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A tissue engineered human endometrial stroma that responds to cues for secretory differentiation, decidualization and menstruation

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Abstract

Objective—To show the responsiveness of a tissue engineered human endometrial stroma to combinations of hormones mimicking the secretory and menstrual phases of the cycle.

Design—In vitro experimental study

Setting—University uterine biology research laboratory

Cells—Telomerase immortalized human endometrial stromal cells

Interventions—The stromal cells were cultured in monolayers (2D) or encapsulated in a collagen I hydrogel (3D) to create a simplified tissue engineered stroma. The cells and tissues were exposed to hormone treatments mimicking early and late secretory phases, decidualization and steroid withdrawal conditions to recapitulate menstruation.

Main Outcome Measure(s)—Morphological and biochemical markers of decidualization and collagenase activity

Result(s)—The 3D tissue is capable of manifesting changes in morphology and biochemical markers of decidualization similar to 2D culture and characteristic of endometrial stroma *in vivo*. Unlike 2D culture, the 3D tissue responded to steroid withdrawal by increased collagenase activity and tissue breakdown.

Conclusion(s)—3D tissue engineered endometrial stroma can mimic secretory and menstrual phases of the cycle and may be useful for studying uterine receptivity and menstruation in a physiological endocrine environment.

Keywords

Human endometrial stromal cell; tissue engineering; uterine receptivity markers; collagenase activity

INTRODUCTION

There is a need for *in vitro* human systems to study physiological and pharmacological aspects of endometrial biology. The menstrual cycle is unique to women and old world primates(1), limiting the utility of rodent models. Monolayer cultures of human cells have been extremely informative, but cannot be used to study complex cell-cell interactions or tissue remodeling and invasion. Tissue explants have been used successfully to study menstruation(2, 3), but tissues contain a highly complex cellular and extracellular matrix environment that make mechanisms and specific cellular contributions difficult to elucidate.

Tissue engineering can provide unique culture models to study endometrial cell biology. The *in vitro* composition can be more tightly controlled than that of explants, while providing a more complex physiological environment than monolayer cultures. Moreover, tissue engineering promises to provide methods to “repair, replace, or enhance tissue function”(4). While there may ultimately be a use for uterine tissue replacement to repair congenital Müllerian anomalies, Asherman’s syndrome or defects following removal of leiomyomas(5–8), engineered tissue is more immediately promising for the study of uterine biology and remodeling. The first tissue engineered stroma was described by Bentin-Ley et al. in 1994(9). This study and some that have followed described the basic tissue architecture including the organization and orientation of ciliated columnar epithelium(9–14). More recently, 3D models have been used to study decidual differentiation(15–17) and trophoblast or embryonic implantation(18–24). While fertility has clearly been a focus, there has also been interesting work using engineered tissue to study epithelial and stromal cell communication(25, 26) and to study invasion in endometrial cancer(27, 28). To date, regulation of endometrial stromal function leading to menstruation has not been a prominent application of these models. The dynamics of the endometrial cycle are precisely controlled by steroid hormones, paracrine factors and a complex array of proteases to effect cell migration and differentiation as well as endometrial remodeling. Defects in this program can adversely affect fertility or lead to abnormal bleeding. In the experiments described herein we set out to develop an *in vitro* system to study endometrial differentiation and remodeling that are required to support early pregnancy, and to evaluate the effects of steroid withdrawal to model menstruation.

MATERIALS AND METHODS

Culture of Cells and Engineered Tissue

Telomerase immortalized human endometrial stromal cells (HESC) were utilized for these studies (29). Monolayer (2D) cultures were seeded on tissue culture treated polystyrene well dishes (Corning Inc., Corning, NY) at a concentration of 30,000 cells/cm² and grown to approximately 90% confluence in cell culture medium consisting of Dulbecco’s Modified Eagle Medium/Ham’s F12 50:50 (DMEM/F12; Mediatech Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Mediatech), 1% non-essential MEM Amino Acids (Mediatech), 1% sodium pyruvate (Mediatech), and 1% penicillin-streptomycin (Hyclone, Logan, UT).

