The isolation and long-term culture of normal human endometrial epithelium and stroma

Expression of mRNAs for angiogenic polypeptides basally and on oestrogen and progesterone challenges

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SUMMARY

A highly reproducible and technically straightforward technique for the isolation and long-term culture of normal human endometrial epithelial cells is described. The essential conditions for long-term culture are that the cells be seeded onto a gelatin matrix and that 'endothelial cell growth supplement' be present in the culture medium. Normal endometrial epithelial cells express cytokeratins and oestrogen receptors. They may be passaged five to six times without change in properties. Growth of normal endometrial epithelial cells was stimulated by 17-β-oestradiol and epidermal growth factor. Expression of the mRNA coding for seven polypeptide angiogenic factors, by normal endometrial epithelial, stromal and three endometrial carcinoma lines, was examined. The endometrial epithelial and stromal cells express mRNA for the polypeptide angiogenic factors, basic fibroblast growth factor, vascular endothelial cell growth factor, transforming growth factor-β1 and pleiotrophin, as well as the cytokine midkine. Expression of the mRNA for both vascular endothelial growth factor and midkine by normal endometrial epithelial cells showed a 2-fold increase on treatment with a physiological dose of 17-β-oestradiol (10⁻¹⁰ M) while, in contrast, the mRNA of transforming growth factor-β1 decreased 4-fold on treatment with 17-β-oestradiol (10⁻¹⁰ M) and was abolished by exposure to progesterone (5×10⁻⁹ M). Expression of the mRNAs for angiogenic polypeptides by the endometrial carcinoma lines was more restricted.

Key words: angiogenesis, endometrium, epithelium

INTRODUCTION

Endometrial development during the menstrual cycle is ultimately under the hormonal control of oestrogen and progesterone. Endometrial development during each cycle involves extensive angiogenesis. Regeneration of the microvasculature is initiated from remaining post-menstrual arteriolar stumps in the basalis and includes well differentiated coiled arterioles, capillaries and venules (Kaiserman-Abramof and Padykula, 1989). Neither oestrogen nor progesterone has intrinsic angiogenic activity. Despite the identification of numerous polypeptide angiogenic factors (Bicknell and Harris, 1991; Folkman and Shing, 1992) there has to date been no clear link between oestrogen or progesterone and a role for the angiogenic polypeptides in endometrial development. The endothelial cells of the endometrium are oestrogen receptor negative (Perrot-Applanat et al., 1988), from which it follows that the angiogenic response to oestrogen must come from the oestrogen receptor positive epithelial or stromal component of the endometrium.

Known angiogenic polypeptides include acidic and basic fibroblast growth factors (aFGF and bFGF; see for example, Folkman and Klagsbrun, 1987), vascular endothelial growth factor (VEGF; see for example, Ferrara et al., 1991) and the related placental growth factor (PIGF; Maglione et al., 1991), transforming growth factor-β1 (TGF-β1; Roberts et al., 1986; Yang and Moses, 1990), pleiotrophin (PT; Courty et al., 1991; Milner, 1991; Wellstein et al., 1992) for which there is a related gene midkine (MK; Tsutsui et al., 1991) and platelet-derived endothelial cell growth factor (PDECGF; Ishikawa et al., 1989), now known to be thymidine phosphorylase (Furukawa et al., 1992; Moghaddam and Bicknell, 1992; Usuki et al., 1992; Finnis et al., 1993).

The aim of the study was to examine angiogenic factor production by the steroid responsive epithelium and stroma. While stromal cells are relatively easy to culture, epithelium has proven more difficult. Previous workers have found it difficult to obtain pure isolates and maintain them in long-term culture (see for example, Satyaswaroop et al., 1979; Trent et al., 1980; Bongso et al., 1988; Fernandez-Shaw et al., 1992). This paper
describes an improved and highly reproducible procedure for the isolation of normal human endometrial epithelium (NEE cells), together with conditions for long-term culture. NEE cells retain epithelial characteristics in that they express epithelial cytokeratins and have functional oestrogen receptors. Expression of the mRNA for seven angiogenic factors by NEE and stromal cells, as well as three endometrial carcinoma lines, is determined both basally and following oestrogen or progesterone challenge.

**MATERIALS AND METHODS**

**Materials**

HEC-1-A (HTB 112), AN3 CA (HTB 111) and RL95-2 (CRL 1671) were from the American Type Culture Collection, Bethesda, MD, USA. All tissue culture medium was prepared at the Imperial Cancer Research Fund Clare Hall Laboratories, London. Foetal calf serum was from J. Bio, Les Ulis, France. Endothelial cell growth supplement (ECGS), collagenase type 1A, EGF, 17-β-oestradiol and progesterone were from Sigma.

