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MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF RABBIT UTERINE EPITHELIAL CELLS GROWN ON FREE FLOATING COLLAGEN GEL

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Abstract : In the present study isolated uterine epithelial cells from normal rabbits were maintained in culture on free floating rat-tail collagen matrix, and the morphological characteristics of these cells were examined. Additionally, the pattern of protein synthesis and secretion by rabbit uterine epithelial cells grown on free floating collagen gels following estradiol and/or progesterone treatment *in vitro* was examined. Isolated epithelial cells cultured on collagen gels in complete medium containing serum attached to form monlayers, and eventually the gels became free floating and contracted giving rise to luminal arrangements. These cells were cytokeratin positive epithelial cells and were ultrastructurally polarized. These cells also exhibited differential upregulation and down regulation in the synthesis and secretion of proteins in response to estradiol, progesterone, and estradiol plus progesterone. Additionally, a permissive action between progesterone and estradiol in the synthesis of two species of secretory proteins was observed. It however remains to be examined whether different species of proteins produced *in vitro* in response to estradiol and progesterone bear any association with physiological states in reproductive cycle in this species.

Key words : collagen, endometrium, epithelial cells, morphology, protein synthesis, three-dimensional culture, protein secretion

INTRODUCTION

The physiological regulation of morphological and functional characteristics of uterine epithelial cells in different phases of mammalian reproductive cycle is not adequately known. To distinguish specialized functions regulated by the direct action of hormones on epithelial cells from those which are controlled indirectly by the action of hormones and regulatory agents on other uterine cell types or delivered via the uterine vasculature, the uterine epithelial cells must be studied as an

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isolated population of cells. The use of primary endometrial cell culture as a tool for study of uterine functions began at the beginning of the last century and it provided significant knowledge about growth and hormonal responsiveness of different cell populations in the uterus. However, in these studies a two-dimensional cell culture system was mainly employed. Paradoxically, in such a system epithelial cells generally do not exhibit physiological differentiation, in terms of morphology and function (1, 2). The continued expression of specialized uterine epithelial functions requires experimental conditions that maintain their polar organization (3). Collagen substrates have been known to enhance the growth as well as differentiation of many cell types in culture as compared to two-dimensional substrates like glass and plastic (3). It is now well recognized that epithelial cells generally achieve differentiated morphology and function when they are grown on an extracellular matrix (4–6). In the present study isolated uterine epithelial cells from normal rabbits were maintained in culture on free floating rat-tail collagen matrix, and the morphological characteristics of these cells were examined. However, it is possible that endometrial cells fail to express differentiated functions of protein synthesis, despite an apparent presentation of differentiated and polarized cellular morphology (7). To this end, we have also examined the pattern of protein synthesis and secretion by rabbit uterine epithelial cells grown on free floating collagen following estradiol and/or progesterone treatment *in vitro*.

METHODS

Animals and supplies

Mature female virgin white New Zealand rabbits weighing 2.5–3.0 kg kept in individual cages and isolated from males were used in this study. The animals were obtained from the Experimental Animal Facility of the All India Institute of Medical Sciences, New Delhi. This study was approved by the Ethics Committee of the Institute.

Hank's balanced salt solution (HBSS) with/without calcium and magnesium, DME-Ham's F12 (1 : 1) mixture, trypsin, bovine serum albumin (BSA), and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY). Methionine-free minimum essential medium (MEM), chemicals for electrophoresis and cell culture and steroid hormones and antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO). Metabolic labeling grade ^{35}S-methionine was purchased from Bhabha Atomic Research Centre (BARC), Mumbai. Chemicals for microscopy were purchased from Electron Microscopy Sciences, Pennsylvania. Chemicals for immunocytochemical staining and radiolabelled molecular weight markers for autoradiography were purchased from Amersham (Arlington, IL). Multiwell tissue culture plates were obtained from Nunc Inc. (Denmark).

