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GROWTH-PROMOTING EFFECTS OF PROGESTERONE IN A HUMAN ENDOMETRIAL CANCER CELL LINE (ISHIKAWA-VAR I)

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Summary—Significant growth responses to progesterone of human endometrial adenocarcinoma cells (Ishikawa-Var I) were observed under *in vitro* culture conditions. Progesterone affected both the rate of exponential proliferation and cell population densities after the exponential phase. In the presence of the hormone, the doubling time of exponentially proliferating cells was reduced from 44 to 35.6 h and cell densities were increased by as much as 2–3 times over those of controls during approx. 2 weeks in culture. The effects of progesterone on cell population growth were dose dependent. Estradiol (10^{-8} M) and testosterone (10^{-6} M) did not affect cell densities and the effects of dexamethasone (10^{-6} M) were small. In contrast, both progesterone and estradiol stimulated colony formation under anchorage-independent conditions in soft agar. These results suggest the possibility that growth of sensitive cell clones in endometrial tumors could be enhanced in some patients during adjuvant progestin therapy.

INTRODUCTION

Contradictory results have been published concerning the effects of progesterone in the growth of tumor cells derived from tissues that are targets to the hormone. Progestins have been reported to inhibit growth in several breast cancer cell lines by antagonizing the action of estrogens or by independent antimitotic effects in the absence of estrogens [1–5]. In contrast, growth-promoting properties of progestins have been demonstrated in experimental rodent mammary tumors, both *in vivo* and *in vitro* [6–8], and in two human breast cancer cell lines [9, 10]. Primary cultures of epithelial cells derived from human breast cancer tissues also exhibited significant growth responses to progesterone [11]. Effects of progestins on the regulation of cell proliferation have been comprehensively reviewed by Clarke and Sutherland [12].

Progestins are used to treat endometrial cancer patients, of whom more than 30% show objective remissions [13, 14]. As a result, considerable experimental effort has been dedicated to the identification of biological properties of endometrial cancer that may serve to predict responsiveness to progestin therapy [15–19]. However, the possibility that progestin treatment may adversely affect the course of tumor development in some patients, suggested by the observation on breast cancer cells cited above, has received little attention.

EXPERIMENTAL

Cells

The origin and maintenance of Ishikawa cells, derived from a well differentiated human endometrial adenocarcinoma [20], have previously been described in detail [21]. The experiments reported here were performed in a variant (Ishikawa-Var I) that spontaneously arose in our laboratories from the estrogen-responsive, progestin-unresponsive Ishikawa line, and in cells from another human endometrial cancer line, HEC-50, established by Kuramoto *et al.* [22] from a moderately differentiated adenocarcinoma. The cells were shown to be free of mycoplasm, as determined by the Gen-Probe Mycoplasma T.C. II Rapid Detection System (Gen-Probe, San Diego, CA).

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Maintenance of cells and experimental procedures

At least 1 week prior to use for experiments, the cells were placed in phenol red-free medium in the presence of fetal bovine serum that had been treated with activated charcoal to remove steroids (ctFBS), as reported previously [21].

Experiments were performed under serum-free conditions using Minimum Essential Medium containing Earle's salts (MEM; Gibco, Grand Island, NY) or basal medium (BM), a phenol red-free mixture of Dulbecco's Minimum Essential Medium (DMEM) and Ham's F-12 medium, 1:1, v/v (prepared by Flow Labs, McLean, VA), supplemented with 10 mM L-glutamine, 15 mM HEPES, and a 1% antibiotic–antimycotic solution (Gibco) to give a final concentration of 100 U/ml penicillin, 100 μg/ml streptocycin and 0.25 μg/ml Fungizone. In some experiments, ctFBS was added to the medium, as indicated. To determine effects of steroids on cell population growth, cells were harvested by 5–10 min exposure to 0.05% trypsin–0.02% EDTA and plated in 6 cm culture dishes (Falcon) in the presence of serum for 1 day. Compounds to be tested were then added (day 0) and cells were maintained in culture for various periods, as indicated. Media were changed every other day, unless otherwise noted.

