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Interactions between oestradiol and epidermal growth factor in endometrial stromal proliferation and differentiation

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The relationship between oestradiol and epidermal growth factor (EGF) in the control of endometrial proliferation and differentiation in cultures of human endometrial stromal cells was investigated. Oestradiol at a concentration of 10 nmol l^{-1} increased the incorporation of both [^{3}H]thymidine and [^{3}H]leucine but the differences were significantly different from control only for [^{3}H]leucine incorporation. Concentrations of 0.16, 1.6 and 16 nmol EGF l^{-1} significantly increased both [^{3}H]thymidine ($P < 0.01$) and [^{3}H]leucine incorporation ($P < 0.01$). The pure steroidal antioestrogen, ICI 182,780, inhibited any increase in [^{3}H]thymidine and [^{3}H]leucine incorporation stimulated by oestradiol in endometrial stroma. The monoclonal antibody, ICR 16, directed against the EGF receptor did not inhibit the oestradiol action in stromal cells, indicating that, in this model system, oestradiol does not act by inducing synthesis or release of EGF. However, ICI 182,780 potently inhibited the incorporation of [^{3}H]thymidine stimulated by EGF in endometrial stromal cells, suggesting interdependence between oestradiol and EGF in the control of endometrial stromal proliferation. Oestrogen-free conditioned medium from endometrial stromal cultures did not stimulate either [^{3}H]thymidine or [^{3}H]leucine incorporation, suggesting that oestradiol did not stimulate the secretion of a trophic factor from endometrial stromal cells.

Introduction

The precise mechanisms involved in endometrial proliferation have been studied intensely in recent years, owing to the emerging consensus that oestrogen may not act only through its receptor. Many workers have failed to demonstrate a proliferative response to oestrogen in endometrial cell cultures *in vitro*, of either epithelial cells or stromal cells (Casimiri *et al.*, 1980; Fleming and Gurpide, 1982; Alkhalaf *et al.*, 1991; Haining *et al.*, 1991; Uchima *et al.*, 1991), although oestrogen will cause proliferation of either epithelial cells or stromal cells under certain culture conditions (Gerschenson *et al.*, 1981; Irwin *et al.*, 1991; Olive *et al.*, 1991). One potential candidate as the agent which controls endometrial proliferation is epidermal growth factor (EGF). Administration of EGF to ovariectomized mice induces uterine and vaginal growth equivalent to that in control mice given oestradiol, and this action of oestradiol is inhibited by an antibody directed against EGF (Nelson *et al.*, 1991). In this model, EGF also induces the expression of lactoferrin, a major oestrogen-inducible secretory protein, in mice. EGF (Haining *et al.*, 1991) and its receptor (EGF-R; Smith *et al.*, 1991) have been identified in human endometrium. Studies have indicated that the concentrations of EGF (Gonzalez *et al.*, 1984) and EGF-R (Bonaccorsi *et al.*, 1989; Stancel *et al.*, 1990; Taketani and Mizuno, 1991; Troche *et al.*, 1991) are regulated by oestrogen in the endometrium. Transforming growth factor α (TGF-α) has also been shown to mediate oestrogen action in the mouse uterus (Nelson *et al.*, 1992).

The aim of this study was to investigate further the relative roles of oestradiol and EGF in endometrial proliferation. Any possible interdependence between oestrogen and EGF in endometrial growth was examined using specific inhibitors of their action in a model system of cultured human endometrial stromal cells. Endometrial stromal cells were chosen because they are known to express oestrogen and EGF receptors, and because they yield an extremely pure population of cells, which facilitates experimental design and interpretation. The actions of oestradiol were inhibited by using the pure specific antioestrogen ICI 182,780 (Wakeling *et al.*, 1991). ICR16, a monoclonal antibody directed against the EGF receptor (Modjtahedi *et al.*, 1993), was used to inhibit EGF action. This strategy enabled examination of two further hypotheses. First, if oestrogen acts on the endometrium to release a trophic factor such as EGF, which then stimulates replication of endometrial stromal cells, then treatment of stromal cultures with ICI 182,780 should inhibit production of this factor and thus any proliferative effect of such a factor should be abolished. Second, if oestrogen causes endometrial growth indirectly via the action of EGF, then treatment of stromal cell cultures with the anti-EGF-R antibody should inhibit any actions of oestrogen.

