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Growth of normal mouse vaginal epithelial cells in and on collagen gels

(serum-free cell culture/estrogen responsiveness/primary epithelial cell culture)

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ABSTRACT **Sustained growth in primary culture of vaginal epithelial cells from ovariectomized adult BALB/cCrgl mice embedded within or seeded on collagen gel matrix was achieved in a serum-free medium composed of Ham's F-12 medium/Dulbecco's modified Eagle's medium, 1:1 (vol/vol), supplemented with insulin, bovine serum albumin fraction V, epidermal growth factor, cholera toxin, and transferrin. Three-dimensional growth of vaginal epithelial cells occurred inside the collagen gel matrix. Cell numbers increased 4- to 8-fold in collagen gel and about 4-fold on collagen gel after 9–10 days in culture. The effect of 17β-estradiol (0.00018–180 nM in gel or 0.018–180 nM on gel) and diethylstilbestrol (DES; 0.0186–186 nM in gel) on the growth of vaginal epithelial cells was examined. The addition of estrogen did not enhance the growth of vaginal epithelial cells during this time period either in the complete medium or in a suboptimal medium. Cultures on floating collagen gels in the serum-free medium are composed of 1–3 cell layers with superficial cornification. Estrogen does not appear to be a direct mitogen for vaginal epithelial cells, at least in this system.**

Estrogens stimulate *in vivo* proliferation and cornification of mouse vaginal epithelium (1). It is not clear, however, whether the effect of estrogens is directly upon the epithelial cells or due to an indirect action on the epithelium by affected stromal cells (2). The responses of the vaginal epithelium of rats and mice to estrogenic hormones have been studied in various organ culture systems. Some investigators showed that explanted rat vaginae (3, 4) and mouse vaginal epithelial outgrowths (2) failed to cornify in response to estradiol, whereas others have reported that the addition of estrogen to the culture medium evoked cornification of vaginal explants from rats (5–8) and mice (9–13). Martin (14) described growth and cornification of cultured mouse vaginal epithelium in the absence of estrogen. The possible role of the stroma and the difficulty of accurate quantification of increases in cell number are among the problems with both organ and cell culture methods. Furthermore, in organ culture, tissues degenerate with time; in cell culture, lack of sustained cell division and decreasing viability occur with time (2). To examine the possible direct effect of estrogen on proliferation and cornification of vaginal epithelium, a system is needed for the *in vitro* culture of isolated epithelial cells.

Recently, a collagen gel culture system was developed for mammary epithelial cells that results in sustained growth of mouse, rat, and human cells in primary culture (15–21). This *in vitro* system has been used successfully in our studies for the growth of mouse vaginal epithelial cells, as judged by an increase in cell number, and for the possible hormonal regulation of differentiation of these cells in the resulting cultures. In the present communication, we report on the sustained growth of cultured vaginal epithelial cells in a serum-free medium and on the failure of estrogen to stimulate further growth.

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MATERIALS AND METHODS

Epithelial Cell Isolation. Normal vaginae dissected from 50- to 60-day-old BALB/cCrgl mice 6–7 days after ovariectomy were transversely sectioned and then incubated in Hank's balanced salt solution containing 0.1% collagenase (CLS III, 175–184 units per mg; Worthington) and 5 mg of bovine serum albumin fraction V per ml for 2 hr at 37°C in a shaking water bath. Epithelial sheets manually separated from stroma were minced into small clumps with a razor blade on a Teflon block. Cell clumps were collected by centrifugation at 1,000 × *g* for 5 min. More than 85% of the cells were found to be viable as determined by the trypan blue exclusion test. Cell number was estimated by mixing 1 vol of cell suspension with 9 vol of 0.02% crystal violet in 0.1 M citric acid and counting stained nuclei in a hemocytometer.

Preparation of Collagen Gels. Collagen solution and gels were prepared as originally described (22). Briefly, 1 g of rat tail collagen fibers was sterilized in ethanol overnight and dissolved in 300 ml of acetic acid in sterile distilled water, 1:1,000 (vol/vol); the supernatant after centrifugation at 10,000 × *g* for 30 min provided the stock collagen solution; 8 vol of stock solution was mixed with 2 vol of 10× Waymouth medium (GIBCO)/0.34 M NaOH, 2:1 (vol/vol), and was kept on ice to prevent immediate gelation. Cells were added to the cold gelatin mixture; 0.5 ml, containing 1.2–2.5 × 10^5 cells, was placed onto 0.3 ml of gelled collagen and allowed to gel at room temperature, or 1–2 × 10^5 cells were plated on 0.5 ml of gelled collagen in each well of Falcon multiwell plates.

