Journal of Cellular Biochemistry 57:262–270 (1995)

# Differentiation in Human Endometrial Cells in Monolayer Culture: Dependence on a Factor in Fetal Bovine Serum

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**Abstract** Human epithelial cells of the Ishikawa endometrial line can be stimulated to differentiate and form multicellular structures in 4–5 day-old monolayer cultures by the addition of a protein factor from fetal bovine serum. Multicellular structures become obvious over an 18–30-h period as the cells enlarge, separate from the dish, and form domes. These structures are similar to those that result from polarization in other epithelial cell lines. Ishikawa dome formation appears to be a multistage process. The appearance of enlarged differentiated cells is detected within hours of adding fetal bovine serum; these enlarged cells lift off the surface of the dish within 6–8 more hours. Domes are observed about 24 h after the addition of fetal bovine serum. Sometimes dome cells migrate into a "bud-like" structure that extends out from the dome. Differentiation of the domes is dependent on a factor from fetal calf serum that behaves similarly to a very large protein or complex of proteins, greater than 300 kd. Progesterone appears to enhance the formation of domes but does not elicit dome formation in the absence of serum factor. 

**Key words:** Ishikawa cells, monolayer, domes, polarization, budding, progesterone, differentiation, endometrium

Epithelial polarization is one of the earliest examples of cellular differentiation in the developing mammalian embryo. As Rodriguez-Boulan and Nelson [1989] discussed in their review, polarization occurs as early as the morula stage (8–16 cells) resulting in the formation of a layer of specialized cells surrounding the internal cavity of the blastocyst. Thereafter, polarization, sometimes accompanied by migration, occurs over and over again in populations of epithelial cells as embryonic development proceeds. A common differentiation event associated with polarized epithelia involves budding of cells from sheets of polarized epithelia into structures that eventually become the tubules and glands of liver, intestine, kidney, pancreas, uterus, and other organs [Gumbiner, 1992].

The singularly important sequence of biochemical and morphological changes whereby nonpolarized epithelia become polarized has actually been found to occur in vitro and has been studied in monolayer cell cultures in kidney lines such as Madin-Darby canine kidney (MDCK) [Cereijido et al., 1981] and LLC-PK1 [Mullin and O'Brien, 1986]; in intestinal lines such as Caco 2 [Pinto et al., 1983] and HT-29 [Zweibaum et al., 1985] and in mammary cell lines [Pourreau-Schneider et al., 1984]. The capacity for polarization was initially detected in monolayers by the regional elevation of cells into fluid-containing domes. This unusual accumulation of fluid is assumed to result from vectorial transport across apical and basal membranes, occurring as a result of cell polarization. The possible morphological significance of dome formation is suggested by the observation that MDCK cells that polarize and form domes on an impermeable dish are able to polarize into tubules [Montesano et al., 1992] in a three-dimensional cell culture environment containing collagen.

Polarization is one of the mechanisms essential to the complex process of differentiation. Epithelial populations must be able to polarize even in adult organisms, since vectorial transport is an essential characteristic of cells lining all of the cavities in the body and these cells must be replaced from time to time. A dramatic example of loss and renewal of polarized epithelia occurs in the endometrial lining of the uterus.

Received May 11, 1994; revised June 26, 1994; accepted June 29, 1994.

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A stem population of epithelial cells proliferates early during each menstrual cycle and becomes differentiated prior to ovulation. An important part of that differentiation involves the invagination of luminal epithelia into glandular structures. That differentiation, resulting in extensively branched glands, must include epithelial polarization, budding, elaboration, and branching of glands deeply into the mesenchyme; it must also be accompanied by increases in the secretory function of the epithelial cells. In the absence of implantation of a fertilized egg, these differentiated cells are sloughed off, and the process begins again.

In this paper we report that the human endometrial epithelial line, called Ishikawa [Nishida et al., 1985; Holinka et al., 1986], retains the ability to differentiate and polarize in response to a protein factor in fetal bovine serum (FBS). Polarization results in the formation of domes with some evidence for "budding" of these structures. The precursors of these "domes" are differentiated clusters of cells. The process of differentiation can be augmented by the addition of physiological concentrations of the steroid progesterone. We have attempted an initial characterization of the differentiation process and of the serum factor involved in that process.

