A novel organotypic culture model for normal human endometrium: regulation of epithelial cell proliferation by estradiol and medroxyprogesterone acetate

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BACKGROUND: A novel organotypic culture system was established for modelling the hormonal responses of the normal human endometrium in vitro. METHODS: Endometrial epithelial cells were cultured as glandular organoids within reconstituted extracellular matrix (Matrigel) in tissue culture inserts and stromal cells on plastic below the epithelial compartment. The effects of estradiol (E2) and E2 together with medroxyprogesterone acetate (MPA) on cell proliferation and the expression of estrogen receptor α (ERα) and progesterone receptor (PR) were studied in 10 epithelial–stromal co-cultures and in three parallel monocultures of epithelial organoids. RESULTS: In co-cultures, E2 was shown to increase the percentage of Ki67-positive cells by 2-fold relative to untreated controls. In the presence of MPA, a significant decrease in cell proliferation was detected. Similar results were obtained when the corresponding percentages of Ki67-positive organoids were calculated instead of individual cells. In the absence of stromal fibroblasts, Ki67 epithelial labelling remained below the control value after both hormonal treatments. Epithelial organoids retained their capacity to express estrogen and progesterone receptors in culture. E2 was shown to markedly increase and MPA to down-regulate the expression of PR. The expression of ERα was only slightly affected by either hormonal treatment. CONCLUSIONS: The present organotypic model provides a novel in vitro system in which to study the effects of steroids in the normal human endometrium both in terms of cell proliferation and gene expression. The culture system holds promise as a useful method to screen novel steroid compounds and may help to circumvent problems related to the use of animal models.

Key words: endometrium/human/Matrigel/organoid/organotypic culture

Introduction

During the normal menstrual cycle, the human endometrium undergoes extensive growth and tissue remodelling in response to ovarian steroid hormones. Follicular estradiol (E2) induces endometrial cell proliferation whereas progesterone from the corpus luteum governs the post-ovulatory phase of cell differentiation and secretory maturation. Together with appropriate hormonal signals, stromal–epithelial interactions are fundamental to normal endometrial growth and function (reviewed in Bigsby, 2002 and Punyadeera et al., 2003). A principal role for the endometrial stroma in mediating the effects of estrogen and progesterone on epithelial cell proliferation has been suggested (Bigsby and Cunha, 1986; Cooke et al., 1997; Kurita et al., 1998; Pierro et al., 2001). That stromal cells are not only involved in epithelial mitogenesis but also modulate the functional differentiation of epithelial cells has been shown in a number of studies (Arnold et al., 2002; Bigsby, 2002; Punyadeera et al., 2003). Reciprocal signals from uterine epithelium to the underlying stroma further emphasize the complex nature of regulatory interactions in endometrial tissue (Bigsby, 2002).

Numerous cell culture models have been designed with an aim to experimentally address questions related to the various cellular and molecular factors underlying human endometrial function and dysfunction. As proliferative disorders (hyperplasia, cancer) of the human endometrium have been linked to hyperestrogenic states and to unopposed estrogen replacement therapy (Rose, 1996), methods to investigate steroid effects on epithelial proliferation in vitro are of particular clinical relevance. In view of the above it is evident that any culture model intended to mimic normal endometrial responses should include both the epithelial and stromal compartments of the tissue.

Explant cultures represent an in vitro model in which tissue integrity is well preserved. Such cultures readily lend themselves to biochemical and histochemical examination.
and have been used to study, for example, progestin effects (Illouz et al., 2003) and tamoxifen biotransformation (Sharma et al., 2003) in human endometrial tissue. The separate contributions of epithelial and stromal cells cannot, however, be directly determined in this type of culture. Moreover, tissue explants are usually unable to maintain their histological structure intact but for a relatively short time in vitro. The assessment of proliferative responses by immunohistochemical means in endometrial explant culture has also proven problematic (Illouz et al., 2003). In order to study stromal–epithelial interactions in the human endometrium, more elaborate culture conditions recombining separated stromal and epithelial cells in different extracellular matrices have been employed (Bentin-Ley et al., 1994; Arnold et al., 2001; Pierro et al., 2001). In these models, epithelial cells have been cultivated as monolayers on top of reconstituted basement membrane matrix (Matrigel) and stromal cells beneath them on plastic or embedded in collagen or Matrigel. Even in this type of organotypic model, in vitro-like proliferative responses to steroid hormones have been rarely documented (Pierro et al., 2001).