**Tissue collection**

Endometrium was collected at hysterectomy into DMEM/HEPES medium containing 10% foetal calf serum. The stage of the menstrual cycle at which the tissue was obtained was determined from the patients menstrual history and endometrial histology (Noyes et al., 1950; Ferenczy, 1987; Buchley and Fox, 1989).

**Isolation of normal human endometrial epithelial cells**

The isolation is based on the methods of Satyaswaroop et al. (1979) and Fernandez-Shaw et al. (1992). Briefly, tissue was cut into 2-3 mm pieces and incubated with 1 mg/ml of collagenase type 1A in DMEM/10% FCS with stirring for 2 hours at 37°C. The suspension was then filtered through a 250 µm nylon sieve. In 10% FCS and on polylysine-coated tissue culture ware the minor contaminating component of NEE cells do not survive beyond a few days. Pure isolates of NEE cells were routinely maintained in culture for 3 to 4 months.

**Cytospins and immunohistochemistry**

Cytospins were prepared from confluent cultures. Slides were air dried for 2 hours, fixed in acetone for 10 minutes, air dried again and stored at −20°C. Antibodies used were: MNF116 and LP34, which stain simple epithelial cytokeratins; JMB2, which is specific for cytokeratin 18 (McGee et al., 1982); ESIVC7 (anti-CD36); C1MEG1 (anti-CD36); V9 (anti-vimentin); W6/32 (anti-human MHC class I); CR3/43 (anti-human MHC class II); JC70 (anti-CD31); and F8/44/20 (anti-von Willebrand factor). The source of antibodies was as follows: MNF116, LP34, V9, W6/32, CR3/43, JC70 and F8/44/20, Dako Ltd, High Wycombe, UK; ESIVC7, E. van der Schoot, Nethrells Red Cross Blood Transfusion Center, Amsterdam, The Netherlands; C1MEG1, G. Pilkington, Peter McCallum Cancer Institute, Melbourne, Australia; and JMB2, J.O. McGee, Nuffield Department of Pathology, John Radcliffe Hospital, Oxford, UK. Cytospin slides were incubated with primary antibody for 30 minutes at room temperature. After washing in PBS for 3 minutes, slides were further incubated with a peroxidase-conjugated rabbit anti-mouse IgG in PBS for 30 minutes, and then washed again in PBS for 3 minutes. Cytospins were developed with diaminobenzidine tetrahydrochloride at 0.6 mg/ml in PBS containing 3 µl/ml of hydrogen peroxide for 10 minutes. All tissue slides were counterstained with haematoxylin and mounted. Negative controls used peroxidase-conjugated rabbit anti-mouse IgG in place of the primary antibody.

**Immunofluorescence staining of cytokeratins**

Cells were seeded onto glass coverslips and left overnight. They were then fixed in 4% paraformaldehyde in PBS at room temperature for 15 minutes, washed with PBS and non-specific binding was blocked with 3% BSA in PBS for 30 minutes. Incubation with the primary antibody in PBS/BSA was for 1 hour, the slides were then washed with PBS (x2), incubated with anti-mouse IgG-FITC in PBS for 1 hour at room temperature, washed with PBS and mounted in 50% (v/v) glycerol/PBS.

**Oestrogen, progesterone and EGF receptor determinations**

The oestrogen and progesterone receptor number in oestrogen-depleted cells were determined by ELISA assay in accord with EORTC (1980) guidelines. Cytosolic fractions were prepared as previously described (Leake et al., 1981) from cells at confluence. EGF (epidermal growth factor) receptors were measured by a competitive binding assay on membrane fractions (Smith et al., 1989).

**[methyl-3H]thymidine uptake determinations**

NEE cells that had been cultured in Phenol Red-free (PRF) DMEM/10% dextran-coated charcoal (DCC)-stripped FCS for 11 days were removed with trypsin and seeded into 96-well gelatin-coated plates at 5,000 cells per well in PRF DMEM/5% DCC-treated FCS. The cells were then left for a further 10 days to quiesce. Fresh PRF DMEM/5% DCC-treated FCS containing the growth stimulus of interest was then added. At 44 hours later the cells were pulse labelled with 0.5 µCi of [methyl-3H]thymidine per well and harvested 4 hours later directly onto filter mats to permit scintillation counting.