The cells were cultured in a medium consisting of Ham's F-12 medium/Dulbecco's modified Eagle's medium (DME medium; GIBCO), 1:1 (vol/vol), containing $NaHCO_3$ at 1.2 g/liter with 50 units of penicillin and 50 μg of streptomycin per ml. This medium was supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), cholera toxin (0.01 μg/ml), and bovine serum albumin (5 mg/ml) from Sigma and with epidermal growth factor (EGF, 10 ng/ml) from Collaborative Research, Waltham, MA. For a suboptimal medium, only insulin (10 μg/ml) and bovine serum albumin (5 mg/ml) were added. Cultures were incubated at 37°C in 95% air/5% CO_2 and fed every 2 days.

Four days after the cells were seeded on collagen gel matrix, some of the gels were released from the plastic substrate by loosening the edges with a forceps and gently shaking the plate. These released gels float beneath the medium surface (floating collagen gels).

Cell number was determined by a DNA fluorometric assay

Abbreviation: DME medium, Dulbecco's modified Eagle's medium.

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(23) utilizing BALB/cfC3H mammary tumor epithelial cells, counted in a hemocytometer, as a standard.

Cultures were fixed in Bouin's fluid, dehydrated in ethanol, and embedded in paraffin. The embedded samples were sectioned perpendicularly to the tissue culture plate at 7 μm and stained with hematoxylin and eosin.

For transmission electron microscopy, cultures were fixed at room temperature for 2 hr in 1% formaldehyde/3% glutaraldehyde/0.1 M sodium cacodylate, postfixed for 1 hr in 1% OsO_4/0.1 M sodium cacodylate, stained 1–2 hr *en bloc* with saturated uranyl acetate dehydrated in ethanol, and embedded in Spurr's resin. Thin sections stained with uranyl acetate and lead citrate were examined in a Siemens Elmiskop 102 electron microscope.

RESULTS

Serum-Free Culture. Freshly-dissociated vaginal epithelial cells were cultured in or on the collagen gel matrix, and their general growth pattern was monitored with phase microscopy. A characteristic and reproducible pattern of growth was observed. Small clumps of cells embedded in the matrix remained as a spherical mass for the first 12 hr. This was followed by a period during which duct-like structures radiated from the mass into the matrix, resulting in three-dimensional outgrowths (Fig. 1 *Upper*). The three-dimensional outgrowths that resulted from the seeded clumps were of various shapes. In general, the outgrowths ranged from duct-like and sheet-like appearance to spherical masses. There were also outgrowths of mixed morphology: duct-like structures projecting from a spherical mass. Histology of the duct-like outgrowths is shown in Fig. 1 *Lower*. Duct-like outgrowths often consisted of a lumen surrounded by epithelial cells. When these outgrowths of varying morphology were recovered by collagenase digestion of the matrix and plated on plastic dishes, they gave rise to islands having typical polygonal epithelial morphology. Small clumps of cells seeded on collagen gel attached to the matrix within 24 hr, then migrated to form islands of polygonal cells and grew as monolayered cell sheets. Monolayer culture of cells seeded on a plastic culture dish were typically polygonal in morphology, and colonies progressively detached from the culture dish.

Growth of cells both embedded in and seeded on collagen gel was apparent by phase microscopy and was confirmed by measuring the total DNA content per well as a function of time. Cells were recovered from the gels by acetic acid dissolution and stored frozen until the DNA assay (24). The growth curves of cells in and on the collagen gel matrix revealed an increase in DNA content as a function of culture period (Fig. 2).

