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HORMONAL CONTROL OF PROLIFERATION IN THE ISHIKAWA ENDOMETRIAL ADENOCARCINOMA CELL LINE

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Summary—Steroid hormone regulation of proliferation of the Ishikawa human endometrial adenocarcinoma cell line was investigated in defined tissue culture medium. Oestradiol increased cell number following treatment for greater than 8 days; 4-OH tamoxifen, used alone, induced growth in a similar manner to oestradiol and was not antagonistic when used in combination with oestradiol. Progesterone decreased cell number 4 days after treatment but thereafter the effect was lost; the effect of progesterone was abolished in the presence of Phenol Red, consistent with the oestrogenic properties of this indicator. Oestradiol together with progesterone for greater than 8 days resulted in maximal growth and was preceded by an apparent increase in synthesis of a protein of molecular weight 36 kDa pI 8.

INTRODUCTION

Endometrial cancer is one of the commonest malignancies of the female genital tract. Progestins are currently used in treatment either as an adjuvant to operable disease, or as part of the therapy of advanced disease. It is thought that progestin action arrests proliferation and induces differentiation [1], the normal end-point of which is cell death. The precise molecular details of these events are currently unknown, although a variety of biochemical end-points of progestin treatment have been described [2–4]. The lack of information concerning steroid regulation of endometrial cancer proliferation has been due, in part, to the absence of a suitable hormone responsive endometrial cell line. The recent availability of the Ishikawa cell line [5], containing both oestrogen and progesterone receptors, in which progesterone inhibits cell growth [6] provides a suitable model.

Impurities in preparations of Phenol Red have oestrogenic properties [7]; hence, cells sensitive to oestrogen when grown in conventional culture media remain subjected to stimulation despite removal of steroids from calf serum. The first aim of this investigation was therefore to establish basal growth conditions for Ishikawa cells in defined serum-free medium without Phenol Red in order to remove the background of oestrogenic stimulation. The effect of the addition of steroid hormones on Ishikawa cell proliferation was then investigated under these conditions as were changes in protein synthesis, by incorporation of ^{35}S-methionine and resolution by two-dimensional PAGE.

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EXPERIMENTAL

Cell culture and hormone treatment

Ishikawa cells were established as a permanent cell line by Nishida *et al.*[5], and were donated to us by Professor Erlio Gurpide, Mount Sinai School of Medicine, New York. In our laboratory, the cells were maintained in log phase growth in Dulbecco's Modified Eagles Medium (DMEM, Gibco) containing 10% Fetal Calf serum (FCS, Flow). The cells were not allowed to grow beyond confluence as this diminishes the hormonal response.

Subconfluent cultures of Ishikawa cells in DMEM–10% FCS were exposed to trypsin (0.05%)–EDTA (0.02%) solution (DIFCO, Michigan, Sigma) for 5–10 min at 37°C. The collected cells were seeded into 12-place multiwell plates (Falcon) at 40×10^3 cells/well in 1 ml of DMEM–10% FCS and left overnight. The medium was replaced with Dulbecco's Modified Eagles Medium F-12 (DMEM/F-12, Sigma) without Phenol Red supplemented with insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenium (6.25 ng/ml), bovine serum albumin (1.25 mg/ml) and linoleic acid (5.35 μg/ml) all from Sigma. These supplements are not an absolute requirement for Ishikawa cell growth, but maintain growth rates in defined conditions that are similar to those in media containing serum.

Oestradiol, Progesterone (both Sigma), medroxyprogesterone acetate (Upjohn) and 4-hydroxytamoxifen (ICI plc) were added in an ethanol vehicle (0.1% v/v) and vehicle alone was added to control wells.

Phenol Red (Northumbria Biologicals Ltd) was added to media to give a final concentration of 28 μM, which is equivalent to that in commercial tissue culture media.

