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Regulation of human endometrial transforming growth factor ß1 and ß3 isoforms through menstrual cycle and medroxyprogesterone acetate treatment

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Summary. The progesterone-induced differentiation of endometrial tissue from proliferative into secretory and decidua seems to be modulated by locally produced hormones and cytokines. Transforming growth factor beta (TGFß), a cytokine produced by endometrial cells, has been shown to modulate endometrial cell proliferation *in vitro*. Our aim was to evaluate the effects of medroxyprogesterone acetate (MPA) and the influence of menstrual cycle on the expression of TGFß1 and TGFß3 in human endometrium *in vivo*. In a double-blind, placebo-controlled trial, 46 healthy women with regular menstrual cycles received either MPA (10 mg/day) or placebo during 10 days. Endometrial and blood samples were collected 8-12 hours after the last MPA or placebo administration. Patients were classified into three groups according to biopsy dating and treatment: proliferative [tissue]/placebo, secretory [tissue]/placebo and secretory [tissue]/MPA. The immunohistochemical distribution of TGFß1 and TGFß1 mRNA was similar in all groups. Immunoreactive TGFß3 was present in the epithelium in 9.1% of proliferative samples, in 41.2% of secretory/placebo samples and in 87.5% of secretory/MPA samples (p=0.001). In the stroma, the frequency of TGFß3 staining was markedly increased after treatment with MPA (62.5%) compared to placebo (proliferative: 9.1%; secretory: 5.9%; p=0.005). The levels of TGFß3 mRNA increased during the secretory phase and were higher in the MPA-treated group, being directly correlated with morphological endometrial differentiation. It is concluded that MPA administration to healthy women increased TGFß3 but did not change TGFß1 gene and protein expression in the endometrium. This finding suggests that TGFß3 may be a local factor mediating progesterone- and progestogen-induced endometrial differentiation.

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Introduction

Transforming growth factor beta (TGFß) is a potent regulator of growth and differentiation in several tissues, including the reproductive system. Three major isoforms have been isolated in mammals: TGFß1 (Derynck et al., 1985); TGFß2 (De-Martin et al., 1987), and TGFß3 (Ten-Dijke et al., 1988). They were subsequently shown to bind to the same receptor (Giudice, 1994), although their gene expression is regulated by independent mechanisms. All three TGFß isoforms are produced and secreted in human endometrium, with particular temporal patterns seen in different endometrial layers and cell types (Chegini et al., 1994; Gold et al., 1994). The role of TGFß on endometrial differentiation during the menstrual cycle is still uncertain. The information that is currently available comes from *in vitro* experiments demonstrating that TGFß1 inhibits the proliferation of isolated epithelial cells of the endometrium (Tang et al., 1994) and enhances their susceptibility to Fos-mediated apoptosis (Tanaka et al., 1998), whereas low concentrations of TGFß1, TGFß2 and TGFß3 may stimulate mitosis in cultured stromal cells (Marshburn et al., 1994).

The expression of TGFß in human endometrium changes during the menstrual cycle, suggesting that endometrial TGFß production may be regulated by cyclic changes in estrogen and progesterone levels (Chegini et al., 1994; Gold et al., 1994). Cross-sectional studies have shown an increase in TGFß3 immunoreactivity and in TGFß1, TGFß2 and TGFß3 mRNA levels during the secretory phase of the menstrual cycle (Kauma et al., 1990; Chegini et al.,

1994; Gold et al., 1994; Marshburn et al., 1994). If TGFß were regulated by progesterone, this would be an important paracrine mechanism to control endometrial growth and differentiation. In the present study, we report the results of a double-blind, placebo-controlled clinical trial designed to evaluate the effects of a progestogen on TGFß gene expression in human endometrium.

Materials and methods

Subjects

Forty-six healthy women with regular menstrual periods, not taking any hormonal medication six months prior to the study were enrolled. Median age was 36 years and median cycle length was 29 days. They were stratified according to menstrual cycle into follicular or luteal phase and randomly assigned to receive either placebo or medroxyprogesterone acetate (MPA):1 10 mg tablet/day at bedtime, for 10 days, in a double-blind fashion.

