Transforming Growth Factor Gene Expression in Human Endometrial Adenocarcinoma Cells: Regulation by Progestins

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ABSTRACT

In an attempt to understand the antiproliferative effects of progestins in endometrial cancer, we have examined the effects of the potent progestin, medroxyprogesterone acetate (MPA), on the cell proliferation and the expression of transforming growth factor (TGF) α and β genes in human endometrial adenocarcinoma cell lines. The two cell lines used were Ishikawa, var 1, and HEC-50. In addition, the effects of exogenous TGF-α and anti-epidermal growth factor (EGF) receptor monoclonal antibody on cell proliferation were determined. Incubation of both cell lines with MPA resulted in a time- and dose-dependent inhibition of cell proliferation. Half-maximal growth inhibition was observed at 0.6 nM. In Ishikawa cells, the relative abundance of TGF-α was significantly reduced by MPA. A significant decrease in TGF-α mRNA was apparent 6 h after exposure to MPA and a further decrease was seen 12-24 h after addition of the progestin. The concentration of TGF-α immunoreactivity in conditioned medium of MPA-treated cells was also significantly reduced compared to control cultures. MPA had no effect on TGF-α expression by HEC-50 cells. EGF mRNA was not detected by Northern blot analysis in either cell type. MPA had no significant effect on EGF receptor mRNA abundance but resulted in a small increase in EGF receptor number in Ishikawa cells. Anti-EGF receptor monoclonal antibody (0.6-5 nM) inhibited Ishikawa cell growth but had no effect on HEC-50 cell proliferation. Exogenous TGF-α stimulated proliferation of both cell lines, but Ishikawa cells were significantly more sensitive to exogenous TGF-α than HEC-50 cells. Furthermore, TGF-α could reverse the growth inhibitory effects of MPA on Ishikawa cells. A decrease in TGF-β mRNA abundance was also observed in MPA-treated Ishikawa and HEC-50 cells. This effect was of small magnitude, variable, and only observed after prolonged exposure to MPA. These observations are consistent with the hypothesis that the antiproliferative effects of progestins on Ishikawa cells are mediated by decreased expression and autocrine action of TGF-α. Since similar growth inhibition is also seen in the HEC-50 cells in which progestins have no effect on TGF-α expression, additional mechanisms are likely to be involved in the antiproliferative effects of progestins in human endometrial cancer.

INTRODUCTION

Progestins are useful adjunctive agents in the treatment of both endometrial and breast cancer. The antiproliferative effects of progestins in human breast cancer cells have been examined by several investigators (1-3); however, there is little data which addresses the mechanism of progestin-induced regression of endometrial adenocarcinoma. It has been proposed that an important component of normal growth and neoplastic transformation may involve the action of locally synthesized growth modulators acting in an autocrine fashion to influence cell proliferation (4). A number of autocrine modulators have been characterized and these include TGF-3 -α, TGF-β, and the insulin-like growth factors. With regard to mammary cancer, it has been demonstrated that several autocrine/paracrine growth factors in human breast cancer cells are regulated by steroid hormones (5-7), and it has been proposed that the growth-modulating effects of estrogens (8) and progestins (9) may be mediated, at least in part, by autocrine growth factors. Although progestins also inhibit proliferation of human endometrial cancer cells (10), the molecular mechanisms involved in this growth inhibition have not been addressed. It is clear, however, that this growth inhibition involves other mechanisms in addition to progestin-induced down-regulation of estrogen receptors (10).

Here we report the results of experiments designed to test the hypothesis that the antiproliferative effects of progestins on human endometrial adenocarcinoma cells are due to the modulation of expression and action of the TGFs, α and β.

MATERIALS AND METHODS

Materials. MPA, dexamethasone, 17β-estradiol, dihydrotestosterone, and phenol red-free Dulbecco's modified Eagle medium plus Ham's F-12 were purchased from Sigma Chemical Co. (St. Louis, MO). R5020 and [32P]dCTP were purchased from Dupont Canada (Lachine, Quebec, Canada). RU486 was a gift from Roussel Uclaf (Romainville, France). TGF-β was purchased from R & D Systems, Inc. (Minneapolis, MN). TGF-α and anti-EGF receptor monoclonal antibody were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Dulbecco's modified Eagle medium plus Ham's F-12 medium and all other cell culture medium ingredients were purchased from GibCO Ltd. (Burlington, Ontario, Canada). HEC-50 cells were obtained from Dr. Kuramoto (Kanagawa-ken, Japan) (11). The original Ishikawa cell line (12) was obtained from the same source. The cell line used in these experiments, designated as Ishikawa var 1, arose spontaneously during continuous culture in the laboratory of Dr. E. Gurpide and differs from the original cell line in terms of estrogen responsiveness (13). The cells were grown in basal medium, a mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, 1:1, 10 mM L-glutamine, 1% antibiotic-antimycotic solution supplemented with 5% FCS. Cells were harvested by scraping the cells off the monolayer with a rubber policeman. After the cells were centrifuged, the cell pellet was snap frozen and stored at −70°C until RNA isolation.

