ABSTRACT

Endometrial cancer is characterized by alterations in the stromal cells and the supporting extracellular matrix in addition to the intrinsic alterations of the malignant epithelial cells. We have developed a cell culture model that demonstrates the role of stromal cells in the regulation of proliferation, hormone responsiveness, and differentiation of an endometrial adenocarcinoma cell line (Ishikawa). Conditioned medium (CM) was collected from normal primary human endometrial stromal cells grown on plastic or within the basement membrane extract, Matrigel. The CM produced by stromal cells cultured in contact with Matrigel markedly inhibited Ishikawa cell proliferation compared with CM from stromal cells cultured on plastic. Ishikawa cell proliferation varied with steroid hormone treatment in the presence of CM from stromal cells embedded in Matrigel. When the Ishikawa cells were placed in coculture in contact with stromal cells in Matrigel, production of a differentiated epithelial secretary product, glycoceptin, was induced. Gene expression of stromal cell hormone receptors, growth factors, and integrins was analyzed by reverse transcription-PCR in the presence of Matrigel to determine the potential factors involved in stromal regulatory function. These combined studies imply that the phenotype of the Ishikawa cells can be induced to differentiate to more closely resemble normal endometrial epithelium by reintroduction of stromal factors and appropriate extracellular matrix. Additionally, the study shows that basement membrane proteins influence the regulatory function of stromal cells as they mediate epithelial cell growth.

INTRODUCTION

Endometrial cancer is the fourth most diagnosed cancer in females with an incidence of 22 of 100,000 and a mortality rate of 3.2 of 100,000 (1). The majority of cases are in postmenopausal women over the age of 60. The causes of endometrial cancer remain unclear but likely include prolonged exposure to unopposed E3 without P to the age of 60. The causes of endometrial cancer remain unclear but likely include prolonged exposure to unopposed E3 without P to the age of 60. The causes of endometrial cancer remain unclear but likely include prolonged exposure to unopposed E3 without P to the age of 60. The causes of endometrial cancer remain unclear but likely include prolonged exposure to unopposed E3 without P to the age of 60.

Endometrial cancers exhibit disruption of normal cell-cell interactions at many levels. Epithelial cancers typically manifest abnormal oncogene or tumor suppressor gene expression and may have lost the ability to be regulated by the local tissue environment (4). This abnormality affects paracrine signals from the mesenchyme/stroma that are present in both embryonic and normal adult tissues leading to the associated changes in the epithelium-ECM-stroma relationships. It has been postulated that such changes lead to dysregulation of growth and eventually to neoplasia (26). The loss of regulatory or suppressive influences over epithelial proliferation may also be the result of aging of the stroma (27), or reversion to a more fetal phenotype, which contributes to the expansion of associated epithelial cells (28). Such observations raise the possibility that some cancers arise by the escape from normal stromal control in addition to epithelial genetic mutations.

This study presents data that support the hypothesis that endometrial stromal cells regulate growth, differentiation, and hormonal responsiveness of endometrial epithelium. Additionally, stromal cells cultured in the appropriate ECM can modify the malignant phenotype of a well-differentiated endometrial cancer cell line (Ishikawa). Using a novel cell-culture system (29), we have attempted to reconstitute the normal paracrine-endocrine-ECM relationships in vitro. The cell culture method described below provides a valuable tool for understanding these complex interactions and may lead to better methods of treatment for this and other epithelial cancers.

MATERIALS AND METHODS

Cells, Medium, and Culture Techniques. Normal primary endometrial stromal cells were isolated from endometrial biopsies obtained from reproductive age women with normal menstrual cycles who were undergoing bilateral tubal ligation. Use of endometrial tissue was approved by the Institutional Review Board (Committee for the Protection of Rights of Human Subjects) at the University of North Carolina. Tissue samples (1–2 grams) were obtained from proliferative phase endometrium between days 5 and 14 of the menstrual cycle, from women on no hormonal therapy within the previous 30 days. Endometrial tissue was transported to the laboratory in DMEM-H culture medium containing high glucose (Life Technologies, Inc., Gaithersburg, MD) and 5% FBS (BioWhittaker, Walkersville, MD) plus 2× antibiotic and antimycotic agents to yield final concentrations of 200 units penicillin, 0.2 mg streptomycin, and 0.5 µg amphotericin-B/ml (Antibiotic/antimycotic solution; Sigma, St. Louis, MO).