Randomization was performed in blocks (Pocock and Simon, 1975): an MPA/placebo ratio of 2:1 was used in order to assure a sufficient number of patients with early, mid, and late secretory endometrium in the placebo group. Four patients received MPA during the follicular phase only to permit a double-blind allocation of treatments, avoiding selection bias. These patients were later excluded because we were specifically interested in the process by which MPA converts the proliferative endometrium into secretory endometrium. Five additional subjects were lost after enrollment because they did not return for biopsy. Thus, 37 patients were included in the study cohort.

In the morning after intake of the last tablet (placebo or MPA), patients underwent aspirative endometrial biopsy and blood collection. Patients studied during the follicular phase started medication (placebo or MPA) between day 25 of the previous cycle and day 5 of the present cycle, and endometrial biopsies were collected between days 5 and 15. Patients studied during the luteal phase started medication between days 13 and 18, with biopsies obtained between days 23 and 28.

The biopsies were dated according to Noyes et al. (1950) and classified as proliferative or secretory [tissue]. Three experimental groups were defined according to endometrial biopsy dating and treatment: proliferative [tissue]/placebo (n=11); secretory/placebo (n=18); and secretory/MPA (n=8). Proliferative endometrium was not observed among patients taking MPA. In some patients, selected at random, biopsy samples were immediately placed in liquid nitrogen for the RT-PCR studies. Thus, 7 RNA samples from each placebo group (proliferative and secretory; a total of 14 samples) and 6 RNA samples from the original secretory/MPA group were submitted to RT-PCR. As the endometrial biopsy samples used for RT-PCR were not microdissected it was not possible to locate TGFß mRNA at different tissue layers or cell types, therefore the results refer to the whole endometrium mRNA. The study protocol was approved by the local Ethics Committee and all patients gave their informed, written consent.

Serum hormone measurements

Serum estradiol and progesterone levels were assayed in duplicate by chemiluminescent enzyme immunoassay (DPC, Los Angeles, CA). The intra- and interassay coefficients of variation were 5% and 12%, respectively.

Immunohistochemistry

Formalin-fixed, paraffin-embedded endometrial samples were cut into 4 μm slices, which were stained by immunohistochemistry using the avidin-biotin-peroxidase method (Hsu et al., 1981), as previously described (Spritzer et al., 1996; Reis et al., 1999). After incubation with 0.1% trypsin to improve antigen retrieval, followed by exposure to 1% H_2O_2 in methanol to block endogenous peroxidase, sections were treated with normal goat serum for 30 min to suppress non-specific binding. Primary antibodies were applied for overnight incubation at 4 °C. Mouse purified immunoglobulin G (IgG) against human TGFß1 (Serotec Ltd, Oxford, UK) was diluted to 5 μg/ml in phosphate-buffered saline (PBS), whereas goat IgG against human TGFß3 (R&D Systems, Minneapolis MN) was diluted to 10 μg/ml in PBS. Sections were treated with biotinylated goat anti-mouse or anti-goat IgG (for TGFß1 and TGFß3, respectively) and incubated with the avidin-biotin-peroxidase complex (Vector, Burlingame, CA) for 60 min. Peroxidase activity was visualized by exposing the slices for 3 min to 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) in PBS containing 0.3% H_2O_2. The sections were counterstained with hematoxylin to enhance color contrast. Negative controls consisted of endometrial sections where the primary antibody was replaced by normal mouse or goat serum. A positive reaction was characterized by the presence of granular brown staining in the cytoplasm. The intensity of immunostaining at the epithelium and stroma was classified as negative, weak, moderate or intense, and converted by a semiquantitative scale into 0-3 arbitrary units (Gold et al., 1994).