Effect of Estrogenic Hormones. An increase of 4- to 8-fold

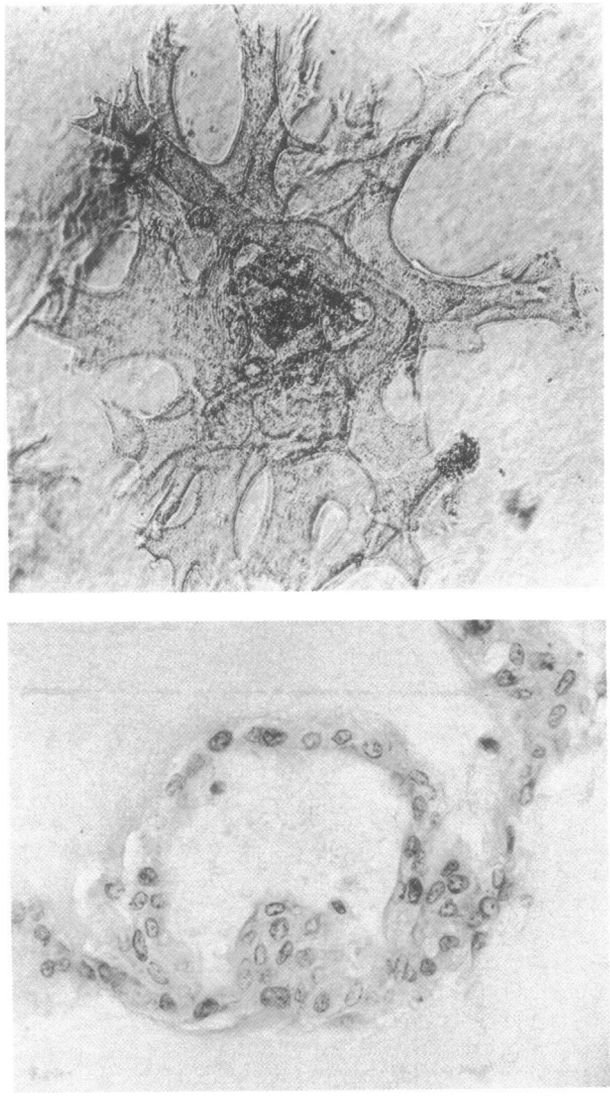

FIG. 1. (*Upper*) Phase micrograph of three-dimensional outgrowth of vaginal epithelial cells in collagen gel. (×83.) (*Lower*) Histology of a duct-like outgrowth of vaginal epithelial cells cultured in serum-free medium in collagen gel. (×330.)

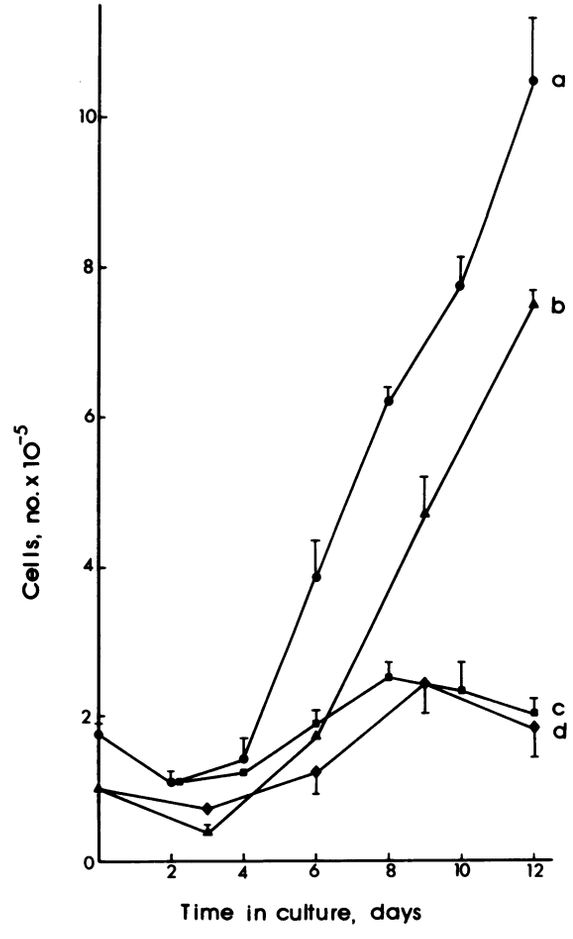

FIG. 2. Growth curves for vaginal epithelial cells cultured in serum-free medium in collagen gel (curve a) or on collagen gel (curve b) or in the presence of 10% swine serum in collagen gel (curve c) or on collagen gel (curve d) (mean ± SD, n = 3).

