Retinoids and steroids regulate menstrual phase histological features in human endometrial organotypic cultures

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Objective: To determine whether organotypic cultures of human endometrium can be manipulated with hormones to exhibit histological features resembling different menstrual cycle phases

Design: Human menstrual cells were collected and cultured in monolayer and organotypic cultures

Setting: Healthy volunteer in an academic research environment.

Patient(s): An individual premenopausal woman

Intervention(s): Endometrial cultures were grown in collagen gels for 4 weeks, and exposed to various steroid and retinoid treatments.

Main Outcome Measure(s): Histological features and expression of cytokeratins, vimentin, and reticulin

Result(s): Cultures developed multiple glands and surface epithelium that exhibited positive cytokeratin and negative vimentin staining Single stromal cells inside the collagen exhibited negative cytokeratin and positive vimentin staining Networks of reticulin fibers produced by the cells were increased by estrogen, decreased by progesterone, and unaffected by retinoids Contraction of the collagen gels was inhibited by retinoids that activated retinoic acid receptors (RARs), but not by a retinoid specific for retinoid X receptors (RXRs). The combination and timing of retinoid and steroid hormone treatments were demonstrated to induce tissue architecture and histological features that resembled either proliferative or secretory phases.

Conclusion(s): Growth of menstrual cells in collagen can be manipulated with retinoids and steroids to resemble histological features of the proliferative and secretory phases. (Fertil Steril® 2002;78:596–602 ©2002 by American Society for Reproductive Medicine.)

Key Words: Endometrium, menstrual cycle, differentiation, retinoids, steroids

The human endometrium consists of a complex mixture of cell types and extracellular matrix that is in a continual state of flux after puberty and before menopause. Greater knowledge of endometrial cycling could be used to enhance our ability to evaluate, prevent, and treat infertility, endometriosis, and cancer. Communication between epithelial cells, stromal cells, and extracellular matrix is fundamental to endometrial cycling and function (1, 2). Furthermore, the ability of the epithelial and stromal cells to communicate through secretion of hormones and growth factors is affected by their proximity to each other (3). This communication is required for their coordination and production of extracellular matrix. Throughout the menstrual cycle, the endometrium exhibits a complex fluctuating pattern of extracellular matrix molecules and membrane bound receptors for these molecules (4, 5).

Therefore, in vitro studies of endometrium should not rely solely on uniform cell lines grown in monolayers, but instead should take into consideration the complex interaction of the different cell types within their extracellular matrix.

During the early proliferative phase of the menstrual cycle, the extracellular matrix surrounding endometrial stromal cells includes a firm network of reticulin fibers that increases in density during the late proliferative phase (6). Just before menstruation, the endometrial granulocytes secrete relaxin, which initiates dissolution of the reticulin fibers (6). Although estrogen and progesterone levels are known to regulate menstrual cycling, it is not known if these steroids directly regulate the production of reticulin by endometrial cells. During the establishment of pregnancy, steroid hormones, growth factors, and cAMP regulate the prolif-

eration and differentiation of stromal cells that occurs during the process of decidualization required for embryo implantation and survival (7).

In addition to steroid hormones, retinoic acid (RA), the natural metabolite of vitamin A, is involved in the maintenance and regulation of differentiation in the cycling endometrium and the decidualization of fibroblasts (8, 9). Synthetic analogs (retinoids) and RA regulate gene expression through nuclear RA receptors (RARs) and retinoid X receptors (RXRs) (10, 11). Cytoplasmic retinoid binding proteins may play a role in the metabolism and transport of RA to the nucleus (10–12). Throughout the menstrual cycle, the intracellular levels of RA and expression of cytoplasmic retinoid binding proteins fluctuate, while the nuclear RAR and RXR receptors remain at similar levels (13, 14).

