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Endometrial stromal cells regulate epithelial cell growth *in vitro*: a new co-culture model

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The regulation of epithelial cell function and morphogenesis by the paracrine effectors from the mesenchyme or stroma has been well established using in-vivo studies. A more complete understanding of these relationships has been delayed due, in part, to a lack of appropriate co-culture models. In this study, we describe a co-culture model which demonstrates that normal paracrine relationships can be reconstituted *in vitro* and that human endometrial stromal cells regulate both growth and differentiation of primary human endometrial epithelial cells. Interesting differences in the proliferation of stromal and epithelial cells were noted in response to the basement membrane extract, Matrigel®. Exposure of stromal cells to Matrigel® enhanced the paracrine capacity of these cells *in vitro*. When epithelial cells were co-cultured in contact with stromal cells embedded in Matrigel®, epithelial cell growth was inhibited by 65–80% compared to controls. Stromal cells in contact with Matrigel® also regulated epithelial cell differentiation, as shown by induction of glycodelin expression. These co-culture studies show great promise as a method to investigate the cellular interactions between endometrial stromal and epithelial cells and their environment and to understand the molecular basis for the regulation of normal growth and differentiation of cells within complex tissues such as the endometrium.

Key words: co-culture/endometrium/epithelium/glycodelin/stroma

Introduction

Mesenchymal cells play a crucial role in directing the development, growth, differentiation, and functioning of the overlying epithelium (Donjacour and Cunha, 1991). As progenitors of connective tissue fibroblasts and stromal cells, the mesenchyme interacts with the endodermal and ectodermal tissue layers that are present during organ formation. These interactions persist well beyond embryonic development and it is now clear that such paracrine interactions are critical in the regulation of proliferation and differentiation of adult tissues as well (Cunha *et al*., 1985; Bigsby and Cunha, 1986).

In the female reproductive tract, stroma can promote epithelial development or reprogramme epithelial differentiation (Cooke *et al*., 1986; Bigsby and Cunha, 1986; Donjacour and Cunha, 1991). The endometrium is composed of mesodermal-derived glandular and luminal epithelia that are supported by a basement membrane and connective tissue stroma. Basement membrane likely plays a key role in promoting an epithelial phenotype (Classen-Linke *et al*., 1997). In addition, stromal cells provide a regulatory role for growth and differentiation of the overlying epithelium (Cunha *et al*., 1985). Mesenchymal regulation of epithelium is especially evident in the cycling endometrium, which undergoes monthly developmental changes in response to ovarian steroids. It now appears that steroid receptors in the stromal cells, but not the epithelium, may be required for the action of oestradiol and progesterone (Cooke *et al*., 1997; Kurita *et al*., 1998), demonstrating the paracrine role for the stromal cells in endometrial function.

The primary objective of the present work was to demonstrate that human stromal cells regulate normal, non-neoplastic human epithelial growth and differentiation *in vitro*. While this concept has been supported in animal models (Chung *et al*., 1992; Cunha and Young, 1992; Cooke *et al*., 1997), there have been few if any successful attempts demonstrating this regulatory role of stroma using in-vitro human cell model systems. The advantages of studying such interactions *in vitro* include: (i) the ability to manipulate cells in a defined environment and to retrieve and study specific cell types, (ii) to identify novel growth or inhibitory paracrine factors involved in regulation of growth, (iii) better to reproduce cell behaviour *in situ* and provide a physiological basis for future studies of molecular and genetic mechanisms of disease, and (iv) by using human cells the mechanisms of cell–cell and cell–matrix interactions involved in human disease can be dissected, avoiding extrapolation of data from studies using other species.

The use of a novel configuration of epithelial and stroma within appropriate extracellular matrices provides new insights into the paracrine role of stroma in directing epithelial cell function.

Materials and methods

Human tissues

Endometrial biopsies were obtained from reproductive age fertile women (ages 20–40 years) with normal menstrual cycles who were undergoing bilateral tubal ligation. Use of endometrial tissue was approved by the Committee for the Protection of Rights of Human Subjects at the University of North Carolina. Tissue samples (1–2 g) were obtained from the proliferative (days 5–14) and secretory phase (days 15–26) from women who received no hormonal therapy within the prior 30 days. Categorization was correlated with histological dating criteria of Noyes *et al*. (Noyes *et al.*, 1950). Endometrial tissue was transported to the laboratory in isolation media consisting of DMEM-H culture media containing high glucose (Gibco Life Technologies, Gaithersburg, MD, USA) and 5% fetal bovine serum (FBS) (lot 14–501F, BioWhittaker, Walkersville, MD, USA) plus ×2 antibiotic and antimycotic agents to yield final concentrations of 200 units penicillin, 0.2 mg streptomycin and 0.5 μg amphotericin-B per ml (antibiotic/antimycotic solution, Sigma, St Louis, MO, USA).

Isolation of primary endometrial epithelial and stromal cells

Endometrial tissue was rinsed in Hanks' balanced salt solution (HBSS) to remove blood and debris. Separation of glandular and stromal components was based on a modification of the work of Satyaswaroop and colleagues (Satyaswaroop *et al.*, 1979). Following gentle centrifugation (600 *g*) the supernatant was removed and the tissue was placed on a 100 mm plastic tissue culture dish (Corning-Costar, Cambridge, MA, USA) under a sterile laminar flow hood. The tissue was minced with sterile scalpels into 1 mm^2 fragments and then digested with collagenase (2 mg/ml, CLS-1, Worthington Biomedical, Freehold, NJ, USA) in isolation medium (as above) for 2.5 h at 37°C on a shaking rotor. The tissue digest was vigorously pipetted to break up any remaining tissue pieces and passed over a stacked sterile wire sieve assembly with number 100 wire cloth sieve (140 μm size, Newark Wire Co., Newark, NJ, USA), followed by a number 400 wire cloth sieve (37 μm) as shown in Figure 1.

After the endometrial digest was added to the top of the sieve assembly, the epithelial glands were retained in the number 100 and number 400 sieves while the stromal cells passed through to the receptacle below. The glands were rinsed with a total of 50 ml of isolation medium before being back-flushed out of the sieves onto a 100 mm sterile dish using the same medium. Any stromal cells remaining with the glands were further separated by selective adherence to plastic tissue culture dishes for 1 h. All cultures were maintained at in 37°C humidified atmosphere of 5% CO_2 in air. Glandular structures were subsequently moved to 100 mm plastic culture dishes coated with 0.5 ml of a 1:8 dilution of basement membrane extract (BME) (Matrigel®, Collaborative Biomedical Products, Bedford, MA, USA) in sterile phosphate buffered saline (PBS) which had been allowed to dry and was rinsed with culture media prior to plating cells. Culture on Matrigel® films allowed expansion of glandular epithelial outgrowths, which were allowed to proliferate for 3–5 days in isolation medium before subsequent co-culture as described below.