Protein labelling and electrophoresis

^{35}S-methionine (NEN) was added to media to a final specific activity of 100 μCi/ml/well and incubated at 37°C for 6 h. Cells were then removed in 100 μl of lysis buffer (9.5 M urea, 2% NP-40, 1.6% ampholine pH 5–7, 0.4% ampholine pH 3–10 and 5% mercaptoethanol) and stored at −70°C until required. Incorporation was determined by TCA precipitation and 100,000 cpm used for electrophoresis. Two-dimensional SDS–PAGE was performed followed by fluorography as previously described [8].

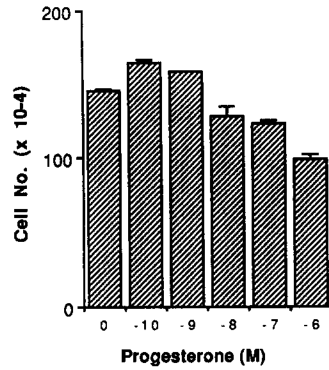

Fig. 2. Progesterone reduces proliferation of Ishikawa cells grown in DMEM/F12, ITS after 5 days of treatment in a dose-dependent manner. P10^{-8} M < C $P < 0.005$. P10^{-6} M < C $P < 0.001$. P10^{-6} M < 10^{-8} M $P < 0.005$. (Student's t-test.)

RESULTS

The potency of Phenol Red as a mitogen in Ishikawa cells is illustrated in Fig. 1, cell numbers were significantly greater than control at 8, 12 and 16 days following treatment. A significant difference between oestradiol-treated and control cultures was observed at 12 and 16 days post-treatment only. Eight days following treatment cell numbers in cultures containing Phenol Red were significantly greater than those treated with oestradiol. Thereafter, although mean values were higher, the difference between Phenol Red and oestradiol was not statistically significant.

In the absence of Phenol Red progesterone inhibited cell growth in a dose-dependent manner (Fig. 2). Progesterone and the synthetic progestin medroxy-progesterone acetate (MPA) consistently decreased cell number after 4 days of treatment in defined medium not containing Phenol Red (Fig. 3). Growth inhibition was also observed in medium supplemented with 10% charcoal stripped serum, however, the effect was abolished in the presence of Phenol Red.

The response of Ishikawa cells to the steroids and their combination was evaluated over a time-course of 17 days, as illustrated in Fig. 4. The reduction in cell number by progesterone is apparent at 4 days of treatment but thereafter the effect is lost. Tissue culture medium was replenished every second day, therefore it is unlikely that metabolism of progesterone accounts for loss of response. The period of preincubation in Phenol Red free medium influenced the inhibitory effect of progesterone (Table 1). Statistically significant effects of progesterone on cell growth after 4 days were observed when added without preincubation, or following 24 h preincubation in ITS medium. At longer preincubation periods the difference between control and progesterone treated culture was not statistically significant.

In the presence of oestradiol the inhibitory effect of progesterone on cell growth on day 4 was abolished (Fig. 4). The stimulatory effect on cell growth of

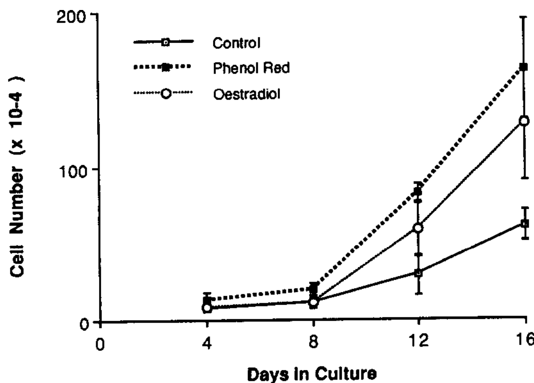

Fig. 1. Growth stimulation of oestradiol (10^{-8} M) and Phenol Red (2.8×10^{-5} M) in Ishikawa cells grown in DMEM/F12, ITS. Each point represents the mean ± SD of three cultures. D8: C < PhR $P < 0.025$, E < PhR $P < 0.025$. D12: C < PhR $P < 0.005$, C < E $P < 0.05$. D16: C < PhR $P < 0.005$, C < E $P < 0.025$. (Student's t-test.)