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Materials and Methods

Materials

Human recombinant EGF was obtained from Boehringer Mannheim (Lewes). Collagenase type XI, insulin, oestradiol, Hepes, L-glutamine, thymidine and leucine were all obtained from Sigma (Poole). Percoll was obtained from Pharmacia (Milton Keynes). Hank's balanced salt solution without calcium, magnesium or phenol red and 10 000 iu penicillin ml^{-1}–10 000 µg streptomycin ml^{-1} were obtained from Northumbria Biologicals Ltd (Cramlington). Dulbecco's modified Eagle's medium, Ham's nutrient mixture F12, amphotericin B (fungizone), gentamycin and nonessential amino acids were obtained from Gibco (Paisley). A custom-made mixture of 1:1 DMEM:F12 without thymidine, leucine or phenol red was also supplied by Gibco. Charcoal-treated fetal calf serum was obtained from Imperial Laboratories (Andover). Trichloroacetic acid (TCA) was provided by BDH (Poole) and Optiscint HiSafe from Wallac (Milton Keynes). All other chemicals were obtained from either BDH or Sigma.

Methyl [^{3}H]thymidine, L-(4,5)[^{3}H]leucine and the Biotrak EGF ELISA kit were all purchased from Amersham International (Amersham, Bucks).

ICI 182,780 (7α[9-(4,4,5,5,5-pentafluoropentylsulphinyl) nonyl]estra-1,3,5(10)-triene-3,17β-diol) has potent and pure antioestrogenic activity both *in vitro* (Wakeling *et al.*, 1991) and *in vivo* (Thomas *et al.*, 1994). The rat monoclonal antibody, ICR16, inhibits EGF activity by blocking the receptor (Modjtahedi *et al.*, 1993). The control antibody used was ALN/11/53, as described by Modjhedi *et al.* (1993).

Tissue samples

Endometrial biopsies were obtained from patients undergoing either hysterectomy or surgical procedures for benign gynaecological disorders using a Pipelle suction curette (Eurosurgical; Cranleigh). None of the patients was receiving any hormonal treatment before surgery, and the operations were performed for menorrhagia or fibroids. The phase of the menstrual cycle at which the specimens were obtained was determined by the date of the last menstrual period. Of the samples used in the experiments, 57% were from day 6 to day 14 of the cycle and the remaining 43% from day 15 to day 28. Tissue samples were collected in Hank's balanced salt solution without calcium, magnesium or phenol red, supplemented with 20 mmol Hepes l^{-1}, 100 iu penicillin ml^{-1}, 100 µg streptomycin ml^{-1} and 5 µg amphotericin B ml^{-1} (medium A). Further processing of the tissue samples occurred in the cell culture laboratory.

All patients gave their consent to the collection of tissue specimens. Ethical permission for this study was granted by Southampton and South West Hampshire Health Authority/ University of Southampton Joint Ethics Committee.