Extraction of RNA and synthesis of cDNA

Endometrial samples stored at -70 °C were homogenized in phenol-guanidine isothiocyanate (Trizol, Gibco BRL, Gaithersburg, MD). Total RNA was extracted with chloroform and precipitated with isopropanol by 12,000 g centrifugation at 4 °C (Chomczynski and Sacchi, 1987). The RNA pellet was washed twice with 75% ethanol, resuspended in diethylpyrocarbonate-treated water and quantified by

light absorbance at 260 nm.

First strand cDNA was synthesized from 2 mg total RNA, using the SuperScript Preamplification System (Gibco BRL, Gaithersburg, MD). After denaturing the template RNA and primers at 70 °C for 10 min, reverse transcriptase was added in the presence of 20 mM Tris-HCl pH 8.4 + 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM dNTP mixture and 10 mM dithiothreitol, and was incubated at 42 °C for 55 min. The mixture was heated at 70 °C to stop the reaction and incubated with *E. coli* RNase for 20 min at 37 °C to destroy untranscribed RNA.

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was carried out in a final volume of 50 μl. Two microliters of the first strand synthesis reaction were denatured at 94 °C for 3 min in the presence of 20 mM Tris-HCl, pH 8.4, + 50 mM KCl and 1.5 mM $MgCl_2$. After this initial heating, 1.25 U of Taq DNA polymerase (Gibco BRL, Gaithersburg, MD) was added together with the same Tris-HCl buffer, 1.5 mM $MgCl_2$, 0.4 μM sense and antisense primers and 0.2 mM dNTP mix.

A 443 bp fragment of the TGFß1 cDNA and a 323 bp fragment of the TGFß3 cDNA were amplified, whereas a 623 bp cDNA fragment corresponding to the ubiquitously expressed protein $ß_2$-microglobulin was used to adjust PCR results to equivalent amounts of cDNA loaded. The localization of the primers in TGFß1, TGFß3, and $ß_2$-microglobulin cDNA sequences, as well as PCR conditions are listed in Table 1. The product of a first strand reaction performed without reverse transcriptase was also amplified to serve as negative control. A sample of the PCR mixture (15 μl) was size-fractioned on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light. The expected bands were quantified by densitometric analysis using an image-processing system (ImageMaster VDS, Pharmacia Biotech, Uppsala, Sweden).

Statistical analysis

Data are reported as medians and ranges. Differences between groups were assessed by the Kruskal-Wallis ANOVA followed by Dunn's test for non-parametric multiple comparisons. The proportions of TGFß-immunopositive samples in different groups were compared using chi-square (χ_2) tests, and correlation between endometrial differentiation and TGFß expression was assessed by calculating Spearman's rank correlation coefficients. A difference of $p < 0.05$ was considered statistically significant.

Results

The serum estradiol levels of all subjects were within the normal range (25-160 pg/ml, median = 72 pg/ml), and their progesterone levels were compatible with the respective endometrial dating (0.9-18.8 ng/ml, median = 7.3 ng/ml in the secretory/placebo group). There was full compliance with the prescription, and all endometrial samples of patients receiving MPA were characterized as mid or late secretory patterns.

Immunoreactive TGFß1 was localized in luminal and glandular epithelium but was more prominent in endometrial stroma (Fig. 1). The intensity of TGFß 1 immunostaining was not altered by the menstrual cycle or by MPA (Fig. 2). The levels of TGFß1 mRNA were also similar in the three experimental groups, as illustrated in Fig. 3.

On the other hand, TGFß3 immunostaining was more intense in the luminal and glandular epithelium than in the stroma (Fig. 4). In the epithelium, TGFß3 was present in 9.1% of proliferative samples, increasing to 41.2% in the secretory/placebo group and to 87.5% in the secretory/MPA group ($\chi^2 = 13.1$, d.f. = 2, $p = 0.001$).

Table 1. Sequence and localization of primers in the TGFß1, TGFß3 and $ß_2$-microglobulin cDNA sequences, expected sizes of the amplified fragments, and PCR conditions.

