EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Suppressed Production of Transforming Growth Factor-β_2 mRNA in Endometrium of Women with Polycystic Ovary Syndrome

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Production of transforming growth factor-β_2 mRNA in the endometrium of women with polycystic ovary syndrome decreased compared to normal and this decrease directly depends on the duration of anovulatory period (from 3 weeks to 4 months). Low production of transforming growth factor-β_2 mRNA probably contributes to the development of endometrial hyperplasia in women with polycystic ovary syndrome.

Key Words: *transforming growth factor-β_2 mRNA; polycystic ovary; hyperplasia; endometrium*

Clinical manifestations of the polycystic ovary syndrome (PCOS) include pronounced biochemical changes, *e.g.*, hyperinsulinemia, luteinizing hormone hypersecretion, hyperandrogenemia, and acyclic estrogen production [1]. Morphological signs of PCOS include the formation of proliferation-type endometrium (structure typical of the proliferative phase, 87% women), asynchronous development of glands (36% women), and hyperplasia (50% women) [2]. Clinical observations indicate that long-term exposure of the endometrium to estrogens increases the risk of adenocarcinoma in women with PCOS [3].

Pathological changes in proliferative activity of the epithelium are related to impaired regulation of cell development and realized via cytokines, including growth factors. Polypeptide transforming growth factors (TGF) of the TGF-β family are important regulators of the cell cycle involved in cyclic endometrial changes. It was shown that TGF-β gene expression is high in the late proliferative and early and mid secretory phases of the menstrual cycle, but decreases during the late secretory phase and premenstrual period [4]. However, production of TGF-β 1, 2, and 3 and the corresponding mRNA in hyperplastic endometrium 5.1-, 3.4-, and 2.6-fold surpasses that in normal endometrial cells at the stage of proliferation, respectively [5]. TGF-β gene expression decreases with the progression of abnormal epithelial cell proliferation from simple to complex hyperplasia.

Here we studied TGB-β_2 mRNA production in the endometrium of women with PCOS and different duration of anovulatory period.

MATERIALS AND METHODS

We studied endometrial biopsy specimens obtained after uterine curettage (under hysteroscopic control) from women with PCOS 21-23 days (n=5) and 4 months (n=3) after the last menstruation-like reaction (MLR). All samples were characterized by pronounced

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signs of hyperplasia. Five endometrial samples were taken from fertile women.

Immediately after surgery, biopsy specimens were placed in sterile tubes and stored in liquid nitrogen, until RNA isolation.

RNA was isolated by routine methods using Tri reagent (Sigma).

Frozen endometrial tissues (100 mg) were homogenized in 1 ml solution in a RiboLyzer homogenizer (Hybaid) for 10 sec. The samples were incubated at room temperature for 5 min, mixed with 0.2 ml chloroform, thoroughly shaken for 15 sec, incubated at room temperature for 10 min, and centrifuged at 15,000*g* and 4°C for 10 min. The upper layer containing RNA was placed in a sterile tube, carefully mixed with 0.5 ml isopropyl alcohol, incubated at room temperature for 10 min, and centrifuged at 17,000*g* and 4°C for 10 min. The water-alcohol phase was removed. RNA precipitate was washed 2 times with 75% ethanol, dried, and dissolved in 200 µl deionized water treated with diethyl pyrocarbonate. RNA concentration was measured electrophoretically and chromatographically. RNA samples were subjected to the reverse transcription (RT) reaction immediately after isolation and measurements of RNA content.

RNA (insoluble precipitate) was stored in 75% ethanol at -20°C.

The procedure of DNA removal from this RNA solution was carried out in 30 µl reaction mixture containing 2 µg RNA, single buffer for reverse transcriptase MMuLV (50 mM Tris-HCl, pH 8.3; 50 mM KCl; 10 mM dithiothreitol; and 4 mM $MgCl_2$), 15 U DNase I without RNase activity (Promega), 2.5 U RNase inhibitor (Sintol), and 250 µM deoxynucleotidyl triphosphates at 37°C for 30 min. DNase I was inactivated by heating of the reaction mixture to 75°C for 5 min. Tubes with RNA solutions treated with DNase I were stored in ice until RT reaction.

Reverse transcriptase MMuLV (1.5 U, Sintol), 2.5 U RNase inhibitor, 0.1 µg oligo-dT primers (12-18 nucleotides, GibcoBRL), and 60 pmol hexarandom primers (Sintol) were added to tubes with 1 µg DNase I-treated RNA. RT reaction was performed at 37°C for 1.5 h. Negative control was incubated without reverse transcriptase.

Polymerase chain reaction (PCR) was performed using a PCR-express amplifier (Hybaid). The RT product (cDNA) was placed in tubes. The reaction was conducted in the presence of single buffer for PCR (6.6 mM Tris-HCl, pH 8.8; 16 mM $(NH_4)_2SO_4$; 0.25 mM $MgCl_2$; 1% gelatin; and 1% Tween 20), 250 µM deoxynucleotidyl triphosphates, 0.2 µM specific primers 5'-AGGCCAACCGCGAGAAGATGAC, 5'-TCGGCCGTGGTGGTGAAGC (β-actin), 5'-GCCTGTCCCAAGATTTAGAACC, and 5'-TCAAGTGAGGCGCGGGATAGG (TGF-β_2, Sintol), and 1 U Taq-polymerase activity over 40 cycles: 15 sec at 95°C, 20 sec at 62°C, and 15 sec at 72°C. PCR results were assayed spectrophotometrically and analyzed using a Gel Doc 1000 software (Bio-Rad). The samples were considered to contain mRNA, if electrophoresis in 2% agarose gel yielded a luminescent band of a known length absent in the negative control.