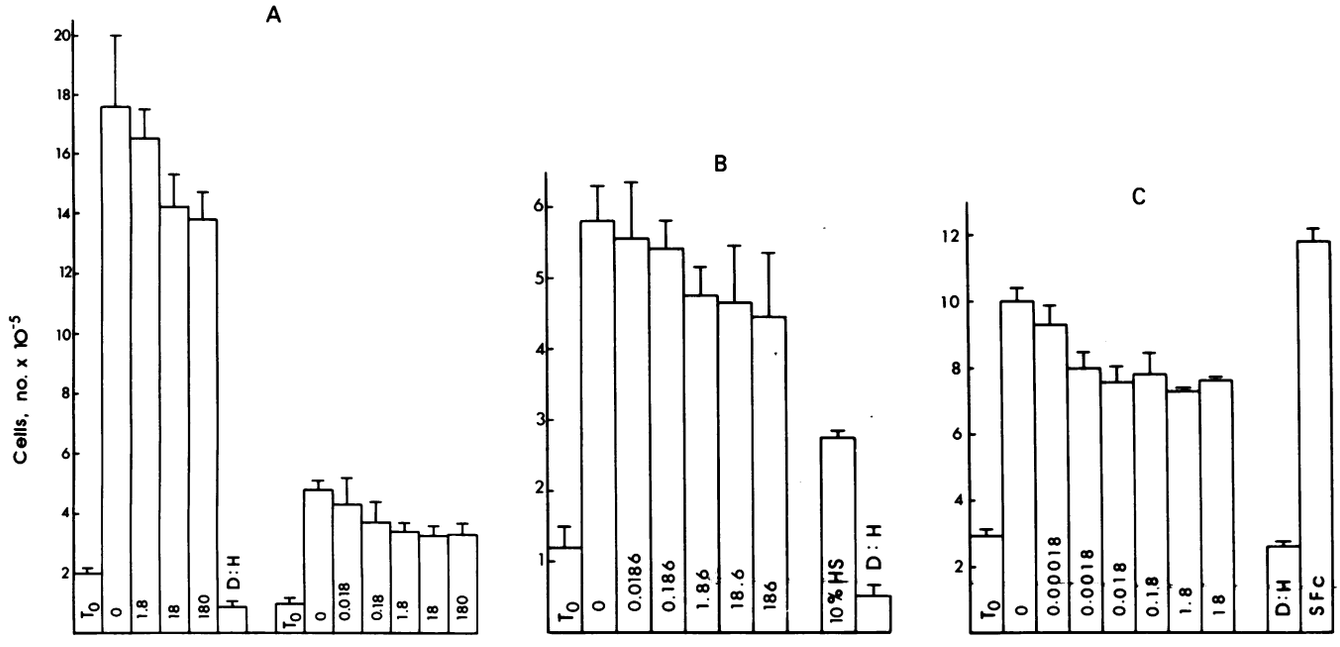

FIG. 3. (*A*) Growth of vaginal epithelial cells (mean ± SD, $n = 3$) cultured for 10 days in serum-free medium with or without 17β-estradiol (nM) in collagen gel (*Left*) or on collagen gel (*Right*). T_0, time 0 values; D:H, unsupplemented medium. (*B*) Growth of vaginal epithelial cells (mean ± SD, $n = 3$) cultured for 10 days in collagen gel in serum-free medium with or without diethylstilbestrol (nM) (*Left*) or in the presence of 10% horse serum (HS) or unsupplemented medium (D:H) (*Right*). T_0, time 0 values. (*C*) Growth of vaginal epithelial cells (mean ± SD, $n = 3$) cultured for 8 days in suboptimal serum-free medium with or without 17β-estradiol (nM) in collagen gel (*Left*) or in unsupplemented medium (D:H) or serum-free complete medium (SFc) (*Right*). T_0, time 0 values.

