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The Epidermal Growth Factor Receptor in the Human Endometrial Adenocarcinoma Cell Line HEC-1-B

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The binding characteristics and steroidal regulation of the EGF receptor were investigated in the human endometrial adenocarcinoma cell line HEC-1-B. The cell line was shown to possess a single, high affinity binding site for epidermal growth factor receptor (EGF) with a K_d of 3.09 ± 1.39 nM (mean ± SD, $n = 6$) and binding of 845 ± 311 fmol/mg protein (mean ± SD, $n = 6$). The protein kinase C activator, phorbol 12-myristate, 13-acetate (PMA) increased the K_d of the EGF receptor in a dose dependent manner (PMA: 0, 1, 10, 100 nM; K_d:4.1, 5, 10, 50 nM, respectively). The effect of PMA (10 nM) was overcome by preincubating the cells with the protein kinase C inhibitor staurosporine (1 μM) prior to the addition of PMA. The effect of the ovarian steroids oestradiol and progresterone on EGF receptor accumulation was studied by pretreating the cells for 6 days with oestradiol or progesterone in phenol red free DMEM:F12, 1:1 supplemented with 5% charcoal stripped fetal calf serum. Both steroids were shown to increase EGF receptor number with a maximum 5- and 7-fold increase in the presence of 1 nM oestradiol or 1 μM progresterone, respectively. The study demonstrates the presence of a high affinity binding site for EGF in HEC-1-B cells which is regulated by oestradiol and progresterone.

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INTRODUCTION

We have previously reported the presence of a single, high affinity EGF receptor in primary endometrial cell cultures of both glands and stromal cells [1] the synthesis of which is regulated by oestradiol and progresterone [2]. However, the use of primary endometrial cell cultures presents several problems, namely in the cyclical variation which exists throughout the menstrual cycle, the lack of tissue homogeneity, a finite life span and loss of steroid receptors. These restrictions are particularly evident when studying glandular epithelium rendering detailed examination of receptor characteristics and regulation difficult in this cell type. One way to overcome these disadvantages is to use an epithelial cell line.

The endometrial adenocarcinoma cell line HEC-1-B, established by Kuramoto *et al.*, in 1972 [3], has been investigated with respect to the action of several growth factors. Presta *et al.* [4] demonstrated that basic fibroblast growth factor (bFGF) increases the synthesis of plasminogen activators and increases cell proliferation. In the same study transforming growth factor-β was shown to have an inhibitory action on both basal and bFGF induced cell proliferation. HEC-1-B cells also produce insulin-like growth factor binding proteins and possess insulin-like growth factor receptors [5]. These studies suggest that the HEC-1-B cell line is suitable for growth factor studies.

The present study was undertaken to evaluate the HEC-1-B cell line as a model for the investigation of the EGF receptor and its regulation by oestradiol and progesterone.

EXPERIMENTAL

Materials

All reagents except culture media were, unless otherwise stated, obtained from the Sigma Chemical Co. Ltd, Poole, U.K. Culture Media, trypsin/EDTA, sodium bicarbonate, fetal calf serum and 'Linbro' multiwell plates were obtained from Flow Laboratories Ltd, Rickmansworth, Herts, WD3 1PQ, U.K.

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HEC-1-B cells

The HEC-1-B human endometrial adenocarcinoma cell line (ATCC NO HTB113), which was isolated in 1968 by Kuramoto *et al.* from a stage 1A endometrial cancer [3], was obtained from the American Type Culture Collection, Rockville, Maryland 20852, U.S.A. The cells were maintained in 75 cm^2 tissue culture flasks at 37°C in Minimum Essential Medium (Modified Eagle's) with Earle's salts (MEM), supplemented with 2 mM glutamine, 1 × non-essential amino acids, 0.075% sodium bicarbonate, 1 mM sodium pyruvate and 10% fetal calf serum, until confluent, after which they were removed by a brief (approx. 2 min) incubation with 3 ml trypsin/EDTA. The trypsin/EDTA was poured off, the cells dislodged with a sharp tap and resuspended in the required volume of MEM, supplemented as described above, but containing 5% fetal calf serum. The resulting cell suspension (dispersed with a pipette to avoid clumping of the cells) was plated into 24-well multiwell plates (20,000 cells per well) and incubated for 24 h prior to use. For experiments not involving steroids, the cell cultures were maintained in supplemented MEM containing 3% fetal calf serum until 70% confluent (usually 2–3 days), after which binding studies were performed. Where the effect of steroids was investigated, the cells were transferred, after the initial 24 h, to phenol red free Dulbecco's Modified Eagle's Medium (DMEM): F12 (1:1 by volume) supplemented with 5% charcoal stripped fetal calf serum. The cells were used between passages 137–140 (calculated from the establishment of the cell line).