Endometrial tissue was rinsed in Hanks BSS to remove blood and debris. After 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). The entire procedure was performed under a sterile laminar flow hood. The tissue was minced with sterile scalpels into 1-mm² fragments and digested with collagenase (2 mg/ml; CLS-1; Worthington Biomedical, Freehold, NJ) in DMEM-H medium (as above) for 2.5 h at 37°C on a shaking rotor. The tissue digest was vigorously pipetted and added to a stacked sterile wire sieve assembly with #100 wire cloth sieve (40 μm size; Newark Wire Co., Newark, NJ), followed by a #400 wire cloth sieve (37 μm). After the endometrial digest was added to the top of the sieve assembly, the epithelial glands were retained in the #100 and #400 sieves while the stromal cells passed through to the recepeta below. Stromal cells were collected from the lower recepta, pelleted by centrifugation, and resuspended in 3 ml of DMEM-H medium. RBCs were removed by carefully layering cell suspension over 3 ml of Ficoll (Pharmacia, Piscataway, NJ) over the cold carbonate. A sterile 15 ml tube was allowed to gel at 37°C for 1–2 h before culture medium was added to each well.

For studies involving steroid hormones, the culture medium was supplemented with 2% charcoal-stripped serum and E, EP, or EPR (as above). Solvent controls (O) included the medium placed on stromal cells on plastic or in Matrigel containing 0.01% ethanol. Cultures were treated with solvent control or hormones for up to 10 days, replacing the medium every 3–4 days. CM was obtained by removing the medium containing hormones and replacing with serum-free, hormone-free culture medium for 24–48 h. The stromal CM was collected, filter-sterilized to remove cellular debris, and stored at −20°C.

Cocultures of stromal and Ishikawa cells were prepared using early passage stromal cells (passage 1–4). Typically, stromal cells were cultured on plastic or embedded in BME (as described above). Stromal cells were plated at 10⁵ cells/well of a 24-well plate (2 cm²) or in BME on MilliCell CM filter inserts (0.4 μm, 12 mm diameter) in triplicate for each data point. Parallel filter inserts were prepared in advance for Ishikawa MCs using the undiluted BME without added stromal cells. Serum-supplemented medium was gently added at 1 ml/well after 1 h. Stromal cells were allowed to incubate overnight. The next day, the Ishikawa cells were seeded at 5 × 10⁴ cells/well onto the filter inserts containing BME alone or BME plus stromal cells. The medium was replaced with the serum-reduced medium, used for CC studies, as described above. [³H]Thymidine Incorporation. Tritiated thymidine ([³H]thymidine) incorporation was used to assess cell proliferation. Ishikawa cells were plated in triplicate in MC or CC, as described previously. After 4 or 7 days of growth, [³H]thymidine was added in culture medium at a final concentration of 2.5 μCi/ml. After 24 h, the medium was removed. Cultures were rinsed twice with HBSS, and trichloroacetic acid (5%) was added to each culture well at 4°C for 20 min to fix cells to plate. The plates were rinsed three times with 5% trichloroacetic acid to remove unincorporated radioactive material. The cells were solubilized with 0.2 m NaOH for 30 min at 37°C before neutralization with 75 mm HCl. The cell solution was added to scintillation vials and counted for 10 s in a Packard Scintillation Counter (Meriden, CT). Thymidine incorporation was standardized to total cell counts (cpm/cell) or to DNA concentration (fmol thymidine/mg DNA). DNA concentrations were determined by isolating DNA from parallel wells using DNAzol (Molecular Research Center, Cincinnati, OH). Isolated DNA was quantified by spectrophotometric absorbency at 260 nm.

Colonies. To determine the effects of medium conditioned by stromal cells on Ishikawa colony growth, Ishikawa cells in culture medium were plated on plastic substrate at clonal density of 500 cells/well of 12-well dish (4 cm²) or 2000 cells/6-well dish (9.5 cm²). After 24 h culture, medium was removed and medium conditioned by stromal cells (CM; see “Materials and Methods,” below) was added to each well at a ratio of 2 parts CM to 1 part CC medium plus 1% charcoal-striped serum. Cultures were grown for 6–7 days, then dishes were fixed and stained with methylene blue (Sigma). Colonies of >75 cells/colony were counted as positive.

Glycoelcin IFMA. To examine the effect of stromal cells on Ishikawa cell differentiation, Ishikawa cells were plated alone or in CC with normal endometrial stromal cells either on plastic or in Matrigel as described previously (see illustrations of experimental configurations in Fig. 6). Hormone treatments included either no added hormones (ethanol control) or EP (10⁻⁸ m, 10⁻⁹ m, respectively). Medium was replaced, and supernatant medium was collected on days 5 and 9 and stored at −80°C. Medium was assayed for glycoelcin using an IFMA. The IFMA used a monoclonal antibody to glycoelcin for coating the microtiter wells and secondary labeling with europium III chelate, as described previously (33). The sensitivity of IFMA is more than...
25 times greater than that of RIA and allows detection and accurate quantitation of glycodelin in samples undetectable by RIA. Experimental samples of 25 μl of cell culture supernatants were added to microtiter plates precoated with antibodies to glycodelin. Plates were prepared for a final volume of 20 μl/tube containing RNase inhibitor (20 units/ml), random primers (3 μg), DTT (10 mM) 5 × buffer, and dNTP (1 mM; all from Life Technologies, Inc.). Samples were incubated at 37°C for 45 min then 90°C for 5 min. cDNA samples were stored at −20°C.