Isolation of uterine epithelial cells

All steps were carried out under aseptic conditions. Animals (n = 6) were sacrificed under ketamine anesthesia by air embolism in the marginal ear vein (8). Uterine horns were collected and cleaned in HBSS containing gentamicin (10 µg/ml) on ice and were slit open and incubated in 0.25% trypsin in HBSS without Ca^{++} and Mg^{++} (HBSS-mod) at room temperature for 15 min and then at 37°C for 30 min. The detached epithelial cells were collected in HBSS-mod on ice by vortexing 3 or 4 times. The pocled supernatant was centrifuged for 10 min at 300x g. The cell pellet was washed in HBSS-mod twice. The final cell pellet was resuspended in a small volume HBSS and it was allowed to sediment at unit gravity through HBSS containing 0.3% BSA for 10 min at 37°C. This step was repeated 3–4 times for obtaining epithelial cell enriched fraction. Finally, cell pellet was resuspended in complete medium, and cellular yield and viability were assessed by trypan blue dye exclusion method.

Preparation of rat-tail collagen and collagen gel matrix

Type I collagen was extracted from tendon bundles of frozen rat-tails according to the method described by Souren et al. (9). The final supernatant containing acid soluble collagen was collected aseptically and used as the stock. Collagen gel matrix was prepared according to method described elsewhere (10). Briefly, a neutralized collagen gel mixture was prepared by mixing stock collagen with 10X DME-Ham's F12 medium and 0.34 N NaOH, and was kept on ice to prevent gelation. Gelation was allowed to occur in an incubator at 37°C and a gas phase of 5% carbon dioxide in air. The mean final collagen concentration in a matrix gel was 2.9 ± 0.1 mg/ml. Each gel matrix was thoroughly washed and equilibrated in complete medium at 37°C in a gas phase of 5% carbon dioxide in air before these were used for cell plating.

Primary culture

Isolated cells were plated at a density of $1 \times 10^5/cm^2$ on soft collagen gel in multiwell tissue culture plates. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Unattached cells were removed after 24 h and fresh medium was added. Complete medium consisted of DME-Ham's F12, 5% FBS, pencillin (100 IU/ml) and streptomycin (100 µg/ml). The medium was changed every 24 h until the cultured cells were used for experiments.

Effect of estradiol and progesterone

5–7 days after plating when cells were nearly confluent, cultures were kept in serum-free medium for 48 h with a change at 24 h interval. Cultures were then maintained in methionine-free MEM medium containing 100 µCi/ml ^{35}S-methionine (1000 Ci/mmol). Cultures were ketp in labeling medium under control condition (group 1), and in the presence of 10^{-9} M 17β-estradiol (group 2), and in the presence of 10^{-9} M progesterone (group 3), and in the presence of both 10^{-9} M 17β-

estradiol and 10^{-9} M progesterone (group 4). Supernatants from cell cultures for each group were collected after 24 h and 48 h intervals. Culture supernatants and gels with cultured cells after thorough washing in phosphate buffered saline (PBS, pH 7.2) were stored at –70°C for further processing.

Electrophoresis and autoradiogram

Culture supernatants as well as cells attached to gels after homogenization in PBS were precipitated with cold ethanol. The precipitates were washed thrice with 10% tricholoroacetic acid followed by three washes with 70% ethanol. The precipitates were air dried and dissolved in sample buffer [Tris-HCI, 0.5 M, pH 6.8: 12.5% (v/v); Glycerol, 10% (v/v); SDS (10% w/v), 20% (v/v); 2β-mercaptoethanol, 5% (v/v), bromophenol blue (0.05%, w/v), 2.5% (v/v)]. Aliquots of redissolved secreted or cellular proteins were added to liquid scintillation fluid in scintillation vials and vortexed thoroughly. The vials were kept at 4°C overnight for stabilization, and radioactivity was measured using a liquid β-scintillation counter (RackBeta, LKB). Aliquots containing equal counts were mixed in sample buffer, heated at 95°C for 5 min and separated by SDS-PAGE with 4% stacking and 10% separating gel (11). On completion of run, gels were fixed, treated with Amplify and exposed to presensitized Hyperfilm MP (Amersham Life Sciences, U.K.) at –70°C for 8 h. The exposed film was developed, fixed and the radio-opaque bands were analyzed by scanning on a densitometer supported by Molecular Analyst software (BioRad, Hercules, CA).

DNA estimation

The collagen gels containing uterine epithelial cells at corresponding time intervals were extracted in PBS, and subjected to trichloracetic acid (TCA) and ethanol wash followed by perchloric acid (PCA) extraction. PCA extracted material was used for DNA estimation by Burton's method (12).