Stromal cells were collected from the lower receptacle, pelleted by centrifugation and resuspended in 3 ml of isolation medium. Red blood cells were removed by carefully layering cell suspension over 3 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ, USA) in a sterile 15 ml polycarbonate tube. The solution was centrifuged for 8–10 min at 400 *g*. The media/Ficoll interface layer containing the stromal cells was plated onto 100 mm plastic tissue culture dishes. Stromal cells were cultured for at least 3 days before being plated for co-culture experiments, as described below. Epithelial cells grew 3–5 days before co-culture, to insure no stromal cell contamination. Purity of epithelial and stromal components was assessed by morphological determination by light microscopy and reassessed by cytokeratin and vimentin staining for epithelial and stromal cells respectively. Each cell population was routinely over 98% pure as assessed by phase microscopy. In parallel studies with primary stromal and epithelial cultures both stromal and epithelial cells were found to respond to oestrogen by increased growth and [^{3}H]-thymidine uptake, but these data are beyond the scope of the present paper.

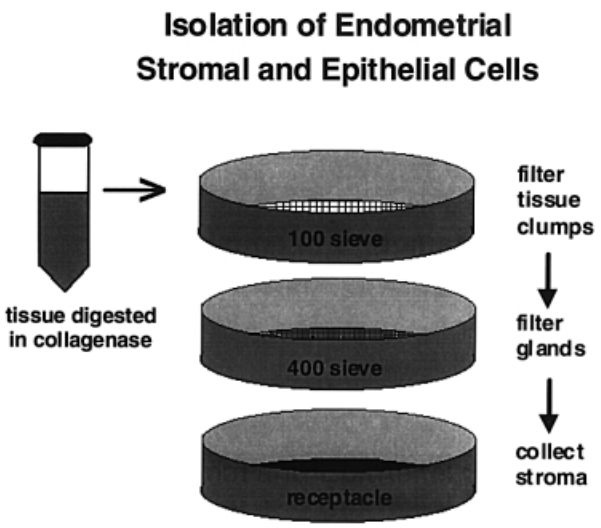

Figure 1. Schematic showing the strategy for isolation of endometrial stromal and epithelial components from primary tissues. This technique of using collagenase followed by sequential sieves to isolate glandular epithelium and stroma is based on the work of Satyaswaroop and colleagues (Satyaswaroop *et al*., 1979). Digested endometrium passed over the sieves effectively separates glandular structures from the individual stromal cells and red blood cells.

Cell culture media

Stromal cells were maintained in medium consisting of a 1:1 mixture of M199:Ham's F12 media (Gibco Life Technologies) supplemented with 4% heat-inactivated FBS, ITS+ [containing insulin (0.62 μg/ml), transferrin (0.62 μg/ml), and selenium (0.62 ng/ml), bovine serum albumin (125 μg/ml) and linoleic acid (52.6 μg/ml) (Collaborative Biomedical Products)] plus 100 units penicillin, 0.1 mg streptomycin and 0.25 μg amphotericin B per ml (antibiotic/antimycotic solution, Sigma). FBS was heat inactivated by incubating in a 56°C water bath for 1 h prior to filter sterilization. Culture medium was routinely changed every 3–4 days. Charcoal-stripped serum was used to remove steroids from culture media.

The primary epithelial gland cells required little or no serum and were cultured in media consisting of M199 and F12 (1:1) with added Mitoplus® (2 ml/l) (Collaborative Biomedical Products), bovine pituitary extract (BPE; 2ml/l) (Collaborative Biomedical Products), ITS+ (concentrations as above), and antibiotic and antimycotic agents as described above.

For co-culture, stromal and epithelial cells were grown in media containing Phenol Red-free M199 and F12 media (1:1) (Gibco) with

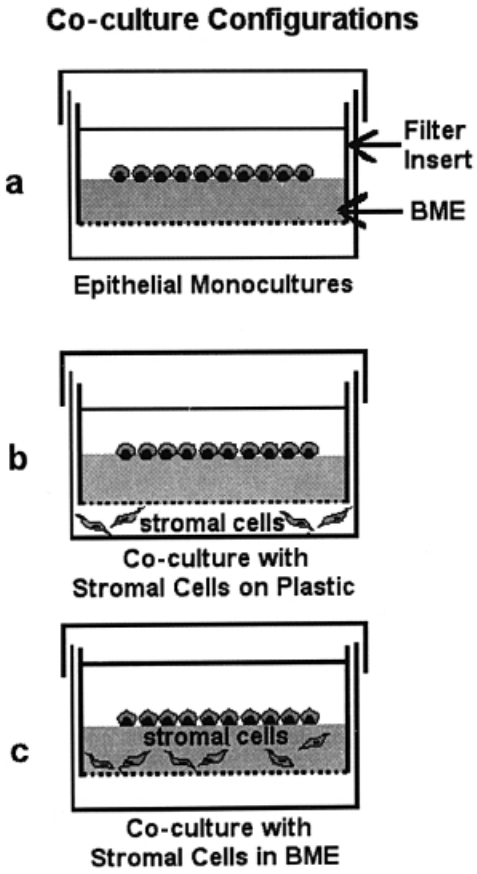

Figure 2. Monoculture and co-culture configurations used to demonstrate paracrine and extracellular matrix mediated effects of stromal cells. Basement membrane extract (BME) alone was used to culture glandular epithelial cells in monoculture (**A**). Addition of stromal cells, cultured on plastic, below the filter insert containing epithelial cells on BME–Matrigel® is shown in **B**. Finally, stromal cells were cultured in direct contact with BME–Matrigel® within the filter insert beneath endometrial epithelium plated on top (**C**). These three conditions were then compared on the basis of cell proliferation and differentiated function.

ITS+ [(insulin (3.1 μg/ml), transferrin (3.1 μg/ml), and selenium (3.1 ng/ml), bovine serum albumin (625 μg/ml) and linoleic acid (263 μg/ml)] plus 0.1 mmol/l phospho-ethanolamine (Sigma) and 1% charcoal-stripped FBS.