**Reverse Transcription Protocol.** For reverse transcription of RNA, 1 μg of RNA sample was added to individual 200-μl microtube vessels. Amounts of DEPC water were adjusted to keep concentrations of RNA consistent between samples. Master mix was prepared for a final volume of 20 μl/tube containing RNasin RNase inhibitor (20 units/ml; Promega, Madison WI). Samples were incubated at 37°C for 5 min. cDNA samples were stored at −37°C.

**PCR Amplification.** PCR amplification of cDNA was performed by adding 2 μl of cDNA sample was added to individual 200-μl microtube vessels. Master mix was prepared for a final volume of 50 μl/sample including Taq polymerase (1.25 units), 10 × buffer (100 mM Tris HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, 0.1% gelatin), and MgCl₂ (1.5 mM). Samples were incubated at 94°C, 1 min; 55°C, 2 min; 72°C, 2 min) × 35 cycles (759 bp) or soak at 4°C before agarose gel analysis. The PCR primer sets along with optimal cycle programs used for stromal cell characterization are detailed in Table 1.

**RESULTS**

The well-differentiated Ishikawa endometrial cancer cells displayed marked differences in morphology when cultured on plastic compared with cells cultured with BME. On plastic surfaces these cells grew as very dense colonies with a high nuclear:cytoplasmic ratio (Fig. 1a). On BME, the cells migrated together within 24 h, despite the fact that they were seeded as individual dispersed cells (Fig. 1b). With time, the colonies formed glandular-like tubular and spherical structures reminiscent of the glandular structure of the tumor from which the Ishikawa cells were derived.

Endometrial stromal cells had the characteristic spindle shape when cultured on tissue culture plastic and progressed to a tight fibroblastic growth pattern at confluence (Fig. 1c). When cultured on Matrigel, stromal cells had a distinct appearance of isolated stellate cells with irregular cell borders (Fig. 1d).

In addition to the distinctions in morphology between the two cell types in CC, we studied the interactions between stromal and Ishikawa cells by immunofluorescent staining using antibodies to cytoskeletal proteins that distinguish between stromal cells and epithelial cancer cells. Stromal cells were identified using an antivimentin antibody and secondary FITC-labeled mouse IgG antibody (green). Ishikawa cells stained positively using an epithelial-specific anticytokeratin antibody with Texas Red-labeled secondary rabbit IgG antibody. Using this immunohistochemical technique to localize the respective cell types, the stromal cells (green) in BME were found to aggregate and intermingle with cells cultured with BME. On plastic surfaces these cells grew as dense colonies (red; Fig. 2, a–c). These photomicrographs document how the Ishikawa cells migrated together within 24 h, despite the fact that they were seeded as individual dispersed cells (Fig. 1b). With time, the colonies formed glandular-like tubular and spherical structures reminiscent of the glandular structure of the tumor from which the Ishikawa cells were derived.