	TGFß1	TGFß3	$ß_2$-MICROGLOBULIN
5' Primer (sense)			
Sequence (5'-3')	CAAGCAGAGTACACACAGCA	GCTTCAATGTGTCCTCAGTGG	ATCCAGCGTACTCCAAAGATTCAG
Nucleotides	1213-1232	660-680	943-1356
3' Primer (antisense)			
Sequence (5'-3')	GATGCTGGGCCCTCTCCAGC	TTCCAGGATATCTCATTGGGC	AAATTGAAAGTTAACTTATGCACGC
Nucleotides	1636-1655	961-982	3826-3850
Size of fragment	443 bp	323 bp	623 bp
PCR conditions			
Denaturation	94 °C, 90"	94 °C, 90"	94 °C 1 min
Annealing	58 °C, 2 min	58 °C, 2 min	55 °C 1 min
Extension	72 °C, 3 min	72 °C, 3 min	72 °C 1 min
Cycles	35	35	30
Final Extension	72 °C, 9 min	72 °C, 9 min	72 °C, 5 min
cDNA sequence source	Derynck et al. (1985)	Ten-Dijke et al. (1988)	Gussow et al. (1987)

The frequency of samples immunostained to TGFß3 in the stroma was markedly increased by treatment with MPA (62.5%) compared to the placebo (proliferative, 9.1%; secretory, 5.9%; $\chi^2 = 10.6$, d.f. = 2, $p = 0.005$). As shown in Fig. 2, the intensity of TGFß3 staining was also significantly increased in the group of women