Iodination of EGF

Epidermal growth factor (5 μg), obtained from Bachem (U.K.) Ltd, Saffron Walden, Essex, was prepared in 5 μl iodination buffer (0.5 M sodium acetate buffer pH 6.0) and reacted with 1 mCi ^{125}I (ICN Biomedicals Ltd, High Wycombe, Bucks., U.K.) and 10 μg lactoperoxidase (in 50 μl iodination buffer) for 6 min. The reaction was terminated with 100 μl saturated tyrosine and 100 μl PBS containing 2% bovine serum albumin (BSA). The reactants were mixed, transferred to a PD10, Sephadex G-10 column (Pharmacia Biosystems Ltd, Milton Keynes) previously equilibrated with 25 ml iodination buffer and 20 × 1 ml fractions collected. ^{125}Epidermal growth factor eluted in fractions 3–5 and the specific activity was 127.6 and 142.9 μCi/μg for the 2 preparations used.

EGF receptor binding studies

Prior to the measurement of EGF receptor binding, the cell cultures were washed 3 times with Hank's Balanced Salt Solution (HBSS) and then maintained for 24 h without serum in either MEM or, for the steroid studies, DMEM: F12, supplemented in each case with 10 mM glutamine, 10 mg/ml insulin, 100 ng/ml hydrocortisone, 10 mg/ml transferrin, and 25 ng/ml sodium selenite. The cells were then washed 3 times with 1 ml of ice cold phosphate buffered saline (PBS) pH 7.5.

Scatchard analysis of EGF receptor binding. Cell cultures were incubated at 37°C for 30 min in MEM containing 0.1% BSA and ^{125}I EGF at concentrations between 0.3 and 12.0 nM. Non-specific binding was determined in the presence of a 500-fold excess of unlabelled EGF. The reaction was terminated by the addition of 1 ml of ice-cold PBS and the cells then washed twice with 0.5 ml ice-cold PBS. The cells were solubilized with 200 μl sodium hydroxide (5 M) and then transferred to polystyrene tubes and counted in a Hewlett-Packard γ counter. Results were transformed by the method of Scatchard [6].

Effect of phorbol 12-myristate 13-acetate (PMA), staurosporine and calphostin C on EGF receptor binding and affinity. Cells cultures were pretreated for 30 min at 37°C with or without the protein kinase C activator PMA (1–100 nM) prior to Scatchard analysis. Cells were also incubated in the presence of absence of the protein kinase C inhibitors staurosporine (1 μM) and calphostin-C (100 nM) for 1 h after which PMA (10 nM) was added and incubation continued for a further 30 min. Scatchard analysis was then performed as described above.

Effect of oestradiol and progesterone on EGF receptor binding. Cell cultures were transferred after 24 h to phenol red-free DMEM/F12 supplemented with 5% charcoal stripped serum and treated with either 10 pM–1 μM oestradiol or 100 pM–10 μM progesterone or vehicle (ethanol) alone for 6 days. Fresh medium and steroids were added daily. Total EGF binding was determined using ^{125}I EGF at a concentration of 8 nM in triplicate for each treatment. Non-specific binding was determined in the presence of a 500-fold excess of unlabelled EGF. Protein content was measured by the method of Lowry *et al.* [17] and the results expressed as fmol EGF bound per mg protein.

RESULTS

HEC-1-B cells were shown to possess a single high affinity binding site for EGF with a K_d of 3.09 ± 1.39 nM (mean $\pm$ SD, $n = 6$) and binding of 845 ± 311 fmol/mg protein (mean $\pm$ SD, $n = 6$). A typical Scatchard plot is shown in Fig. 1. Pretreatment of HEC-1-B cells for 30 min with PMA (1–100 nM) caused a shift in the affinity of the EGF receptor from a K_d of 4 nM to 50 nM (Fig. 2) but no change in the number of binding sites (Fig. 3). This effect of PMA was overcome by preincubation of the cells with the protein kinase C inhibitor staurosporine (1 μM) prior to the addition of PMA (Fig. 3). However, preincubation with calphostin-C (100 nM) had no effect