in gel or approximately 4-fold on gel occurred after 9–10 days in the basal serum-free medium [DME medium/Ham's F-12 medium, 1:1 (vol/vol), supplemented with insulin (10 μg/ml), transferrin (10 μg/ml), cholera toxin (0.01 μg/ml), bovine serum albumin (5 mg/ml), and epidermal growth factor (10 ng/ml)]. The effect of 17β-estradiol (1.8–180 nM in gel or 0.018–180 nM on gel) and diethylstilbestrol (DES;0.0186–186 nM in gel) in serum-free medium on the growth of vaginal epithelial cells from ovariectomized mice was examined. Addition of either 17β-estradiol or diethylstilbestrol failed to enhance vaginal epithelial cell growth when cultured either in or on collagen gel (Fig. 3 *A* and *B*). The effect of 17β-estradiol (0.00018–180 nM) on the growth of vaginal epithelial cells was tested in suboptimal serum-free medium [DME medium/Ham's F-12 medium, 1:1 (vol/vol), supplemented with insulin (10 μg/ml) and bovine serum albumin (5 mg/ml)]. In this medium, cell number increased about 2- to 3-fold in the collagen gel; however, 17β-estradiol did not enhance vaginal epithelial cell growth (Fig. 3*C*). Histological examination of epithelial cells growing in or on collagen gel revealed no marked keratinization even in the presence of estradiol (Fig. 4 *Upper*).

Floating Collagen Gel Culture. Examination by light microscopy revealed that the cells grown on detached collagen gels (floating gel culture) in serum-free medium showed an enhanced organization. The most obvious difference between the detached and intact cultures was the development of some superficial keratinized cell layers in the former (Fig. 4 *Lower*).

Electron-Microscope Observations. Transmission electron micrograph of cells cultured in collagen in serum-free medium revealed ultrastructure similar to that previously described for vaginal epithelial cells *in vivo* (25, 26). The cells contained numerous free ribosomes, rod-shaped mitochondria, cytoplasmic processes projecting into the intercellular space, tonofilaments and desmosomes connecting adjacent cells (Fig. 5). Tonofilaments and desmosomes are characteristic features of epithelial cells.

DISCUSSION

Floating collagen gels have been used to study hormonal regulation of mammary epithelial cell differentiation (27–32); primary cultures embedded in gel have shown growth of normal and neoplastic mouse, rat, and human mammary epithelial cells (15–21) and normal mouse submandibular epithelial cells (24, 33). In the present study, which adopts these collagen gel culture systems, we report successful, sustained primary culture of mouse vaginal epithelial cells. This system can be further utilized to analyze the regulation of growth and differentiation of isolated mouse vaginal epithelial cells.

The results indicate the successful growth of mouse vaginal epithelial cells. Cells plated on conventional plastic culture dishes did not grow (unpublished data); however, when coated with collagen gel, epithelial cell proliferation as judged by 4- to 8-fold increase in DNA content was observed. Vaginal epithelial cell shapes are different in collagen gels compared with those in conventional monolayers. Evidence exists for an important role of cell shape in the control of growth (34, 35) and elicitation of hormone responsiveness (31, 36–39).

Estrogenic hormones failed to stimulate growth of normal vaginal epithelial cells both in and on collagen gel. The lack of a proliferative response to estrogenic hormones corresponds to some recent findings that seem to challenge the classical concept that trophic hormones directly stimulate their target cells to proliferate. For example, corticotropin, thyrotropin, and gonadotropins, thought to be responsible for both the growth and functioning of the adrenal cortex, thyroid, and ovary, respectively, were found to have no effect or actually to inhibit the growth of their target cells *in vitro* (40–42). The reason for the lack of vaginal epithelial cell response to estrogen in the present study is not clear, but several explanations may be proposed. (*i*) The number of receptor sites for estradiol is affected by ovarian factors (43) and increased by prolactin (44). It is also possible that these cells may lack sufficient estrogen receptor