Co-culture of stromal and epithelial cells

For purposes of co-culture, cells were plated either on plastic or on Millicell® CM filter inserts (Millipore, Bedford, MA, USA) containing Matrigel®. Endometrial epithelium was cultured either alone or in co-culture with stromal cells. Prior to plating, BME was kept at 4°C and was added undiluted to pre-cooled MilliCell® CM filter inserts (0.4 μm, 12 mm diameter).

Stromal and epithelial cell co-cultures were prepared from freshly isolated primary epithelial and early passage stromal cells (passage 1–4). Frozen cells were not used as new primary cell lots were prepared for each co-culture. Co-cultures mostly consisted of stromal and epithelial cells from the same patient. Typically, stromal cells were plated at 10^5 cells per well of a 24 well plate and all conditions were performed in triplicate. Stromal cells were either grown within the Matrigel® on the filter insert or beneath the filter insert on the plastic culture dish. These culture conditions are depicted in Figure 2, comparing epithelial monoculture (a) to co-culture with stromal cells on plastic below the filter insert (b) or embedded in Matrigel® within the filter insert (c). When suspended within the BME, stromal cells were pooled, pelleted and resuspended in the total volume of undiluted Matrigel® at 4°C (liquid phase). Aliquots of 100 μl Matrigel® plus 10^5 stromal cells per well were added to the cold inserts, spread evenly over the inserts and allowed to gel at 37°C for 1 h. Parallel inserts were prepared for epithelial monocultures using the undiluted Matrigel® in the absence of added stromal cells. Culture medium was gently added over the Matrigel® coated inserts at 1 ml/well after 1 h at 37°C (gel phase).

Immunohistochemistry for cytoskeletal proteins

Stromal and epithelial cells in co-culture were identified and visualized by cell specific staining of cytoskeletal proteins. Primary antibodies to cytokeratin (rabbit anti-human number NCL-5D3, Novocastra, Newcastle upon Tyne, UK), or vimentin (mouse anti-human, number M0725, Dako Corp., Carpinteria, CA, USA) were used to examine epithelial (Texas Red secondary fluorescent) and stromal (FITC-green) specific markers respectively. Nuclei were labelled an immuno-fluorescent blue by 4′6-diamidino-2-phenylindole (DAPI) (Sigma). Cultures or tissue sections were rinsed with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. They were then treated with ice cold 100% methanol for 10 min, then 100% acetone for 1 min, then 0.4% Triton X-100 in PBS for 10 min, with triple PBS rinses for 5 min each between treatments. Samples were treated with 2% horse serum and 2% goat serum in PBS containing 4% BSA (PBS/BSA) for 30 min at 37°C to block non-specific binding of primary antibodies. Antibodies were diluted in PBS/BSA plus 2% horse and 2% goat sera at 1:50 for cytokeratin and 1:100 for vimentin. Controls included samples treated with no primary antibody, but treated with secondary fluorescent antibodies. The primary antibodies were incubated on cells or tissue sections for 45 min at 37°C, then overnight at 4°C. Samples were rinsed three times with PBS. Fluorescent secondary antibodies were added concurrently: fluorescein anti-mouse IgG diluted 1:75, and Texas Red anti-rabbit IgG diluted 1:75 (Vector Labs, Burlingame, CA, USA) in PBS/BSA plus 2% horse and 2% goat sera for 30 min at 37°C. Samples were rinsed three times with PBS and fresh DAPI was added at 0.2 μg/ml PBS for 2 min at room temperature. Slides were rinsed with PBS and coverslips added using an anti-fade mounting medium (courtesy of Department of Pathology and Laboratory Medicine, Microscopy Services Laboratory, UNC). Fluorescence was visualized using a Nikon Microphot FXA fluorescent microscope with triple-band-pass filter cube containing ultraviolet, rhodamine and fluorescein filters (Omega Optical Inc., Brattleboro, VT, USA, part number XF-63).

Cell proliferation assays

Isolated primary cells were plated at 2×10^5 cells per well in six well dishes. Co-cultures were prepared as previously described using filter inserts and Matrigel®. After 7 days in culture, cells were released from the Matrigel® by digesting with a 1:1 mixture of trypsin-EDTA (Gibco Life Technologies) and Matrisperse® (Collaborative Biomedical Products) on ice for 30–60 min. Cell number was quantified using a haemocytometer and/or Coulter cell counter (Coulter Corp., Miami, FL, USA).

DNA synthesis was determined using tritiated thymidine incorporation, as a measure of cell proliferation. Epithelial cells were plated in triplicate in monoculture or co-cultured, as described. After 4 or 7 days of growth, tritiated (^{3}H) thymidine was added in culture media at a final concentration of 2.5 μCi/ml. After 24 h, the medium was removed. Cultures were rinsed twice with HBSS and trichloro-acetic acid (TCA; 5%) was added to each culture well at 4°C for 20 min, to fix cells to the plates. The plates were rinsed three times with 5%

TCA to remove unincorporated radioactive material. The cells were solublized with 0.2 mol/l NaOH, for 30 min at 37°C, before neutralization with 75 mmol/l HCl. The cell solution was added to scintillation vials and counted for 10 s in a Packard Scintillation Counter (Packard, Meriden, CT, USA). Thymidine incorporation was standardized to total cell counts (CPM/cell) or to DNA concentration (fmol/mg DNA). DNA concentrations were determined by isolating DNA from parallel wells using DNAzol (Molecular Research Center, OH, USA). Isolated DNA was quantified by spectrophotometric absorbency at 260 nm.

Glycodelin immunofluorometric assay (IFMA)

To compare indices of differentiated function in mono- and co-cultures of human endometrial epithelium with or without stromal cells, we used the epithelial gene product glycodelin (PP14). Primary endometrial epithelial cells (obtained only from proliferative phase endometrium to avoid pre-existing glycodelin expression) were plated alone or in co-culture with stromal cells either on plastic or in Matrigel® as previously described. Supernatant media were collected on days 2, 5, 9, and 12 at the time of media change and stored at –80°C. Media were assayed for glycodelin using an immunofluorometric assay (Koistinen *et al.*, 1996). The immunofluorometric assay used monoclonal antibodies to glycodelin for coating the microtitre wells and secondary labelling with europium III chelate, as previously described (Rittinen *et al.*, 1989). The sensitivity of the immunofluorometric assay is more than ×25 greater than that of radioimmunoassay and allows detection and accurate quantification of glycodelin in samples undetectable by radioimmunoassay. Experimental samples of 25 µl of cell culture supernatants were added to microtitre plates precoated with antibodies to glycodelin. Plates were incubated overnight at room temperature. Samples were washed and the secondary antibody, europium III chelate, was added to each well and incubated at room temperature for 2 h. The wells were washed and a fluorescence enhancement solution containing 2-naphtoyltrifluoroacetone and tri-n-octylphosphine oxide was added. Fluorescence was measured using a fluorometer. Results are presented as means of triplicate determinations in units of ng glycodelin/µg DNA.