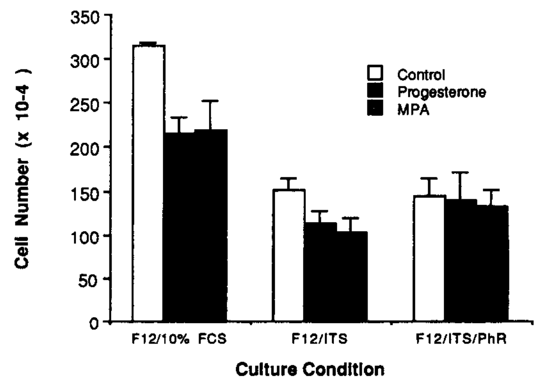

Fig. 3. Progesterone reduces proliferation of Ishikawa cells after 4 days in media containing 10% DCC–FCS or serum substitute ITS but not when Phenol Red (PhR) is present. F12/10%FCS: P < C $P < 0.0005$, MPA < C $P < 0.005$. F12/ITS: P < C $P < 0.025$, MPA < C $P < 0.01$. (Student's t-test.)

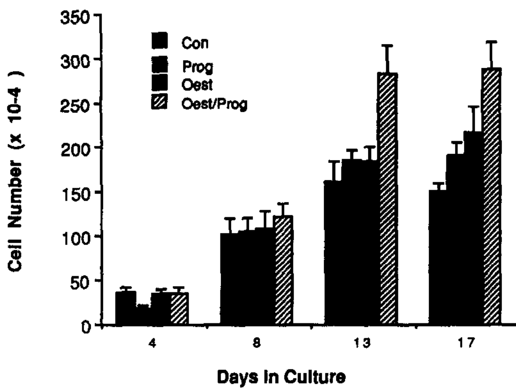

Fig. 4. Effect of progesterone (10^{-6} M) and oestradiol (10^{-8} M) on Ishikawa cells grown in DMEM/F12, ITS over 17 days. Media was changed every second day. Each bar represents the mean ± SD of three cultures. D4: P < C $P < 0.01$. D17: C < E $P < 0.025$, C < E + P $P < 0.005$, E < E + P $P < 0.025$. (Student's t-test.)

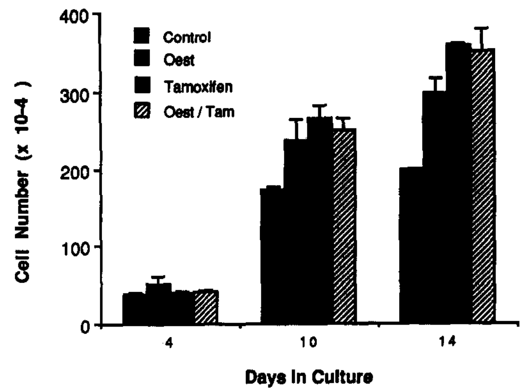

Fig. 5. Effect of tamoxifen (10^{-6} M) and oestradiol (10^{-8} M) on Ishikawa cells grown in DMEM/F12, ITS over 14 days. Media was changed every second day. Each bar represents the mean ± SD of three determinations. D10: C < E $P < 0.01$, C < T $P < 0.005$, C < E + T $P < 0.005$. D14: C < E $P < 0.005$, C < T $P < 0.005$, C < E + T $P < 0.005$. (Student's t-test.)

oestradiol at later time points in culture was increased in the copresence of progesterone (Fig. 4). The antioestrogen 4-OH tamoxifen increased cell number when used alone (Fig. 5), and in combination with oestradiol there was no evidence of an antioestrogenic effect.

The profile of labelled cellular proteins was evaluated after 4 days of steroid treatment, by 2-dimensional SDS–PAGE (Fig. 6), and was similar in each culture. However, in those cultures treated with oestrogen plus progesterone (E + P) where subsequently cell number increased most dramatically, the intensity of a protein of 36 kDa pI8 was increased. In progesterone-treated cultures the synthesis of this protein was apparently decreased (Fig. 6).