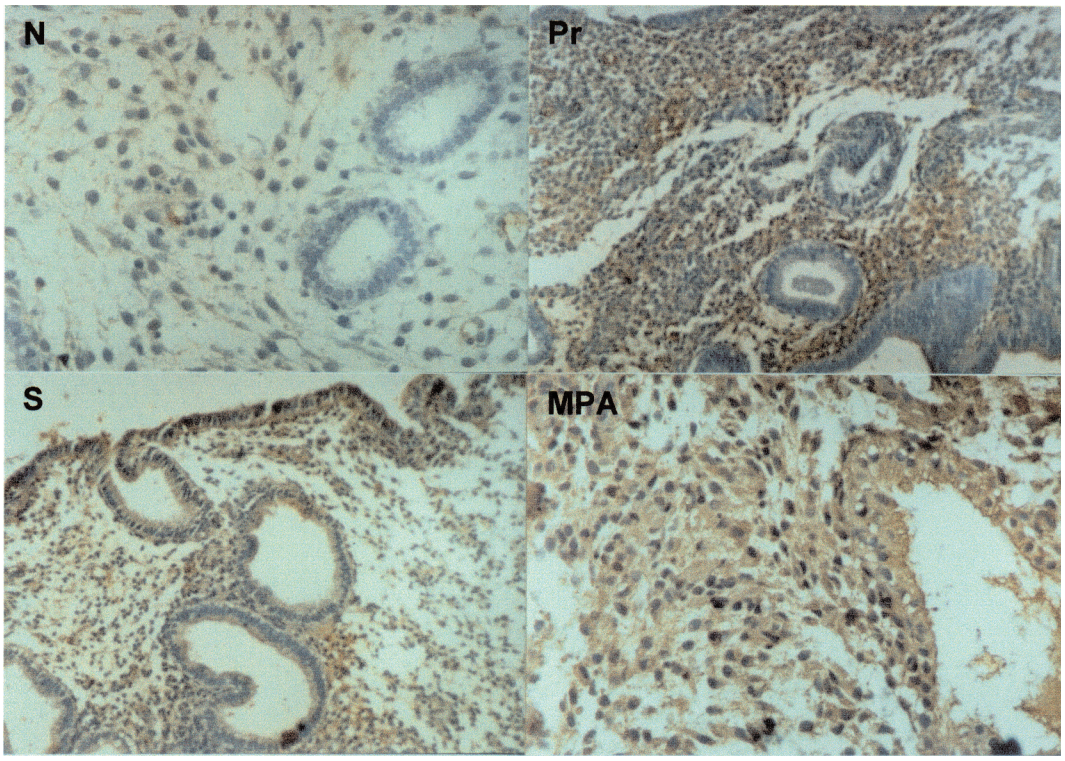

Fig. 1. Immunostaining of TGFß1 in human endometrium. Specific staining is indicated by the brown color and is confined to the cytoplasm. N. Negative control. Pr. Proliferative/placebo endometrium. S. Secretory/placebo endometrium. MPA. Secretory/MPA endometrium. Magnification: Pr and S, x 200; N and MPA, x 400

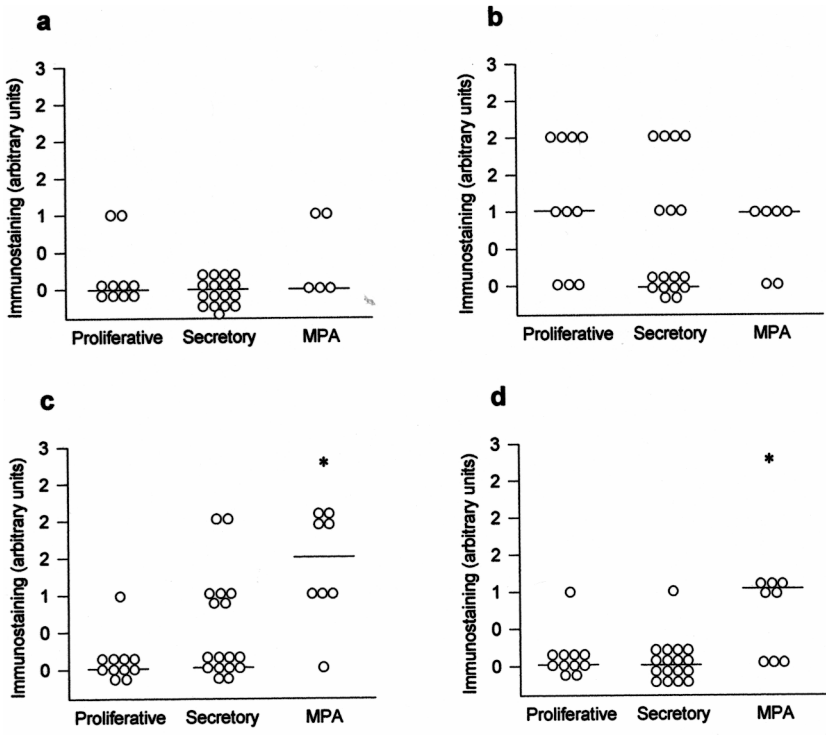

Fig. 2. Intensity of immunostaining for TGFß1 and TGFß3 on epithelial and stromal cells of human endometrium after administration of placebo in the proliferative and secretory phases of menstrual cycle or MPA in the secretory phase. **a.** Epithelium, TFGß1. **b.** Stroma, TGFß1. **c.** Epithelium, TGFß3. **d.** Stroma, TGFß3. Individual values are represented by circles and medians are indicated by horizontal bars. * $p<0.05$ vs. proliferative/placebo (Dunn's test).

receiving MPA, both in epithelial and stromal tissues ($p<0.05$, Dunn's test).