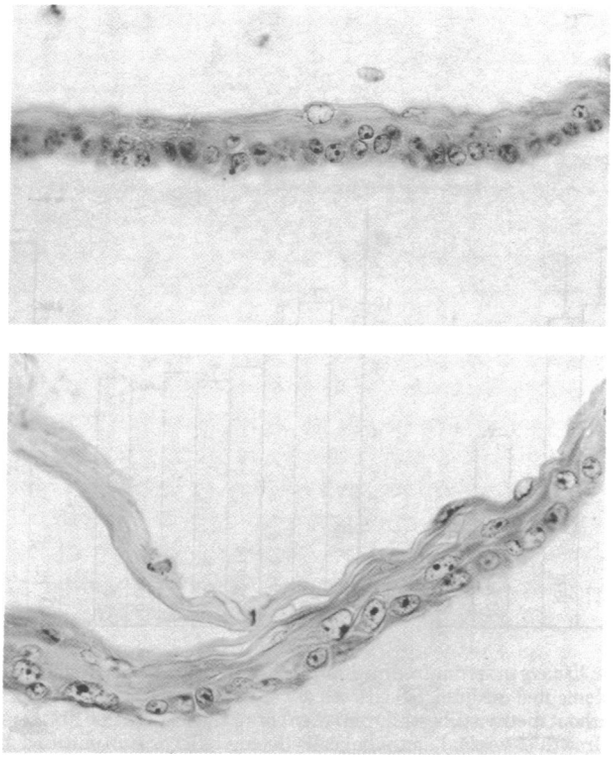

FIG. 4. (*Upper*) Histology of cultured vaginal epithelial cells in serum-free medium on attached collagen gel revealing absence of keratinization. (×330.) (*Lower*) Histology of cultured vaginal epithelial cells in serum-free medium on floating collagen gel showing superficial keratinized cell layers. (×330.)

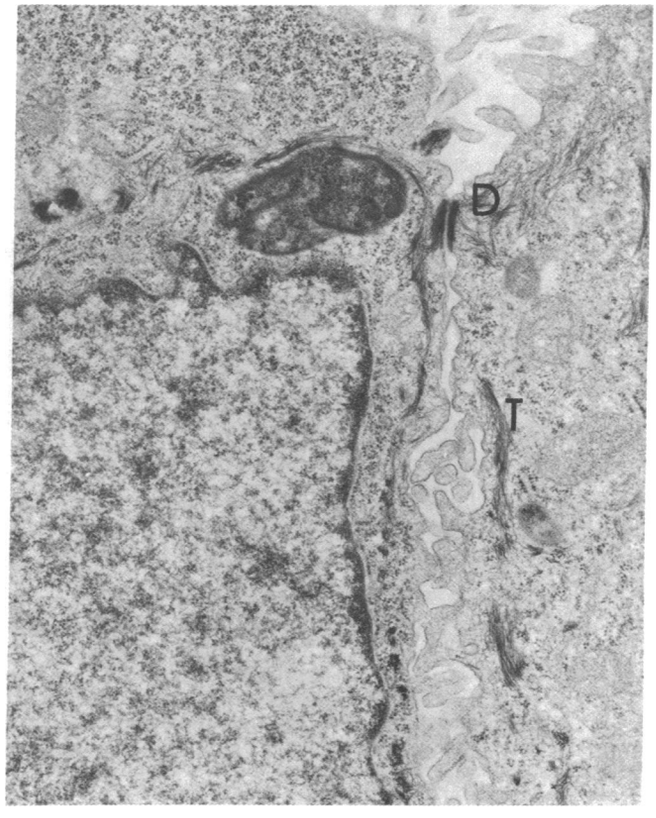

FIG. 5. Transmission electron micrograph of cultured vaginal epithelial cells in serum-free medium in collagen for 7 days showing desmosome (D) and tonofilaments (T). (×20,000.)

sites in the cytoplasm and nucleus because of deficiencies in the *in vitro* environment (45). (*ii*) The presence of estrogen on a continuous basis may alter cell function and mitotic activity *in vivo* so that a decrease in growth is observed (46). However, in organ cultures of mouse vagina, where the epithelium is reported to differentiate in response to estradiol (9–13), hormone was present for extended periods and obviously was not inhibitory. Our lot of albumin fraction V was assayed for steroids by radioimmunoassay (20) and was found to contain picogram amounts of estradiol and progesterone per gram of albumin. Therefore, the albumin is not believed to be a significant source of ovarian steroids in our system. It is not likely that lack of growth response is due to the continuous presence of estrogen. (*iii*) Associated connective tissue factor(s) may be required for epithelial cell response. Both during development and in the adult state, epithelial cell proliferation and differentiation are known to be influenced by underlying connective tissue factors (47). Specific aspects of epithelial cell differentiation in developing hormone-sensitive tissues may result from a combination of direct hormonal and connective tissue-mediated interactions (48–50). In organ culture studies of mouse vagina, where keratinization has been demonstrated in response to estrogen alone, the epithelium enjoys a normal relationship with the underlying connective tissue (9–13). In this study, examination of epithelial cells growing in or on collagen gel showed no marked keratinization even in the presence of estradiol. The keratinization seen in cultures grown on detached gels is minimal and not increased by estrogen. The growth-promoting action of estrogenic hormones on a target tissue may be an indirect one, mediated by an estrogen-induced growth factor from a different tissue (51–53), including possibly the vaginal stroma.