**Determination of the effect of oestrogen and EGF on NEE cell growth**

NEE cells were grown to confluence and then allowed to quiesce for either one or two weeks in PRF DMEM/10% double DCC-stripped FCS. Over these two weeks medium was replaced every two days. Cells were then seeded into 6-well plates in PRF DMEM/2% double DCC-stripped FCS with the indicated (see Fig. 3) concentrations of 17-β-oestradiol or EGF. Cells were then re-fed every second day with
Endometrial angiogenesis

PRF DMEM/2% double DCC-stripped FCS with the indicated concentrations of 17-β-oestradiol or EGF. Cells were counted (Coulter counter) on day 9 following release by treatment with trypsin.

**Determination of the expression of the mRNA of angiogenic factors by RNase protection analysis**

RNA was prepared according to Chomczynski and Sacchi (1987). Briefly, NEE or NES cells were cultured to confluence and then left to quiesce for one week in oestrogen-free medium (PRF DMEM/10% DCC-stripped FCS). Cells were then treated with fresh PRF DMEM/10% DCC-stripped FCS containing either 5×10⁻¹⁰ M 17-β-oestradiol or 5×10⁻⁹ M progesterone. Total RNA was prepared 18 hours later. RNase protection analysis was carried out as described (Ausubel et al., 1990). Constructs to generate probes for TGF-β1 and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control have been described (McCarthy and Bicknell, 1992). Constructs to generate probes for aFGF, bFGF, VEGF, PlGF, PT and MK will be described elsewhere (Relf et al., unpublished data). Similar results were obtained whether the cells quiesced for one or two weeks. mRNA expression was quantified by laser densitometry (Bio Image, Milligen/Bioscience). Expression is given as the ratio to that of a GAPDH control, which was included in each determination.

**RESULTS**

**Isolation and culture of normal human endometrial epithelial (NEE) cells**

In Materials and Methods we have described a highly reproducible and simple procedure to isolate and culture pure normal human endometrial epithelium. The technique involves a standard collagenase digest, followed by the technique of differential sieving (Satyaswaroop et al., 1979) to enrich the isolate in endometrial glands. The glands are then seeded to give a 10–40% confluent culture after 2 days of spreading. Between 2 and 4 days later the cultures either comprise pure epithelial colonies or, in most cases, stromal colonies appear as a minor component. In the latter case the stromal colonies are identified by their characteristic morphology and removed by scraping. The advance that we describe here is that these pure cultures of endometrial epithelium may be maintained in culture without change in properties for periods of up to six months. Two modifications of earlier culture techniques enable this substantial extension of the epithelial life span: namely, culture on gelatin-coated, as opposed to standard (polysine-coated), tissue culture plasticware, and addition of the so-called ‘endothelial cell growth supplement’ to the culture medium. Isolates from the proliferative phase of the endometrium showed a markedly greater viability in vitro than did those from the secretory phase.

**Characterisation of normal endometrial epithelial cells**

**Morphology**

The original isolate comprises gland structures as described by
Satyaswaroop et al. (1979). These attach to the gelatin-coated plates and in the first few days cells grow out in characteristic epithelial whorls. As confluence the cells form a homogeneous, but not particularly cobblestone, monolayer (Fig. 1A). NEE cells exhibit strong contact inhibition and may be maintained as a confluent monolayer for several weeks provided that they are fed with fresh medium at least every 1 to 2 days. If the cells are not fed they senesce (but see comments below).

Removal of ECGS from the culture medium resulted in no immediate morphological change but in premature senescence. In contrast, if both ECGS and oestrogens were removed from the culture medium (i.e. Phenol Red-free DMEM/10% DCC-stripped serum) the cells underwent a striking morphological change over a 4 day period, adopting a classic epithelial cobblestone morphology (Fig. 1B). This state the cells are quiescent and require feeding only once every 1-2 weeks without apparent cell death. This morphological change has so far proven irreversible. Thus, adding back ECGS and oestrogens failed to return the cells to a proliferative state and we conclude that the cells may well have terminally differentiated.