The levels of TGFß3 mRNA increased during the secretory phase and were higher in the MPA-treated group (Fig. 3). The expression of TGFß3 (mRNA and protein) was directly correlated with the grade of

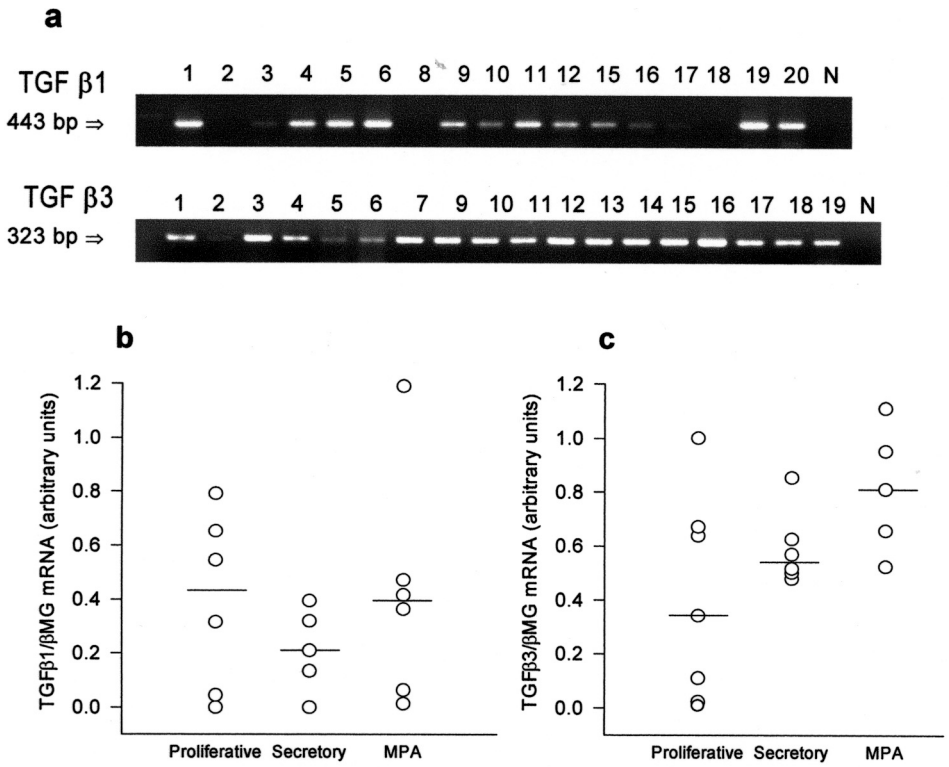

Fig. 3. RT-PCR assessment of TGFß1 and TGFß3 gene expression in human endometrium. **a)** PCR products visualized in agarose gel stained with ethidium bromide. Samples 1–7: prolife rative/placebo; samples 8–14, secretory / placebo: samples 15–20, secretory / MPA; N, negative controls; **b** and **c**. Densitometric analysis of TGFß1. **b** and TGFß3. **c** amplification bands, expressed in arbitrary units after correction by $ß_2$-microglobulin expression. Circles represent individual values. Medians are indicated by horizontal bars.