Soft agar experiments were performed as described previously [23]. Briefly, agar (Difco Labs, Detroit, MI) solutions were prepared in BM containing the indicated hormones to give a final agar concentration of 0.6% for the bottom layer and 0.3% for the feeder layer. One and one-half milliliters of the 0.6% solution was placed into 6 cm plastic culture dishes and allowed to solidify at 4°C. Cells were suspended in cold growth medium to yield a final density of 50,000 cells/dish. The cell suspension was quickly mixed with culture medium containing 0.6% agar at 42°C, to achieve a final agar concentration of 0.3%. Under these conditions, the temperature never exceeded 37°C. The 0.3% agar cell suspension was quickly pipetted as an overlay into dishes containing the 0.6% agar bottom layers. Hormones were added in ethanol to give a final ethanol concentration of 0.2%. Only vehicle was added to control dishes.

Twice weekly, 1.5 ml of culture medium containing 0.3% agar and appropriate hormones was added to the dishes. Colonies $>30\,\mu$m in diameter were counted under a phase contrast microscope 18–21 days after plating. Colonies in 10 fields of each of 3 dishes were evaluated, representing a total area of 28.4 mm^2.

Statistical analyses

Significance of differences between means was analyzed by the Student's t-test. The significance of differences in the rates of proliferation during the exponential growth phase was tested using the computerized Statistical Analysis System to evaluate the difference in the slopes of the regression lines corresponding to treatment and control groups.

RESULTS

Addition of progesterone to serum-free BM resulted in marked growth stimulation in the Ishikawa variant. As shown in Fig. 1, cell numbers in cultures containing progesterone were consistently greater than those in control dishes, and significant differences in cell numbers were evident thereafter, until the end of the 13th day culture period. Estradiol neither increased cell population growth in this variant of Ishikawa cells, in agreement with previous results [24], nor enhanced the effect of progesterone.

Table 1 shows similar stimulatory effects of progesterone on cell numbers in cultures containing 1% ctFBS in BM.

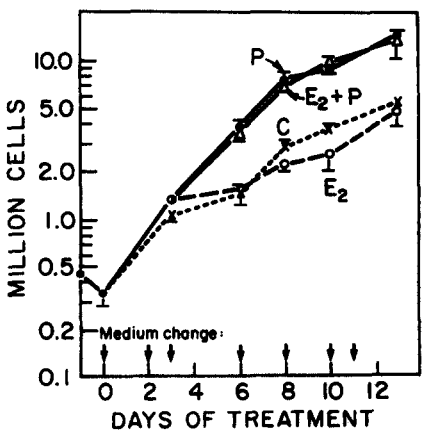

Fig. 1. Effects of progesterone and estradiol on Ishikawa-Var I cell numbers during a 2 week culture period. Cells (0.5 million/6 cm dish) were seeded in serum-free BM. On the following day (day 0), the medium was replaced with BM containing the indicated hormones dissolved in ethanol to obtain final concentrations in the medium of 10 nM estradiol (E_2) progesterone (P), 1 μM and 0.2% ethanol. Control cultures received ethanol only. Medium was renewed as shown. On the indicated days, cell numbers in 3 dishes for each experimental group was quantified by counting in a hemocytometer under an inverted microscope. The bars indicate standard deviations.