Because of the paucity of in vitro models for the human endometrium able to simulate steroid hormonal responses, we set out to develop and validate culture conditions for this purpose. Here, we describe an organotypic model in which epithelial cells of the normal human endometrium are cultivated as glandular organoids within Matrigel matrix in co-culture with stromal cells seeded on plastic. We report in vivo-like effects of E2 and medroxyprogesterone acetate (MPA) on epithelial cell proliferation in this system. The concomitant expression and regulation of estrogen receptor (ER) and progesterone receptor (PR) is also evaluated.

**Materials and methods**

**Tissues**

Endometrial specimens were obtained from pre-menopausal women undergoing hysterectomy for benign uterine leiomyomata. None of the patients had received any hormonal therapy for ≥6 months prior to operation. Tissue biopsies from the uterine cavity were taken with a suction cannula before surgery. For transport to the laboratory, the tissue specimens were placed in ice-cold Dulbecco’s modified Eagle’s medium (DMEM/F12 medium (Gibco BRL, UK) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin (penicillin/streptomycin solution, Gibco BRL) and 2.5 mg/ml amphotericin B (Sigma, USA). Fourteen samples from women with a mean age of 42 years (range 38–48) were processed for validation purposes. Here, we set out to develop and validate culture conditions for this purpose. However, we describe an organotypic model in which epithelial cells of the normal human endometrium are cultivated as glandular organoids within Matrigel matrix in co-culture with stromal cells seeded on plastic. We report in vivo-like effects of E2 and medroxyprogesterone acetate (MPA) on epithelial cell proliferation in this system. The concomitant expression and regulation of estrogen receptor (ER) and progesterone receptor (PR) is also evaluated.

**Isolation of epithelial organoids and stromal cells**

Endometrial glands and stromal cells were isolated by a modification of the method described by Laird et al. (1997). To remove blood and debris the tissue was first rinsed in culture medium consisting of phenol red-free DMEM/F12 (Gibco BRL) supplemented with 2% dextran-coated, charcoal-treated fetal calf serum (Gibco) and antibiotic/antimycotic agents as described above. The tissue was then minced finely with a sterile scalpel and after rinsing and removal of the medium by centrifugation at 50 g for 1 min, the tissue pieces were incubated in culture medium containing 0.1% collagenase A (Roche, Germany) for 1 h at 37°C. To aid digestion, the tissue was gently pipetted several times during the incubation period. The digest was thereafter passed through a wire sieve to remove any undigested material and centrifuged at 250 g for 2 min. After resuspending the pellet in culture medium, glandular structures were separated from stromal cells by centrifugation at 50 g for 1 min. The red layer, mainly of vascular organoids on top of the white glandular sediment, was carefully removed by pipetting. The supernatant was collected and further centrifuged at 250 g for 2 min to pellet the stromal cells. The stromal pellet was freed of red blood cells by exposing it to 1 ml distilled water for not longer than 30 s, after which time 9 ml culture medium was added and the stromal cells were repelleted at 250 g. Further purification of the isolated glandular and stromal cells was done by gently pipetting the fractions, both suspended in 1 ml culture medium, onto 9 ml medium each and letting them sediment at unit gravity for 5 (epithelial glands) or 30 min (stromal cells). The procedure was repeated once or twice for organoids. Stromal cells were harvested from the top 9 ml and used for cell culture. The viability of the stromal cells thus obtained was between 80 and 95% as assessed by Trypan Blue exclusion. Only the sedimented epithelial glands were collected for organoid culture.

**Organoid culture**

Ten organoid/stromal cell co-cultures and in three cases parallel organoid cultures without stromal cells were prepared from freshly isolated epithelial organoids and stromal cells (Figure 1). The co-cultures always consisted of organoids and stromal cells from the same individual.