Several approaches to develop experimental model systems for the study of endometrium have been attempted. The earliest studies of endometrium were performed using animal models such as the Rhesus monkey (15). In human studies, organ cultures cut from hysterectomy specimens have been used as a model system, but were limited by variability between specimens and inability to cycle the endometrium in vitro (16). Cultured endometrial cells collected from hysterectomy specimens, peritoneal fluid, or curettage of the endometrium have also been used after separating the stromal cells from the epithelial glands (3, 17–19). More recently, cells collected from menstrual secretions have been successfully grown in tissue culture (20). Although these studies have been successful at culturing normal endometrial cells in monolayers, the systems used are highly artificial and do not mimic the in vivo architecture or intercellular communication of the human endometrium.

Three-dimensional organotypic cultures of endometrial cells isolated from surgical specimens and grown in collagen I or basement membrane material (matrigel) have been used in attempts to develop experimental systems for the study of human endometrium (21–23). These culture systems demonstrated characteristics of endometrial architecture but were limited by only occasional formation of gland-like structures and considerable shrinkage of the collagen gels over prolonged treatment times (22, 23). The objectives of this study were to determine [1] if cultures of menstruated human endometrial cells grown in organotypic culture resemble normal endometrium, [2] if retinoids regulate collagen gel contraction by endometrial cells, and [3] if steroid hormones regulate production of reticulin and the histological features in endometrial organotypic cultures in patterns resembling different menstrual cycle phases.

MATERIALS AND METHODS

Primary Cultures of Normal Human Endometrium

The University of Oklahoma Health Sciences Center Institutional Review Board approved the collection of men-

strual cells exuded from one individual premenopausal female. During the 2-year period in which cultures were established for the experiments presented in this report, the individual was 40 and 41 years of age and exhibited regular 28-day menstrual cycles regulated by OrthoTri-Cyclen oral contraceptive pills (norgestimate/ethinyl estradiol). The cells mixed with blood were collected in a 50-mL conical tube containing 10 mL of minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, sodium pyruvate, antibiotics and antimycotics (complete MEM). A single lot of FBS with undetectable levels ($<10^{-8}$ M) of retinol and RA isomers as determined by high-performance liquid chromatography was used for all experiments and repeat experiments presented in this report. The specimens were vortexed vigorously and plated onto 100-mm tissue culture dishes containing complete MEM media. The cells and cell clumps were allowed to attach overnight and the media was changed the next day. It was not possible to determine the viability of the cells plated, because, in addition to adherence of the single cells, many viable cells grew out of the clumps of cells that adhered to the bottom of the plate No effort was made to remove the blood cells from the media The monolayer cultures were passaged once before cryogenic preservation of a portion of the culture. Seven separate primary cultures were established, each from a separate menstrual cycle.

Endometrial Organotypic Cultures

Single-cell suspensions of the endometrial cells were used to generate organotypic cultures. No efforts were made to separate the different cell types or remove the blood cells. A concentration of 10^5 cells were pelleted and resuspended in a 4°C solution containing complete MEM media and rat tail collagen I (Collaborative Biomedical Products, Bedford, MA), which remains liquid at 4°C

The cell suspensions were transferred to Falcon cell culture inserts with transparent membranes (0.4 μm pore size, Becton Dickinson, Franklin Lakes, NJ) and then placed in tissue culture plates. After allowing the collagen to solidify at 37°C, a layer of 3×10^5 cells were placed on top of the collagen gels. The media inside and surrounding the insert was replenished every 2 days.

After 2 to 4 weeks of hormonal and/or retinoid treatment, the cultures were cut in half One half was fixed in formalin, and subsequently embedded in paraffin and sectioned; the other half was snap-frozen in liquid nitrogen. Each experiment was repeated at least two times to confirm consistent results.

Treatment With Hormones and Retinoids

The E_2 and progesterone (Sigma Chemical Company, St. Louis, MO) were dissolved in ethanol and stored at -80°C. The heteroarotinoid retinoids were synthesized by Dr. K. Darrell Berlin (Oklahoma State University, Stillwater, OK). The Fenretinide (4-HPR) compound was a gift from R.W.