Statistical analysis

Statistical significance of data was tested using analysis of variance (ANOVA) with Scheffé's correction to compare data from multiple conditions. For comparison of two populations, a two-sample Student's *t*-test was used.

Results

The regulation by stromal cells of epithelial cell growth and differentiated function was established using a novel in-vitro cell culture system, and optimized with respect to culture conditions, choice of media, the presence of the BME, Matrigel® and the arrangement of cells within these co-culture configurations. This model system, as presented, provided the opportunity to examine stromal effects on epithelial growth and differentiation that have not previously been reported.

To investigate the contributions of extracellular matrix on epithelial gland–stroma interactions, we initially analysed the growth characteristics of isolated stromal or epithelial cells in monoculture when cultured with Matrigel® as compared with culture on plastic. The effect of the BME substrate on stromal and epithelial cell proliferation after 5 days in culture is depicted in Figure 3. Endometrial epithelial cells displayed less proliferation on plastic surfaces compared to Matrigel® ($P < 0.05$). Conversely, stromal cells exhibited greater proliferation on plastic surfaces as compared to when they were cultured in contact with Matrigel® ($P < 0.05$). The stromal cells remain viable as assessed by Trypan Blue exclusion, yet do not proliferate when embedded in the Matrigel®.

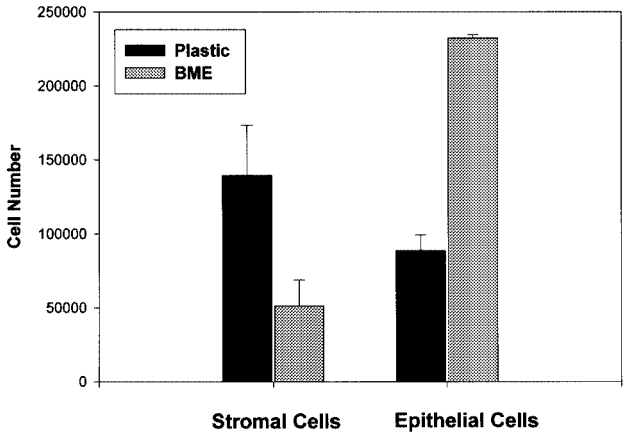

Figure 3. Effect of substrate on cell proliferation of endometrial epithelial and stromal cells. Endometrial stromal were plated at 50 000 cells per well and epithelial cells were plated at 100 000 cells per well on plastic or Matrigel® substrate (BME) and cell counts were determined after 5 days of growth. Stromal cells were proliferative on plastic but showed reduced proliferation on Matrigel® ($P < 0.05$) while epithelial cells grew better on Matrigel® than plastic ($P < 0.05$). Cell number $\times 10^4$. e = epithelial cells.

In addition to differences in rate of growth, the morphology of epithelial and stromal cells was dependent on the substrate provided. On plastic, endometrial stromal cells had a characteristic spindled shape, which progressed to a tight fibroblastic growth pattern at confluence (Figure 4a). When cultured on Matrigel®, stromal cells had a distinct stellate appearance with irregular cell borders (Figure 4b). This appearance is similar to that seen in endometrial tissue sections by immunofluorescence (Figure 4c, immunofluorescent). Stromal cells within the tissue also appear singular and stellate with irregular cell borders. Stromal cells exposed to Matrigel® remained individually distinguishable or grew together to form three-dimensional lattice-like structures.

Epithelial cells cultured on plastic initially maintained a polyhedral or whorled pattern typical of epithelial cells (Figure 4d) (Satyaswaroop *et al.*, 1979; Varma *et al.*, 1982). Culture of primary endometrial epithelial cells was limited to less than 2 weeks when cultured on plastic; cell integrity deteriorated as evidenced by intracellular vacuoles and squamoid morphology. When cultured in contact with Matrigel®, glandular epithelium formed tight complex structures that extended into different planes of the Matrigel® (Figure 4e). These cells assumed three-dimensional spherical and tubular structures (Figure 4f).

Since a distinction between cell types in co-culture was difficult using phase microscopy alone (Figure 5a), epithelial cells were labelled using anti-cytokeratin antibodies and Texas