The organoids were rinsed with serum-free medium and suspended in undiluted Matrigel basement membrane matrix (BD Biosciences, USA). Aliquots of 200 ml suspension were pipetted into pre-cooled tissue culture inserts (3 µm pore size, 10 mm diameter; Nunc, Denmark) and allowed to gel for 30 min at 37°C. To prevent the organoids from sedimenting on the insert filter, Matrigel with sufficiently high protein content (≥ 10 mg/ml) was chosen. Epithelial cells growing on the filter exhibited uncontrolled growth characteristics and were excluded from this study. Depending on the size of the sample, four or eight inserts were prepared from each tissue specimen. Stromal cells were plated into four wells of a 24-well tissue culture plate at a density of 30 000 viable cells/well in a culture specimen. Stromal cells were plated into four wells of a 24-well tissue culture plate at a density of 30 000 viable cells/well in a volume of 500 µl culture medium. After gelling, the Matrigel-coated inserts were transferred into the wells containing stromal cells and 200 µl medium was added into the inserts. When sample size allowed, a parallel set of four inserts was prepared without stromal cells.

The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. After 2 days, the culture medium was renewed and after another 24 h the cultures were subjected to hormonal treatment. One insert of each set was removed and fixed in 4% paraformaldehyde to serve as the day 0 control. The other three were cultured for a further 7 days with 10 nmol/l 17β-E2 (Sigma) or 10 nmol/l E2 together with 100 nmol/l MPA (Sigma); the day 7 control insert received a similar amount of 100% ethanol used to dissolve the steroids. The medium was renewed every 2 days and fresh steroids were added. Seven days after treatment with hormones,
the inserts were collected, fixed in 4% paraformaldehyde overnight and thereafter immersed in 70% ethanol. Under a stereomicroscope, the insert membrane was partially detached from its housing with an injection needle and the fixed Matrigel bed was carefully detached from the membrane. The gel was cut with a razor blade into four pieces, which were then closed into an embedding cassette and automatically infiltrated with paraffin (Tissue-Tek V.I.P.; Miles Inc., USA). The gel pieces were thereafter manually aligned with their cut sides up in a base mould and embedded in paraffin.

Immunohistochemistry

Immunohistochemical analysis was used to characterize and validate the culture method. Staining was performed using the broad spectrum Histostain Plus kit from Zymed (South San Francisco, USA). Deparaffinized and rehydrated sections were first immersed for 10 min in methanol with 0.5% H₂O₂ to inactivate endogenous peroxidases. Subsequently, the sections were boiled in a microwave oven (1000 W) in 0.01 mol/l citrate buffer (pH 6.0) for 10 min followed by incubation in the same buffer for a further 20 min. After rinsing in Tris-buffered saline, blocking reagent was applied for 20 min at room temperature. Sections were then incubated with primary antibodies overnight at 4°C. The antibodies were diluted in phosphate-buffered saline (PBS) containing 0.5% BSA and 0.01% Tween. The following monoclonal anti-human antibodies were applied at the dilutions indicated: anti-ER (1:100) and PR (1:300) (NovoCastra, UK), anti-cytokeratin 18 (1:500) and anti-vimentin (1:500) (Dako, Denmark), anti-E-cadherin (1:1000; Santa Cruz) and anti-Ki67 (1:800; Roche, Germany). A polyclonal rabbit anti-human von Willebrand factor was used (1:5000; Sigma). After extensive rinsing in PBS, biotinylated second antibody was applied for 20 min at room temperature. After rinsing, the sections were incubated with enzyme conjugate for 10 min at room temperature. After a further wash step, the sections were covered with 3,3-diaminobenzidine chromogen for 10 min. The sections were counterstained with haematoxylin. Controls were performed by replacing primary antibodies with PBS or normal mouse (2.5 mg/ml) or rabbit (2 mg/ml) immunoglobulin G.