Three dimensional (3D) endometrial stroma (TES) were created by encapsulating HESC in type I collagen hydrogels. Collagen hydrogels were constructed by neutralizing the pH of acid solubilized type I dermal bovine collagen (MP Biomedicals, Solon, OH) and combining it with HESC and culture medium for final concentrations of 2 mg/ml and 2×10⁶ cells/ml, respectively, and placed into molds (12-well dishes). After gelation, the tissue was gently released from the sides of the wells and cell culture medium supplemented with 50 µg/ml L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma, St. Louis, MO). Ascorbic acid is a cofactor for prolyl and lysyl hydroxylases that promote collagen cross-

linking and stabilization of the hydrogels (30, 31). 24 hours later the gels were transferred into larger wells for an additional 24 hours prior to addition of hormones. This step allowed the majority of hydrogel contraction to occur prior to hormone treatments.

Hormone Treatments

Hormone treatments to mimic four stages of secretory and menstrual endometrium were administered over a period of 10 days. After the initial 48 hours of culture, the cells or tissues were rinsed with PBS and media with defined steroid concentrations were added. Basal medium for the hormone treatments was phenol red-free DMEM/F12 with 1% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential MEM Amino Acids and 5% or 10% charcoal stripped fetal calf serum (cFCS, Hyclone) for 2D or 3D culture, respectively. 3D cultures were also supplemented with 50 µg/ml ascorbic acid. The early secretory phase was mimicked by the addition of 10 nM 17β-estradiol (E₂, Sigma) and 10 nM progesterone (P, Sigma); the late secretory phase by the addition of 10 nM E₂ and 100 nM P; decidualization was induced by the addition of 10 nM E₂, 100 nM P and 500 µM dibutyl cAMP (Sigma); menstruation was mimicked by 6 days of 10 nM E₂ and 100 nM P followed by 4 days with no hormones for steroid withdrawal. Two-thirds of each culture medium was replaced every 2 days in all cultures.

DNA Quantification

DNA content was determined by Hoechst nuclear dye assay. 2D and 3D cultures were rinsed in PBS and dried in an oven. The samples were digested in 0.5 mg/ml Proteinase K in Tris-HCl buffer with EDTA at 60°C. A DNA assay was performed by loading samples in duplicate into a 96-well plate along with calf thymus DNA standards (Sigma). The samples were incubated for 15 minutes at room temperature with 0.1 µg/ml Hoechst 33258 dye (Sigma) suspended in 10 mM Tris-HCl buffer with 1 mM EDTA and 0.2 M NaCl. The plate was read using an excitation wavelength of 365 nm and emission wavelength of 458 nm. DNA quantity was determined by comparison to a standard curve. The results for 2D cultures are expressed as µg DNA; the 3D DNA content was normalized to dry weight and is expressed as µg DNA/mg dry weight.

Histology and cytology

The 3D tissue was rinsed in PBS and fixed in buffered formalin at room temperature for 24 hours. After twenty four hours the samples were rinsed and transferred to 70% alcohol at 4°C until processing. The samples were paraffin embedded; 7 µm sections were cut and sections were deparaffinized prior to staining. Histological evaluation was done with Hematoxylin & Eosin (H&E) using standard protocols. 2D samples were rinsed with PBS and images were taken immediately at the end of culture under phase contrast microscopy.

Shape Index

Cell shape index was determined for 2D cultures whereas nuclear shape index was determined for 3D cultures. Two frames from each phase contrast micrograph for the monolayer cultures and H&E stained light micrograph from the 3D cultures were evaluated for cell or nuclear circularity using ImageJ software(32). The shape index (SI) is calculated using the following formula: $SI = 4\pi \times \text{Area}/\text{Perimeter}^2$, which is an established method, originally reported to determine vascular cell shape(33). A circle would have a shape index of 1; a straight line an index of 0. Shape index was used to quantitate the cell and nuclear shape changes associated with hormone treatments.

ELISAs for Decidualization Markers

The concentrations of the decidual markers prolactin (PRL), insulin like growth factor binding protein 1 (IGFBP-1) and vascular endothelial growth factor (VEGF), were assessed in the spent medium. ELISA kits for PRL and IGFBP-1 were purchased from Alpha Diagnostic (San Antonio, TX) and VEGF from R&D systems (Minneapolis, MN). The volumetric protein concentrations were determined by use of standard curves and presented as protein content per μg DNA.