Immunocytochemistry

Immunocytochemical staining for simple epithelial marker cytokeratin was performed with cells cultured on collagen-coated cell culture grade cover slips, which were fixed in neutral buffered formaldehyde. The methodological details have been given elsewhere (13).

Microscopic examination

Cultures were fixed using 2.5% (w/v) glutaraldehyde in sodium cacodylate buffer, followed by routine processing for thick section light microscopy, thin section transmission electron microscopy and scanning electron microscopy as described previously (14).

Statistical analysis

Data of incorporated ^{35}S-methionine in cell lysates and spent media for different treatment groups were statistically analysed using Kruskal-Wallis test for non-parametric one-way analysis of variance and Mann-Whitney *U* test (15).

RESULTS

Viability, growth and maintenance of cells

Rabbit uterine epithelial cells isolated using 0.25% trypsin and enriched using HBSS containing 0.3% BSA consistently resulted in good cell yield with ~90% viability. Isolated epithelial cells cultured on collagen gel in complete medium containing serum attached within 24 h at an initial density of ~1.5×10^5 cells per well. The gels became free floating by 48 h of culture. They proliferated to a cell density of ~2.5×10^5 cells per well on day 5. As shown in Figure 1A, immunocytochemical staining for cytokeratins revealed that a high percentage (>95%) of cells isolated and grown on collagen was cytokeratin positive epithelial cells.

Morphological characteristics

Various morphological characteristics of rabbit uterine epithelial cells grown on floating collagen gel are shown in Figures 1 to 3. The epithelial cells in three-dimensional culture were found to form monolayer and clusters of cells