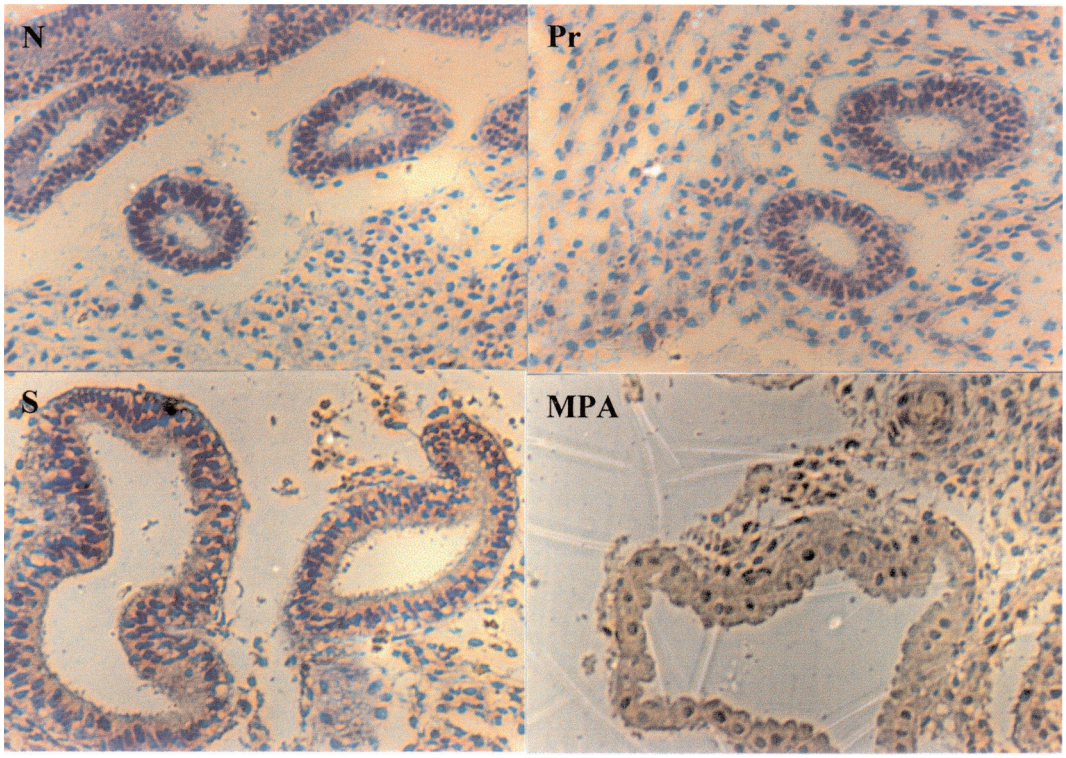

Fig. 4. Immunostaining of TGFß3 in human endometrium. Specific staining is indicated by the brown color and is confined to the cytoplasm. **N.** Negative control. **Pr.** Proliferative/placebo endometrium. **S.** Secretory/placebo endometrium. **MPA.** Secretory/MPA endometrium. x 400

endometrial differentiation, being minimal in early proliferative and maximal in mid and late secretory endometrial samples (Fig. 5).

Discussion

The present results demonstrate that TGFß1 and TGFß3 gene and protein expression are independently regulated in human endometrium. While TGFß1 expression was stronger in stroma than in epithelium and did not change either during the menstrual cycle or after progestogen administration, TGFß3 expression was higher in secretory than in proliferative endometrium and even higher following the use of MPA. In a cross-sectional study, Gold et al. (1994) also observed that TGFß3 (but not TGFß1 or TGFß2) immunostaining was more intense in secretory than in proliferative endometrial tissues. These observations support the concept that despite the local production of different TGFß isoforms, TGFß3 is specifically involved in the control of endometrial growth and differentiation during a normal menstrual cycle, whereas the role of TGFß1 seems to be related to anti-neoplastic mechanisms, because endometrial carcinomas express very low amounts of TGFß1 mRNA (Perlino et al., 1998). Although TGFß2 was not studied in the present work, other investigators have not observed any differences in TGFß2 expression in proliferative or secretory endometrium (Gold et al., 1994). On the other hand, preliminary data on mRNA levels suggest that TGFß2 might behave similarly to TGFß3, with increased gene expression in secretory/MPA endometrium (Reis, 1998). Further studies are still required to determine the specific role of TGFß2 in the regulation of the endometrium cycle.