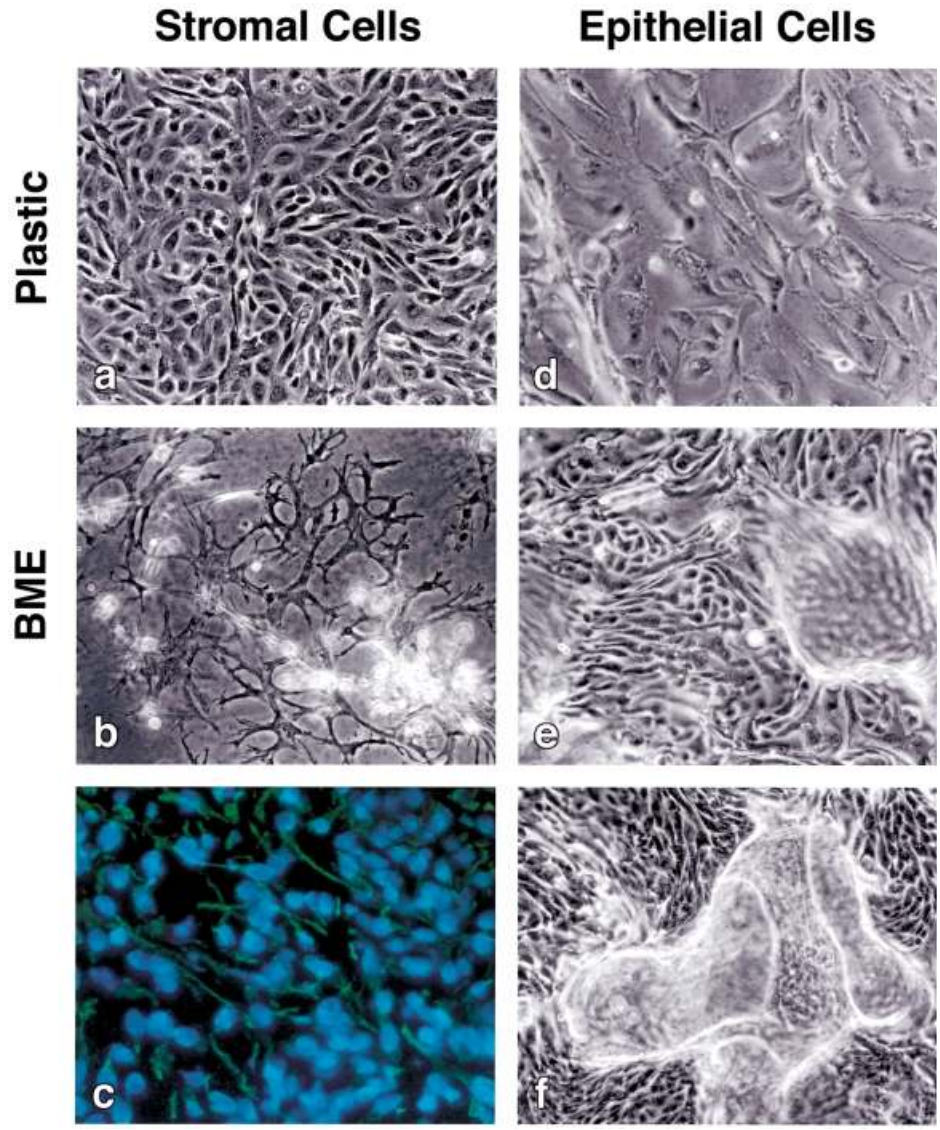

Figure 4. Photomicrographs of endometrial epithelial and stromal cells under different culture conditions. Stromal cells cultured on plastic typically appear spindle shaped and reach confluence within a short period of time (**a**). When cultured in contact with the BME Matrigel®, stromal cells take on a different appearance with an individual stellate pattern and do not appear to proliferate (**b**). This appearance is similar to that seen in endometrial tissues, as shown by immunohistochemical staining for vimentin (green) with DAPI labelling of the nuclei (blue) (**c**). On plastic, epithelial cells derived from the glandular epithelium initially presented a whorled morphology but within 2 weeks cell integrity deteriorated as evidenced by intracellular vacuoles and squamoid morphology (**d**). When cultured on BME–Matrigel®, glandular epithelium formed tight complex structures that extended into different planes of the Matrigel® (**e**). These cells assumed three-dimensional spherical and tubular structures (**f**), not generally seen when cultured on plastic surfaces.

Red fluorochrome and stromal cells with vimentin antibodies and using fluorescein (FITC-green fluorochrome). The nuclei of all cells were stained blue with DAPI. As shown in Figure 5b–f, the dynamic nature of epithelial cell–stromal cell interaction is mediated in part by the presence of this extracellular matrix. As shown in Figure 5b, c and d, epithelial cells (shown in red) formed spherical structures surrounded by stromal cells (green staining) that had adopted a position around the perimeter when cultured on Matrigel®. In cross-section and at higher power this was more evident (Figure 5d). The cytoplasmic projections from single stromal cells sometimes appeared to project towards specific epithelial cells (Figure 5e). When endometrial epithelial and stromal cells were co-cultured on plastic, this higher order of organized association was not apparent (Figure 5g).

Proliferation of epithelial cells isolated from either proliferative or secretory endometrium was examined when cultured in the presence and absence of stromal cells (from proliferative endometrium) and Matrigel®. There were no substantial differences in response to stromal cells between primary cultures obtained from proliferative and secretory endometrium. Cell proliferation of epithelial cells as measured by [^{3}H]-thymidine incorporation decreased by 70% when proliferative phase epithelial cells were co-cultured with stromal cells on Matrigel®, compared to epithelial cells cultured alone (Figure 6, $P < 0.02$). Samples obtained in the secretory phase had a