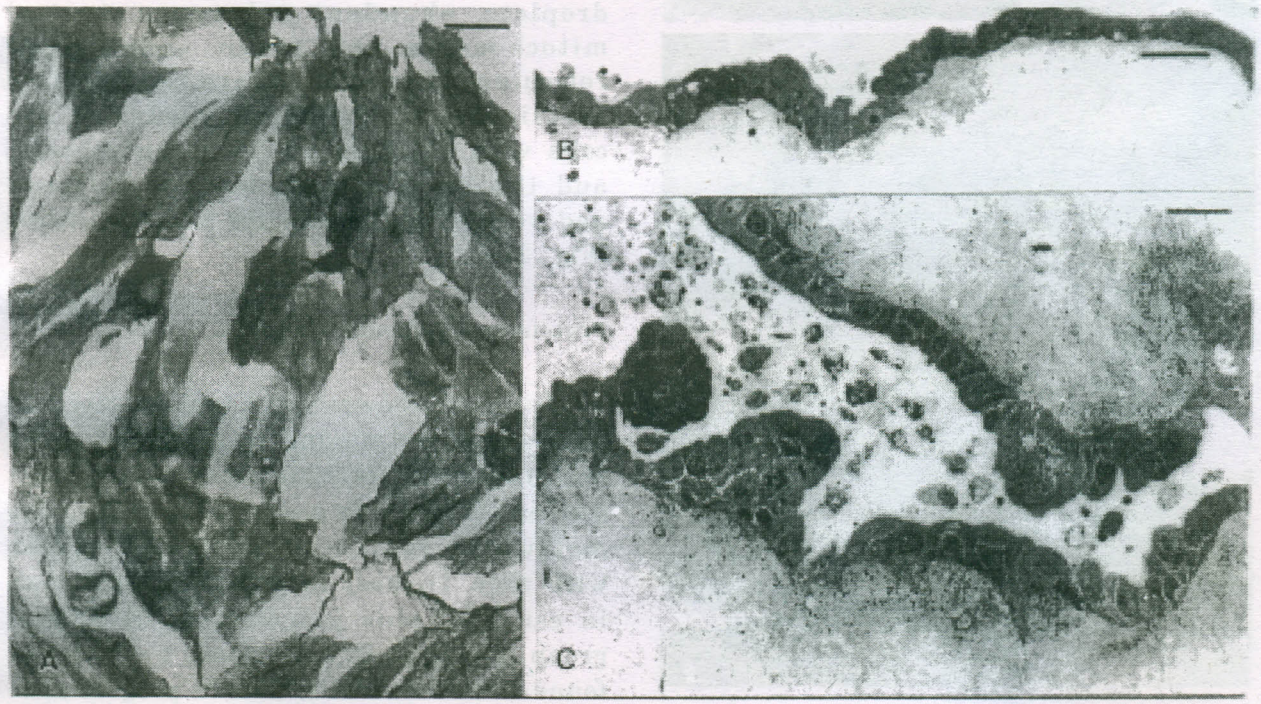

Fig. 1: Light microscopic characteristics of isolated rabbit endometrial epithelial cells grown on rat-tail collagen matrix. These cells are cytokeratin positive as detected in immunocytochemical staining (A), and they become attached to the matrix giving rise to monolayer (B). This is followed by contraction and release of collagen gels when cells become more columnar, and it gives rise to a lumen-like structure (C). Bars = 20 µm (A), 40 µm (B, C).

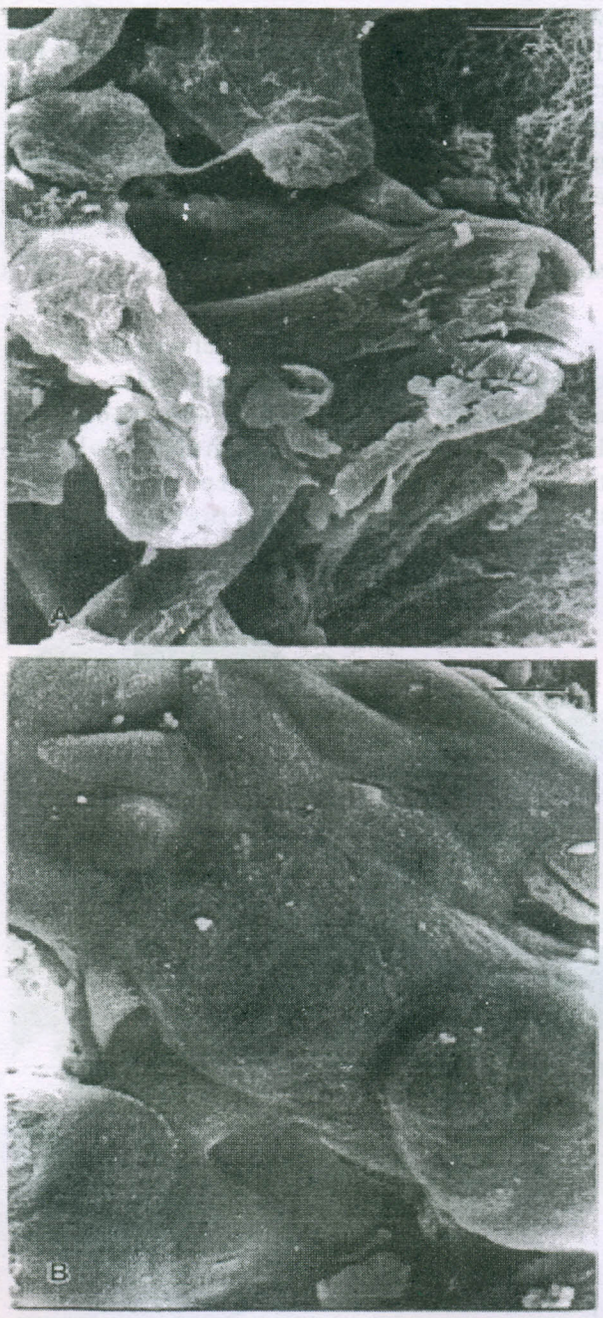

Fig. 2: Scanning electron microscopy of rabbit endometrial epithelial cells grown on rat-tail collagen matrix. During 3 days after plating, cells spread on the collagen (A). At portions, uncovered collagen matrix (**) is seen. Five days after plating, cells stop spreading and start showing near confluency state (B). Bar = 10 µm.

(Fig. 1B, C). The collagen gels were found to contract resulting in lumen-like structures with clusters of cells lining the gels (Fig. 1C). Scanning electron microscopic examination revealed that the plated cells spreaded over the substratum (Fig. 2A), and then these cells eventually form mounds through close apposition and interaction among cells (Fig. 2B), and also microvilli on the cell surface became evident (Fig. 2B). Transmission electron microscopy revealed that apical borders of cells facing the medium were covered with villi and cilia, while the lateral borders had interdigitating plicated processes and apical junctional complexes (Fig. 3). These cells also contained lipid droplets, abundant polysomes, typical mitochondria and rough endoplasmic reticulum (Fig. 3A). There was a close association of the basal border of the cells with the collagen fibrils underneath, and the basal domain became flattened and void of villi and cilia like structures (Fig. 3B).