Johnson Pharmaceutical Research Institute (Raritan, NJ), and 9-*cis*-RA was obtained from a commercial source (Bio-Mol, Plymouth Meeting, PA). All retinoids were dissolved in dimethyl sulfoxide (DMSO) and stored as aliquots of 1000× stocks at −80°C.

Because the retinoids were light sensitive, all manipulations involving retinoids were performed under subdued lighting. Each experiment included control cultures that received the same volumes of ethanol and/or DMSO solvent that were administered in the drug treatment. The concentration of DMSO in each experiment was always equal to or less than 0.01%, which is not toxic and does not induce differentiation

Histochemical and Immunohistochemical Analysis

Upon completion of treatment, the organotypic cultures were fixed in paraformaldehyde and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H & E) and with antibodies to cytokeratins, vimentin, or reticulin using standard immunohistochemical techniques by a commercial laboratory (Precision Histology, Oklahoma City, OK). The Ventana Immunostainer and Ventana kits were used for detection of estrogen receptor (ER) and progesterone receptor (PR) according to the manufacturer's instructions (Ventana, Tucson, AZ).

RESULTS

### Primary Cultures of Menstruated Endometrial Cells

Monolayer primary cultures were established from exuded normal menstrual endometrial cells. The cells in these cultures exhibited a uniform morphologic appearance with flat, stellate bodies and dendritic appendages (Fig. 1). Upon continuous passage, however, the morphologic appearance gradually changed. At passage 11, areas of the cultures that contained cells with a more rounded epithelial appearance developed; by passage 13, the entire culture uniformly consisted of rounded epithelial cells (see Fig. 1). The cells with epithelial morphologic characteristics had a propensity to fuse together in a polarized circular fashion surrounding a central lumen (see Fig. 1, passages 11 and 16). Progression to epithelial morphologic characteristics occurred in all of seven separate primary cultures, which were each isolated from a different menstrual cycle.

Retinoid Regulation of Collagen Gel Contraction

Endometrial cultures grown inside collagen gels caused the gels to contract and pull away from the sides of the tissue culture inserts within 48 hours of incubation. Initiation of retinoid treatment after 11 days in culture inhibited the contraction, whereas treatment with the same volume of solvent (DMSO) used to administer the retinoids in the treated cultures had no effect. A natural isomer of RA,

Progression of primary endometrial cultures from fibroblast to epithelial morphologic character. Photomicrographs of the same primary culture taken at increasing passages plated a low density (*top row*) and high density (*bottom row*) were taken at magnification ×10.

Passage 3   Passage 11   Passage 16
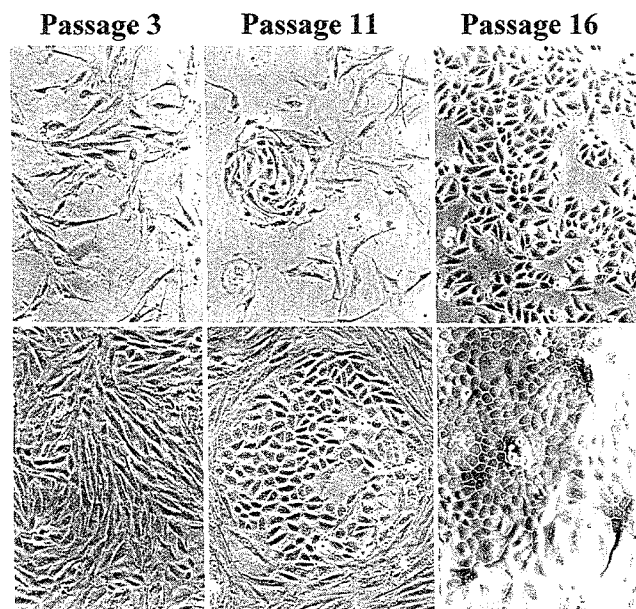
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9-*cis*-RA, and synthetic retinoids, including Fenretinide (4-HPR) and several heteroarotinoids, were equally effective in inhibiting contraction, while DMSO did not prevent contraction. Cultures treated with DMSO continually decreased in size, and after 1 month of culturing, they were no longer visible. A concentration of 100 nM was found equally effective as 1 μM in the inhibition of gel shrinkage

