In Vivo Assessment of the Regulation of Transforming Growth Factor Alpha, Epidermal Growth Factor (EGF), and EGF Receptor in the Human Endometrium by Medroxyprogesterone Acetate

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Purpose: The present study evaluated the in vivo effect of medroxyprogesterone acetate (MPA) on the localization of immunoreactive transforming growth factor alpha (TGF α), epidermal growth factor (EGF), and their common receptor (EGF-R) in the human endometrium.

Methods: The study design was a randomized clinical trial enrolling 36 healthy women with regular menstrual cycles. The participants were randomly assigned into three groups: groups 1 ($n = 11$) and 2 ($n = 17$) received placebo and were submitted to endometrial biopsy during the proliferative and secretory phases of menstrual cycle, respectively; group 3 ($n = 8$) received MPA (10 mg/day) for 10 days followed by endometrial biopsy, which was performed during the secretory phase. Immunohistochemistry was used to localize TGF α , EGF, and EGF-R in the endometrial tissue.

Results: TGF α was present markedly in the luminal and glandular epithelia but also in the periglandular stroma, with a distribution pattern similar in the three experimental groups. EGF immunostaining was equally distributed in epithelial and stromal layers of the endometrium and remained unchanged in endometrial samples from women treated with MPA compared to placebo. EGF-R was expressed only in the epithelium. The intensity of EGF-R immunostaining was higher in secretory than in proliferative endometrium and was further increased by administration of MPA ($p < 0.05$, chi-square test).

Conclusion: The present results suggest that the progestogen-induced in vivo differentiation of secretory endometrium does not require dramatic changes in the expression of EGF or TGF α , whereas EGF-R may be up regulated.

KEY WORDS: Endometrium; growth factor; medroxyprogesterone acetate; menstrual cycle.

INTRODUCTION

Progesterone and synthetic compounds with progesterone-like effect initiate their action by binding to the intracellular progesterone receptor.

The molecular events triggered by the hormone-progesterone receptor complex leading to morphological and functional changes in the uterus are not fully understood. Progress in this area is hampered

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by the limited number of clinical trials assessing the mechanisms of hormone action *in vivo*. Although many potential mediators of progesterone signal have been identified by the observation of isolated cells and tissues, whether these mediators are active in the living organism can only be ascertained by *in vivo* experiments in different animal species, comprising the human.

Growth factors are thought to mediate the effects of steroid hormones on target tissues along the female reproductive tract. Transforming growth factor alpha (TGF α) and epidermal growth factor (EGF) have emerged from *in vitro* studies as potential mediators of estrogen and progesterone actions leading to the cyclic phenomena of growth, differentiation and shedding of human endometrium (1–3). TGF α and EGF are the natural ligands of EGF receptor (EGF-R), a membrane glycoprotein receptor with intrinsic ligand-dependent tyrosine kinase activity.

TGF α and EGF are produced by the human endometrium across the menstrual cycle and by the decidua henceforth, and both act via paracrine and autocrine mechanisms to regulate endometrial growth (1,2,4–6). Immunoreactive TGF α has been detected in the luminal surface epithelium of endometrial samples during all phases of menstrual cycle, but a diminished expression has been observed during the midsecretory phase (7). A possible cyclic variation in the expression of EGF and EGF-R in the endometrium is controversial, being observed (8–10) or not (4,11,12) in different studies.

In spite of increasing evidence that estradiol and progesterone interact with EGF and TGF α to stimulate cell proliferation in mouse (6,13) and human (1,14) uterine tissues, the effect of sex steroid hormones on endometrial expression of these growth factors has not been assessed by controlled clinical trials *in vivo*.

The present study was designed to investigate the effect of an orally active progestogen, medroxyprogesterone acetate (MPA) on the localization of TGF α , EGF, and EGF-R in human endometrium. The effect of MPA was compared with placebo administered during both proliferative and secretory phases of menstrual cycle.

METHODS

The study included 36 healthy female volunteers with median age of 36 years (22–45 years) and median cycle length of 29 days (21–37 days). None

had used hormonal medication in the last 6 months. The study protocol was approved by the local Ethics Committee and the participants provided written informed consent. They were randomly assigned to three experimental groups. Groups 1 ($n = 11$) and 2 ($n = 17$) received placebo tablets and were studied during the proliferative and secretory phases of menstrual cycle, respectively, while group 3 ($n = 8$) received MPA (10 mg/day, 10 days, orally) for 10 days and was studied during the secretory phase. In the morning after intake of the last tablet (placebo or MPA), patients underwent endometrial biopsy and blood sampling. Patients studied during the proliferative phase (group 1) started medication between day 25 of the previous cycle and day 5 of present cycle, and endometrial biopsies and blood samples were collected between days 5 and 15. Patients studied during the secretory phase (groups 2 and 3) started medication between days 13 and 18, with biopsies and blood samples obtained between days 23 and 28.