DISCUSSION

The oestrogenic properties of Phenol Red have been reported in the hormone sensitive breast cancer cell line, MCF-7 [9]. Our observations indicate a similar effect in an endometrial carcinoma cell line and suggest that the mitogenic properties of commercial preparations of Phenol Red may contribute to the failure to demonstrate antiproliferative effects of progesterone and synthetic progestins on Ishikawa cells [10].

Under Phenol Red free conditions progesterone treatment reduced cell number at 4 days of treatment,

Table 1. Effect of pre-incubation of Ishikawa cells in DMEM/F12, ITS before 4 days of progesterone (10^{-6} M) treatment

Preincubation (days)	Cell number ($\times 10^{-4}$) Control	Progesterone
0	13.3 ± 1.9	9.2 ± 1.7
1	13.5 ± 0.8	9.8 ± 1.0
2	22.5 ± 2.4	19.0 ± 1.8
3	26.0 ± 3.5	25.0 ± 3.5
4	40.0 ± 8.0	32.0 ± 11.0

Each value represents the mean ± SD of three cultures. Day 0: P < C $P < 0.025$, Day 1: P < C $P < 0.01$. (Student's t-test.)

but thereafter the effect was lost. This contrasts with observations made on a variant subline of Ishikawa (IK-90), where a reduction in cell number was apparent at 7 days of treatment with progesterone [11]. However, the IK-90 subline express progesterone receptor constitutively independent of oestrogenic stimulation. The progesterone receptor content of the cells used in the present study decrease in the absence of Phenol Red (Jamil *et al.*, manuscript in preparation). Such a decrease, together with progesterone induced down-regulation [3], may explain the loss of sensitivity to progesterone at later time points in culture in the absence of Phenol Red and oestrogenic stimulation.

The apparent synergism between oestradiol and progesterone in inducing cell growth at later time points in culture is consistent with similar effects on mitosis in mouse uterine epithelium [13], and in the stimulation of uterine concentrations of colony stimulating factor-1 (CSF-1) [14]. A role for CSF-1 in the regulation of placental growth has been suggested [14], a growth factor response that may be applicable to other sex-steroid responsive tissue.

In agreement with other reports oestradiol [12] and tamoxifen [18, 19] stimulate Ishikawa cell growth. Gottardis *et al.*[15], observed that tamoxifen stimulated growth of endometrial cancer cells (EnCa101) but inhibited growth of breast cancer cells (MCF-7) when innoculated in opposite flanks of the same nude mouse. These observations, and those reported here, would suggest the effects of tamoxifen on oestrogen sensitive cells to be cell-type specific. Katzenellenbogen *et al.*[16], have described however that MCF-7 cells grown *in vitro* in the absence of Phenol Red are stimulated weakly by low concentrations of tamoxifen, but tamoxifen inhibits the stimulation by oestradiol. Similarly Wakeling *et al.*[17], demonstrated the oestrogenic properties of 4-OH tamoxifen in MCF-7 cells, the antagonism of this response by a