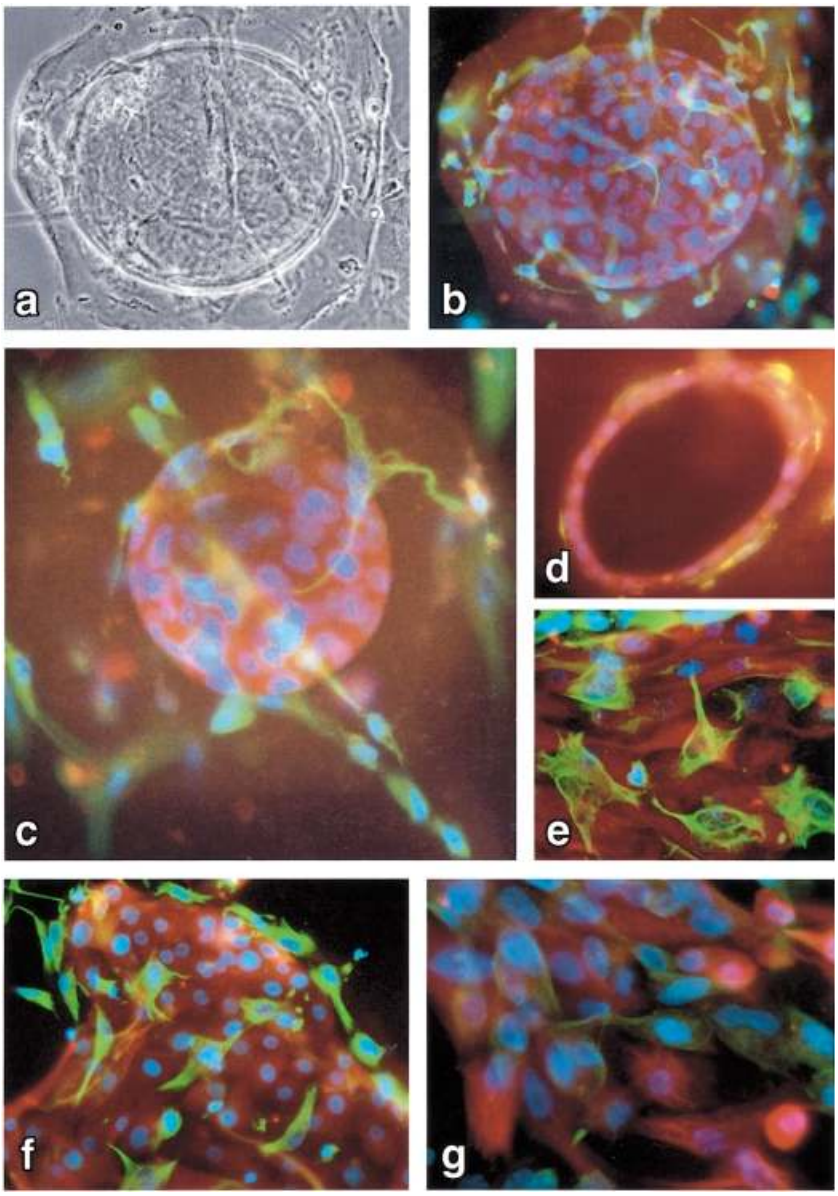

Figure 5. Primary endometrial epithelial and stromal cells were co-cultured in Matrigel® for 10 days. Analysis of co-cultures by phase microscopy (**a** ×400) suggested a complexity of interactions between the cell types but cannot distinguish between cell types. Immunofluorescent staining of the same section (**b** ×400) provides unique view into stromal–epithelial cell interactions. Epithelial cells formed spherical structures in Matrigel® and were stained by immunofluorescence for cytokeratin (Texas Red) (**b** ×400). Stromal cells immunostained for vimentin (FITC-green) appeared to surround the epithelial structures (**f** ×400). Nuclei were stained blue using DAPI. Stromal cells were seen to localize towards the epithelial spherical structures and were seen within a 'halo' of Matrigel® (**c** ×400). A cross-section of the epithelial sphere indicated that a central lumen or cavity was formed with stromal cells (**d** ×400) lining up along the perimeter of the epithelial structure. The cytoplasmic projections from single stromal cells sometimes appeared to project towards specific epithelial cells (**e** ×600). When stromal and epithelial cells were co-cultured on plastic alone, there were no apparent physical interactions (**g** ×400).

comparable reduction in proliferation (80%; $P = 0.047$). Values from epithelial cells in co-culture were obtained by the following formula :

$$\text{epithelial } [^3\text{H}]\text{-thymidine incorporation} = \frac{\text{co-culture CPM} - \text{stromal CPM}}{\text{co-culture cell number} - \text{stromal cell number}}$$

Stromal cells cultured alone in Matrigel® were not proliferative, as described above (Figure 3), thus there was minimal thymidine incorporation noted for stromal cell monoculture that would interfere with the co-culture results.

To evaluate the role of Matrigel® in this apparent stromal-mediated decline in epithelial cell proliferation, epithelial cells were plated alone, or with stromal cells plated beneath the filter insert, on plastic or within the Matrigel® as shown in Figure 7. In these studies, epithelial cell [^{3}H]thymidine incorporation was decreased by 33% when stromal co-culture

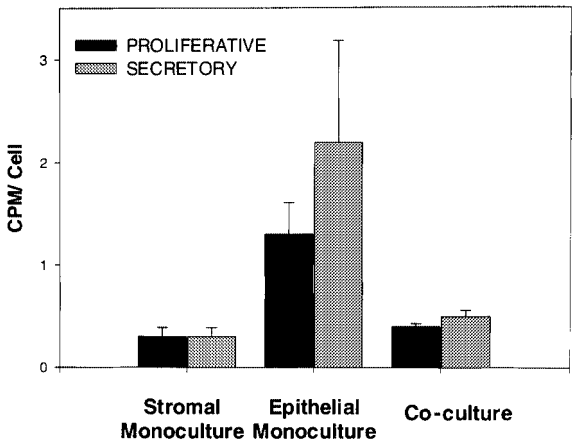

Figure 6. Epithelial cell proliferation is inhibited by the presence of stromal cells and basement membrane extract (Matrigel®). Primary endometrial cells were isolated from surgical specimens obtained during the proliferative and secretory phases of the menstrual cycle. Stromal and epithelial components were isolated and grown in monocultures or in co-culture in association with Matrigel® as described in the text. [^{3}H]-thymidine incorporation was measured after 3 days of culture and compared between epithelial cells in monoculture or in the presence of stromal cells in co-culture. Each data point represents the average ± standard deviation of three determinations. Stromal cells on BME–Matrigel® contributed little to the degree of proliferation as shown by monoculture of stromal cells alone on BME. The [^{3}H]-thymidine incorporation values for epithelial cells in co-culture have stromal cell values subtracted out.

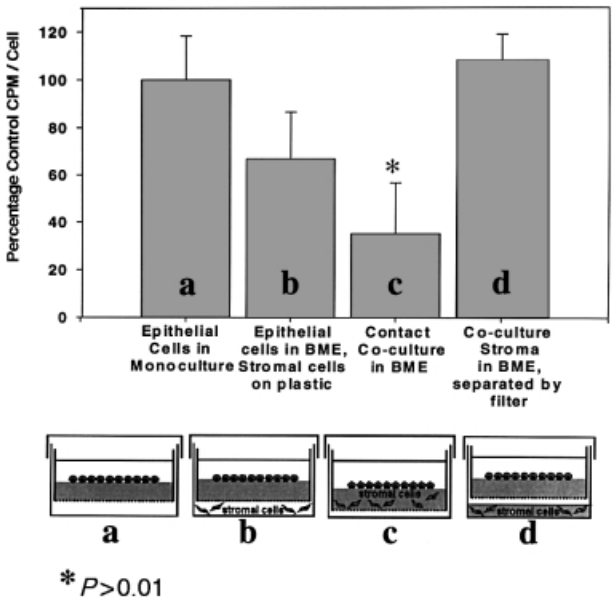

Figure 7. Stromal cell inhibition of [^{3}H]-thymidine incorporation in epithelial cells: stromal cells cultured on plastic versus embedded in basement membrane extract (BME), Matrigel®. Epithelial cells seeded on BME were grown alone in monoculture (**a**), or in co-culture with stromal cells either plated on plastic below filter insert (**b**), or within the BME (**c**). The cultures were grown for 8 days and thymidine incorporation was assessed. Epithelial cell [^{3}H]-thymidine incorporation was decreased 33% ($P = 0.07$, not significant) when grown in co-culture with stromal cells on plastic (**b**) compared with growing in monoculture (**a**). When epithelial cells were grown in contact co-culture with stromal cells embedded in BME (**c**), the [^{3}H]-thymidine incorporation was decreased by 65% ($P = 0.004$). In an additional configuration, stromal cells were embedded in BME but separated from epithelial cells by a filter insert (monoculture, **a**, or in co-culture). Each data point represents the average ± SD of three determinations. Statistical values were analysed by ANOVA with Scheffe's correction, and referenced to thymidine incorporation in epithelial monocultures.