Isolation and culture of endometrial stromal cells

Endometrial stromal cells uncontaminated with glandular

Mellor and Thomas (1994). Endometrial tissue was minced and digested with 1 mg collagenase type XI ml^{-1} until individual endometrial glands were released from the preparation. Undigested pieces of tissue and endometrial glands were removed from the digest by a two-step filtration procedure through 45 µm and 10 µm nylon meshes (Lockertex, Warrington). The filtrate was centrifuged (at 500 ***g*** for 15 min) over 60% Percoll to remove contaminating red blood cells. After washing the cells contained in the band at the interface of the Percoll and medium A, cells were resuspended in 1:1 DMEM:F12 containing 20 mmol Hepes l^{-1}, 100 µg streptomycin ml^{-1}, 100 iu penicillin ml^{-1}, 5 µg amphotericin B ml^{-1}, 1% (v/v) nonessential amino acids, 50 µg gentamycin ml^{-1}, 10 mmol L-glutamine l^{-1}, 10 µg insulin ml^{-1} and 5% (v/v) charcoal-treated fetal calf serum (medium C). After counting by haemocytometry, cells were plated out at a concentration of 1×10^5 ml^{-1} in 24-well plates (Nunc, Paisley). The stromal cells were allowed to adhere overnight in a humid atmosphere of 95% CO_2:5% O_2 at 37°C. On the following day, this medium was removed and replaced with the custom-made phenol red-free 1:1 DMEM:F12 containing all the additions listed above. Cells were incubated in this medium for a further 3 days until experiments began. The medium was replenished every 3 days.

[^{3}H]thymidine and [^{3}H]leucine incorporation studies

All experiments were performed in phenol red-free medium C. Oestradiol and ICI 182,780 were dissolved in ethanol, and appropriate dilutions prepared in culture medium. The final concentration of ethanol in all wells was adjusted to 0.01% (v/v). Experiments were commenced on day 4 of culture. Stromal cells were incubated with the agent(s) of interest (oestradiol, ICI 182,780, EGF or anti-EGF-R antibody) for 48 h. Experimental media are replaced after 24 h. Methyl [^{3}H]thymidine or L-4,5-[^{3}H]leucine was added for the final 18 h of the experiment at a concentration of 5 µCi ml^{-1} (185 kBq ml^{-1}). At the conclusion of the experiment, the medium was removed; the cells were washed in medium A and removed from the plates with 0.05% (v/v) trypsin–0.02% (v/v) EDTA and by scraping. The radioactive macromolecules were precipitated by the addition of TCA to a final concentration of 5% (w/v) ([^{3}H]thymidine experiments) or 10% (w/v) ([^{3}H]leucine experiments). After at least 1 h at 4°C, the precipitates were collected on Whatman GF/C filters (BDH) and air-dried. The liquid scintillant Optiscint HiSafe was added and the radioactivity on the filters determined by scintillation counting (LKB). For the ICR16 antibody experiments, the stromal cells were preincubated with the antibody for 2 h before to the addition of EGF.

Preparation of conditioned media

Cells were plated out in 75 cm^2 flasks (Nunc) at an equivalent cell density to that used for the experiments in 24-well plates. The same experimental protocol as in the labelling studies described above was used for the preparation of conditioned media. Cells were incubated in phenol red-free medium C under the following experimental conditions: con-

Table 1. Effect of various concentrations of oestradiol and epidermal growth factor (EGF) on [^{3}H]thymidine and [^{3}H]leucine incorporation in endometrial stromal cell cultures

Parameter	Oestradiol (nmol l^{-1})	Percentage of control (SEM)	EGF (nmol l^{-1})	Percentage of control (SEM)
[^{3}H]thymidine incorporation	0.01	75.6 (10.9)	0.16	248 (29)*
	0.1	94 (4.1)	1.6	238 (29)**
	1	130.1 (35)	16	274 (48)***
	10	129 (19.7)		
[^{3}H]leucine incorporation	0.01	95 (8.8)	0.16	134.5 (4.6)
	0.1	107.5 (5.8)	1.6	142.2 (5)**
	1	101.4 (3.3)	16	133.9 (4)**
	10	111.7 (6.2)*		

Data are expressed as percentage of the control value (control = 100%) and are a mean of four experiments for oestradiol and three for EGF.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

oestradiol l^{-1} plus 100 nmol ICI 182,780 l^{-1}, or 100 nmol ICI 182,780 l^{-1} alone. The media for each treatment were collected after 24 and 48 h and then pooled. Residual oestradiol and ICI 182,780, which might have interfered with the interpretation of experimental data, were removed from the conditioned media by ultrafiltration using Centriprep concentrators (Amicon, Stonehouse, Gloucestershire) with a membrane cut-off of 3 kDa. After ultrafiltration, the concentrate was diluted to the original volume using phenol red-free, insulin-free medium C that contained only 1% (w/v) charcoal-treated fetal calf serum, and filtered through 0.22 µm filters. Oestradiol assay (Serono Diagnostics, Fleet) performed on the conditioned media that initially contained 10 nmol oestradiol l^{-1} revealed that, after ultrafiltration, the concentration of oestradiol was 400 pmol l^{-1}, a concentration that was ineffective on [^{3}H]thymidine or [^{3}H]leucine incorporation in the assay system described here.