Several observations indicated strongly that the cell outgrowths are epithelial in origin: (*i*) When embedded cells were recovered and transferred to monolayer culture on plastic or collagen gel, they formed a continuous sheet of polygonal cells. There was no obvious contamination of fibroblasts in the monolayers. Fibroblasts in the collagen gels give rise to characteristic morphology (15), and the extent of fibroblastic contamination can be assessed by microscopic observation. There were no obvious fibroblastic outgrowths in the collagen gels. (*ii*) Cells plated on collagen gels were organized into a sheet of polygonal cells. When these gels were released, the cells stratified and formed superficial keratinized layers. This histology is characteristic of vaginal epithelium *in vivo*. (*iii*) Ultrastructural observations of the outgrowths cultured in serum-free medium in collagen gel demonstrated tonofilaments and desmosomes characteristic of vaginal epithelial cells.

The authors honor the accomplishments of Professor Kiyoshi Takewaki, pioneer in the study of vaginal responses to hormones, on the occasion of his 77th birthday. Professor S. Nandi kindly reviewed the paper and made important suggestions. We thank Dr. Yasuhiro Tomooka for valuable discussions; Karen Tanada Mills, Aniko Mos, and Lionel Thomas for assistance; John Underhill for photographic expertise; and Susie Castillo for typing the manuscript. This investigation was supported by Public Health Services Grants CA05388 and CA09041 awarded by the National Cancer Institute.

1. Gardner, W. U. (1959) *Ann. N.Y. Acad. Sci.* **83**, 145–159.
2. Flaxman, B. A., Copra, D. P. & Newman, D. (1973) *In Vitro* **9**, 194–201.
3. Rosenthal, W. (1934) *Acta Brevia Neerl. Physiol. Pharmacol. Microbiol.* **4**, 13–15.
4. Emmens, C. W. & Ludford, R. J. (1940) *Nature (London)* **145**, 746–747.
5. Dux, C. (1941) *Ann. Endocrinol.* **2**, 39–59.
6. Coujard, R. (1943) *Bull. Biol. Fr. Belg.* **77**, 120–223.