Endometrial biopsies were obtained by catheter aspiration as an outpatient procedure. Samples were thoroughly rinsed in sterile saline, fixed in 10% buffered formaldehyde, embedded in paraffin, and sectioned into 4 μ m sections that were mounted on slides coated with (3-Aminopropyl) triethoxysilane (Sigma Chemical Co., St. Louis, MO). All samples were dated according to the criteria of Noyes *et al.* (15).

Immunohistochemistry was performed using the avidin-biotin-peroxidase method, as previously described (16). In the immunohistochemistry for TGF α and EGF-R, antigen retrieval was enhanced by boiling the slides for 10 min in 0.01 M citrate buffer, pH 6.0, followed by incubation at room temperature and PBS washing. After exposure to 1% H₂O₂ in methanol to block endogenous peroxidase, sections were treated with normal goat serum for 30 min to suppress nonspecific binding. Rabbit anti-mouse EGF antiserum (Serotec Ltd, Oxford, UK) was diluted 1:600 and applied for overnight incubation at 4°C. For TGF α and EGF-R immunostaining, monoclonal antibodies purchased from Oncogene Research Products (Cambridge, MA) were applied at the concentration of 10 μ g/mL overnight at room temperature. The anti-TGF α is a mouse monoclonal IgG_{2a} that reacts with native and some denatured forms of human TGF α but has no cross-reactivity with EGF. The anti EGF-R is a monoclonal antibody generated by immunizing mice with partially purified EGF-R from human epidermal carcinoma cells.

Sections were treated with biotinylated goat anti-rabbit IgG (EGF) or anti-mouse IgG (TGF α and EGF-R) and incubated with the avidin-biotin-peroxidase complex (Vector, Burlingame, CA) for 60 min. Peroxidase reaction was developed by exposing the slices for 3 min to 1 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in PBS containing 0.3% H₂O₂. Sections were counterstained with hematoxylin to enhance color contrast. A positive reaction was characterized by the presence of granular brown staining in the cytoplasm.

All samples were run together, including negative controls in which the primary antibody was replaced by nonimmune rabbit (EGF) or mouse (TGF α and EGF-R) serum. The intensity of immunostaining at the epithelium and stroma was evaluated by two independent observers and classified as absent/weak, moderate or strong and analyzed by nonparametric statistics.

Serum progesterone concentrations were assayed in samples from the placebo groups (groups 1 and 2) by chemiluminescent enzyme immunoassay (DPC, Los Angeles, CA), in order to confirm the presence of menstrual cycle related differences in serum progesterone levels. All samples were run together and the intraassay coefficient of variation was 5%. The results were expressed as medians and interquartile ranges and statistical significance was assessed by the Mann-Whitney *U* test.

RESULTS

Immunoreactive TGF α was predominantly expressed in the epithelium (Fig. 1(a)) but it was also present in the stroma, and its distribution pattern was similar in the three experimental groups. The intensity of TGF α immunostaining did not change significantly across the menstrual cycle in the placebo groups, neither was modified by MPA treatment (Fig. 2).

EGF was present in all stages of endometrial proliferation and differentiation in women receiving placebo, with no significant change between the two phases of menstrual cycle. The regional analysis of EGF localization revealed equal distribution of EGF staining in the endometrial stroma and in luminal and glandular epithelial layers (Fig. 1(b)). The distribution and intensity of EGF immunostaining in the endometria of women taking MPA was similar to that observed in the placebo-treated groups (Fig. 2).

Fig. 1. Immunostaining of (a) TGF α , (b) EGF, and (c) EGF-R in representative endometrial samples from patients treated with MPA. The spatial distribution of each protein was similar in the remaining patient groups. Specific staining is indicated by the brown color in the cytoplasm. Magnification: 200 $\times$.

EGF-R immunostaining was mostly confined to the glandular epithelium, whereas some weakly positive cells were scattered through the stroma (Fig. 1(c)). The intensity of EGF-R immunostaining in the glandular epithelium increased significantly during the secretory phase and was further increased in endometrial specimens from women treated with MPA ($p < 0.05$, chi-square test, Fig. 2).

Serum progesterone levels were, as expected, significantly higher in the secretory phase group

Fig. 2. Frequency of each intensity of immunostaining in the three groups of endometrial biopsies: placebo/proliferative (P), placebo/secretory (S) and treated with medroxyprogesterone acetate (MPA). Immunostaining was classified as absent or weak (white bars), moderate (hatched bars), and strong (black bars). (a) TGFα, epithelium; (b) TGFα, stroma; (c) EGF, epithelium; (d) EGF, stroma; (e), EGF-R, epithelium; (f) EGF-R, stroma.