Ki67 labelling index and statistical analysis

The percentage of Ki67 expression (the number of stained cells divided by the total number of cells) was calculated in representative 4 mm sections. All intact organoids in each section were counted. Sections from different levels of each sample were analysed to attain ≈500 counted cells in ≈20 individual organoids per sample. The percentage of Ki-positive organoids (bearing at least one Ki-positive cell) was also calculated. The means ± SEM were calculated for each treatment and statistical differences between means were assessed using the Wilcoxon signed rank test. Correlation was considered statistically significant if P < 0.05.

Assessment of stromal cell proliferation

In six cases, stromal cells from each hormonal treatment were trypsinized at day 7 and their number was counted using a Bürker chamber. Statistical analysis was performed as above.

Results

Establishment of the culture system

A novel organotypic culture model was developed for studying the hormonal responses of normal human endometrial epithelial cells in vitro (Figure 1a). The overall procedure was optimized with regard to methods of cell separation, medium constituents and the volume of Matrigel.

Isolation and purification of the two cellular compartments from primary tissues was done by selective centrifugation and sedimentation. Epithelial organoids with a minimum of contaminating vascular fragments were obtained by careful removal of vascular elements from the top of the first organoid pellet and by reducing the time for organoid sedimentation to 5 min. Cellular purity of the epithelial compartment was assessed by immunohistochemical analysis. All organoids were shown to express the epithelial cell markers cytokeratin 18 and E-cadherin, with no detectable changes in staining intensity during cultivation (Figure 2). Occasional strands of endothelial cells positive for von Willebrand factor were found in some samples together with adhering fibroblasts
As previously reported (Marshburn et al., 1992; Matthews et al., 1992; Nisolle et al., 1995; Classen-Linke et al., 1997), anti-vimentin staining was inconsistently positive also in epithelial cells and was, therefore, used in parallel with anti-cytokeratin 18 to reveal contaminating fibroblasts. In this manner, only a few vimentin-positive but cytokeratin 18-negative spindle-shaped cells or bundles thereof were detected in each sample (data not shown).

Prior to hormonal challenge, the cultures were incubated for 3 days in plain culture medium. During this adaptation period the isolated epithelial fragments reorganized into gland-like arrangements of different sizes (Figure 1b,c). Cell death was also involved as demonstrated by cell debris and apoptotic cells within some epithelial spheres (data not shown). Hormonal treatment appeared not to affect cytokeratin 18 or E-cadherin expression in the cultivated organoids. Four samples were rejected because of delayed gland formation and/or abnormal growth characteristics with long extensions protruding from the organoids.

Cell proliferation in cultured organoids

The Ki67 nuclear antigen was used as a marker to immunohistochemically determine the fraction of proliferating epithelial cells in each sample (Figures 3 and 5a–d). After 3 days in culture, at the time of hormone supplementation (day 0), the Ki67 index in epithelial organoids was 6.2 ± 2.4 (mean ± SEM), the control values after 10 days of culture (day 7 of hormone treatment) being somewhat lower, with a mean value of 4.6 ± 0.8. There was individual variation in the percentage of proliferating cells in cultured organoids. The percentages in untreated controls (at day 7) varied between 1.8 and 9.3 with no apparent correlation with menstrual cycle phase.

A significant ($P = 0.005$) increase in the relative number of proliferating epithelial cells was detected in co-cultures treated with E$_2$ (10 nmol/l) (Figure 3a). When E$_2$ was added together with MPA (100 nmol/l), cell proliferation was minimal, the percentage of Ki67-positive cells being below that of controls in most samples ($P = 0.005$). Similar results were
obtained by calculating the percentage of organoids showing one or several Ki67-positive cells in their cross-section (Figure 3b) \( (P = 0.005 \text{ and } P < 0.01 \) for samples treated with \( E_2 \) and \( E_2 + \text{MPA} \), respectively, as compared with controls). No substantial differences in hormonal responses were detected between organoids obtained from proliferative and secretory endometrium.

In three samples, the hormonal effects were compared in parallel organoid cultures with or without stromal cells (Figure 4). Whereas in co-cultures \( E_2 \) was shown to increase and MPA to decrease the Ki67 index, in the absence of stromal cells, both hormonal treatments resulted in similar low Ki67 indices, which were below the control value. Due to the limited number of cases, the differences did not reach statistical significance.