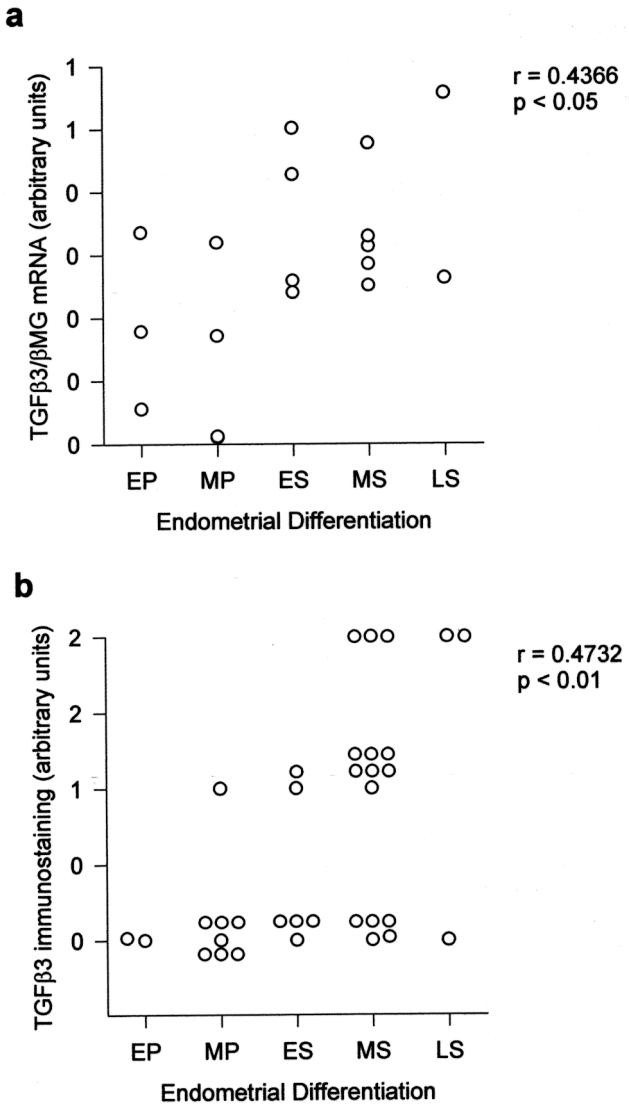

Fig. 5. Correlation between endometrial differentiation and TGFß3 gene expression. Endometrial biopsy dating was stratified in 5 sequential groups and correlated with mRNA levels **(a)** or with the intensity of immunostaining in the epithelium **(b)** by the Spearman's rank correlation coefficient (r). EP= early proliferative; MP= midproliferative; ES= early secretory; MS= midsecretory; LS= late secretory.

Our data show an increase in endometrial TGFß3 gene and protein expression after progestogen administration, compared with control subjects studied in both proliferative and secretory phases of the menstrual cycle. Among spontaneously cycling controls, TGFß3 expression was most pronounced during the intermediate secretory phase, when the endometrium showed maximal signs of progesterone stimulation. These findings suggest that the cyclic changes observed in endometrial TGFß3 expression might be determined by the post-ovulatory progesterone surge. In accordance with this hypothesis, an increment of TGFß3 mRNA hybridization signal has been observed at early and mid secretory phases (Chegini et al., 1994), which coincides with higher circulating progesterone levels. One recent study employing northern blot analysis suggested that TGFß3 mRNA levels were higher during the first phase of the menstrual cycle (Arici et al., 1996). However, this paradoxical observation was not confirmed by morphological or biochemical data to certify that the biopsies collected in the second half of the cycle were true secretory endometrium.

The molecular mechanisms underlying the progestogen-induced stimulation of TGFß3 gene expression in endometrial cells are still unknown. Nevertheless, at least two basic pathways can be hypothesized: the complex hormone-progesterone receptor may bind directly to the regulatory region of TGFß3 gene, or may release TGFß3 gene expression from the inhibitory effect of estrogen. In agreement with the former mechanism is the fact that TGFß mediates the progesterone suppression of an endometrial metalloproteinase *in vitro* (Bruner et al., 1995). The latter mechanism is supported by the fact that estradiol suppresses TGFß2 and TGFß3 production by cultured human breast cancer cell lines (Arrick et al., 1990).

The present study demonstrated that TGFß3 gene

and protein expression in human endometrium is increased by progestin administration *in vivo*. Furthermore, we concluded that the local expression of TGFß3 was parallel to the process of endometrial differentiation from proliferative into secretory. These findings indicate that TGFß3 is a paracrine factor secreted by the differentiating endometrium and may be an important local mediator of progestin-induced endometrial differentiation.

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