For quantitative analyses of TGF-β_2 mRNA production under normal and pathological conditions, cDNA was titrated by amplifying 5, 1, and 0.2 µl cDNA

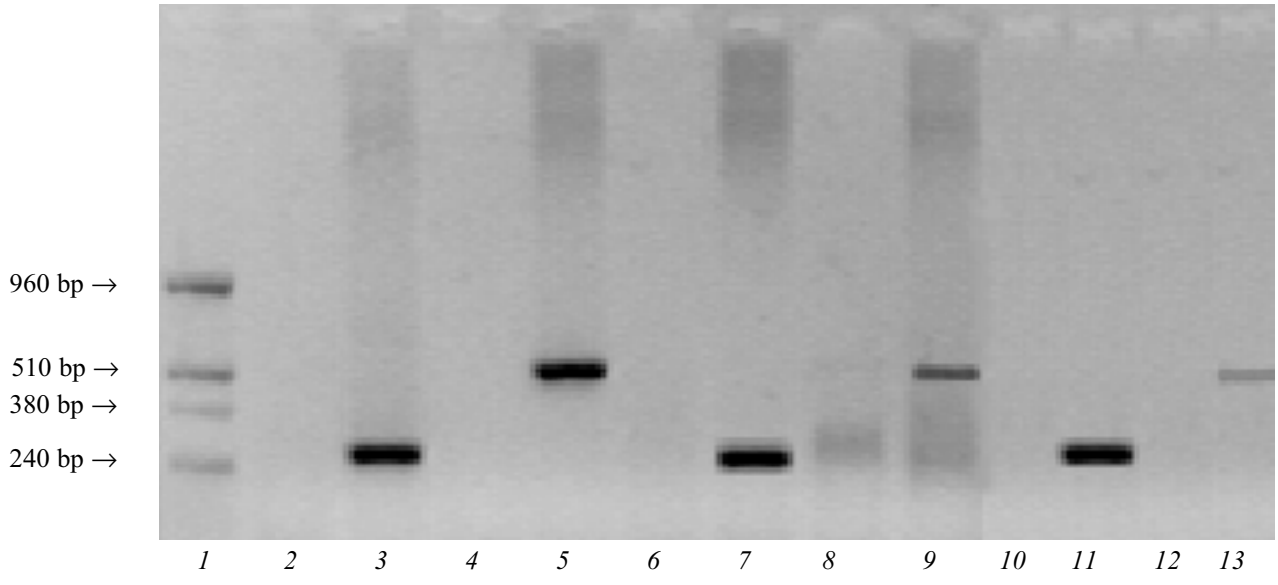

Fig. 1. Production of transforming growth factor-β_2 mRNA in normal endometrium (*2-5*) and in polycystic ovary syndrome after 3-week (*6-9*) and 4-month (*10-13)* anovulatory periods (electrophoregram of reverse transcription PCR product in 2% agarose gel): molecular weight marker (*1*), β-actin (*2*, *3*, *6*, *7*, *10*, *11*), and TGF-β_2 (*4*, *5*, *8*, *9*, *12*, *13*). Even numbers: negative control.

aliquots. Previous studied showed that 2-fold decrease in the content of RT-PCR product corresponds to a 10-fold inhibition of mRNA production. For standardization of our results, RT-PCR was performed using primers specific for constitutively expressed human β-actin gene.

Semiquantitative analyses of suppressed cytokine production were performed using a Molecular Analyst gel electrophoresis software (Bio-Rad). Intergroup differences were analyzed by nonparametric Wilcoxon test. The differences were significant at $p<0.05$.

RESULTS

In healthy women (control), production of TGF-β_2 mRNA was high and corresponded to β-actin gene expression. TGF-β_2 mRNA production tended to decrease during the mid-secretory phase (implantation period, day 21) compared to that in the mid-menstrual cycle (day 14), which was consistent with published data [4].

RT-PCR showed that TGF-β_2 mRNA production by immunocompetent cells of hyperplastic endometrium in women with PCOS decreased on days 21-23 after the last MLR compared to that in normal endometrium (1.6±0.1 and 3.5±0.2, respectively, $p<0.05$), which was probably related to impaired hormonal regulation of TGF-β_2 secretion. After a 4-month anovulatory period, TGF-β_2 mRNA production decreased to 1.0±0.1 arb. units ($p<0.05$ compared to normal and days 21-23 after the last MLR).

We revealed no causal relationship between suppressed cell growth, changes in TGF-β_2 production, and abnormal hormonal status in women with PCOS. Our findings suggest that decreased production of TGF-β_2 promotes pathological changes in proliferative activity of epithelial cells in the endometrium.

Thus, suppression of TGF-β_2 gene expression in the endometrium directly depends on the duration of anovulatory period in women with PCOS and probably contributes to the development of endometrial hyperplasia.

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