Protein synthesis and secretion

Table I shows the incorporation of radioactive methionine in cells and spent media after different treatments. There was a steady increase in precipitable radioactive methionine incorporation in cells grown with time. Highest incorporation was seen in estradiol plus progesterone treatment group (group 4). The secretory activity was found to increase with time in progesterone treated group (group 3) and estrogen plus progesterone treated group (group 4), however, with a characteristic differences in the time sequence of their responses; in group 3 significant increase was observed

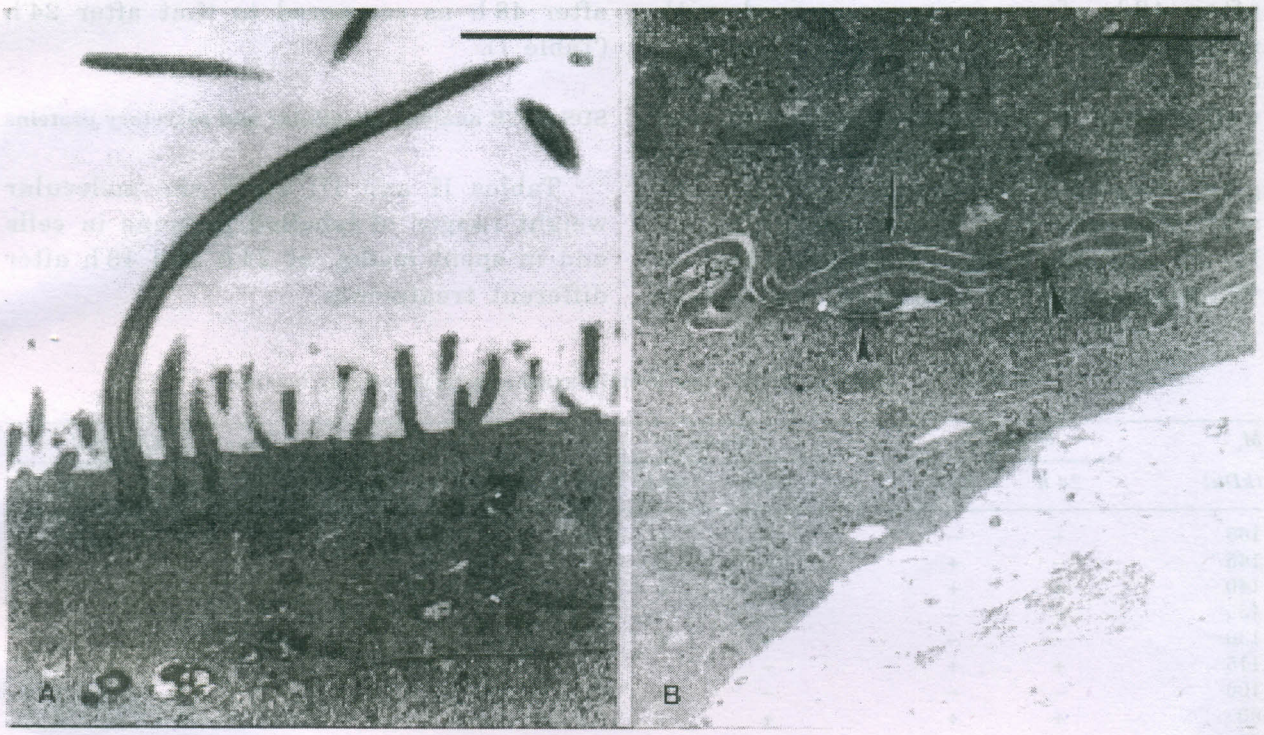

Fig. 3: Transmission electron microscopy of post-confluent rabbit endometrial epithelial cells cultured for 9 cells on free-floating three-dimensional rat-tail collagen matrix. In the apical side, the cells show villi and cilia, numerous mitochondria, endoplasmic reticulum and Golgi complex (A), and in the basal side the cell surface is flattened with organized matrix underneath (B). Intercellular membrane plication (*arrow*) and desmosomal junctional complexes (*arrow heads*) are seen. Bar = 1 µm.