A small aliquot of each conditioned medium was taken for EGF assay. The remainder was applied to fresh stromal cell cultures for [^{3}H]thymidine incorporation studies using the basic experimental protocol.

EGF assay

Aliquots of the conditioned media were assayed for EGF with the EGF Biotrak ELISA.

Western blotting

Endometrial stromal cells in 75 cm^2 flasks were treated with 10 nmol oestradiol l^{-1} either alone or in combination with 100 nmol ICI 182,780 l^{-1}, as in the protocol described above, for 48 h. At the end of the experiments, cells were removed by trypsinization and scraping and collected by centrifugation at 110 g for 5 min. The approximate volume of the pellet was determined and an 8 times excess of Tris–EDTA–molybdate buffer (10 mmol Tris l^{-1}, 1.5 mmol EDTA l^{-1}, 5.0 mmol sodium molybdate l^{-1}) containing 1 mmol monothioglycerol

centrifuged at 4°C for 20 min at 2500 g. Cytosols were analysed by western blotting to detect the oestrogen receptor by S. Dauvois and M. G. Parker (Imperial Cancer Research Fund, London).

Statistical analysis

All the data were analysed statistically using Student's paired t test.

Results

Culture of endometrial stromal cells

Characterization of endometrial stromal cells isolated using a two-step filtration method is discussed by Matthews *et al.* (1992). Filtration of the tissue digest through a 10 µm filter results in preparations of stromal cells virtually uncontaminated with glandular epithelial cells. Cells were plated out in phenol red-containing medium C. After the cells had adhered to the plastic of the culture vessels (overnight), the cells were washed in medium A and incubated in phenol red-free medium C. The brief period in which the cells were exposed to phenol red did not affect cellular responses to oestradiol, since cells spent a further 3 days in phenol red-free medium C. The stage of the menstrual cycle at which the cells were isolated did not appear to affect the experimental data, although the small numbers used in each experiment may have concealed differences.

Effect of oestradiol and EGF on [^{3}H]thymidine and [^{3}H]leucine incorporation in endometrial stromal cells in culture

Table 1 shows that 10 nmol oestradiol l^{-1} increased both [^{3}H]thymidine and [^{3}H]leucine incorporation, but that only the increase in [^{3}H]leucine incorporation was statistically significant ($P = 0.15$ and $P = 0.04$, respectively). EGF significantly stimu-