**Immunocytochemical staining**

Table 1 summarises the immunocytochemical properties of both NEE cells and endometrial sections. Strong staining of NEE cells with three different antibodies to cytokeratins is consistent with their epithelial origin. In endometrial tissue sections only epithelium stained with these antibodies. NEE cells are negative for the endothelial markers von Willebrand factor and CD31. Fig. 1C and D shows cytokeratin staining of NEE cells by antibodies LP34 and MNF116, respectively.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Cytospin</th>
<th>Ep</th>
<th>S</th>
<th>En</th>
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<tr>
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<td>4+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LS34</td>
<td>Cytokeratins</td>
<td>2+/3+</td>
<td>4+</td>
<td>0</td>
<td>0</td>
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<tr>
<td>JMB2</td>
<td>Cytokeratin 18</td>
<td>3+</td>
<td>4+</td>
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</tr>
<tr>
<td>ESHV/7</td>
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<td>1+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C1Meg1</td>
<td>CD36</td>
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<tr>
<td>V9</td>
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<td>4+</td>
<td>2+</td>
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<td>Von Willebrand</td>
<td>factor</td>
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Abbreviations: Ep, epithelial; S, stromal and En, endothelial components of the endometrium.

Expression of oestrogen and EGF receptors by NEE cells

Table 2 gives values for oestrogen and EGF receptors determined by ligand binding assay for NEE cells and the three endometrial carcinoma lines HEC-1-A, AN3 CA and RL95-2. All cells showed a comparatively high level of EGF receptor expression but only NEE cells expressed detectable oestrogen receptors. The variation between the two determinations for NEE cells, of 9.4 and 26.3 fmol/mg protein, possibly reflects patient variability in that these were two separate isolates. RL95-2 cells express oestrogen receptors at low passage (<30) but not at high passage (Sundareshan and Hendrix, 1992). The cells used in this study were of passage >126 and it was not unexpected for them to be oestrogen receptor negative.

**Growth response to 17-β-oestradiol and EGF**

Table 3 summarises expression of mRNAs for angiogenic factors in NEE, stromal and endometrial carcinoma lines. Table 3 summarises expression of mRNAs for angiogenic factors in NEE, stromal and endometrial carcinoma lines as determined by RNase protection analysis. NEE cells were found to express mRNA for several angiogenic polypeptides including aFGF, bFGF, VEGF, TGF-β and PT, and the cytokine MK. The only factor examined that they did not express was placental growth factor (PIGF). Neither was PIGF expression detected in stromal isolates or any of the carcinoma lines examined. 17-β-Oestradiol treatment of oestrogen-
depleted (1 or 2 weeks) NEE cultures induced a 2-fold increase in the level of mRNA for VEGF and MK (Fig. 4A and B) but did not affect expression of the other factors. Induction of the mRNA for VEGF and MK by 17-β-oestradiol was also seen in NES cells (data not shown). Progesterone promoted a reproducible fall in the expression of MK mRNA in NEE cells. Stromal isolates expressed the same profile of angiogenic polypeptide mRNAs as the epithelial isolates but particularly strongly expressed the mRNA for VEGF (Fig. 4A). The level of TGF-β1 fell in NEE cells on treatment with 17-β-oestradiol or progesterone but was unchanged in NES cells (Fig. 4C).

**DISCUSSION**

This paper describes the isolation and long-term culture of normal endometrial epithelium and stroma, and their expression of mRNAs for angiogenic polypeptides. Several reports of the isolation and culture of normal human endometrial epithelium are extant but none describes long-term culture. Amongst the first was that by Trent et al. (1986), who obtained cultures simply by outgrowth from endometrial tissue. It has proven difficult to obtain reliably pure isolates using this technique. A slightly earlier paper also described cultures that were greatly enriched in epithelial cells (Kirk et al., 1978). Enrichment was achieved by a combination of collagenase digestion and selective attachment to plastic, the epithelial component taking considerably longer to attach to the plastic than did the stromal component. However, these authors were also unable to maintain cultures of the epithelial cells for more than 10 to 15 days and the epithelial cultures were in addition oestrogen unresponsive. However, by far the most utilised isolation procedure to date has been that of Satyaswaroop et al. (1979), which involves differential sieving. More recent isolation procedures have used antibodies coupled to magnetic microspheres, e.g. Fernandez-Shaw et al. (1992) employed anti-Thy-1 to obtain 98% pure isolates of stromal cells and 82% pure isolates of epithelial cells; however, these cultures could only be maintained for a week or so. The significant advance in the culture of normal human endometrial epithelium that we describe here is that seeding of the epithelial isolates (enriched by the differential sieving technique of Satyaswaroop et al., 1979) onto gelatin-coated plates in the presence of ECGS permits sustained growth for