Cell Growth Experiments. In experiments in which cells were treated with various compounds, the cells were plated in basal medium; 2 days later, while the cells were still subconfluent, the medium was replaced with fresh medium containing 5% FCS. The steroid hormones were added directly from a 1000x stock solution in ethanol to achieve the concentrations indicated. For cell growth experiments, cells were plated at approximately 104 cells/35-mm dish. On day 2, the medium was replaced with fresh medium containing varying concentrations of the progestins.
drugs to be tested. Fresh medium was added every other day, and the cells were counted on the days indicated. In experiments in which the effects of TGF-α and anti-EGF receptor monoclonal antibody were examined, the basal medium was replaced with medium containing 3% charcoal-treated FBS (14). Cell counts were performed in duplicate or triplicate using an electronic cell counter. Each experiment was completely replicated on two or more occasions.

TGF-α Radioimmunoassay. Confluent cells were grown for 24 h in serum-free medium in the presence or absence of 100 nM MPA. The conditioned medium was dialyzed against deionized water and concentrated 200-fold. TGF-α was measured by radioimmunoassay using the methods and reagents supplied by Biomedical Technologies Inc. (Stoughton, MA).

EGF Receptor Number. Cells were grown to confluence in 24-well tissue culture plates and were then grown for 24 h in serum-free medium in the presence or absence of 100 nM MPA. For determination of EGF receptor concentration, 30 fmol of 125I-mouse EGF (specific activity, 160-180 μCi/μg; Biomedical Technologies) was added to each well in 0.5 ml in serum-free culture medium containing 0.1% bovine serum albumin. The incubation was continued for 2 h at 22°C in the presence of varying amounts of unlabeled mouse EGF. Receptor number and affinity were calculated from Scatchard plots.

RNA Extraction and Northern Blot Analysis. RNA was isolated by the guanidinium thiocyanate/cesium chloride method (15) and enriched for poly(A)+ RNA by one cycle of oligo(dT) cellulose chromatography (16). Poly(A)+ RNA, 10-15 μg, was denatured in 50% formamide and 2.2 M formaldehyde, size separated by electrophoresis on 1% (w/v) agarose gels containing 2.2 M formaldehyde, and then blotted onto nitrocellulose. Filters were baked for 2 h at 80°C under vacuum and then prehybridized in hybridization solution for at least 3 h. The filters were then hybridized with the human TGF-α cDNA probe as described previously (5). After the hybridization signal had decayed, the same filters were hybridized with a human TGF-β-1 cDNA probe (5). Hybridizations, usually for 24 h, were performed at 42°C in the presence of 50% (v/v) deionized formamide, 5× Denhardt’s solution (1× Denhardt’s = 0.02%, w/v, each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 5× SSPE (1× SSPE = 1.15 M NaCl, 0.01 M NaH2PO4, 1 mM EDTA), 250 μg/ml denatured salmon sperm DNA, and 0.1% SDS. At the end of the hybridization period, the blots were washed twice in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS for 15-30 min at room temperature, followed by one wash in 0.1× standard sodium citrate, 0.1% SDS for 15-30 min at 65°C. Filters were also hybridized with NB-29 (17), a cDNA which encodes a constitutively expressed heat shock-like protein. The signal obtained with this cDNA was used as a control for gel loading. Filters were exposed to Kodak XAR film at —70°C with an intensifying screen.

Statistical Analysis. An analysis of variance and Dunnett’s t test were used to determine the statistical significance of differences between control and treatment groups.