Table 1

<table>
<thead>
<tr>
<th>PCR primer sets</th>
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<tr>
<td><strong>Growth factors</strong></td>
<td><strong>PCR primer sets</strong></td>
</tr>
<tr>
<td>TGF-β1 (57)</td>
<td>5’ CTG-CCA-GAA-GCC-GTA-CCT-GAA-C-3’</td>
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<td></td>
<td>5’ CAC-TTG-CAG-TGG-ATT-ATC-CCT-3’ (288 bp)</td>
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<tr>
<td></td>
<td>(94°C, 1 min; 60°C, 2 min; 72°C, 3 min) × 35 cycles – 72°C, 5 min; 4°C, soak</td>
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<tr>
<td>PDGF (58)</td>
<td>5’ AAG-AGA-AGC-ATG-GAG-GAA-GCT-GTC-3’</td>
</tr>
<tr>
<td></td>
<td>5’ TTC-CTG-GTC-CTC-TTC-CCG-ATA-ATC-3’ (330 bp)</td>
</tr>
<tr>
<td></td>
<td>(94°C, 1 min; 68°C, 2 min) × 45 cycles – 72°C, 8 min; 4°C, soak</td>
</tr>
<tr>
<td><strong>Integrins</strong></td>
<td><strong>PCR primer sets</strong></td>
</tr>
<tr>
<td>α-2</td>
<td>5’ TGG-AGT-GGC-TTT-CCT-GAC-3’</td>
</tr>
<tr>
<td></td>
<td>5’ CCG-AAT-GTG-TTT-AGG-TCC-C-3’ (556 bp)</td>
</tr>
<tr>
<td>α-3</td>
<td>5’ AAC-ATG-CTG-GTC-CCT-3’</td>
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<tr>
<td></td>
<td>5’ GGC-ATG-ATC-ATA-TAG-CCG-G-3’ (441 bp)</td>
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<tr>
<td>α-4</td>
<td>5’ AGA-CCG-TGG-TGG-TGG-TGG-G-3’</td>
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<tr>
<td></td>
<td>5’ CCA-CCG-TGG-AGG-AGG-CTA-TTC-C-3’ (438 bp)</td>
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<tr>
<td>α-6</td>
<td>5’ TGT-GTC-CAT-CTC-ACC-GTC-TTC-3’</td>
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<tr>
<td></td>
<td>5’ TAG-AGG-AGG-AAA-CAA-AAC-3’ (692 bp)</td>
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<tr>
<td>β3</td>
<td>5’ GGA-AAG-ATT-GGC-TGG-AGG-AA-3’</td>
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<tr>
<td></td>
<td>5’ GCC-ATA-CCC-CAC-CTC-CAA-AAG-3’ (662 bp)</td>
</tr>
<tr>
<td><strong>Steroid hormone receptors</strong></td>
<td><strong>PCR primer sets</strong></td>
</tr>
<tr>
<td>ER</td>
<td>5’ CTC-TGT-GCT-CTC-GTC-ACG-C-3’</td>
</tr>
<tr>
<td></td>
<td>5’ ATG-AGA-AAA-GAG-GTC-AG-3’ (360 bp)</td>
</tr>
<tr>
<td>PR (59)</td>
<td>5’ CTA-CGG-CCC-TAT-CTC-AA-3’</td>
</tr>
<tr>
<td></td>
<td>5’ AAT-GAA-CAG-CGG-ATG-AAA-3’ (759 bp)</td>
</tr>
<tr>
<td></td>
<td>(94°C, 1 min; 55°C, 2 min; 35°C, 3 min) × 15 cycles – 72°C, 8 min; 4°C, soak</td>
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<tr>
<td><strong>PCG cycle parameters</strong></td>
<td><strong>PCR primer sets</strong></td>
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<tr>
<td></td>
<td>(94°C, 1 min; 55°C, 1 min; 72°C, 3 min) × 20 cycles – 72°C, 8 min; 4°C, soak</td>
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ikawa cells, having been plated as single cells on top of the gel of Matrigel, appear to migrate into the gel, coalescing to form dense structures where bridges of cells create honeycombed patterns (Fig. 2b). The stromal cells also having been plated in homogeneous lawn of cells in the Matrigel, appear to congregate or migrate toward the epithelial structures (Fig. 2a), and surround them making contact via cellular processes (Fig. 2, b and c). This cell-specific migration and association of stromal cells with Ishikawa cells did not occur when plastic was used as a substrate for CC (Fig. 2d). On the plastic culture plate, the cells randomly intermixed on the culture plate suggesting that BME mediates a cellular behavior that favors specific cell-cell interaction.

Fig. 1. Ishikawa and stromal cell morphology in plastic and BME. Cell morphology was dependent on growth substrate. On plastic, Ishikawa cells grew in a dense monolayer with a high nuclear:cytoplasmic ratio (×200; a). When plated on BME, the Ishikawa cells aggregated to form glandular-appearing tubular and spherical structures (×100; b). Endometrial stromal cells grew in a characteristic spindle shape pattern progressing to a tight fibroblastic monolayer at confluence (×200; c). When cultured on BME, stromal cells had a distinct appearance of isolated stellate cells with irregular cell borders (×200; d).

Fig. 2. Ishikawa and stromal cells in CC. When Ishikawa cells were cocultured with stromal cells, immunofluorescent labels and morphology were used to distinguish the two cell types. Ishikawa cells fluoresced red using a Texas Red-conjugated secondary antibody to an epithelial specific anticytokeratin antibody. Stromal cells fluoresced green with an FITC-conjugated secondary antibody to antivimentin primary antibody. Nuclei were stained by DAPI (blue). Morphological distinction was based on growth patterns, which tend to be dense colonies with tight cell-cell connections for the Ishikawa cells, whereas stromal cells are more spindled and solitary with cytoplasmic projections. After 5 days in CC, the Ishikawa cells formed three-dimensional honeycombed patterns in which the individual cells were too small to be distinguished. Both cell types had been plated as single cells in a homogeneous lawn. The stromal cells (green and solitary) were found aggregated around the Ishikawa cell structures (red) and appeared to interperse between the cells [(a and b, see arrows) (a, ×600; b, ×400)]. c, apparent congregating pattern of the stromal cells (see arrow) toward the Ishikawa cell structures (×600). When plastic was used as a substrate for CC, this migration and association of stromal cells with Ishikawa cells did not occur (d; ×400x). On the plastic culture plate, the Ishikawa cells formed tight colonies but were large enough to distinguish individual cells and nuclei. The stromal cells (arrow) showed no association with the Ishikawa cells, and the two cell types were randomly intermixed on the culture plate.