To further evaluate the retinoid antagonism of contraction, the effects of retinoids on the diameters of the organotypic culture gels were measured over time. Retinoids that activate specific subsets of their nuclear receptors were evaluated. The all-*trans* isoform of RA (*t*-RA) that only activates the RAR receptors, 9-*cis*-RA that activates both RAR and RXR receptors, and a synthetic rexinoid called OHet72 that activates only RXR receptors (24) were compared. Both isoforms of RA inhibited contraction, and OHet72 had no effect (Fig. 2).

Development of Histologic Features Resembling the Proliferative Phase

The timing of initiation of retinoid treatment in combination with estrogen treatment was found to regulate the degree of collagen gel contraction and the histologic features observed. Initiation of retinoid treatment on day 2 limited the amount of contraction to a consistent curl around the edges.

Retinoid inhibition of organotypic culture contraction. The diameters of organotypic cultures were measured over time. The diameters of the cultures treated with control solvent (□) were identical to the cultures treated with the rexinoid initiated at day 0 or at day 7 (◆). Cultures treated with either all-*trans*-RA or 9-*cis*-RA from day 0 (RA, ▲) and from day 7 (RA, ▼) were the same size.
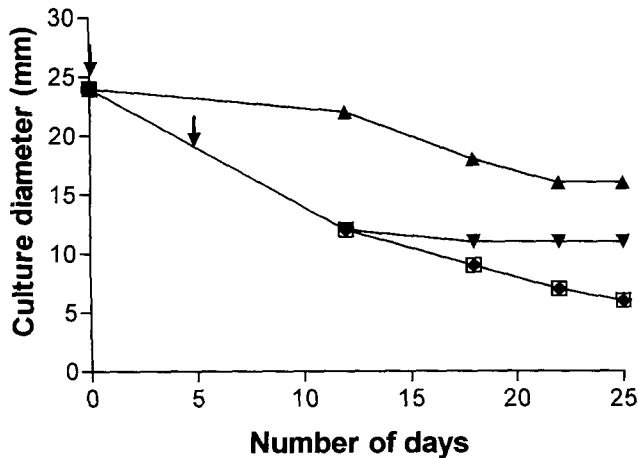
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When combined with low-dose estrogen treatment that was initiated on day 7, the edges of the curls formed central lumens that were surrounded by collagen (Fig. 3). The collagen was filled with multiple glands and single stromal cells, and the surfaces of the collagen and the glands were lined with epithelium. Positive cytokeratin staining and weak vimentin staining confirmed the epithelial differentiation of the glandular and surface cells. Positive vimentin staining and negative cytokeratin staining confirmed the stromal differentiation of the single cells inside the collagen. A culture that did not receive retinoid treatment until day 7 exhibited an extensive invagination filled with epithelium that resembled the endometrial stripe that is observed in ultrasound evaluations of the uterus (Fig. 3, upper left picture). Networks of reticulin fibers developed around all of the cells (Figs. 3 and 4). Both the stromal and epithelial components stained positive for estrogen receptor and progesterone receptor (data not shown).

Development of Histologic Features Resembling the Secretory Phase

If retinoid treatment was delayed by 11 days and no exogenous steroids were added, the gels rounded up into balls containing lumens lined with epithelial cells surrounded by collagen containing single stromal cells (see Fig. 3). Multiple glands formed in the folds of the gel and networks of reticulin fibers were expressed around all cells. The lumen, which formed where the two sides of the curled

up gel came together, was lined with cells and reticulin fibers to seal the opening. Inside the central cavity of the curled up gel, loose aggregates of glands and stroma were observed that resembled changes that occur during the secretory phase of the endometrial cycle (see Fig. 3). Specifically, stromal cells that were undergoing changes from "small dark" spindled cells to cells with abundant cytoplasm were observed forming sheet-like areas (decidualization). Positive cytokeratin staining and weak vimentin staining confirmed the epithelial differentiation of the glandular and surface cells. Positive vimentin staining and negative cytokeratin staining confirmed the stromal differentiation of the single cells inside the collagen. Greater levels of reticulin expression were noted surrounding stromal cells in comparison to epithelial cells.