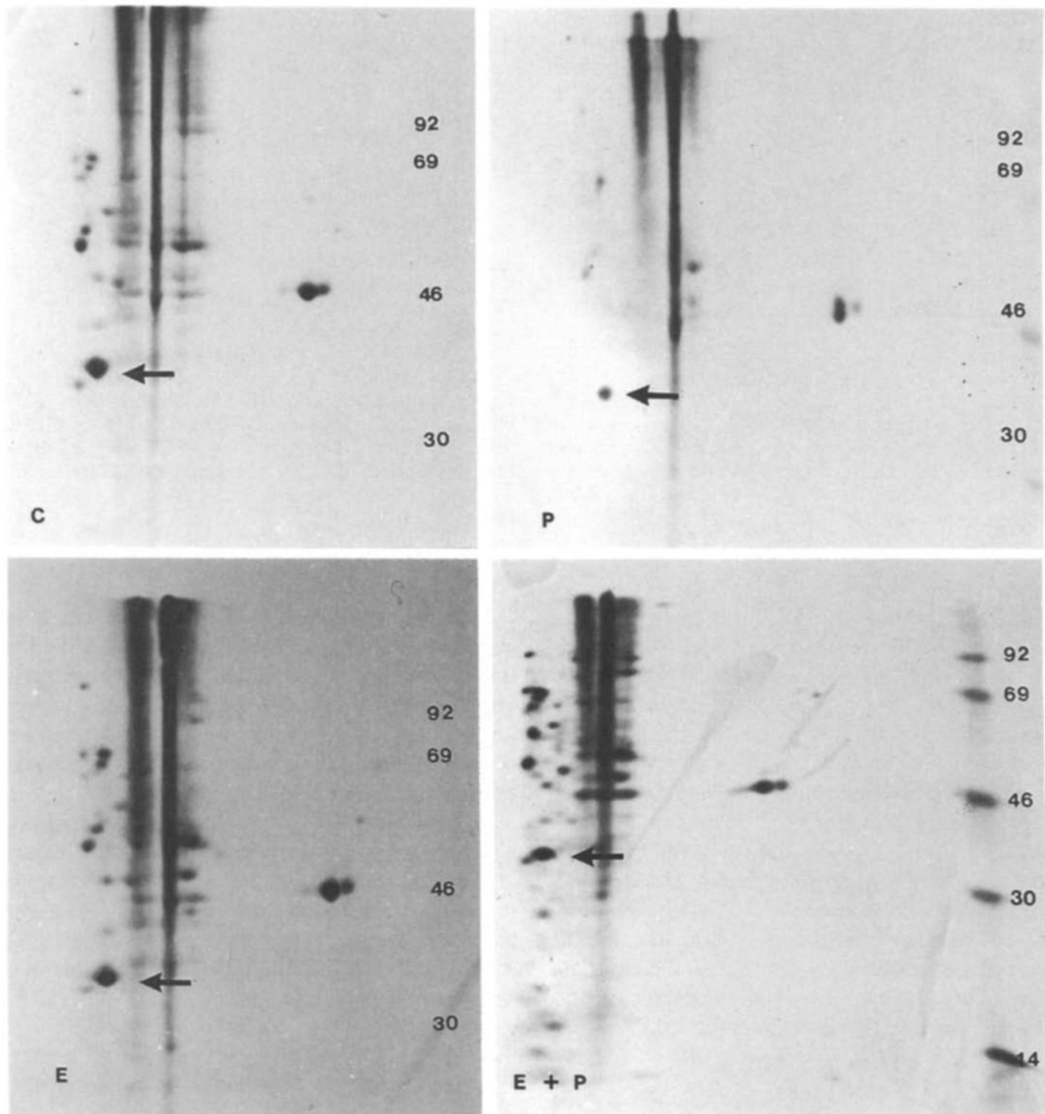

Fig. 6. Fluorographs of the incorporation of ^{35}S-methionine over 6 h into Ishikawa cells as resolved by 2D SDS PAGE. Cultures were treated for 4 days with (C) control, (E) 10^{-8} M oestradiol, (P) 10^{-6} M progesterone. Arrowed spot changes in intensity.

pure antioestrogen and the ability of antioestrogens to antagonize the marked response of breast cancer cells to oestradiol. In the experiments reported here tamoxifen failed to act as an antioestrogen at molar ratios relative to oestradiol that inhibit oestrogen induced growth of breast cancer cells. Thus the growth response of Ishikawa endometrial cells, that contain oestradiol and progesterone receptor [5], differ from that of breast cancer cells following treatment with tamoxifen.

Changes in protein synthesis associated with these steroid treatments have revealed a protein of 36 kDa, pI8 to be increased following oestrogen plus progesterone treatment and decreased following progesterone treatment. The synthesis of a protein with similar co-ordinates was also decreased in primary cultures of endometrial cancer following 6 h exposure to progesterone [2]. The synthesis of this protein therefore may be an index of the cellular response to progestin in endometrial cancer and its identity is currently being investigated.

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