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To examine the role of stromal factors in Ishikawa cell growth, Ishikawa cells were cultured in the presence or absence of CM from stromal cells grown on plastic or embedded in BME. Proliferation of Ishikawa cells was measured by \(^{3}\)H\)thymidine incorporation. CM from stromal cells growing in BME caused a reduction of \(^{3}\)H\)thymidine incorporation in Ishikawa cells by 75% (Fig. 3). The CM from stromal cells cultured on plastic had no apparent inhibitory effect. These findings suggest that normal endometrial stromal cells exert a paracrine inhibitory influence on the Ishikawa cells that is dependent on exposure to BME components.

Similar stromal regulation of cell growth was observed using Ishikawa colony counts as the proliferation index. The CM was collected from stromal cells growing on plastic or embedded in BME (as described above). As shown in Fig. 4, Ishikawa colony counts were reduced by 29% when CM was added to stromal cells cultured on plastic \((P < 0.01)\) compared with unconditioned medium controls. When CM from stromal cells embedded in BME was added to Ishikawa cultures, the colony counts were inhibited by 82% \((P < 0.001)\).

To explore the ability of the stromal cells to mediate hormonal growth response, Ishikawa cells were treated with CM from stromal cells cultured in BME and chronically treated with steroid hormones. CM was also collected from a parallel culture of normal human foreskin fibroblasts (NHF-1), which had been cultured embedded in Matrigel. The stromal and NHF-1 cells were pretreated 10 days with CC test medium containing 1% charcoal-stripped serum plus solvent control \((O)\), E at \(10^{-8} \text{M}\), EP \((P\) at \(10^{-8}\) M), or EPR \((R\) at \(10^{-6}\) M). To collect the CM, the hormone-containing medium was removed, and replaced with serum-free, hormone-free culture medium for 24–48 h.

This CM was then used to determine colony formation in Ishikawa cells. When Ishikawa cells were treated in the absence of stromal cell CM, there were no significant changes noted in cell proliferation in response to E, EP, or EPR (Fig. 5) compared with solvent control \((O)\). When Ishikawa cells were treated with CM from hormone-treated stromal cells grown in BME plus fresh aliquots of the same hormones, a 2.5-fold increase in colony numbers was seen in response to E plus stromal factors \((P < 0.001)\). The addition of EP plus EP-treated stromal medium diminished the cell colony count to 64% compared with E alone \((P < 0.05)\), whereas EPR reversed the EP inhibitory effect, bringing the colony counts back to the level of E alone \((P < 0.05)\). The CM from steroid-treated NHF-1 cells showed no growth inhibition or hormonal regulation of Ishikawa cell colony formation. Instead, the CM from the NHF-1 cells was shown to increase Ishikawa colony counts compared with unconditioned medium controls, the colonies being too numerous to count (one representative count shown per group). These data indicate that factors from hormone-treated endometrial stromal cells restore hormone growth responsiveness to the Ishikawa cell line. This hormone-induced growth pattern was repeatedly found in multiple experiments, using not only stromal CM but also direct CC of stroma and Ishikawa cells (data not shown).

Glycodelin is a major secretory product of the normal endometrial epithelium. To investigate the role of stromal cells in restoring a more normal phenotype to the well-differentiated endometrial cancer line, we performed studies in which Ishikawa cells were cultured in the presence or absence of normal endometrial stromal cells on plastic or...
in the presence of Matrigel-BME for 5 days. As shown in Fig. 6, Ishikawa cells growing alone in MCs on either plastic or BME showed little detectable glycodelin expression. Glycodelin was not expressed in Ishikawa cells growing on BME and cocultured with stromal cells on plastic. When Ishikawa cells were in contact with stromal cells in BME, glycodelin expression was induced to 3.4 ng/ml ($P = 0.02$) and to 4.7 ng/ml ($P = 0.0002$) with EP treatment compared with controls of Ishikawa cells in MC on BME; bars, ±SE.

The expression of integrin subunits $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_6$, and $\beta_3$ by RT-PCR is shown in Fig. 7 and is standardized against actin. One notable finding was that the laminin-collagen receptor $\alpha_2$ was decreased with E and EP when stromal cells were cultured on tissue culture plastic. Less difference was seen in stromal cells in BME with only a slight decrease in the EP-treated cells. The $\alpha_3\beta_1$ integrin, a receptor for laminin, collagen, and fibronectin, remained consistent in all of the hormone treatments and substrates as assessed by RT-PCR of the $\alpha_3$ subunit. The fibronectin receptor $\alpha_4$ subunit was suppressed by EP in stromal cells when cultured on in both plastic and BME. The laminin receptor $\alpha_6\beta_1$ was assessed through the $\alpha_6$ subunit. Expression was decreased in control and E treatment when stromal cells were cultured on plastic and was absent with EP treatment. Stromal cells cultured in BME maintained this integrin except for diminished expression with EP treatment. The fibronectin and vitronectin receptor subunit $\beta_3$ was present in all of the conditions but showed increased expression in E-treated stromal cells when cultured in BME.