Modulation of Histologic Features and Reticulin Expression With Steroids

Further manipulation of the cultures by treatment with exogenous progesterone alone, or estrogen alone at low and high concentrations was performed. In addition, estrogen and progesterone were administered to cultures at the varying concentrations that occur during the menstrual cycle (Table 1). All of the cultures were treated with 9-*cis*-RA starting on day 2. Although there were little differences in the overall histological features of the cultures treated with these steroids, cultures treated with high-dose estrogen were noted to consistently develop a thicker cell layer on the surface of the gels, in comparison to low-dose estrogen (see Fig. 4). The timing of initiation of estrogen treatment was also noted to affect the histological features of the cultures. Initiation of estrogen treatment on the day the cultures were made decreased the number of cells in the epithelial layer in comparison with initiation of estrogen treatment on day 7 (data not shown). Therefore, initiation of estrogen on day 0 appeared to be toxic to the cells.

The expression of reticulin fibers in endometrial cultures was modulated by hormones in a manner that was consistent with the build up and breakdown of reticulin fibers during the menstrual cycle (see Fig. 4). A network of reticulin was observed surrounding the cells and glands in the cultures treated with low-dose estrogen and much higher levels of reticulin expression occurred in the cultures treated with high-dose estrogen. Reticulin expression was absent in the cultures that were treated with progesterone only. Cultures treated with cycling estrogen and progesterone exhibited reduced reticulin expression in comparison to that treated with low-dose estrogen alone. Cultures grown with 9-*cis*-RA alone in the absence of exogenous steroids expressed reticulin at higher levels than that observed in the presence of added exogenous progesterone.

Effect of Passage on Differentiation

The ratio of epithelial cells to stromal cells that developed in the organotypic cultures reflected the passage number of the monolayer cultures from which they were derived. As

Histologic features of organotypic cultures resembling proliferative (*top panel*) and secretory (*bottom panel*) phases of the menstrual cycle. The proliferative phase cultures were treated with 100 pg/mL E_2 starting on day 7 and 100 nM 9-*cis*-RA starting on day 2, with the exception of the picture in the top left, which initiated both retinoid and E_2 treatment on day 7. Sections of organotypic cultures were stained with H & E (HE), antibodies to a cytokeratin cocktail (CK), or antibodies to vimentin (VM). A didydnium filter was used to take the photomicrographs, resulting in the positive stain for VM and CK appearing as brown. Arrows indicate stromal cells. (Photomicrograph magnification: ×4 top left, ×12.5, top right HE; ×20 CK and VM.) The secretory phase cultures (*bottom panel*) were treated with 100 nM 9-*cis*-RA from day 11 and no estrogen, and were stained for reticulin (RET), CK, and VM. A didydnium filter was not used for the photomicrographs of secretory phase, resulting in the positive stain appearing as dark green and the positive RET stain appearing as black. (Photomicrograph magnification: ×4 RET, ×12.5 CK and VM.)
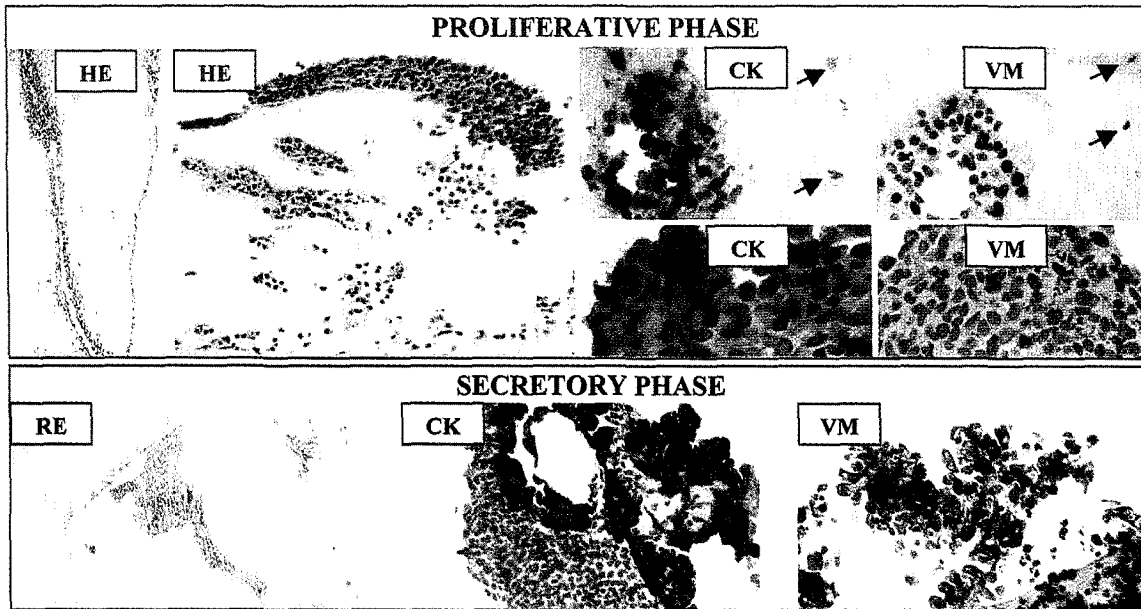
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mentioned previously, the number of cells with epithelial morphologic characteristics gradually increased with passage in monolayer cultures. Organotypic cultures generated from primary cultures that were passaged more than 15 times exhibited markedly reduced numbers of stromal cells inside the collagen (data not shown). The optimal resemblance of normal endometrial histologic features was derived from passages that had not yet demonstrated epithelial morphologic characteristics in monolayer culture (≤ passage 10).