was employed with stromal cells on plastic beneath the filter (Figure 7b), compared with epithelial cells in monoculture (Figure 7a; $P = 0.07$). When the epithelial cells were cultured in contact with the stromal cells that were embedded in the Matrigel®, incorporation of thymidine by epithelial cells was decreased by 65% compared with monoculture conditions (Figure 7c; $P = 0.004$). To test whether both direct contact and Matrigel® were necessary, an additional configuration for co-culture was evaluated as shown in Figure 7d. The effect of suppression of proliferation was not seen if the stromal cells were cultured below the filter in Matrigel® but not in contact with the epithelial cells (Figure 7d). Finally, to determine if the presence of stromal cells could modulate specific gene expression by endometrial epithelial cells, we studied the secretion patterns of glycodelin as a biomarker of endometrial epithelial cell differentiation. Glycodelin was measured in media obtained from endometrial epithelial cells in both monoculture and in co-culture. As shown in Figure 8 there was no glycodelin expression in the stromal cells cultured alone in Matrigel®. Epithelial cells in monoculture on Matrigel® or in co-culture with stromal cells plated on plastic produced little glycodelin (1–2 ng/μg DNA). Glycodelin production was significantly increased when epithelial cells were co-cultured with stromal cells growing in Matrigel®. Under these conditions, glycodelin secretion into the culture media increased to 8 ng/μg DNA ($P = 0.024$). These results are further evidence for a role of Matrigel® and stromal cells in modifying endometrial differentiation and induction of an endometrial epithelium gene product.

Discussion

In the present study, we have utilized the now established methods of isolation and culture of primary endometrial epithelial and stromal cells, but applied to those methods a novel modification that restores a more normal and physiological cell–cell interaction. Using this model we have demonstrated three-dimensionally organized epithelial–stromal interactions, regulation of cell proliferation, and induction of differentiated epithelial gene expression. By exposing stromal cells to basement membrane material, we find that the adjacent epithelial cells display a reduction in proliferation and an increased expression of the epithelial gene product, glycodelin. Not only does the Matrigel® appear to induce the stromal regulatory phenotype, but also may act as the mediator to present the regulatory message to the epithelial cells. By restoring the three-dimensional milieu of the extracellular matrix, we believe that we have recreated what occurs in living tissues, namely a paracrine relationship between stromal and epithelial cells both interacting with a basement membrane.

Attempts to demonstrate stromal regulation of epithelial cell function have been explored in other tissues with the development of various cell culture models. In collagen gels,

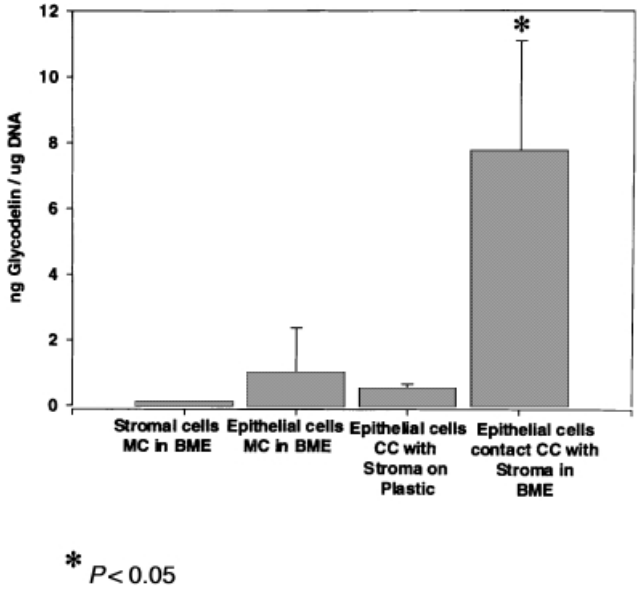

Figure 8. Glycodelin expression in human primary epithelial cell cultures in the presence or absence of basement membrane extract, Matrigel®. Stromal cells were seeded in triplicate onto plastic dishes or in Matrigel® in filter inserts. Epithelial cells were added to Matrigel® alone or Matrigel® plus stromal cells and treated for 5 days. Conditions were varied with stromal cells or epithelial cells in monoculture on Matrigel®, or stromal and epithelial cells in co-culture on plastic or on Matrigel® as described in Materials and methods. Supernatant media were collected on day 5 and assayed for glycodelin using an immunofluorometric assay. Glycodelin concentrations were standardized to DNA concentrations for each sample. Each bar represents the mean glycodelin concentration ± SD of three determinations. Co-culture on Matrigel® resulted in a significant increase in glycodelin secretion (asterisk; $P < 0.024$). MC = monoculture; BME = basement membrane extract (Matrigel®); CC = co-culture.

fibroblasts induced epithelial tubular morphogenesis in MDCK kidney cells (Montesano *et al.*, 1991). Media conditioned by stromal cells was shown to inhibit proliferation of prostate epithelial cells *in vitro* (Kooistra *et al.*, 1995). Stromal induction of prostatic epithelial differentiated function has also been studied by Chung *et al.* (Chung *et al.*, 1992) in an in-vitro–in-vivo model and in three-dimensional organoid cultures (Zhau *et al.*, 1997). Co-culture models have also been developed for keratinocytes (Konig and Bruckner-Tderman, 1991; Fusenig, 1992) and for mammary cells in which stromal cells were found to regulate epithelial function through interaction with extracellular matrix constituents (Haslam, 1986; Haslam and Counterman, 1991). Few in-vitro studies using human endometrial cells have been successful at demonstrating such differences in epithelial proliferation or regulation of differentiated cell function that are dependent on the presence of stromal cells. As revealed in the current study, contact with basement membrane extract (Matrigel®) provides a key modification to published methods which successfully demonstrates in-vitro regulation of endometrial epithelial growth and differentiation that is mediated by endometrial stroma.

The human endometrium is a very relevant tissue for investigating the influence of the tissue micro-environment on epithelial growth and differentiation. In the adult, the endometrium undergoes cyclic developmental changes that can be studied in the context of endocrine, paracrine and extracellular influences. It is a tissue that is readily accessible and many of the regulated genes have now been identified (Bell and Drife, 1989; Seppälä *et al.*, 1992). Methods to separate and culture endometrial stromal and epithelial elements have now been well established, and the purity and morphology have been described (Kirk *et al.*, 1978; Satyaswaroop *et al.*, 1979; Varma *et al.*, 1982; Osteen *et al.*, 1989; Classen-Linke *et al.*, 1997). The importance of the extracellular matrix for epithelial polarization has previously been demonstrated for endometrial cells (Schatz *et al.*, 1990) as well as for re-establishment of three-dimensional morphology (Kaufman *et al.*, 1980; Rinehart *et al.*, 1988; Bentin-Ley *et al.*, 1994; Classen-Linke *et al.*, 1997). Epithelial cells in culture express endometrial specific secretory products (Wegner and Carson, 1992) and can respond to the ovarian steroid hormones (Astrahantseff and Morris, 1994; Osteen *et al.*, 1994; Classen-Linke *et al.*, 1997). With the exception of work by Osteen demonstrating stromal regulation of epithelial metalloproteases, mediated by tumour growth factor (TGF)-β (Osteen *et al.*, 1994), stromal regulation of epithelial function had not been reproduced in cell co-culture for human endometrium.