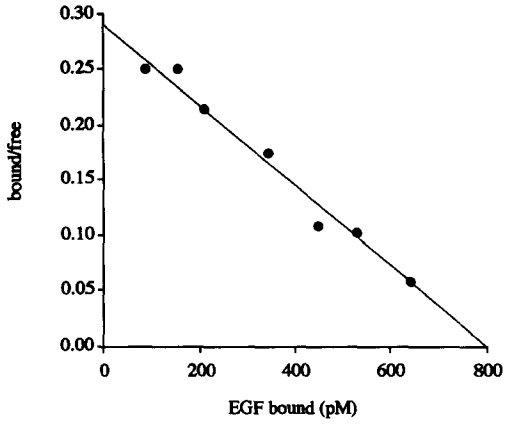

Fig. 1. Scatchard analysis of ^{125}I-EGF binding in HEC-1-B cells. The K_d was 2.8 nM.

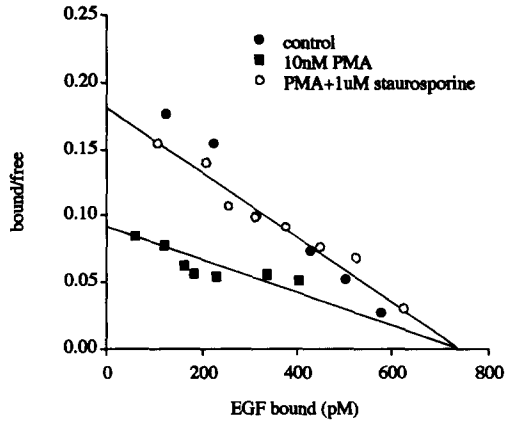

Fig. 3. The effect of PMA (10 nM) and staurosporine (1 μM) on EGF receptor affinity and binding in HEC-1-B cells.

on the response to PMA (data not shown). Neither staurosporine nor calphostin-C alone had an effect on EGF receptor binding (data not shown).

Prolonged treatment with oestradiol stimulated a significant increase in EGF receptor binding (Fig. 4) which was dose related and reached a maximum 5.5-fold increase in response to 1 nM oestradiol ($P < 0.01$ compared with untreated cells). The effective range of oestradiol was 100 pM–10 nM while at higher concentrations (100 nM and 1 μM) the stimulatory effect was lost.

Progesterone also caused a marked increase in EGF receptor binding when tested using the same 6 day treatment schedule (Fig. 5). A significant increase in EGF receptor binding was seen over the concentration range 10 nM–10 μM progesterone with a maximum 7-fold effect at 100 nM–1 μM ($P < 0.001$ compared with untreated cells). There was a small increase in EGF receptor binding at the lower doses tested (0.1 and 1 nM) but the effect was not significant.

DISCUSSION

The present study demonstrates the presence of a single, high affinity receptor for EGF in the endometrial carcinoma cell line HEC-1-B. The mean value of the K_d for 6 experiments was 3.09 ± 1.39 nM which is of the same magnitude as demonstrated in primary cultures of endometrial stromal cells and glandular epithelium (1.1 and 0.9 nM, respectively) [1]. The number of binding sites for the HEC-1-B cell line (mean number for 6 experiments: 845 ± 311 fmol/mg protein) is also similar to that observed in our primary cell cultures (510 and 260 fmol/mg protein for stromal cells and epithelial cells, respectively) [1]. The similarity between the cell line and normal primary endometrial cells in terms of the number of EGF binding sites measured suggests that HEC-1-B cells do not overexpress the EGF receptor and points to their suitability as a model for the study of EGF receptor function in the endometrium.

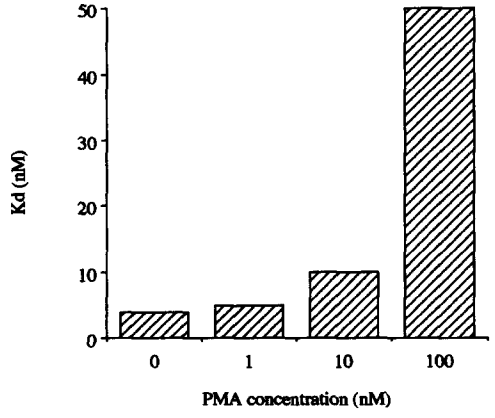

Fig. 2. The effect of PMA on EGF receptor affinity in HEC-1-B cells. Each bar represents the dissociation constant obtained by Scatchard analysis using a 7 point assay at the specified concentration of PMA.