(group 2, median 9.2 ng/mL, interquartile range 5.9–14.2 ng/mL) than in the proliferative phase group (group 1, median 0.85 ng/mL, interquartile range 0.6–1.8 ng/mL, $p < 0.001$).

DISCUSSION

The present clinical trial investigated the effect of a progestogen, MPA, on TGFα, EGF, and EGF-R expression in human endometrium. This *in vivo* approach was chosen because the findings of previous

in vitro and observational studies were disagreeing about the possible effect of sex steroids, particularly progesterone and progestins, on the expression of endometrial EGF-R and its ligands.

Our findings suggest that TGFα expression is not regulated by progesterone in human endometrium *in vivo*. This observation comes from prospective, randomized administration of a very specific progesterone receptor agonist, MPA, to healthy voluntaries during the luteal phase of menstrual cycle, and does not support the findings of previous observational studies (7,17) showing that TGFα expression was lower in secretory than in proliferative endometrium. In the current study, the administration of an exogenous progestogen created the opportunity to observe the endometrial response to a homogeneous progesterone-like stimulation, which is difficult to obtain in spontaneous cycles due to interindividual variability of progesterone levels. Our results argue against a cyclic variation in TGFα expression in the human endometrium and suggest that this peptide is not regulated by progesterone stimulation during the luteal phase.

We observed that the tissue distribution and abundance of immunoreactive EGF in the endometrium was not affected by MPA administration. This finding suggests that modulating EGF expression is not a key step of progestogen action in controlling endometrial growth and differentiation. Since estradiol and EGF are synergic in stimulating endometrial cell proliferation *in vitro* (1), it may be argued that estradiol and progesterone modify EGF expression as part of their molecular control of endometrial growth and differentiation. However, our findings suggest that basal EGF expression in the endometrium *in vivo* does not require persistent, unopposed estrogen stimulus, because it persisted after endometrial decidualization in the presence of MPA. Although estradiol seems to stimulate EGF secretion by cultured endometrial cells (13,18) and progesterone may modulate this estrogenic effect in some *in vitro* models (1), our data indicate that *in vivo* EGF expression is too complex to be predicted simply by considering the changes in estrogen/progesterone balance during menstrual cycle.

In previous studies, the regulation of EGF-R by ovarian steroids has been inferred from indirect observation through changes over the menstrual cycle or extrapolated from *in vitro* experiments. While EGF-R affinity appears to be higher

during the late proliferative and late secretory phases of menstrual cycle (8,18), cultured endometrial cells stimulated with estrogen or progesterone increase their EGF-R content (8,19) and binding activity (8,20). In the present study, EGF-R immunostaining was stronger in the glandular epithelium of secretory endometrium, especially in the group treated with MPA, suggesting that stimulation of progesterone receptor may indeed play a role in the control of endometrial EGF-R expression. However, further studies should clarify whether MPA treatment has any effect on the functioning of the EGF-R system in the endometrium. This is important because MPA has many therapeutic uses including chemotherapy of metastatic endometrial cancer (21), and overexpression of EGF-R is probably an important feature of endometrial carcinomas (22).

Although the expression of EGF-R in the stroma is much weaker than in the endometrial glands, Chobotova *et al.* (23) recently demonstrated that heparin-binding EGF, which is another member of the EGF family, stimulates DNA synthesis in endometrial stroma and modulates stromal cell proliferation across the menstrual cycle, acting through EGF-R. In addition to the proliferation of endometrial glands and stroma, EGF, and EGF-R may also be involved in the control of endometrial angiogenesis during the menstrual cycle (10). Clinical evidence suggests that the expression of angiogenic factors in the endometrial vessels increases under strong progestin stimulation, as observed in levonorgestrel intrauterine system users (24), and this concept may be expanded to EGF-R as suggested by the present study. Thus, EGF-R is a candidate mediator of progestin action in more than one target within the differentiating endometrium.

The regulation of EGF-R and its ligands by ovarian steroid hormones in the human endometrium remains open to debate since observational (8,9) and in vitro (14,18,20) studies have produced conflicting results regarding the effects of estradiol and progesterone on EGF-R level. Here we show that administration of MPA increased EGF-R immunoreactivity in the endometrium, whereas TGF α and EGF did not change from proliferative to secretory phases of menstrual cycle and were not modified by treatment with MPA. We conclude that the progestogen-induced in vivo differentiation of secretory endometrium does not require dramatic changes in the expression of TGF α or EGF, whereas EGF-R may be up regulated.

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