<table>
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<tr>
<th>Cell line</th>
<th>aFGF</th>
<th>bFGF</th>
<th>VEGF</th>
<th>PI GF</th>
<th>TGF-β1</th>
<th>PT</th>
<th>MK</th>
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<tr>
<td>NEE</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>−</td>
<td>4</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>NEE+E</td>
<td>n.d.</td>
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<td>4</td>
<td>−</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>NEE+P</td>
<td>n.d.</td>
<td>2</td>
<td>2</td>
<td>−</td>
<td>+/-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AN3 CA</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>−</td>
<td>−</td>
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<td>+/−</td>
</tr>
<tr>
<td>RL.95-2</td>
<td>−</td>
<td>−</td>
<td>1</td>
<td>−</td>
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<td>−</td>
<td>10</td>
<td>−</td>
<td>−</td>
<td>+/-</td>
<td>+/−</td>
</tr>
</tbody>
</table>

The ratio to GAPDH control is given. E, 17-β-oestradiol; P, progesterone; n.d. not determined; +/- indicates trace detection.
2-3 months encompassing five to six passages. When quiescent, NEE cells may be kept in culture for periods of up to 6 months. ECGS is a crude protein extract of bovine brain. We have not yet identified the component present in ECGS that stimulates NEE cell growth.

The morphology of NEE cells under the culture conditions

**Fig. 4.** RNase protection analysis of mRNA expression of angiogenic polypeptides by epithelial, stromal and endometrial carcinoma cells. Expression was quantified by laser densitometry. The ratio of the mRNA for the angiogenic peptide to that of GAPDH control is given. (A) VEGF, (B) MK and (C) TGF-β1. Each experiment was repeated three times. The effects of oestradiol and progesterone were reproduced in each experiment.
described, i.e. in the presence of ECGS, was not that of a classic epithelial cobblestone (Fig. 1A); however, they readily adopted a cobblestone morphology when deprived of ECGS and oestrogens (Fig. 1B).

The cells were identified as epithelial by the presence of epithelial marker cytokeratins. Cytokeratin 18 is present in NEE cells. The cytokeratin family is extensive, with at least 20 members (Moll et al., 1982, 1983). Cytokeratins 7, 8, 18 and 19 have been detected in proliferative endometrium (Moll et al., 1983). Vimentin, the intermediate filament protein, was present in NEE cells, but was also detected in endometrial epithelial tissue sections as previously reported (Fernandez-Shaw et al., 1992; Freshney, 1992). NEE cells express oestrogen receptors, as determined by ligand binding, and the stimulation of both growth of oestrogen-deprived cells when treated with 17-β-oestradiol and a 20% induction of progesterone receptor expression confirms that the oestrogen receptors are functional. Thus, we conclude that NEE cells together with the much more readily isolated and cultured oestrogen receptor positive stromal cells provide a useful system in which to examine basal and oestrogen-stimulated release of angiogenic polypeptides. NEE cells also expressed EGF receptors and a growth response to EGF (Figs 2B and 3B) showed these to be functional. The detection of EGF receptors in NEE cells is in agreement with their previous detection on the surface of endometrial glandular epithelium (Smith et al., 1991).

Examination of the mRNA for polypeptide angiogenic factors revealed that NEE cells express RNA coding for six of the seven factors examined: namely, aFGF, bFGF, VEGF, TGF-β1, PT and MK. The only mRNA not detected was PlGF. NES cells expressed the same range of factors as NEE cells. Our findings concur with the detection of FGF and VEGF in intact human endometrium (Rusnati et al., 1990; Charnock-Jones et al., 1993; Ferriani et al., 1993).