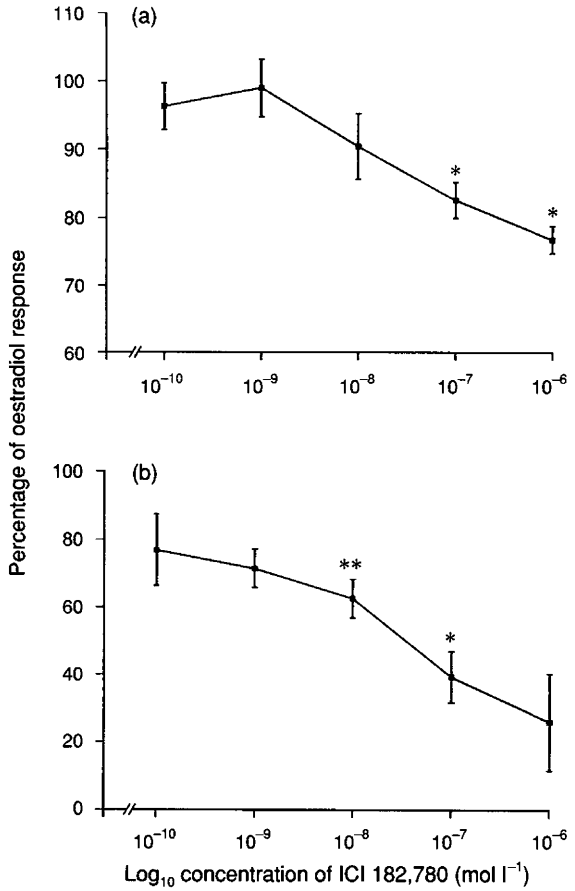

Fig. 1. Effect of various concentrations of ICI 182,780 and 10 nmol oestradiol l^{-1} on (a) [^{3}H]leucine and (b) [^{3}H]thymidine incorporation in endometrial stromal cell cultures. Values are mean (± SEM) and represent three experiments ([^{3}H]thymidine) or six experiments ([^{3}H]leucine). *$P < 0.05$, **$P < 0.01$ compared with 10 nmol oestradiol l^{-1} alone.

16 nmol l^{-1} ($P < 0.01$), but 0.16 nmol EGF l^{-1} augmented only [^{3}H]thymidine incorporation ($P < 0.05$).

Inhibition of oestradiol-stimulated [^{3}H]thymidine and [^{3}H]leucine incorporation by ICI 182,780

When stromal cells were incubated with various concentrations of ICI 182,780 in the absence of oestradiol, [^{3}H]thymidine and [^{3}H]leucine incorporation did not differ from control values. Co-incubation with various doses of ICI 182,780 inhibited any stimulation of 10 nmol oestradiol l^{-1} on both [^{3}H]leucine and [^{3}H]thymidine incorporation (Fig. 1). Furthermore, there was a suppression of this incorporation below control values, which was statistically significant at concentrations of ICI 182,780 of 100 nmol l^{-1} and 10 nmol l^{-1} for the [^{3}H]thymidine experiments and 1000 nmol ICI 182,780 l^{-1} for the [^{3}H]leucine experiments ($P < 0.05$). Figure 2 is a western blot analysis for oestrogen receptors showing that oestrogen receptors are absent from stromal cells treated with vehicle alone, but are induced by treatment of cells with 10 nmol