7. Hardy, M. H., Biggers, J. D. & Claringbold, P. J. (1953) *Nature (London)* **172,** 1196–1197.
8. Kahn, R. H. (1954) *Nature (London)* **174,** 317.
9. Kahn, R. H. (1959) *Ann. N.Y. Acad. Sci.* **83,** 347–355.
10. Biggers, J. D., Claringbold, P. J. & Hardy, M. H. (1956) *J. Physiol. (London)* **131,** 497–515.
11. Lasnitzki, I. (1961) *Br. J. Radiol.* **34,** 356–361.
12. Lasnitzki, I. (1961) *Exp. Cell Res.* **24,** 37–45.
13. Mori, T. & Kimura, T. (1970) *J. Fac. Sci. Univ. Tokyo Sect. 4* **12,** 23–36.
14. Martin, L. (1959) *J. Endocrinol.* **18,** 334–342.
15. Yang, J., Richards, J., Bowman, P., Guzman, R., Enami, J., McCormick, K., Hamamoto, S., Pitelka, D. & Nandi, S. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3401–3405.
16. Yang, J., Guzman, R., Richards, J., Imagawa, W., McCormick, K. & Nandi, S. (1980) *Endocrinology* **107,** 35–41.
17. Yang, J., Richards, J., Guzman, R., Imagawa, W. & Nandi, S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2088–2092.
18. Yang, J., Guzman, R., Richards, J., Jentoft, V., DeVault, M. R., Wellings, S. R. & Nandi, S. (1980) *J. Natl. Cancer Inst.* **65,** 337–343.
19. Yang, J., Elias, J. J. Petrakis, N. L., Wellings, S. R. & Nandi, S. (1981) *Cancer Res.* **41,** 1021–1027.
20. Imagawa, W., Tomooka, Y. & Nandi, S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4074–4077.
21. Pasco, D., Quan, A., Smith, S. & Nandi, S. (1982) *Exp. Cell Res.* **141,** 313–324.
22. Michalopoulos, G. & Pitot, H. C. (1975) *Exp. Cell Res.* **94,** 70–78.
23. Hinegardner, R. T. (1971) *Anal. Biochem.* **39,** 197–201.
24. Yang, J., Flynn, D., Larson, L. & Hamamoto, S. (1982) *In Vitro* **18,** 435–442.
25. Mori, T., Nagahama, Y. & Bern, H. A. (1974) *Anat. Rec.* **179,** 225–240.
26. Iguchi, T. & Ohta, Y. (1980) *Acta Anat.* **108,** 469–480.
27. Emerman, J. T. & Pitelka, D. R. (1972) *In Vitro* **13,** 316–328.
28. Emerman, J. T., Enami, J., Pitelka, D. R. & Nandi, S. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4466–4470.
29. Enami, J. & Nandi, S. (1977) *J. Dairy Sci.* **61,** 729–732.
30. Emerman, J. T., Burwen, S. J. & Pitelka, D. R. (1979) *Tissue Cell* **11,** 109–119.
31. Burwen, S. J. & Pitelka, D. R. (1980) *Exp. Cell Res.* **126,** 249–262.
32. Shannon, J. M. & Pitelka, D. R. (1981) *In Vitro* **17,** 1016–1028.
33. Yang, J., Larson, L. & Nandi, S. (1982) *Exp. Cell Res.* **137,** 481–485.
34. Folkman, J. & Moscona, A. (1978) *Nature (London)* **273,** 345–349.
35. Gospodarowicz, D., Greenburg, G. & Birdwell, C. R. (1978) *Cancer Res.* **38,** 4155–4171.
36. Yang, J., Enami, J. & Nandi, S. (1977) *Cancer Res.* **37,** 3644–3647.
37. Sakai, S., Bowman, P. D., Yang, J., McCormick, K. & Nandi, S. (1979) *Endocrinology* **104,** 1447–1449.
38. Enami, J., Yang, J. & Nandi, S. (1979) *Cancer Lett.* **6,** 99–105.
39. Michalopoulos, G., Sattler, G. I. & Pitot, H. C. (1978) *Cancer Res.* **38,** 1550–1555.
40. Ramachandran, J. & Suyama, A. T. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 113–117.
41. Gospodarowicz, D., Ill, C. R. & Birdwell, C. R. (1977) *Endocrinology* **100,** 1108–1120.
42. Westermark, B., Karlsson, F. A. & Walinder, O. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2022–2026.
43. McGuire, J. L. & Lisk, R. D. (1968) *Proc. Natl. Acad. Sci. USA* **61,** 497–503.
44. Shafie, S. & Brooks, S. C. (1977) *Cancer Res.* **37,** 792–799.
45. Fleming, H., Blumenthal, R. & Gurpide, E. (1982) *Endocrinology* **111,** 1671–1677.
46. Epifanova, O. I. (1971) in *The Cell Cycle and Cancer*, ed. Baserga, R. (Dekker, New York), Vol. 1, pp. 145–190.
47. Fleischmajer, R. & Billingham, R. E., eds. (1968) *Epithelial-Mesenchymal Interactions*, 18th Hahnemann Symposium (Williams & Wilkins, Baltimore), p. 326.
48. Franks, L. M., Riddle, P. N., Carbonell, A. W. & Gey, G. O. (1970) *J. Pathol.* **100,** 113–119.
49. Cunha, G. R. (1976) *Int. Rev. Cytol.* **47,** 137–194.
50. Cunha, G. R., Shannon, J. M., Neubauer, B. L., Sawyer, L. M., Fujii, H., Taguchi, O. & Chung, L. W. K. (1981) *Hum. Genet.* **58,** 68–77.
51. Sirbasku, D. A. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3786–3790.
52. Sirbasku, D. A. (1979) *Cold Spring Harbor Conf. Cell Proliferation* **6,** 477–497.
53. Sirbasku, D. A., Officer, J. B., Leland, F. E. & Ito, M. (1982) *Cold Spring Harbor Conf. Cell Proliferation* **9,** 763–778.