DISCUSSION

This report describes a new organotypic culture model of endometrium that overcomes limitations of in vitro models reported by others. In addition, this model demonstrated responses to estrogen and progesterone treatments that reflect the morphologic changes that occur during the menstrual cycle. Primary cultures established from exuded menstrual cells provided the variety of cell types found in the endometrium. Cytokeratin and vimentin staining confirmed

the epithelial differentiation of cells lining lumens and the surface of the collagen and the stromal differentiation of single cells located inside the collagen. Expression of reticulin fibers surrounding the cells was modulated by steroid hormones to resemble the pattern of change that occurs during the menstrual cycle. Networks of reticulin fibers are built up during the proliferative phase as estrogen levels increase and then broken down during the secretory phase as progesterone concentrations rise. The dose-responsive induction of reticulin by estrogen and the prevention of reticulin expression by progesterone in our model are consistent with this pattern. The intermediate levels of reticulin expression in cultures treated with estrogen and progesterone reflects the opposing nature of these two hormones. Therefore, estrogen and progesterone appeared to directly regulate reticulin expression by the endometrial cells in this organotypic model in a biologically relevant manner.

The greater amount of epithelium in cultures treated with high-dose estrogen in comparison to low-dose estrogen also

Effects of steroid hormones on reticulin expression. Organo-typic cultures treated with 9-*cis*-RA only (RA) starting on day 2 and continuing for 23 days without or with additional treatments of 100 pg/mL E_2 (100 E), 250 pg/mL E_2 (250 E), varying E_2 and progesterone concentrations that occur throughout the human menstrual cycle (EP), or 10 ng/mL of progesterone (P) only. (All magnifications, ×25.)
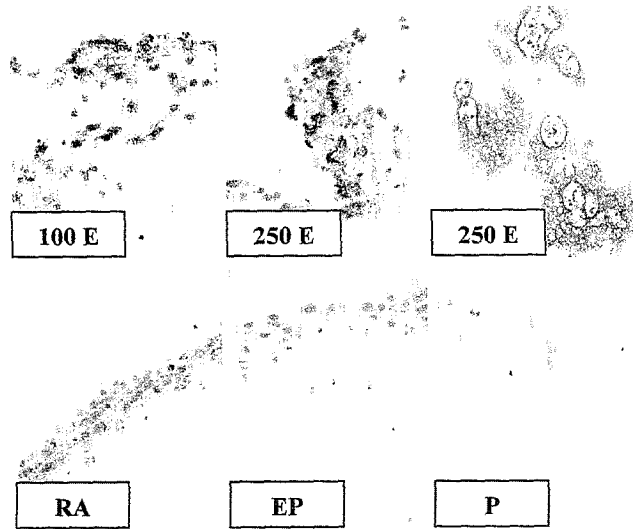
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mimics the effects of estrogen during the menstrual cycle This result also suggests that prolonged high-dose estrogen may be able to induce hyperplasia in this model. Other published models could not use long treatment times because the gels were lost to excessive contraction. The ability to treat these cultures indefinitely allows the study of the effects of long-term unopposed estrogen, selective estrogen response modifiers (SERMs), and over-the-counter birth control pills (OCPs) on endometrial histologic features.

The final estrogenic activity in the culture medium of this model was affected by the phenol red and/or the fetal bovine serum in the tissue culture media, both of which are known to have estrogenic activity (25). This did not prevent the ability to modulate the histological features with added hormones and retinoids to resemble two different phases of the menstrual cycle. Future studies are planned to evaluate hormonal modulation in this model using defined medium to control the levels of estrogen and progesterone more precisely.