Collagenase Activity Assay

Collagenase activity in the spent medium was determined using a quantitative collagenase activity assay (Chondrex, Inc, Redmond, WA). Briefly, samples were incubated for 60 minutes at 35°C with or without the organomercurial agent 4-aminophenylmercuric acetate (APMA), to activate latent MMPs. FITC-labeled type I collagen was added to the medium and incubated for 90 minutes at 35°C. The cleaved collagen was further denatured by elastase during incubation at 35°C for 10 minutes. The solution was spun down and fluorescence intensity (FI) of fragments released into the supernatant used to calculate collagenase activity according to the equation:

$$\text{Units} = (\text{FI}_{\text{sample}} - \text{FI}_{\text{blank}}) \times 100 \text{ ug} / (\text{FI}_{\text{control}} - \text{FI}_{\text{buffer}}) \times \text{Reaction Time (min)} \times \text{Sample Volume (mL)}.$$

. Collagenase activity was expressed as Units per μg DNA.

Gelatin Zymography

Gelatin zymography was completed on spent medium samples in order to qualitatively visualize the latent and active forms of the gelatinases MMP-2 and MMP-9. The protein content of the samples was determined by the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). 25 μg of protein were loaded per well in a gelatin zymogram gel (Invitrogen, Carlsbad, CA). After electrophoresis, the gels were renatured in renaturing buffer (Invitrogen), developed overnight at 37°C in developing buffer (Invitrogen) and stained with colloidal blue (Invitrogen) to visualize the bands. The gelatin infused gel stains blue, whereas areas of zymogen activity are unstained. A molecular weight ladder was used to identify the bands.

Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean. One way ANOVA with *post hoc* Tukey's t-test was used with a 95% confidence interval to determine significance. The number of independent experiments performed under each condition is described in the Results section.

RESULTS

Effects on secretory transformation

After 10 days of hormone treatments, the different cultures were observed and morphological changes were compared ($n=6$) (Fig. 1A). The shape index was calculated to determine if there was a statistically significant change in cell or nuclear morphology ($n=6$) (Fig. 1B). The addition of steroid hormones alone had no discernible effect on cell morphology in 2D cultures. However, incubation in the decidualization cocktail resulted in the classical epithelioid transformation described by others(34). The shape index was significantly higher, indicating rounder cells, in cultures exposed to E_2 , P and cAMP, whereas other conditions exhibited more elongated shapes. In the 3D model, decidualization hormones also resulted in more rounded cells, as indicated by the larger nuclear shape indices. The nuclei were significantly less rounded under conditions mimicking the early secretory phase; however, no reversal in shape index was observed after steroid withdrawal.

This may be due to lower sensitivity of nuclear circularity or due to the fact that the cells cultured in 3D are more sensitive to the effects of P.

Evaluation of the decidualization markers PRL and IGFBP-1 was assessed along with VEGF, a growth factor critical to differentiated secretory function (Fig. 2). Sample sizes were $n=6$ for PRL, VEGF and IGFBP-1. The same trends were seen with all three proteins. In 2D cultures, PRL, IGFBP-1 and VEGF secretion was significantly increased in HESC treated with decidualization hormones. No differences were seen with the other hormone treatments. In 3D cultures, PRL, IGFBP-1 and VEGF content was significantly higher following exposure to decidualization hormones as compared to early secretory or menstrual conditions. The late secretory condition showed more variability and was not significantly different from the other conditions, with mean protein levels falling between the early secretory and decidual conditions. PRL levels were noted to be higher in the 2D cultures exposed to the decidualization hormones than 3D cultures under the same hormonal conditions. This effect may be directly related to cellular production, or may be due to reduced diffusion or increased binding of PRL in the engineered tissue. By contrast, VEGF levels were higher in all of the 3D cultures than the 2D monolayers.