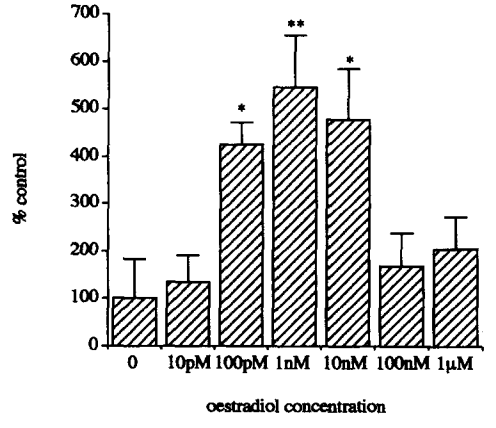

Fig. 4. The effect of a 6-day treatment with oestradiol on EGF receptor synthesis in HEC-1-B cells. Each bar represents mean ± SD of triplicate estimations. Results were expressed as fmol EGF bound per mg protein, then as % control where the control value was designated to be 100%. Data were analysed by unpaired t-test ($^*P < 0.05$,$^{**}P < 0.01$).

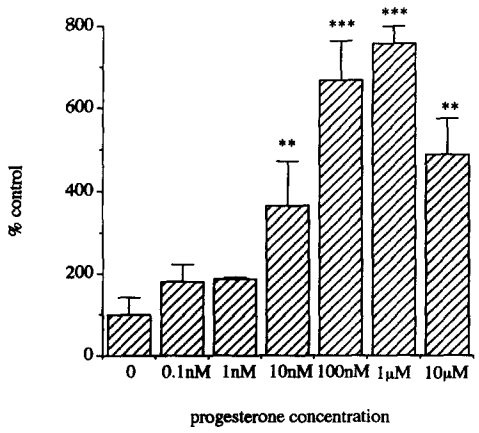

Fig. 5. The effect of a 6-day treatment with progesterone on EGF receptor synthesis in HEC-1-B cells. Each bar represents the mean ± SD of triplicate estimations. Results were expressed as fmol EGF bound per mg protein, then as % control where the control value was designated to be 100%. Data were analysed by unpaired *t*-test ($P < 0.01$, ***$P < 0.001$).**

The affinity of the EGF receptor in HEC-1-B cells was decreased following treatment with PMA without any concommitant alteration in EGF receptor binding. Phosphorylation of the EGF receptor by protein kinase C is considered to regulate EGF receptor affinity thus explaining the decrease in affinity invoked by the protein kinase C activator PMA [8, 9]. The inhibition of this effect by the protein kinase C inhibitor staurosporine lends weight to this observation, although calphostin-C, reputed to be a more specific inhibitor than staurosporine, had no effect. However, it is possible that the concentration of calphostin-C used (100 nM) was too low to cause inhibition. Studies with other cells (e.g. A431 cells) have shown that a concentration of calphostin-C of 1000 nM is required to achieve inhibition [10]. The cytotoxic effect of calphostin-C to HEC-1-B cells at concentrations above 100 nM (unpublished data) prevented further investigation. Our studies of the EGF receptor with primary cultures of endometrial stromal cells have demonstrated a similar effect of PMA [1].

Oestradiol is known to stimulate endometrial growth but the exact mechanism by which this occurs is still unclear. Epidermal growth factor has been shown to stimulate proliferation in normal endometrial stromal cells [11] and it is now becoming apparent that one way in which oestrogen regulates uterine growth is by inducing the synthesis of EGF [12] or its receptor [13–15]. Our studies with primary cultures of endometrial stromal cells have demonstrated that treatment with oestradiol or progesterone increases EGF receptor number and that this effect is enhanced when both steroids are present [2]. In the present study we have demonstrated that both oestradiol and progesterone cause a dose dependent increase in EGF receptor number in HEC-1-B adenocarcinoma cells. This suggests that ovarian steroids may also regulate the growth and differentiation of these cells by increasing EGF receptor synthesis. However, further work is required to determine whether this is a direct effect on receptor synthesis or whether other mechanisms are involved. Studies with normal endometrial epithelial cells are also required in order to clarify whether the stimulatory effects of oestradiol and progesterone are characteristic of endometrial epithelial cells in general or solely a feature of HEC-1-B cells.

The results obtained in this study indicate that the HEC-1-B endometrial adenocarcinoma cell line possesses an EGF receptor with similar properties to that of primary endometrial cells in culture. On this basis, the suitability of the cell line as a model for studying the regulation of the EGF receptor in endometrial epithelial cells is promising, but ultimately its usefulness will depend on whether endometrial epithelial cells respond to steroids in the same manner.

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