To additionally investigate these phenomena, we examined steroid receptor and potential paracrine mediators using RT-PCR. RT-PCR analysis of ER and PR was performed in steroid hormone-treated stromal cells cultured on plastic or BME (Fig. 8). Increased expression of PR was noted in BME culture compared with plastic independent of hormone treatment. ER expression increased with E treatment and decreased with EP treatment in both matrix conditions.

Stromal cell expression of TGF-$\beta$1 and PDGF, potential mediators of stromal paracrine effect, was analyzed by RT-PCR in cells cultured on plastic or in BME, and treated with hormones. TGF-$\beta$1 expression was increased with E treatment over control in stromal cells both on plastic and in BME (Fig. 8). TGF-$\beta$1 was present in stromal cells on BME treated with EP but was diminished in stromal cells on plastic. PDGF appeared to be slightly increased in stromal cells in BME.

DISCUSSION

The importance of stromal cells in directing growth and differentiation of the overlying epithelium has been well established. What is less clear are the mechanisms of alterations in epithelial-stromal interactions in the development of neoplasms. To test the hypothesis that cancers may result from the disruption of normal regulatory relationships between stromal cells, epithelial cells, and ECM, a...
unique cell culture model has been used to investigate interactions among cancer cells and both normal stromal cells and basement membrane proteins present in Matrigel. The restoration of normal paracrine relationships in cell culture that mimic the in vivo state requires both the presence of normal stromal cells and the presence of basement membrane proteins. The basement membrane surrounding the endometrial epithelial glands has two planar surfaces, with epithelial cells on one side and stromal cells on the other. Because stromal cells closest to the epithelial component also contact this material, then models to study paracrine effects in vitro should include Matrigel in cultures containing stromal cells. This laboratory has demonstrated the importance of the ECM in directing paracrine activity of the stroma (29). These earlier studies demonstrated that stromal cells cultured in BME were able to regulate the growth of primary noncancerous endometrial epithelial cells and achieve stromal and ECM-dependent glycodelin expression in vitro. In the present model, similar though even more dramatic findings were obtained.

The present study examined the effect of stromal cells and Matrigel on the regulation of endometrial cancer cell (Ishikawa) growth and differentiation using a CC method with normal endometrial stromal cells or medium conditioned by stromal cells. The Ishikawa cell line used in this study was first developed by Nishida et al. (35) and contains functional ERs and PRs, as well as most of the endometrial epithelial integrins found in normal endometrial epithelium (30, 31, 36).

Morphological observations indicated a dynamic interaction between the stromal cells and the Ishikawa cells as shown in the immunofluorescent photomicrographs. In the combined CC both cell types within the structures appear to be embedded within the Matrigel with proliferation suppressed in both stromal and Ishikawa cells. When cocultured in the Matrigel, it is likely that stromal (and Ishikawa) cells remodel the ECM around them. It appears that stromal cells migrate out of the Matrigel and assume positions in close contact with the Ishikawa cells. This chemotactic tendency is an interesting phenomenon and one that we plan to additionally investigate.

Ishikawa colony counts and [3H]thymidine incorporation were repeatedly and significantly inhibited in the presence of CM from stromal cells in contact with BME compared with proliferation in the absence of stromal factors. CM from stromal cells cultured on plastic was partially inhibitory to Ishikawa colony growth but showed no inhibition of [3H]thymidine incorporation in Ishikawa cells. These data demonstrate that not only do stromal cells modulate the growth of neoplastic cells in vitro but also that stromal cells in contact with basement membrane proteins provide a greater inhibitory effect on Ishikawa cells growth than the stromal cells not connected to basement membrane. These data suggest that in the endometrium, subepithelial stromal cells may produce unique paracrine factors or different quantities of paracrine factors involved in growth suppression of the epithelial cells compared with factors produced by interstitial stromal cells.