TABLE I: Incorporation of ^{35}S-methionine in cells and spent media.

Duration of treatment	*Treatment groups Mean ± SEM (10^5; DPM per mg DNA)*							
	1		*2*		*3*		*4*	
	Cells	*Media*	*Cells*	*Media*	*Cells*	*Media*	*Cells*	*Media*
24 h	2.8±0.03	1.7±0.03	4.7±0.02[a]	2.4±0.02	3.9±0.03[a]	1.7±0.03	6.8±0.03[d]	4.6±0.05[b]
48 h	3.2±0.05	0.9±0.02	6.9±0.04[c]	3.6±0.04[e]	9.1±0.04[d]	7.6±0.03[d,f]	9.2±0.04[d]	2.4±0.03[c,g]
Significance (P<)	0.05	0.05	0.01	0.05	0.001	0.001	0.01	0.01

[a]P<0.01 compared with group 1 and group 4. [b]P<0.01 compared with all other groups. [c]P<0.01, and [d]P<0.001 compared with group 1. [e]P<0.01 compared with group 1. [f]P<0.01 compared with group 2 and group 4. [g]P<0.05 compared with group 2. n = 6 in triplicate for each group.

after 48 h of exposure compared with radioactive methionine incorporation observed after 24 h in the same group, as well as, with those observed after 24 h and 48 h in all other groups (Table I). In group 4 however an increase was observed after 24 h and then it declined (Table I). It is notable that incorporation of ^{35}S-methionine in group 1 was lower after 48 h as compared to that after 24 h (Table I).

SDS-PAGE analysis of cellular and secretory proteins

Tables II and III show the molecular weight ranges of labelled proteins in cells and in spent media, at 24 h and 48 h after different treatments.

TABLE II: Analysis of *de novo* protein synthesis from cellular lysates.

M_r	Group 1		Group 2		Group 3		Group 4	
(kDa)	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
165	+	–	+	+	–	–	–	–
145	–	+	+	+	+	+	+	+
140	+	+	+	+	–	–	–	–
135	–	–	–	–	+	+	+	+
130	–	–	+	+	–	–	+	+
115	+	+	–	–	–	+	–	–
100	–	–	–	–	–	+	+	+
90	+	+	+	+	+	+	+	+
80	+	+	–	–	+	+	+	+
75	–	–	+	+	+	+	+	+
65	+	+	+	+	+	+	–	+
60	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+
45	+	+	+	+	+	+	+	+
35	–	–	–	–	–	+	+	+
30	–	–	–	–	+	+	+	+
25	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+

TABLE III: Analysis of proteins secreted by endometrial epithelial cells in primary culture.

M_r	Group 1		Group 2		Group 3		Group 4	
(kDa)	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
150	+	+	+	+	+	+	+	+
130	+	+	+	+	+	+	+	+
110	+	–	–	–	–	–	–	–
100	+	+	+	+	+	+	+	+
85	–	–	–	+	–	+	–	+
70	+	+	+	–	–	–	–	–
65	–	–	+	+	+	+	+	+
60	+	+	+	+	+	+	+	+
50	–	–	–	–	–	+	+	+
30	+	+	+	+	+	+	+	+
20	–	–	–	–	–	+	+	+

In SDS-PAGE analysis of cellular proteins, bands in the ranges of molecular weight 130 and 75 kDa were seen to be upregulated in presence of estradiol as in groups 2 and 3. Of these two bands, the band in the range of 75 kDa was also upregulated by progesterone. On the other hand, protein bands in the ranges of 135, 35, and 30 kDa were specifically upregulated by progesterone as in groups 3 and 4. Two protein bands in the ranges of 165 and 140 kDa were down regulated by progesterone, while estradiol was seen to down regulate a protein band in the molecular weight range of 80 kDa.

The protein band observed in spent media at 24 h and 48 h after the treatment with steroid hormones separately (groups 2 and 3) and in combination (group 4), which was not seen in control group (group 1) was in the molecular weight range of 65 kDa, while the protein band observed in spend media only at 48 h after the treatment with steroid hormones separately (groups 2 and 3) and in combination (group 4), which was not seen in control group (group 1), was in the molecular weight range of 85 kDa. Two protein bands in the ranges of 50 and 20 kDa were seen in group 3 (progesterone) at 48 h, and in group 4 (estradiol and progesterone) at both 24 and 48 h.