RESULTS

The effect of MPA on Ishikawa and HEC-50 cell growth is shown in Fig. 1. Significant growth inhibition was observed at concentrations as low as 1 nM. Maximal growth inhibition was observed at 10-100 nM MPA; however, both cell types proliferated, albeit at a reduced rate, in the presence of 100 nM MPA (Fig. 1, A and B). Ishikawa and HEC-50 cell lines were equally sensitive to the growth-inhibiting effects of MPA with half-maximal reduction in proliferation rate observed at 0.6 μM.

The effects of MPA on TGF-α and TGF-β mRNA abundance in Ishikawa and HEC-50 cells are shown in Fig. 2. Both cell lines expressed TGF-α and TGF-β. TGF-α mRNA was relatively more abundant in Ishikawa cells, whereas TGF-β mRNA was more abundant in HEC-50 cells. In Ishikawa cells,
MPA reduced both TGF-α and TGF-β1 mRNA (Fig. 2). The reduction in TGF-β1 mRNA was of small magnitude and was only consistently observed after prolonged exposures to high concentrations of MPA. Exposure to 100 nM MPA for 24 h reduced TGF-β1 mRNA levels to 78.3 ± 9.2% (mean ± SEM) of controls, P < 0.05 (Fig. 2). A further reduction in TGF-β1 mRNA abundance was observed after 5 days: 54 ± 1.5% of controls, P < 0.05 (data not shown). MPA had no effect on TGF-α mRNA in HEC-50 cells but reduced the abundance of TGF-β1 mRNA (Fig. 2).

The time course of MPA action on TGF-α mRNA abundance in Ishikawa cells is shown in Fig. 3A. A significant reduction in TGF-α expression was apparent 6 h after exposure to MPA. The effect of increasing concentrations of MPA on TGF-α expression in Ishikawa cells is shown in Fig. 3B. Concentrations as low as 0.1 nM MPA caused a significant reduction in TGF-α mRNA abundance. Higher concentrations of MPA were only marginally more potent. Although a slight reduction in TGF-β1 mRNA was observed with increasing concentrations of MPA, in these experiments this decline in TGF-β1 mRNA did not achieve statistical significance when compared to control cultures. The time course and dose response for the effect of MPA on TGF-α and TGF-β1 expression in HEC-50 cells are shown in Fig. 4. Consistent with results shown in Fig. 2, MPA had no significant effect on TGF-α mRNA abundance in HEC-50 cells. MPA reduced TGF-β1 expression, but this effect was only seen after prolonged exposure to MPA. When TGF-α protein was measured by radioimmunoassay in conditioned medium from MPA-treated Ishikawa cells, it was found to be significantly
Table 1 Effect of medroxyprogesterone acetate on TGF-α immunoreactivity in conditioned medium

<table>
<thead>
<tr>
<th>TGF-α concentration is expressed as ng/10⁵ cell/24 h.</th>
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<tr>
<td><strong>Cell line</strong></td>
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<td>Ishikawa cells</td>
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<td>HEC-50</td>
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* Mean ± SEM, N = 4.  
NS, not significant.

![Graph](image)

**DISCUSSION**

In this study we examined the effects of the potent progestin, MPA, on two endometrial cell lines. The Ishikawa cell line used in these experiments is a variant of the original cell line described by Nishida et al. (12). Unlike the parent cell line, estrogen-induced growth stimulation can no longer be demonstrated; however, in this variant line alkaline phosphatase activity remains estrogen responsive (13). HEC-50 cells do not lower than in control cultures (Table 1). In contrast, the TGF-α concentration in MPA-treated HEC-50 cells was not significantly different from control cultures.

Other progestins, such as progesterone and R5020, also reduce TGF-α mRNA in Ishikawa cells (Fig. 5). The antiprogestin, RU486, was able to reverse the MPA-induced decrease in TGF-α mRNA. The other steroid hormones which were tested, 17β-estradiol, dexamethasone, and dihydrotestosterone, had no significant effect on TGF-α mRNA.

The effects of exogenous TGF-α and anti-EGF receptor monoclonal antibody on the Ishikawa and HEC-50 cell growth are shown in Fig. 6. These experiments were performed in medium containing 3% charcoal-treated FCS to reduce the concentrations of serum-derived growth factors. Exogenous TGF-α resulted in significant stimulation of Ishikawa and HEC-50 cell growth. In Ishikawa cells, significant stimulation was seen at concentrations of TGF-α as low as 2 nM, whereas higher concentrations were required for significant growth stimulation in HEC-50 cells. The EGF-receptor number as determined by ligand binding was significantly higher in HEC-50 cells compared to Ishikawa cells. In the latter cell line, MPA treatment resulted in a small but nonsignificant (P = 0.06) increase in EGF receptor number (Table 2). MPA had no effect on EGF receptor mRNA in either cell line (data not shown). Anti-EGF receptor monoclonal antibody inhibited Ishikawa cell growth but had no significant effect on HEC-50 cell proliferation (Fig. 6).