Determination of the hormone dependence of expression in NEE cells showed the 17-β-oestradiol stimulated a 2-fold increase in the mRNA expression of VEGF (Fig. 4A) and of MK (Fig. 4B) but not of the other factors. 17-β-Oestradiol also increased VEGF and MK mRNAs in NES cells (data not shown). Our results with steroid stimulation concur with three recent reports that VEGF expression is regulated by oestrogens. In the first of these the pattern of expression of VEGF and its receptors in mice was consistent with a role for VEGF in hormonally regulated angiogenesis (Shweiki et al., 1993). Four tissues in which active angiogenesis occurs during the female reproductive cycle were examined: namely, ovarian follicles, corpus luteum, sites of embryonic implantation and, of relevance to the studies described here, endometrial regrowth. In each case VEGF mRNA was found to be expressed in areas of expanding vasculature. By in situ hybridisation, VEGF was shown to be expressed in 10 steroidogenic and/or steroid responsive cell types. In some cells upregulation of VEGF expression was concurrent with steroidogenic activity. The second study involved whole rat uterus (Cullinan-Bove and Koos, 1993) and showed by PCR analysis that both oestrogen and progesterone stimulated VEGF mRNA upregulation. However, endometrium and myometrium were not studied individually. The third study (Charnock-Jones et al., 1993) reported expression of VEGF in human uterine tissues and that the mRNA for VEGF is upregulated in HEC-1-A cells on exposure to 17-β-oestradiol. The latter observation is difficult to rationalise, however, in view of the fact that, as shown in Table 2, HEC-1-A cells do not have oestrogen receptors. Indeed, nowhere in the literature has it been reported that they do. Furthermore, an unphysiologically high dose of 17-β-oestradiol of 10−8 M was used. Similar comments apply to the studies of 17-β-oestradiol stimulation of bFGF synthesis in HEC-1-A, HEC-1-B and AN3 CA cells described by Presta (1988). Thus, concentrations of 17-β-oestradiol between 1×10−5 and 3×10−4 M were required to observe a stimulation of bFGF expression. (The AN3 CA cells used in Presta’s study were from the American Type Culture Collection, as were the ones used here. These cells are of late passage and are oestrogen receptor negative (Table 1).) We are led to conclude that these inductions of angiogenic polypeptides by superphysiological levels of oestrogens are not of relevance to the endocrinology of oestrogen.

Our finding that 17-β-oestradiol induces mRNAs for VEGF and MK is the first report of angiogenic factor induction by a physiological dose of oestrogen. This is consistent with analysis of the promoters of the VEGF and MK genes. Thus, the presence of three upstream half-palindromic 5′-TGACC-3′ (Kato et al., 1992) oestrogen response elements upstream of the VEGF initiation site and a further five downstream (Tischer et al., 1991) point to oestrogen-mediated expression. Likewise, the MK promoter region contains 10 such motifs (Uehara et al., 1992). Expression of MK, with the exception of kidney (Tsutsui et al., 1991), has been thought to be largely restricted to embryonic tissue, although Huang et al. (1990) detected mRNA in the murine uterus. Pleiotrophin, the other member of the MK family, is also present in uterine tissue from which it was originally isolated (Möllner et al., 1989).

In contrast to the upregulation of the mRNA for VEGF and MK by 17-β-oestradiol in NEE cells, we found that the level of mRNA for TGF-β1 decreased. Furthermore, hormone regulation of TGF-β1 mRNA expression was observed in NEE but not NES cells (Fig. 4C), a clear difference between the epithelial and stromal cells. The TGF-β1 promoter is complex. Studies have identified two negative, in addition to two positive, regulatory regions, and an enhancer region of the promoter (Kim et al., 1989). Two half-palindromic response elements were present in the −731 to −453 negative regulatory region, one in the enhancer region and one in a positive regulatory region. Thus, again a decrease in mRNA for TGF-β1 on treatment with 17-β-oestradiol is not inconsistent with the analysis of its promoter.

Two potential models of endometrial angiogenesis under the control of the female steroid hormones are readily envisaged. In the first of these, oestrogens promote a dramatic burst of angiogenic factor release from the oestrogen receptor positive epithelial and stromal components, which gives rise to the extensive angiogenesis at the start of endometrial development. Alternatively, endometrial epithelium may secrete a basal comparatively low level of angiogenic polypeptides. The effect of oestrogen is then to stimulate proliferation of endometrial epithelia and stroma; these continue to secrete a basal level of angiogenic factors, which initiates vascular development that occurs synchronously with epithelial and stromal expansion. The results presented here lend support to the latter of the two. Thus, NEE and NES cells contain message for at least six angiogenic polypeptides but in no case was there a dramatic
increase in mRNA expression on oestrogen challenge. Why these cells express so many angiogenic peptides is not clear, although others have postulated that angiogenesis is such a fundamental physiological process that there exists much redundancy in the mechanism whereby development of new blood vessels is brought about.

This study has identified six angiogenic factors that could play a role in endometrial angiogenesis and we think that it would be particularly interesting to examine VEGF and MK further. We are currently examining expression of mRNA for angiogenic factors by in situ hybridisation and of polypeptides by immunohistochemistry (as antibodies become available) on endometrial sections throughout the cycle in order to elucidate further which factors play a role in in vivo endometrial angiogenesis.

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