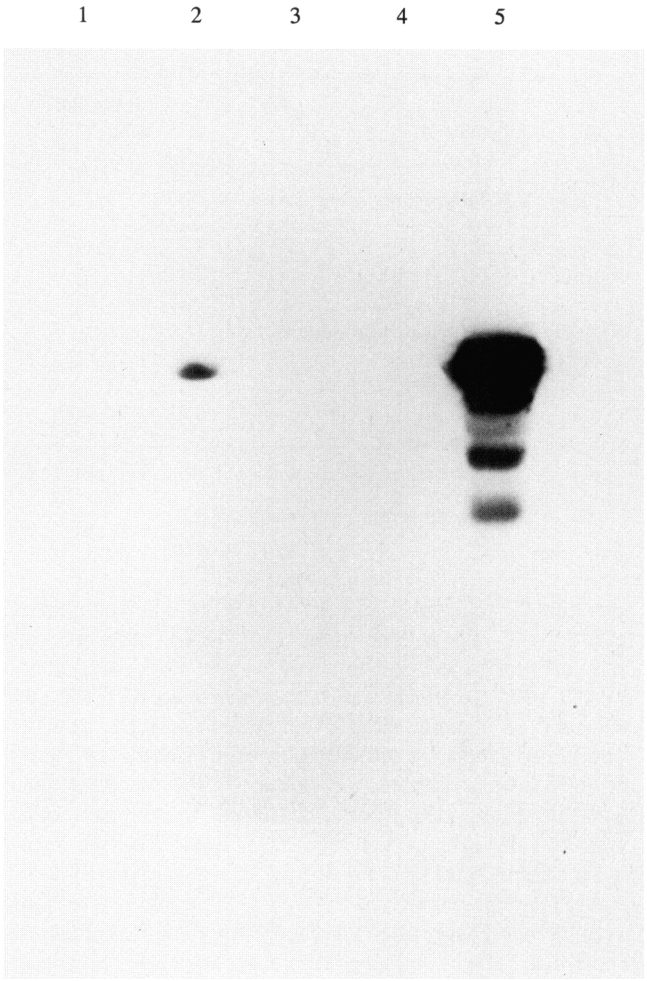

Fig. 2. Western blot of cytosolic extracts of human endometrial stromal cells and immunodetection of the oestrogen receptor. Track 1, control cells; track 2, 10 nmol oestradiol l^{-1}; track 3, 10 nmol oestradiol l^{-1} and 100 nmol ICI 182,780 l^{-1}; track 4, 100 nmol ICI 182,780 l^{-1}; track 5, positive control corresponding to approximately 200 fmol oestrogen receptor l^{-1}.

This induction of the oestrogen receptor was completely blocked by co-incubation of 10 nmol oestradiol l^{-1} and 100 nmol ICI 182,780 l^{-1}. Administration of 100 nmol ICI 182,780 l^{-1} alone did not induce expression of the oestrogen receptor.

Co-incubation of EGF with the anti-EGF-R antibody ICR16

Table 2 shows that 5 μg ICR16 ml^{-1} inhibited by 50% the incorporation of [^{3}H]thymidine stimulated by 1.6 nmol EGF l^{-1} and that the response was completely abolished with 10 μg ICR16 ml^{-1}. Because of the large quantities of antibodies required, a limited number of control wells were included to conserve supplies. A concentration of 5 μg ml^{-1} of a control antibody in combination with 1.6 nmol EGF l^{-1} did not diminish the response to 1.6 nmol EGF l^{-1}. However, 10 μg ml^{-1} of the control antibody decreased the amount of

Table 2. Effect of the antibody ICR16 on EGF-induced [^{3}H]thymidine incorporation in endometrial stromal cultures

Addition	[^{3}H]thymidine incorporation: percentage of control (SEM)
1.6 nmol EGF l^{-1}	199 (24.0)**
1.6 nmol EGF l^{-1}/5 μg ICR16 ml^{-1}	141 (11.7)*
1.6 nmol EGF l^{-1}/10 μg ICR16 ml^{-1}	98 (3.8)
1.6 nmol EGF l^{-1}/5 μg control antibody ml^{-1}	163 (4.6)***
10 μg control antibody ml^{-1}	41.5 (0.45)****

Data are expressed as percentage of the control value (control = 100%) and are means of three experiments. *$P < 0.05$, **$P < 0.02$, ***$P < 0.01$, ****$P < 0.001$.

Co-incubation of oestradiol and ICR16 anti-EGF-R antibody

Endometrial stromal cells were preincubated with 10 μg ICR16 ml^{-1} for 2 h before 10 nmol oestradiol l^{-1} was added to the cultures. It was not possible to demonstrate any blockage of oestradiol-induced increase in radiolabelled incorporation by the presence of the anti-EGF-R antibody at concentrations that effectively abolished the effects of 1.6 nmol EGF l^{-1}. This may well have occurred because of the difficulty of demonstrating any stimulatory effects of 10 nmol oestradiol l^{-1} on [^{3}H]thymidine incorporation. However, in [^{3}H]leucine incorporation experiments, a concentration of 10 μg ICR16 ml^{-1} did not reduce the stimulation of protein synthesis caused by 10 nmol oestradiol l^{-1} (Table 3).