Stromal cell proliferation was not significantly affected by either hormonal treatment (data not shown).

**Regulation of ER and PR in cultured epithelial organoids**

Immunohistochemical analysis was used to study the regulation of ER and PR by \( E_2 \) and MPA in cultured endometrial organoids. Apart from minor differences in staining intensity in some samples, ER expression resembled the *in vivo* situation throughout the experiment with no apparent regulation with either hormonal treatment (Figure 5f–h). PR expression, on the other hand, declined rapidly compared with that in non-cultured tissue, being markedly reduced or undetectable after the first 3 days of culture. After 10 days of culture, PR expression was no longer detected in any of the untreated controls (Figure 5j). PR expression was markedly increased under \( E_2 \) treatment in all samples studied (Figure 5k). MPA counteracted the PR-inducing effect of \( E_2 \), the PR expression being undetectable in organoids under simultaneous treatment with \( E_2 \) and MPA (Figure 5l). The expression and regulation of ER and PR was similar in the presence and absence of stromal cells (data not shown).

**Discussion**

We have established a novel organotypic model for the human endometrium in which primary epithelial cells retain their steroid hormone responsiveness. In this model, \( E_2 \) was shown to increase and MPA to decrease epithelial cell proliferation. The two steroids also regulated PR protein expression in an *in vivo*-like manner.
Contacts with the extracellular matrix and with neighbouring cells are essential for epithelial cells to display differentiated morphology and function in vivo and in vitro. The present model was designed to mimic the in vivo epithelial organization by providing an extracellular matrix for epithelial organoids to grow in glandular arrangements with (or without) the inclusion of a separate compartment of stromal cells. Reconstituted basement membrane extract (Matrigel) has been widely used to grow epithelial cells in vitro and was chosen also here to serve as the supportive matrix for the organoids. Due to its protein composition, including the two main components of natural basement membranes, laminin and type IV collagen, Matrigel is able to promote epithelial cell attachment and differentiation in vitro. In previous studies, monolayers of endometrial epithelial cells on Matrigel have been shown to become polarized (Schatz et al., 1990; Bentin-Ley et al., 1994; Classen-Linke et al., 1997) and to form glandular structures within the gel (Rinehart et al., 1988; White et al., 1990). On bare cell culture plastic, on the other hand, primary endometrial epithelial cells tend to lose their epithelial phenotype and grow in whorl-like patterns with a loss of lateral junctions (Schatz et al., 1990; Arnold et al., 2001).

Instead of growing dissociated epithelial cells on Matrigel surface as in previous organotypic models (Bentin-Ley et al., 1994; Arnold et al., 2001; Pierro et al., 2001), we embedded fragments of epithelium directly inside the matrix (Hopfer et al., 1994), an approach previously successfully used to grow human mammary gland epithelial organoids in vivo (Parmar et al., 2002). With intact cell–cell contacts, most organoids readily adapted to the culture environment and glandular structures could be detected within 24 h. The organoids maintained their glandular morphology and epithelial phenotype throughout the experimental period as indicated by their in vivo-like expression of cytokeratin 18 and E-cadherin, whose immunohistochemical expression has been shown to decrease with dedifferentiation in many tissues, including the human endometrium (Pötter et al., 1999).

Besides structural integrity, the organoids retained their ability to respond to hormonal cues under the optimized culture conditions. No substantial differences in hormonal responses between organoids from different phases of the menstrual cycle were detected. It appears that when brought into standardized culture conditions with a 3 day hormone-free period, the cells retain their inherent capacity to respond to steroid hormones regardless of their original hormonal milieu in vivo. Previously, comparable changes in the proliferation of epithelial cells isolated from proliferative and secretory epithelium in response to stromal cells have been described (Arnold et al., 2001).