DISCUSSION

Culture of endometrial epithelial cells in serum free medium containing defined supplements and preserving an apparently normal morphology has been successfully reported in mouse and in human (14, 16–18). In the present study rabbit uterine epithelial cells were found to display morphological differentiation and polarization in primary culture on floating three-dimensional rat-tail collagen gels. Scanning and transmission electron microscopy of cells grown on collagen gels revealed that these cells had villi, secretory droplets and cilia, and other features of morphological polarization. The close morphological association between cells was also observed.

Manners (7) has demonstrated that endometrial cells may fail to express differentiated functions of protein synthesis, despite an apparent presentation of differentiated cellular morphology. Thus, in the present study the differentiated function of protein synthesis and secretion was also studied. It was observed that the protein synthesis by rabbit uterine epithelial cells grown on free floating collagen gel was quantitatively affected in the presence of ovarian steroid hormones. Autoradiographic analysis revealed that morphologically differentiated rabbit uterine epithelial cells exhibited differential upregulation and down regulation in the synthesis and secretion of proteins in response to estradiol, progesterone, and estradiol plus progesterone. Additionally, we also observed a permissive action between progesterone and estradiol in the synthesis of secretory proteins in the molecular weight ranges of 50 and 20 kDa, which were seen only after 48 h of progesterone treatment; while these bands are discernible by 24 h when progesterone was supplemented with estradiol; estradiol alone could not induce these proteins. Interestingly, the protein secretory activity decreased in control treatment at 48 h compared with 24 h, suggesting that the protein secretion

by polarized endometrial epithelial cells is primarily regulated by hormones and other mediators, and is not a constitutive property of these cells.

Previously, Mani et al. (19) reported that in immature rabbit uterine epithelial cells cultured on matrigel (basement membrane extract) coated inserts progesterone treatment induced secretion of uteroglobin. The observed differentiation in protein synthesis by rabbit uterine epithelial cells grown on biomatrices appear interesting in view of the fact that progesterone (10^{-5} M) and estradiol (10^{-7} M) did not significantly alter the initial uteroglobin secretion rate of rabbit uterine epithelial cells cultured on plastic. Other studies with rabbit endometrial cells in two-dimensional primary culture have also revealed that their differential synthesis and secretion of proteins correlate with the growth status, rather than their differential responses to hormones (19–23). Carson et al (24) observed in immature rat uterine epithelial cultures on basal membrane extract that estrogen had a profound effect on the synthesis of glycoproteins. Furthermore, it has been shown that cultured mouse mammary cells respond in a differentiated manner to estradiol or prolactin only if they are cultured in the presence of fibroblasts (25, 26). Thus, it appears from the present study and other reports that presence of fibroblasts and/or extracellular matrix macromolecules render physiological environment to epithelial cells, which in turn help these cells to exhibit differential responses to hormones and physiological agents. Although it was observed in the present study that rabbit endometrial epitehlial cells grown on free floating collagen gels exhibited morphological polarity and differential responses to estradiol and progesterone in terms of synthesis and secretion of polypeptides, it remains to be examined whether different species of proteins produced *in vitro* in response to estradiol and progesterone bear any association with physiological states in reproductive cycle in this species.

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REFERENCES

1. Freshney RI. Culture of Animal Cells: A Manual of Basic Technique. New York: Alan R Liss, 1987; 298–307.
2. Yang J, Elias JJ, Petrakis NL, Willings SR, Nandi S. Effects of hormones and growth factors on human mammary epitehlial cells in collagen gel culture. *Cancer Res* 1981; 41: 1021–1027.
3. Freshney RI. Culture of Epithelial Cells. New York: Wiley-Liss, 1992; 25–57.
4. Michalopoulos G, Pitot. HC. Primary parenchymal liver cells on collagen membranes. *Exp Cell Res* 1975; 94: 70–78.
5. Burwen S, Pitelka DR. Secretory function of lactating mouse mammary epithelial cells cultured on collagen gels. *Exp Cell Res* 1980; 126: 249–262.
6. White TEK, Agnese PAS, Miller RK. Human endometrial cells grown on an extracellular matrix forms simple columnar epithelia and glands. *In Vitro Cell Dev Biol* 1990; 26: 636–642.