The timing of initiation of retinoid treatment had a considerable effect on the control of gel shrinkage, which could be modulated to generate a range of three dimensional structures from spheres with small opening(s) filled with endometrium to discs containing curled up edges creating a lumen. Combination of retinoid and hormone treatment appeared to determine the menstrual cycle phase mimicked by the endometrium that developed. Initiation of retinoid treatment on day 2 and estrogen treatment on day 7 limited the gel contraction and the type of histological features to the proliferative phase, while preventing completed closure of gels and formation of clumps resembling secretory phase cells The clumps of secretory phase cells were only noted when retinoid treatment was delayed until day 11 and no additional steroid hormones were administered to the cultures

The specificity of the retinoid used for the different subclasses of nuclear receptors also affected the control of gel shrinkage. The lack of effect of the RXR-specific retinoid OHet 72 indicates that RXR activation is not sufficient to inhibit contraction. The equal efficacy of the RAR-specific all-*trans*-RA, and the panagonist RAR and RXR activator, 9-*cis*-RA, suggests that activation of RXRs is not involved in the mechanism. The mechanism of retinoid inhibition of collagen gel contraction is unknown, but could involve inhibition of metalloproteinase expression through AP-1 sites in the metalloproteinase gene promoters (26, 27)

Although the monolayer primary endometrial cultures appeared uniform in morphologic appearance, both epithelial and stromal cells developed in the organotypic cultures. This indicates that cells already committed to epithelial or stromal differentiation were present in the primary cultures. The increase in the proportion of cells with epithelial-type morphologic character with passage in monolayer culture could be due to selection for these types of cells caused by the culture conditions. The possibility still remains, however, that the menstrual cells may contain adult stem cells capable

Final concentrations of E_2 and P administered to endometrial organotypic cultures to mimic the human menstrual cycle.

Treatment day	8	10	12	14	16	18	20	22	24	26	28
E_2 (pg/ml)	70	80	150	250	80	80	100	125	100	90	50
P (ng/ml)	0	0	0	1	3	6	12	15	10	6	3

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of differentiating into either epithelial or stromal cells, a possibility that we are currently investigating.

In conclusion, organotypic cultures made with normal human menstruated cells can be manipulated with steroids and retinoids to develop histological features resembling the proliferative and secretory phases of the menstrual cycle. This model has considerable potential to be further modulated by hormones, retinoids, and carcinogens to resemble hyperplasia, cancer, endometriosis, and pregnancy, thus offering service to all fields of obstetrics and gynecology.

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