Although endometrial epithelial cells respond to follicular E2 by a marked increase in cell proliferation in vitro, their estrogen responsiveness has been difficult to reproduce in vivo. The results from monocultures of uterine epithelial cells, both rodent and human, imply that E2 may not have a direct mitogenic effect on these cells (Alkhalaf et al., 1991; Uchima et al., 1991; Marshburn et al., 1992; Classen-Linke et al., 1997, 1998). That E2-induced epithelial mitogenesis in the female reproductive tract is rather an indirect process mediated by stromal ERα has been proposed by Cooke et al.
A paracrine mode of action for progestins in uterine tissue has also been suggested (Kurita et al., 1998). Comparison of the three mono- and co-cultures in the present study appears to be in agreement with the above findings. The limited number of replicates and lack of statistical significance do not, however, allow any definite conclusions to be made as to the significance of the stromal compartment to the proliferative effects observed in epithelial organoids. Moreover, the presence of steroid receptors in the stromal compartment, a prerequisite for stromal-mediated effects, was not investigated in the present study. In co-cultures, E2 doubled the number of Ki67-positive cells relative to controls. A somewhat smaller but statistically significant effect was observed when the relative number of Ki67-positive organoids was calculated, providing a convenient means to assess proliferative hormone effects in the present model. MPA was shown not only to counteract the effect of E2, but also to reduce cell proliferation to half of that in controls. As for E2 effects, our findings are in agreement with those of Pierro et al. (2001) who used co-cultures of epithelial cell monolayers on Matrigel-coated inserts and stromal cells on plastic to study estrogen responsiveness in normal human endometrium. Conversely, in the three organoid monocultures analysed in the present study, E2 both alone and together with MPA decreased the Ki67 index by approximately half. A similar phenomenon has previously been reported in rodent epithelial monocultures (Astrahantseff and Morris, 1994), whereas in the human model of Pierro et al. (2001) no marked change compared to controls was detected. The difference between the two human models may reflect differences in their respective culture environments but, because of the limitations associated with the present monoculture data, the significance of these observations remains unclear.

The effect of stromal-produced factors on epithelial cell proliferation has also been shown in cultures without hormonal stimuli. Stromal cell-conditioned medium has been reported to increase the replication of human endometrial epithelial cells in monolayer cultures (Akoum et al., 1996). Also Pierro et al. (2001) showed an enhanced epithelial proliferation in control co-cultures as compared with parallel epithelial monocultures and the same trend was seen, albeit less reproducibly, in the present study. In the study of Arnold et al. (2001), on the other hand, stromal cells were reported to inhibit epithelial cell growth especially when embedded in Matrigel in contact co-culture. Thus, it appears likely that proteins of the basement membrane may modulate the regulatory capacity of adhering stromal cells. Considering the potential impact of Matrigel on stromal cell function in our culture conditions, adequate cellular purity of the epithelial compartment is important to accomplish.

The expression of ER and PR in the human endometrial epithelium changes characteristically during the normal endometrial cycle, being maximal in the proliferative phase and diminishing in the mid-secretory phase (Snijders et al., 1992; Noe et al., 1999; Vienonen et al., 2004). In agreement with the known effects of estrogen and progestins on PR expression, E2 was found to induce and MPA to down-regulate PR expression in the present organoid culture. Previously, hormonal regulation of PR has been reported in human primary endometrial epithelial cells cultured on top of Matrigel (Classen-Linke et al., 1997, 1998) and in explant cultures of the human proliferative endometrium (Illouz et al., 2003). In both these models, MPA was shown to decrease not only PR but also ER expression. Also, different types of progestins have been shown to have different in vitro effects. (Illouz et al., 2003). With regard to ERα, only minor changes in the staining intensity of positive cells could be detected in our study after steroid treatment. The high level of ERα detected in epithelial organoids without hormonal stimuli resembles their constitutive expression in the post-menopausal uterus (Snijders et al., 1992; Noe et al., 1999).

In conclusion, most of our current knowledge concerning the effects of steroids on the human endometrium derives from clinical data, and in vitro methods allowing experimentation on human endometrial tissue have been limited. The organotypic model presented here provides an in vitro setting in which to study steroid hormone effects on the normal human endometrium both in terms of cell proliferation and gene expression. The model facilitates the manipulation of the culture environment and may help to elucidate the molecular basis of the effects of steroids and other biological agents in human endometrial cells, thereby obviating the need of extrapolation from animal models. The culture system may have potential uses in preclinical screening of hormones for therapeutic use.

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