Effects on MMP activity

The menstrual condition was mimicked by culturing the TES for 6 days in late secretory hormones followed by 4 days of steroid withdrawal. Within 24 hours of steroid withdrawal, noticeable deterioration of the 3D tissue could be seen, which progressed over the 4 days after steroid withdrawal when images were taken (Fig. 3A). Collagenase activity was assessed quantitatively using a FITC conjugated collagen degradation assay (Fig 3B). No changes in collagenase activity were seen within the 2D cultures ($n=6$), even under menstruation conditions. There was a significant increase in collagenase activity in the 3D cultures ($n=6$) subjected to menstrual conditions as compared to the early secretory and late secretory cultures. 3D cultures exposed to decidualization conditions had collagenase activity levels that were not significantly different than either of the secretory conditions. APMA stimulation increased the amount of active collagenase. The trends in 2D and 3D cultures were similar, with more latent, as well as spontaneously active, enzyme activity in the 3D cultures, particularly under menstrual conditions. MMP-2 and MMP-9 latent and active forms were visualized using gelatin zymography. Five different samples from the 2D and 3D conditions were run and a representative image is shown (Fig 3C). MMP-9 protein levels were low and no differences were seen in MMP-9 in either the 2D or 3D conditions. There were large amounts of MMP-2 in the 2D cultures with more in the latent form. No differences were seen between the different hormonal conditions in the 2D cultures. In the 3D condition there is more latent and active MMP-2 in the menstruation mimetic condition than in the other conditions. In all batches the menstrual condition had significant MMP-2 activation. In some, but not all samples, MMP-2 activation was seen in samples exposed to decidualization hormones.

Culture Conditions

The media utilized for 2D and 3D were different in order to optimize culture conditions for the cells and tissue. The 2D culture conditions were chosen so that the results could be compared with the literature. The 3D conditions were chosen to promote collagen deposition and cross-linking and to improve tissue integrity (30, 31). In addition to the data reported here, pilot studies ($n=2$) were performed culturing monolayers in 3D tissue culture medium which contains ascorbic acid and 10% cFCS. The results were not significantly different than what was observed in 2D culture medium. PRL levels ranged between 0.006–0.017 ng/ μ g DNA for the early secretory, late secretory and menstrual conditions, but was 1.9 ng/ μ g DNA for the decidual condition. IGFBP-1 ranged between 0.007–0.028 μ g/ μ g DNA for the

early secretory, late secretory and menstrual conditions, but was 1.5 $\mu\text{g}/\mu\text{g}$ DNA respectively for the decidual condition. VEGF levels were similar ranging from 0–0.8 $\text{pg}/\mu\text{g}$ DNA for the early secretory, late secretory and menstrual conditions while the decidual samples had an average of 145.2 $\text{pg}/\mu\text{g}$ DNA, within the range of results in 2D medium (Fig. 2). Collagenase activity varied between 0.18 and 0.25 $\text{U}/\mu\text{g}$ DNA in all the hormonal conditions and the menstrual condition did not generate the highest activity. No differences in MMP-2 or MMP-9 activation were seen between the two culture media.

DNA Content

DNA was determined at the end of each culture period (Fig. 4). Cells and tissues exposed to the decidualization hormones had lower DNA content, but that effect only reached significance in 2D culture, where a 31% reduction compared to early secretory conditions was observed ($n=6$).

DISCUSSION

We have developed a tissue engineered human endometrial stroma that mimics secretory and menstrual phases of the cycle. Our findings suggest that 3D culture is a more physiological model than 2D cell monolayers. However, despite its mimetic morphology and biochemistry, our 3D model remains a simplified representation of native endometrial stroma. For example, the cell density is much lower in the engineered tissue than *in vivo* to allow for nutrient and oxygen diffusion to the center of the hydrogel. Also, for these studies, we chose to use telomerase immortalized cells rather than primary cells in order to reduce interpatient variability as well as differences due to dedifferentiation with passage number (29). Only a single cell type was used in these experiments. Other cell types, such as epithelial cells, vascular cells, and leukocytes can be added to increase the complexity as some groups have reported (9, 10, 12–18, 20, 22, 23, 25, 27). For this study we chose the simplest configuration so that in future studies other cell types can be added in a controlled fashion to understand their individual contributions and interactions.

HESC cells previously were shown to be progesterone responsive in monolayer culture, resulting in morphological changes and expression of decidual and angiogenic proteins(29, 35). We observed similar responses within the 3D tissue. While PRL and IGFBP-1 responses were more subtle in the 3D cultures than 2D, the levels of VEGF were higher in engineered endometrial stroma. This finding may reflect lower oxygen tensions within the hydrogel and may in fact be more physiological(36). Cellular and nuclear shape index has been used for over thirty years to quantify morphological changes in vascular endothelial (33) and smooth muscle cells(37). Using this technique we found that nuclear and cellular “rounding” occurs in concert with decidualization biomarkers secreted during the late secretory phase.