7. Manners CV. Endometrial assessment in a group of infertile women stimulated cycle for IVF: Immunohistochemical findings. *Hum Reprod* 1990; 5: 128–132.

8. Iwarsson K, Lindberg, Waller T. Common non-surgical techniques and procedures. In Handbook of Laboratory Animal Science. Eds. Svendsen P, Hau J. Boca Raton: CRC Press, 1994: 229–272.

9. Souren JEM, Ponec M, van Wijk R. Contraction of collagen by human fibroblasts and keratinocytes. *In Vitro Cell Dev Biol* 1989; 25: 1039–1045.

10. Iguchi T, Uchima FA, Ostrander PL, Bern HA. Growth of normal mouse vaginal epithelial cells in and on collagen gels. *Proc Natl Acad Sci USA* 1983; 80: 3743–3747.

11. Andrews AT. Electrophoresis. Theory, Techniques, and Biochemical and Clinical Applications. Oxford: Clarendon Press, 1987: 5–78.

12. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 1956; 62: 315–322.

13. Ghosh D, Dhara S, Kumar A, Sengupta J. Immunohostochemical localization of receptors for progesterone and estradiol-17β in the implantation site of the rhesus monkey. *Hum Reprod* 1999; 14: 505–514.

14. Sengupta J, Given RL, Carey JB et al. Primary culture of mouse endometrium on floating gels: a potential *in vitro* model for implantation. *Ann NY Acad Sci* 1986; 476: 75–94.

15. Siegel S, Castellan NJ Jr. Nonparametric Statistics for Behavioral Sciences. Singapore: McGraw-Hill, 1988: 206–216.

16. Ghosh D, Danielson KG, Alston JT, Heyner S. Functional differentiation of mouse uterine epithelial cells grown on collagen gels or reconstituted basement membranes. *In Vitro Cell Dev Biol* 1991; 27A: 713–719.

17. Bentin-Ley U, Pederson B, Lindenberg S, Falck Larsen J, Hamberger L, Horn. T. Isolation and culture of human endometrial cells in a three dimensional culture system. *J Reprod Fertil* 1994; 101: 327–332.

18. Ghosh D, Sengupta J. Morphological characteristics of human endometrial epithelial cells cultured on rat tail collagen matrix. *Hum Reprod* 1995; 10: 785–790.

19. Mani SK, Decker GL, Glasser SR. Hormonal responsiveness by immature rabbit uterine epithelial cells polarised *in vitro*. *Endocrinology* 1991; 128: 1563–1573.

20. Mani SK, Carson DD, Glasser SR. Steroid hormones differentially modulate glycoconjugate synthesis and vectorial secretion by polarised uterine epithelial cells *in vitro*. *Endocrinology* 1992; 130: 240–248.

21. Mulholland J, Winterhager E, Beier HM. Changes in proteins synthesised by rabbit endometrial epitehlial cells following primary culture. *Cell Tissue Res* 1988; 252: 123–132.

22. Gerschenson LE, Berliner J, Yang JJ. Diethylstilbesterol and progesterone regulation of cultured rabbit endometrial cell growth. *Cancer Res* 1974; 34: 2873–2880.

23. Gerschenson LE, Depaoli JR, Murai JT. Inhibition of estrogen induced proliferation of cultured rabbit uterine epithelial cells by a cell density-dependent factor produced by the same cells. *J Steroid Biochem* 1981; 15: 959–969.

24. Casslen BG, Harper MJK. Human endometrial epithelial cells grown on collagen in serum free medium. Estrogen responsiveness and morphology. *Acta Endocrinologica* 1991; 125: 101–108.

25. Haslam SZ, Levely ML. Estrogen responsiveness of normal mouse mammary cells in primary cell culture: association of mammary fibroblasts with estrogenic regulation of progesterone receptors. *Endocrinology* 1985; 116: 1835–1843.

26. Levine JF, Stockdale FE. Cell-cell interactions promote mammary epithelial cell differentiation. *J Cell Biol* 1985; 100: 1415–1422.