Medium conditioned by stromal cells cultured in the presence of BME not only inhibited the growth of Ishikawa cells but also restored hormonal responsiveness. Steroid hormones E and P, and the antiprogestin R regulated growth of Ishikawa cells in the presence of CM containing diffusible factors released by steroid-treated stromal cells embedded in BME. Steroid hormones had little growth effect on Ishikawa cells in the absence of stromal-induced factors contained in CM. The pattern of growth of the Ishikawa cells with stromal CM reflects the variations in growth of epithelial cells occurring with hormonal changes during the natural endometrial cycle. In the hormonal cycle, the glandular epithelial cells proliferate in the E-dominant phase and exhibit decreased proliferation in response to P in the secretory phase. This pattern of hormonal response was repeated in multiple experiments, which included either stromal CM or direct CC of cells (data not shown). It is important to note that R has multiple complex mechanisms of action. In addition to its functioning as an antiprogestin, it also activates the glucocorticoid receptor. The restoration in colony formation, after addition of EPR, is likely attributable to the negative effect on P and PR activity but could be because of R binding to the glucocorticoid receptor. The R interactions with the glucocorticoid receptor are important, and we are currently working toward a better understanding of glucocorticoid receptors in normal endometrium and Ishikawa cells.

An important point illustrated by these studies was that the hormonal regulation by stromal cells was specific to endometrial stroma. As expected, neither the growth inhibition nor the hormonal response in colony growth was observed when Ishikawa cells were treated with CM from the foreskin fibroblast cell line NHF-1 cultured in BME. This indicated that endometrial stromal cells were unique in producing hormone-induced growth and inhibitory factors. The NHF-1 cells also provided a control for potential competition of nutrients in CM. The Ishikawa colony counts were increased with NHF-1 CM suggesting that this CC protocol supplies sufficient nutrients for both cell types. Thus, Ishikawa cell growth inhibition by stromal cell CM is not attributable to lack of sufficient nutrients. In our model, endometrial stromal cells cultured in BME were unique in that they provided regulation of Ishikawa proliferation via paracrine factors yet to be identified.

The stromal cells cultured in BME in the presence of E and P induced the expression of glycodelin in the Ishikawa cells. Neither neoplastic endometrium nor Ishikawa cells in standard cell culture conditions express glycodelin (37), suggesting the loss of this differentiated function is an early event in the transformation process. Hackenberg et al. (38) reported glycodelin expression by another endometrial cell line derived from a poorly differentiated endometrial cancer and from several primary tumors. It is interesting to speculate that regulated expression of glycodelin in the well-differentiated Ishikawa cells represents a higher level of cellular organization requiring stromal paracrine signals. It is perhaps fortuitous that these cells,
which maintained regulated expression of other hormone-responsive genes (30, 36), demonstrated the role of stromal cells in regulating specific gene products.

Glycodelin expression appears to be complex. Whereas considered to be a P-induced protein, its expression is delayed relative to the onset of P secretion, and the timing of glycodelin does not correlate well with serum P levels (39). Investigators have reported no difference in expression in the endometrium of women with “out-of-phase” endometrium compared with “in-phase” cycles (40). Recently the peptide hormone relaxin, which is derived from the corpus luteum as well as the endometrium, has been shown to induce glycodelin (39). Because stromal cells are a target cell for relaxin (41, 42), perhaps the effect we observed is mediated indirectly by relaxin through the stromal component of our CC model.

The induction of Ishikawa glycodelin secretion by stromal cells in BME suggests for the first time that CC with stromal cells under the proper conditions may induce differentiation an endometrial cancer cell line. These results suggest that some normal functioning of neoplastic cells remains possible and may be restored when stromal factors and ECM are present. The lack of glycodelin expression in neoplastic endometrium and Ishikawa cells in standard culture conditions may reflect altered gland-stromal interactions that occur in endometrial tumors. Furthermore, these data support the concept that loss of normal epithelial-stromal cell contact and/or loss of basement membrane may contribute to the abnormal phenotype of cancer cells in vivo. This change in phenotype toward a more normal phenotype may have broad implications for the treatment of endometrial cancers in the future.

The initial characterization of molecular differences in stromal cells in plastic and BME included RT-PCR for integrins, steroid receptors, the inhibitory factor TGF-β1, as well as an important stimulatory factor, PDGF. Stromal cultures were treated with estradiol (E) or EP to represent proliferative and secretory cycle variations, respectively. Whereas the stromal cell morphology varied greatly between plastic and BME, these gene expression data suggest several subtle differences but reveal no striking differences between stromal cells on these substrata. This result is surprising given the great difference in morphology and effects of the stromal culture conditions on epithelial growth. Cycle-specific changes in integrins found in vivo by Lessey et al. (43) were reflected in the EP suppression of α2, α4, and α6 in stromal cells on both substrates. The increased β3 expression in E-treated stromal cells in BME is in contrast with the regulation of αvβ3 in endometrial epithelial cells (44).

The steroid receptors ER and PR were studied by RT-PCR. In the normal endometrium, E induces an increase in stromal ER during the proliferative phase, whereas the P in the secretory phase down-regulates ER (45). This regulation of ER was reflected in in vitro cultures of stromal cells grown on both growth substrates. Estrogen also induces PR in the proliferative stage, but contrary to ER, the stromal cell PR levels are maintained during the secretory stage (45). The stromal cells in culture with BME maintained PR expression compared with stromal cells cultured on plastic independent of steroid treatment.