Steroid withdrawal, designed to recapitulate the menstrual phase, demonstrated significant differences between 2D and 3D cultures. Steroid hormone withdrawal did not have a noticeable effect on the 2D cultures, which is consistent with previous reports that phorbol myristyl acetate or interleukin-1 α was needed to induce MMP-1 expression after steroid withdrawal(38–40). By contrast, 3D tissues responded to steroid withdrawal with increased collagenase activity, MMP-2 activity and tissue fragmentation. The fact that no changes in MMP-9 activity were seen, unlike in tissue(41), is most likely due to the fact that our 3D ECM is a processed collagen I hydrogel. In 3D cultures increased collagenase activity was also found in tissues exposed to the decidualization hormones. These observations are consistent with *in vivo* conditions where decidual cell morphology changes are associated with extracellular matrix remodeling, transforming from a collagenous environment to a more basement membrane-like matrix.

DNA content was determined for each culture in order to normalize the effects of hormone treatments to cellularity. The only significant difference found among all conditions was a reduced DNA content in 2D cultures exposed to decidualization hormones. While cell cycle dynamics and apoptosis were not evaluated directly, it is our impression that the HESC stop proliferating when the decidualized cell phenotype is induced. The fact that no differences were seen in the 3D culture may be due to the fact that cells encapsulated within type I collagen have reduced proliferative responses compared to those cultured in 2D(42).

Even with the simple, unicellular model presented here, we have demonstrated that 3D engineered endometrial stroma responds to cues for decidualization and menstruation. In both cases the 3D effects compare favorably to 2D cultures. In the case of biochemical markers of decidualization, the 3D model resembles monolayer culture, whereas after steroid withdrawal only 3D cultures undergo fragmentation and breakdown in a physiologic manner that recapitulates menstruation. This 3D endometrial stroma is a useful tool for studies of trophoblast receptivity and implantation, and in other conditions where endometrial remodeling occurs such as menstruation or inflammation(43). 3D cultures can complement the 2D cell and *in situ* work by providing an intermediate model that is more complex than monolayer cultures, yet more controlled and simplified than whole tissue explants.

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Figure 1.

A change in HESC morphology is seen in response to hormone treatment. Images of the cells in 2D with phase contrast or 3D histological sections stained with H&E are shown (A). Scale bars represent 50 μ m. Only images of the early secretory (ES) and decidual (D) conditions are shown as there are no visible differences noticeable between early secretory, late secretory (LS), and menstruation/steroid withdrawal (M) conditions ($n=6$). In order to quantify morphological differences, cellular (2D) and nuclear (3D) shape indices were calculated from cells and nuclei in $n=6$ samples (B). * indicates $p<0.05$ compared to all other conditions within the culture condition (2D or 3D). In 2D culture, decidualization induced a rounder cell shape as compared to the other hormone treatments. In 3D culture, the early secretory phase nuclei were significantly flatter than those in the other hormone treatments, but late secretory, decidual and menstruation conditions were not significantly different.

Figure 2.

ELISAs were performed to determine the secretion of decidualization biomarkers PRL (A), IGFBP-1 (B), and VEGF (C) which has been shown to increase during stromal differentiation (n=6). The results were consistent across all three proteins. In 2D and 3D cultures, cells secreted significantly higher amounts of these biomarkers when given the decidualization hormones (D). The late secretory (LS) stimulus responses were not significantly different from any of the other conditions in 3D culture.

Figure 3.

Steroid withdrawal led to visible degradation of the 3D tissues. Images of the 2D and 3D cultures at day 10 are shown (A). Scale bars indicate 50 μ m in the 2D frames and 20 μ m in the 3D frames. Examples of late secretory (LS) and menstrual/steroid withdrawal (M) conditions are shown, the menstrual condition is the only one that showed evidence of breakdown in 3D tissues. An increase in active collagenase was seen following steroid withdrawal in 3D tissues, but was not significantly affected in 2D cultures (n=6) (B). Gelatin zymography was performed to visualize latent and active gelatinases (n=5). MMP-2 and MMP-9 were identified by their molecular weights. The lanes from left to right are 3D: ES,

LS, D, M followed by 2D: ES, LS, D, M. The menstrual conditions lead to increase MMP-2 activation in 3D culture, however, no differences are seen in 2D culture (C).

Figure 4.

The hormone treatments resulted in subtle differences in stromal cell numbers as indicated by DNA content. The content for 3D was normalized to dry weight in order to take into consideration differences in hydrogel size. The only significant finding was the lower DNA content detected in the 2D cultures exposed to decidualization hormones (n=6).