Table 1. Comparison of effects of progesterone (P) on population growth of cells cultured under serum-free conditions (BM) or in the presence ctFBS (BM + 1% ctFBS)

Days in culture under experimental conditions	Cell numbers (millions) + SD					
	BM			BM + 1%ctFBS		
	Control	+P	P vs C	Control	+P	P vs C
0	0.37 ± 0.04	—	—	0.37 ± 0.04	—	—
5	1.61 ± 0.19	2.47 ± 0.68	NS	3.83 ± 0.18	4.05 ± 0.49	NS
7	0.90 ± 0.21	2.77 ± 0.37	$P < 0.005$	4.96 ± 0.54	6.00 ± 0.25	$P < 0.05$
9	1.16 ± 0.20	4.91 ± 0.42	$P < 0.001$	4.18 ± 0.67	8.39 ± 1.67	$P < 0.02$

Figure 2 presents data illustrating differences in rates of cell proliferation during the period of exponential growth in serum-free BM in the absence or presence of progesterone. The differences in growth rates were significant at the $P < 0.05$ level. Calculations based on measurements of doubling time (44 h in controls vs 35.6 h in the presence of progesterone) and assumptions of constant length of the S, G_2 and M cell cycle phases (11, 1 and 3 h, respectively, as described in a previous report [25]), indicate a shortening of the G_1 phase from 29 to 20 h.

Results from 3 separate experiments shown in Fig. 3, indicate a concentration dependence in the effects of progesterone. Significant increases over control values were observed at 10^{-7} M but they were smaller than those obtained at 10^{-6} M; increases noted at 10^{-8} M were not statistically significant. Metabolic degradation of progesterone during the culture period may have resulted in lower actual concentrations of the hormone.

Table 2 provides data on effects of various steroids on Ishikawa-Var I cell population growth, as determined in a series of parallel experiments. Highly significant increases in cell numbers were observed in the presence of 10^{-7} or 10^{-6} M progesterone. By comparison, the responses to dexamethasone or testosterone were small, even at 10^{-6} M concentrations. As expected from this variant, no responses to estradiol were noted.

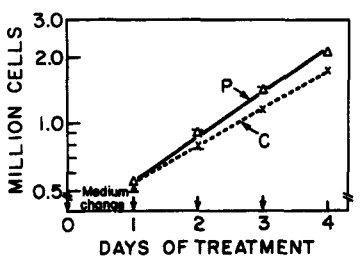

Fig. 2. Effects of progesterone (P) on proliferaton during the initial exponential growth period. The experimental conditions are given in the legend to Fig. 1. Progesterone was added to the culture medium to obtain 1 μM concentrations.

The growth-promoting effects of progesterone were not observed in cells of another human endometrial adenocarcinoma line, HEC-50, tested under culture conditions identical to those employed for Ishikawa-Var I cells, both in the presence or absence of 1% ctFBS.

Progesterone also enhanced colony formation in soft agar. As shown in Fig. 4, progesterone significantly increased the number of colonies in 4 of 5 experiments. Estradiol (10 nM) stimulated colony formation in all experiments and enhanced the effect of progesterone, notably in Exp. No. 1, likely by enhancing progesterone receptor levels, a known estrogenic action in other systems.

DISCUSSION

The present study shows a dose-dependent stimulatory effect of progesterone on population growth of human endometrial adenocarcinoma cells in culture. Growth promotion was apparent both in cells attached to the substratum of culture dishes and in cells forming colonies in soft agar under anchorage-independent conditions. The unexpected growth-promoting action of progesterone in Ishikawa-Var I cells contrasts with the antimitogenic properties observed in human endometrium *in vivo* and in several cell lines in culture, including the endometrial adenocarcinoma IK-90 line, also a variant of the original Ishikawa cells [26]. It is noteworthy, however, that Padykula *et al.* [27] have reported autoradiographically evident increases of thymidine incorporation in glandular epithelial cells adjacent to the myometrium of the Rhesus monkey both during the periods of estradiol and of progesterone dominance, suggesting a growth-promoting effect of endogenous progesterone.

