of estrogen and progesterone, and extend into the lamina propria to form various small glands. Lamina propria is connective tissue containing blood vessels and stellate stromal cells. When the endometrial tissue function is abnormal, it will increase the incidence of infertility. However, TGF-β1 intervention could increase cell proliferation, induce abnormal uterine cell cycle, and promote the activity of the endometrial matrix.

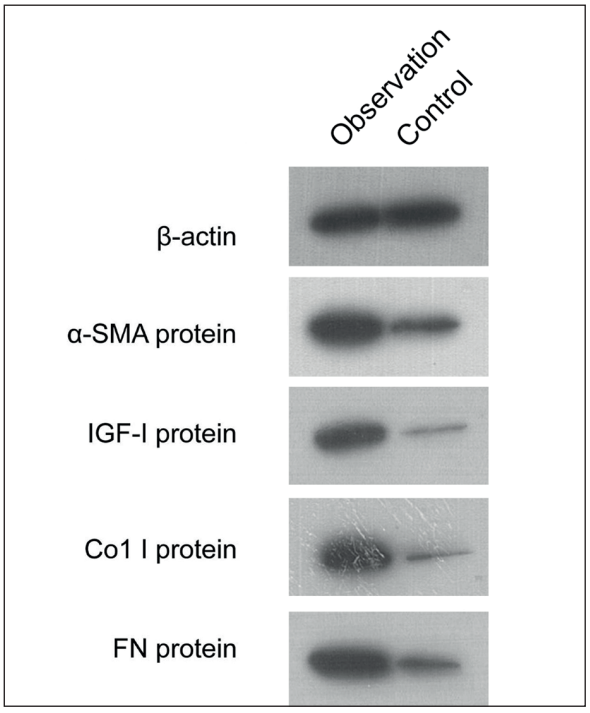

Figure 3. Comparison two groups of α-SMA, IGF-I, Col I, and FN protein.

α-SMA is a microfilament-forming globular multifunctional protein that is found in all eukaryotic cells and can be present at a concentration of over 100 mM. Kumar and Prabhu[16] have shown that the interaction of α-SMA structural protein is based on the adhesion and connection of cadherin, and actin filaments interact with α-SMA, which is an essential component of the cytoskeleton and can provide mechanical support for cell proliferation and growth. Signal transduction can also be facilitated by cytoplasmic transport pathways. IGF-I is a kind of growth-promoting polypeptide and is an important growth factor in childhood. Meanwhile, IGF-I secretion has an effect on the maintenance of muscle volume, strength, body composition, and nutrient metabolism[15]. Col I is a natural protein of the body with a strong affinity for skin surface proteins, relatively weak antigenicity, and good biocompatibility and biodegradation. At the same time, Col I also has good smoothness and elasticity with a hemostatic effect. FN protein has a variety of biological functions and is widely found in animal tissues and tissue fluids. FN is highly conserved in the evolutionary process with similar structure, properties, and biological functions in the body fluids of various animals. It regulates cell adhesion and migration and participates in embryonic development, differentiation, and growth[17]. In this research, the real-time fluorescent PCR results show that observation group after TGF-β1 intervention showed higher α-SMA, IGF-I, Col I, and FN mRNA levels than the control group

($p < 0.05$). The Western blot also found higher α-SMA, IGF-I, Col I, and FN protein levels in the observation group after TGF-β1 intervention ($p < 0.05$), indicating that TGF-β1 affects endometrial stromal cell activity possibly through regulation of α-SMA, IGF-I, Col I, and FN mRNA and protein levels, which is the novelty of our investigation. However, the exact molecular mechanism needs further investigation in the future.

Conclusions

From our investigation, we found that TGF-β1 can stimulate endometrial stromal cell proliferation, which may be related to regulate α-SMA, IGF-I, Col I, and FN mRNA and protein levels.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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