In phenol red-free media supplemented with 3% charcoal-treated FCS, the doubling time of the Ishikawa cells was 39 ± 0.5 h. This was significantly prolonged compared to the doubling time of 30 ± 1.2 h in medium containing 5% FCS (Fig. 7). While the effect of MPA on Ishikawa cell growth was more marked in basal medium containing 5% FCS, MPA was still inhibitory in the presence of media containing the lower concentrations of serum. Exogenous TGF-α was able to stimulate Ishikawa cell proliferation in medium containing 3% charcoal-treated FCS and was able to partly reverse the MPA-induced inhibition of cell proliferation (Fig. 7). A qualitatively similar result was obtained when 5% serum was used instead of 3% charcoal-treated FCS (data not shown).
appear to be estrogen responsive (11).

Under various in vitro experimental conditions, progestins can both stimulate and inhibit proliferation of human breast and endometrial cancer cells (2, 9, 10). Multiple mechanisms appear to be involved in the progestin modulation of growth of these steroid hormone-responsive cells. In our experiments in which both basal medium containing 5% FCS and estrogen-depleted medium were used, MPA significantly inhibited growth of each of the cell lines. In Ishikawa cells, but not in HEC-50 cells, this inhibition was associated with a reduction in TGF-α expression. This effect was mediated via the progestosterone receptor since this phenomenon was seen only with progestins and the anti-progestin, RU486, inhibited the MPA-induced attenuation of TGF-α expression.

Under basal conditions, TGF-α, EGF, or some other similar ligand which interacts with the EGF receptor appear to be important in stimulating Ishikawa cell growth. Blockade of the EGF receptor with a monoclonal antibody inhibited cell growth. Since EGF mRNA was undetectable by Northern blot analysis, TGF-α is likely to be the functionally more important ligand for the EGF receptor in these cells. The ability of exogenous TGF-α to reverse the growth-inhibiting effects of MPA is consistent with the hypothesis that this effect of MPA could be mediated by the reduction in TGF-α expression. Thus, it is possible that, in the Ishikawa cells, MPA-induced growth inhibition may be due in part to a reduction in the autocrine stimulation by TGF-α. Although HEC-50 cells were just as sensitive to the antiproliferative effects of MPA as Ishikawa cells, this agent had no effect on TGF-α expression in HEC-50 cells. Thus, the antiproliferative effect of progestins in this cell line cannot be explained by an action on TGF-α expression. In both cell lines, MPA resulted in a small but significant reduction in TGF-β1 expression. Exogenous TGF-β1 has very different effects on growth of various human endometrial cell lines. Boyd and Kaufman (18) demonstrated that in a series of 8 endometrial carcinoma cells TGF-β1 inhibited growth in 5 cell lines, while a small stimulation of growth was seen in 3 cell lines. Interestingly, the cell lines in which TGF-β1 stimulated growth demonstrated the highest levels of TGF-β1 mRNA levels. Of the two cell lines examined, HEC-50 cell had the highest levels of TGF-β1 mRNA. While exogenous TGF-β1 inhibits proliferation of Ishikawa cells, HEC-50 cell proliferation is stimulated by TGF-β1. If TGF-β functions in an autocrine fashion in HEC-50 cells, then inhibition of TGF-β1 expression may be one possible mechanism by which progestins inhibit proliferation of HEC-50 cells.

The observations reported here suggest that multiple mechanisms may be involved in progestin-induced growth inhibition. Although it is tempting to explain the growth inhibitory effects of MPA in terms of the effects on the transforming growth factors, these responses may be secondary phenomena related to the growth inhibition itself. For example we have previously reported that progestins enhanced expression of TGF-α and reduced TGF-β1 mRNA levels in human breast cancer under conditions in which growth inhibition was observed (5, 9). These findings in breast cancer cells could be considered to be a compensatory response to counteract the growth inhibitory effects of MPA. A further note of caution is the observation that many cells produce a diverse variety of growth factor activity, and it may be naive to attempt to explain gross perturbations in growth rate in terms of a single class of growth factors.

REFERENCES


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