The effect of progesterone on proliferation of endometrial cancer cells may be limited to particular cell clones since the hormone did not affect growth of the parent Ishikawa cell line [20] and did not alter the proliferation rates of

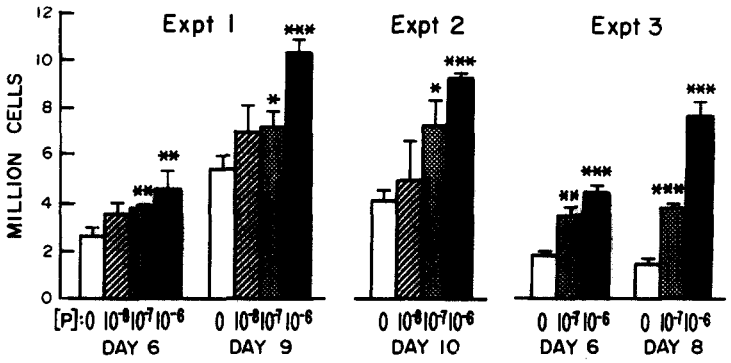

Fig. 3. Concentration dependence and increases in cell numbers in response to addition of progesterone to the medium. Cells (0.5 million/6 cm dish) were seeded in BM. On the following day (day 0), the medium was replaced with BM containing progesterone dissolved in ethanol to obtain the indicated final concentrations in the medium. Control cultures received only ethanol (0.2%). Medium was renewed every second day. On the indicated days, cell numbers in 3 dishes for each experimental group were counted in a hemocytometer under an inverted light microscope. The bars indicate standard deviations. Significance of differences from control dishes: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

the human endometrial cancer cell line HEC-50 (data not shown). The difference in responsiveness to progesterone between the parent cell line and Ishikawa-Var I cells may be the result of one of several mutagenic events that occurred *in vitro* in an originally homogeneous cell population; alternatively, the difference may reflect the expansion of one of several initially small subpopulations to form a cell line, a possibility that has been documented in a variety of tumors [28]. While growth promotion by progesterone may only occur under culture conditions, it is nevertheless possible that cell clones in some endometrial adenocarcinomas may, in rare instances, respond *in vivo* to progestins and be adversely affected by adjuvant progestin therapy. Synthetic progestins, such as medroxyprogesterone acetate (MPA), can be expected to behave as progesterone and even have a higher potency due to their metabolic stability, as clearly demonstrated in cells of another endometrial adenocarcinoma line [29]. Rapid metabolism may account for the lack of maximal response on cell proliferation observed at even 10^{-7} M concentration of progesterone, well above its dissociation constant for the progesterone receptor.

Two interesting properties related to hormonal responsiveness characterize the present Ishikawa-Var I line. First, the cells fail to respond to growth stimulation by estradiol under regular culture conditions in plastic dishes; in contrast, when growing under anchorage-independent conditions in soft agar, Ishikawa-VAR I cells clearly respond to estrogens, as apparent by comparison of Figs 1 and 4. A possible explanation, albeit speculative, would have to consider the existence of a small subpopulation capable at once to grow on soft agar and to

Table 2. Specificity of growth promoting effects of progesterone[a]

Addition to medium	Day after start of treatment	
	6	8
C (0.1% ethanol)	1.72 ± 0.23[b]	1.58 ± 0.38[b]
E_2 [10^{-8} M]	1.68 ± 0.17	1.72 ± 0.05
T [10^{-6} M]	2.11 ± 0.18	2.64 ± 0.44
DEX [10^{-6} M]	2.77 ± 0.16**,c	2.95 ± 0.21
P [10^{-7} M]	3.50 ± 0.41**	3.80 ± 0.16***
[10^{-6} M]	4.46 ± 0.45***	7.80 ± 0.69***

[a]The cells were grown in serum-free BM.
[b]Cell numbers in million: mean of 3 dishes ± SD.
[c]Significant differences from control values, determined by the Student t-test: $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$.