Stromal cell expression of TGF-β1, TGF-β2, and PDGF was analyzed by RT-PCR. These growth factors were chosen as potential mediators of stromal paracrine effect based on previous studies (46–48). Also, TGF-β was shown to be involved in P-induced stromal regulation of epithelial metalloproteases (21). Similar to the RT-PCR results with integrins, the expression of TGF-β1 and PDGF showed more sensitivity to steroid presence than to substrate. TGF-β1 showed increased expression with E treatment in both substrates. The lack of expression of TGF-β in EP-treated stromal cells on plastic may contribute to the loss of regulation of epithelial growth by this culture condition. It is not clear from these studies whether TGF-β produced by the stromal cells is present in the latent or active form (49). It is possible that stromal cells could produce an additional protease involved with the activation of TGF-β (50) resulting in an inhibitory signal to the epithelial cells.

This screening of gene expression of known mediators was a preliminary step in determining factors involved in stromal paracrine regulation. There was not any single integrin, growth factor, or hormone receptor that had markedly different expression in stromal cells when grown in BME. These results reflect the complex “vocabulary” of signaling that takes place between integrin receptors, hormone receptors, and growth factor receptors. (3, 51). It may be that the combination of increased expression of the α-6 integrin, PR, and TGF-β1 found in stromal cells in Matrigel treated with EP are necessary for the growth inhibition and induction of differentiation.

The mechanisms of stromal cell inhibition of epithelial cell growth and induction of differentiation may also include the proteolytic release by the stromal cells of growth (or inhibitory) factors present in the Matrigel. This is a very important potential mechanism, especially as the ECM is a storehouse of multiple peptide growth factors (52). The hormonal induction of stromal metalloproteases plays a critical role in the cyclical growth and sloughing of the endometrium (53). Additional studies will determine not only the involvement of this mechanism of stromal regulation but also what potential growth or inhibitory factors are involved.

Disruption of the delicate balance of signaling results in dramatic changes in the way cells interact with each other and with the ECM. Bissel and Hall (54) hypothesized that the unit of function in higher organisms is not the individual cell but the tissue itself. Tissue architecture is intimately involved in controlling the signaling processes within cells. Future studies will provide a better understanding of tissue architecture and may reveal new concepts of tissue function.

This work has revealed an additional layer of complexity in cell-cell communications by demonstrating the role of ECM in regulating the paracrine function of stromal cells. This provides a hint that stromal cells immediately next to the basement membrane, the “subepithelial stroma,” may function differently than the interstitial stromal cells more remote from the basement membrane, which are in contact with other collagen and fibronectin matrix proteins. Indeed, a similar cellular subpopulation was reported for prostate peripithelial stromal cells that, in the proximity of the basement membrane, had the lost phenotypic expression of androgen receptor in the progression of prostate cancer (55).

The concept of stromal cell association with basement membrane proteins provides insights into potential mechanisms involved in neoplastic progression. During tumor metastasis, interruptions are commonly observed in the basal lamina. Abnormal morphology of the basal lamina in some tumors is one indication that stromal-epithelial interactions are perturbed (12). If stromal cells lose their basement membrane-associated regulating functions as the basement membrane is altered in neoplastic lesions, they may also lose the ability to synthesize appropriate inhibitory factors, thus promoting progression of preneoplastic epithelial lesions.

The results presented here are consistent with data we published previously using normal endometrial epithelial cells (29). Very similar results were found including stromal-induced inhibition of growth and induction of glycodelin in normal epithelial cells using the same CC configuration. The predominant difference between the CC of normal endometrial cells and the endometrial cancer cells, as described in the current report, was the inability to document a stromal-induced hormonal growth response in normal endometrial cells. Data from primary cells had shown a modest effect by stromal cells on hormonal-induced changes in growth patterns that were repeatable but never
strongly significant. This could be attributable to variations in lots of primary cells from their previous hormonal milieu. Growth of the Ishikawa cells was reproducibly and significantly modulated by the stromal factors and hormones.

The development of CC models such as the one presented here is timely and urgently needed if we are to understand the course of cancer induction and progression. Many investigators are recognizing the importance of stroma in the epithelial neoplastic process. It is necessary to rebuild the parts of a tissue to understand cancer as a sum of the interactions of each component. This dilemma in cancer research was stated by Smithers: “Reduction gains precision about parts but at each step loses information about the larger organization it leaves behind” (56). The CC model presented here represents a tool to explore the next layer of complexity of cellular and tissue functioning. It provides the opportunity to examine communications within the epithelial-stromal-ECM axis between normal and neoplastic tissues and to elucidate how abnormalities in these communications may contribute to disease.

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