The configuration of cell co-culture used in this study is the first that we are aware of to place stromal cells in direct contact with basement membrane material. Our data suggest that optimal configuration for stromal growth inhibitory effect in co-culture was when stromal cells were not only in the presence of BME but also in direct proximity to the epithelial cells. Diffusible factors created by the stromal cells in Matrigel® were not sufficient for inhibition. Whereas much of the extracellular matrix of the interstitial stroma is primarily comprised of fibronectin (Fazleabas *et al.*, 1997), this study offers the hypothesis of a potential unique regulatory role for the periglandular subpopulation of stromal cells which are in contact with the basement membrane.

BME or Matrigel® has been used in studies of epithelial cells *in vitro* for several tissues, including the endometrium (Rinehart *et al.*, 1988; Kliman and Feinberg, 1990; Mahfoudi *et al.*, 1991; Streuli *et al.*, 1991; Bentin-Ley *et al.*, 1994; Baatout, 1997; Classen-Linke *et al.*, 1997). This material is derived from the Engelbreth-Holm-Swarm (EHS) tumour cell line (Kleinman *et al.*, 1986). This reconstituted basement membrane extract contains laminin (60%), type IV collagen (30%), heparan sulphate proteoglycan (8%) entactin or nidogen (1%) and resembles the basal lamina at the electron microscopic level when polymerized. Endometrial epithelial cells attach to the basement membrane proteins and become polarized through specific interactions with cell adhesion molecules (Davis and Camarillo, 1995; Strunck and Vollmer, 1996; Fukushima *et al.*, 1998). In a model using fetal mouse mammary cells, just the presence of Matrigel® was enough to restore hormone-dependent gene expression (Streuli *et al.*, 1991; Streuli and Bissell, 1991), but Matrigel® alone does not appear to be sufficient for full differentiation of adult human endometrial epithelial cells, as demonstrated by our study.

What has not been appreciated is the effect basement membrane proteins may have on the cells that reside on the side opposite to the epithelial cells, namely the stroma. Since

stromal cells are now suspected of having major roles in epithelial growth and differentiation in adult tissues (Cunha *et al.*, 1985), we reasoned that stromal cells closest to the epithelial cells would be the most likely to be involved in emitting regulatory signals. Basement membrane itself, which has two sides, may be the stimulus required for a differentiation of stromal cells toward this paracrine role. We have previously demonstrated this phenomenon *in situ* by examining HB–epidermal growth factor (EGF). Only the stromal cells adjacent to basement membrane expressed this growth factor, while stromal cells away from the basement membrane (and epithelial cells) displayed little if any HB–EGF expression (B.A.Lessey *et al*., unpublished data). As witnessed by the significant morphological changes that occur in both cell types shown in the present study, it appears that Matrigel® may activate a collection of genes that alter cellular phenotype *in vitro* by direct and indirect effects.

To investigate further the paracrine effects of stromal cells on epithelial cell function, several gene products were considered as potential markers of differentiation. Glycodelin, also known as placental protein-14 (PP-14) or α2-PEG, is an epithelial secretory product present in the late luteal phase and in the decidualized endometrium during early pregnancy (Julkunen *et al.*, 1986a, b; Seppälä *et al.*, 1994). This glycoprotein has an extensive carbohydrate content and may have contraceptive potential by inhibiting binding of human spermatozoa to the zona pellucida *in vitro* (Dell *et al.*, 1995; Oehninger *et al.*, 1995; Clark *et al.*, 1996). Glycodelin has also been demonstrated to exhibit immunosuppressive activity (Okamoto *et al.*, 1991; Dell *et al.*, 1995). While the exact function of glycodelin remains to be elucidated, its synthesis increases in endometrium around the time of implantation and early pregnancy, suggesting a role in the establishment of pregnancy (Klentzeris *et al.*, 1994). Glycodelin can be assayed readily using immunofluorometric techniques as described in the present study. As such, the use of this hormone-regulated endometrial gene product was ideal for examining the effect of stromal cells on epithelial differentiation in our co-culture model system.

The regulation of glycodelin expression has previously been used to assess differentiation in endometrial epithelial cells *in vitro*. Preliminary data by White *et al.* (White *et al.*, 1990) demonstrated that a mixed culture of endometrial stromal and epithelial cells in Matrigel® expressed glycodelin (PP14) more than epithelial cells growing in a monolayer without Matrigel®. Mixed endometrial cell cultures grown on a plastic substrate were reported to exhibit glycodelin concentrations that rapidly declined in culture within the first week (Chatzaki *et al.*, 1994). It was noted that this decrease in glycodelin was associated with a parallel increase in epithelial cell senescence. With successful separation of epithelial and stromal cells in culture we have further demonstrated that the increase in glycodelin concentrations was greatly facilitated by co-culture with stromal cells in the presence of Matrigel®.

As with any model, this co-culture system has both advantages as well as limitations. While we would like to recreate conditions *in vivo* as much as possible, we have not included other endometrial cellular components such as endothelial cells, immunological cells or myometrium; all of these cells may play a role in the function of this complex tissue. It was, perhaps, fortuitous that inclusion of only isolated epithelial and stromal cells demonstrated the restoration of epithelial responsiveness. Stromal cells, epithelial cells and contact with basement membrane proteins may be the minimal requirements for in-vitro restoration of endometrial growth and differentiation.

For endometrium, this model will have significant impact for the investigation of implantation and the establishment of uterine receptivity as well as elucidating factors that favour the development of endometrial hyperplasia or cancer. Furthermore, the important relationships between cells, endocrine and paracrine responses, and the extracellular matrix can now be more logically dissected and analysed. The model proposed here, in which stromal cells encounter BME, illustrates the importance of the tissue micro-environment and its effect on cell function. This simple alteration to the traditional methods for endometrial cell culture yields striking new results and provides potentially new insights into important questions in cell biology.

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