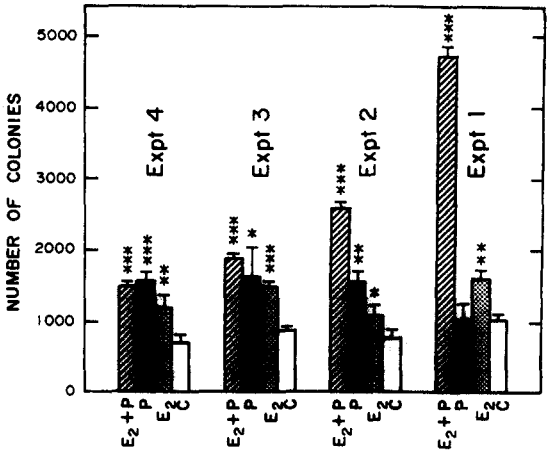

Fig. 4. Stimulation of colony formation by ovarian steroids. Cells (50,000) were plated in BM + 1% ctFBS containing 0.3% agar. Fresh medium (2 ml) was added twice a week. Colonies were counted under an inverted microscope 18–21 days after seeding. Concentrations of estradiol (E_2) and progesterone (P), alone or in combination, were 10 nM and 1 μM, respectively. Bars indicate mean and SD values from 3 dishes in parallel cultures. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

respond to estrogen. Alternatively, each of the two culture systems may provide unique conditions that inhibit or enhance hormonal growth regulation. For example, interactions between cells and the substratum are known to mediate growth responses [30], and different types of soft agar have been shown to mediate anchorage-independent growth regulation depending on the extent of sulfurylation of the agar [31].

The second noteworthy characteristic of the Ishikawa cell variant is the retention of responsiveness of alkaline phosphatase to estradiol, characteristic of the parent line. The retention of estrogen regulation of this enzyme suggests that the basic structural and functional properties of the estrogen receptor have remained intact and that the loss of growth responsiveness to estradiol is due to a specific alteration in growth regulation rather than to a deficiency in the activation of the receptor system.

To explore the possibility that the differences in responsiveness to progesterone between the Ishikawa-Var I and the HEC-50 cell lines were due to different progestin binding profiles, we determined binding properties by multiple point analysis of total receptors by the hydroxylapatite method, as reported previously [23]. Analysis of the data by the Fourier-derived affinity spectrum [32] revealed the presence of high and low affinity progesterone binding (J. Mechanick *et al.*, unpublished). From the absence of major differences in binding between the two cell lines it appears that the differences in growth responses to progesterone are not primarily related to the binding profiles measured under these conditions.

A relation between the actions of steroid hormones and growth factors is a topic of current interest. It has been shown that progesterone enhances proliferation of stromal cells from normal human endometrium when cultured in serum-free medium containing insulin and epidermal growth factor (EGF) [33]. Further *in vitro* experiments showed that EGF, basic fibroblastic growth factor or platelet-derived growth factor, which did not have by themselves a stimulatory effect on cell proliferation, increased the effects of progesterone on stromal cell population growth [34]. These findings indicate an interaction between steroids and growth factors. EGF is expressed by some human endometrium adenocarcinoma biopsy samples and cell lines [35, 36] and is able to stimulate proliferation of the HEC-50 human endometrial adenocarcinoma line [36]. In the human endometrium immunohistochemical localization of the EGF receptor suggests that it is expressed predominantly in epithelial cells [37].

In recent years, the importance of cell death regulation in overall growth has been emphasized [38]. Increases in cell numbers observed in the presence of hormones could therefore reflect hormonal stimulation of cell proliferation, hormonal inhibition of cell death, or a combination of both. However, cell death is likely to be minimal during conditions of exponential proliferation and the significant increases in growth rates by progesterone observed under those conditions can be expected to reflect true mitogenic effects.

One may speculate from the present results that while growth promotion by progesterone may only occur under culture conditions, it is nevertheless possible that some endometrial adenocarcinomas may, in rare instances, express *in vivo* a progestin-responsive phenotype and be adversely affected by adjuvant progestin therapy.

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