Conditioned medium experiments

Conditioned media were prepared from stromal cells treated with either vehicle alone, 10 nmol oestradiol l^{-1}, 10 nmol oestradiol l^{-1} plus 100 nmol ICI 182,780 l^{-1} or 100 nmol ICI 182,780 l^{-1} alone. These conditioned media were then applied to fresh stromal cultures and the effect of the conditioned media on [^{3}H]thymidine incorporation was determined according to the standard experimental protocol. There was no difference in the values for the amount of [^{3}H]thymidine between stromal cells incubated in conditioned medium obtained from cells treated with ethanolic vehicle alone and those from cells treated with 10 nmol oestradiol l^{-1}. No EGF was detected in any of the conditioned media (Table 4). Since the limit of sensitivity of the assay was 0.2 pg ml^{-1}, this confirms that the lack of stimulation of [^{3}H]thymidine counts above control values by conditioned media from oestradiol-treated stromal cells is due to the absence of any oestradiol-induced synthesis or release of EGF. Conditioned media from cells treated with a combination of 10 nmol oestradiol l^{-1} and 100 nmol ICI 182,780 l^{-1} seemed to depress the amount of radioactivity present to below that of cells treated with vehicle alone.

Co-incubation of EGF and ICI 182,780

Stromal cells were incubated with 1.6 nmol EGF l^{-1} and [illegible] experimental protocol. ICI 182,780 inhibited EGF action in endometrial stromal cells (Fig. 3). All the concentrations of ICI 182,780 tested were effective at reducing the stimulation of [^{3}H]thymidine incorporation into DNA by 1.6 nmol EGF l^{-1} ($P < 0.05$). There was no significant reduction in EGF-induced stimulation of [^{3}H]leucine incorporation by ICI 182,780.

Discussion

This study describes a series of experiments carried out to clarify the interactions and interdependence between oestradiol and EGF in the endometrium by delineating their individual actions and interactive roles, using human endometrial stromal cells as a model. By using specific inhibitors of the action of oestradiol and EGF, the hypothesis that oestrogen causes growth and differentiation of the human endometrium by inducing the synthesis or release of the polypeptide growth factor, EGF, was tested.

In pure cultures of human endometrial stroma cells, 10 nmol oestradiol l^{-1} had a small stimulatory effect on DNA and protein synthesis. However, in these cells, EGF was potently mitogenic. Western blotting demonstrated that oestradiol administration induced oestrogen receptor expression in the stromal cells, so it appears that the absence of a mitogenic response to oestradiol was not due to a deficiency in oestrogen receptors. Uchima *et al.* (1991) were unable to demonstrate a proliferative response to oestradiol in uterine epithelial cells despite the presence of functional oestrogen receptors. Other authors have reported the apparent dichotomy that oestrogen can induce protein synthesis or progesterone receptor expression, without a concomitant increase in cellular proliferation (Casimiri *et al.*, 1980; Aronica and Katzenellenbogen, 1991; Uchima *et al.*, 1991).

It was not possible to demonstrate that the activity of oestradiol in endometrial stromal cells was inhibited by an antibody directed against the EGF-R. Under the same experimental conditions in which the mitogenic activity of EGF was completely abolished, no inhibition of oestradiol action by the anti-EGF-R antibody was observed. Similarly, there was no evidence that the action of oestradiol induced synthesis or release of EGF, since EGF was not detected in conditioned media from stromal cells that had been treated with 10 nmol oestradiol l^{-1}. If oestradiol acted through a cascade mechanism, then it might be expected that a small amount of EGF would have a large effect on endometrial growth. However, any EGF in the oestradiol-conditioned medium would have been present at concentrations of less than 0.2 pg ml^{-1}, the limit of sensitivity of the kit used. Since the conditioned medium had no effect on [^{3}H]thymidine incorporation when applied to fresh stromal cell cultures, either EGF can cause proliferation only at concentrations of greater than 0.2 pg ml^{-1} or no suitable stimulatory growth factors were induced by oestradiol treatment under these experimental conditions.

The experiments performed with ICI 182,780 alone confirm the classification of ICI 182,780 as a pure steroidal antioestrogen (Wakeling *et al.*, 1989, 1991; Dukes *et al.*, 1992, 1993; Wade *et al.*, 1993). ICI 182,780 alone in stromal cell culture [illegible]

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