## MATERIALS AND METHODS

Ishikawa cells were cultured in phenol-red free minimum essential medium (MEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg amphotericin B (Gibco, Grand Island, N.Y.). The cells were obtained from Dr. Erlio Gurpide at Mt. Sinai Hospital (New York, NY) and were originally derived from an endometrial adenocarcinoma line developed by Nishida et al. [1985], who demonstrated the presence of receptors for both estradiol and progesterone. Holinka and colleagues showed that these cells are responsive to estradiol with regard to proliferation [Holinka et al., 1986a] and as measured by the induction of alkaline phosphatase [Holinka et al., 1986b].

Experiments to quantitate differentiation were performed with cells seeded at an approximate density of $5 \times 10^5$ cells/cm$^2$, grown for approximately 3–4 days in 5% FBS and then transferred to medium containing 1% FBS. Assays for dome formation were done in confluent cultures, although differentiation has been observed in nonconfluent cultures. Cultures left in MEM with 1% FBS could survive for an additional 3–5 days with little proliferation. Experiments had to be done in cultures with a minimum of 1% FBS to keep the cells alive, but at this concentration of FBS there was no dome differentiation.

Differentiation started with dramatic changes in individual cells or clusters of cells and culminated in multicellular structures that looked like domes. These structures were visible and easily quantified using an Olympus inverted-stage microscope at a power of ×40. Magnifications of ×100 and ×200 were used to examine the differentiating cells. Many of the experiments were done in multiwell dishes, containing either 24 or 48 wells. Some experiments were done in larger dishes. Differentiated structures or domes were always counted over the entire area of the dish or the well, since domes sometimes form in clusters. The numbers counted in each experiment are reported, together with the area surveyed. It quickly became obvious that cultures needed to be examined several times over a 3–4 day period after the addition of FBS factor. Peak levels of "dome formation" often did not appear until 24–48 h after the addition of FBS and frequently the domes subsided after another 24–48 h. Progesterone and/or estradiol (Sigma, St. Louis, MO) were added to the cells at physiological concentrations of 10 nM.

In a first step to characterize the factor responsible for dome formation, the FBS was fractionated by centrifugation through a series of exclusion filters. The experiment included in this paper shows the results of placing FBS into a 15-ml "Macrosep" centrifugal concentrator (Filtron, MA) designed to exclude macromolecules larger than 300,000 daltons. The concentrator was placed in a Sorvall SS 34 fixed-angle rotor and centrifuged at 6,000 *g* for 30 min. The material retained by the filter as well as the material that passed through the filter was assayed by adding it with fresh medium containing 1% FBS to confluent cells.

## RESULTS

Differentiation, culminating in dome formation, can be detected within hours in cultures to which FBS has been added. Figures 1 and 2 illustrate the stages of differentiation as observed at two different magnifications. At the relatively low magnification of ×40, individual cells are not visible, but it is possible to see that the addition of 15% FBS quickly (within 8 h), causes the appearance of gaps in the grainy

surface of the monolayer (Fig. 1a). After 16 or more hours, numerous multicellular dome structures can be detected in a field of cells (Fig. 1b). At low magnification, the salient feature of these structures is the "ring" of differentiated cells. After an additional 16–24 h, these characteristic rings become more irregular (Fig. 1c), but some are still visible. At higher magnifications, it is sometimes possible to detect that the dome cells are no longer elevated. After another day or two any evidence of differentiation will have completely disappeared.

Under higher magnification (×200), the "gaps" appearing in the culture within the first 10 h can be seen to be differentiating cells, or clusters of cells (Fig. 2a,b) that have become enlarged and have lost the granular appearance characteristic of nondifferentiated cells. Some of the differentiating cells appear to be three or four times larger than surrounding monolayer cells. It is not clear whether individual cells have swollen or cells have fused to make these very large cells.

These enlarged cells, or clusters of cells, lift off the surface of the dish during the next several hours so that it becomes necessary to focus above the monolayer to clearly see differentiated cell structure. The cells that have become elevated (Fig. 2c) look very different from the cells in the surrounding monolayer (Fig. 2d). A lumen is clearly visible in the monolayer (Fig. 2d).

The ring structures seen at lower magnification, approximately 20 hr after the addition of 15% FBS, are shown at higher magnifications to be due to the differentiation of cuboidal or even columnar cells surrounding the lumen, at the base of the dome (Fig. 2f). Processes frequently extend from the bordering cells into the lumen. Focusing up on the dome reveals that dome cells are smaller and more numerous (Fig. 2e) than the cells initially observed to detach from the surface of the dish (Fig. 2c). The decrease in cell size suggests cell division, but it is also possible that cells migrate up from the monolayer into the dome structure.

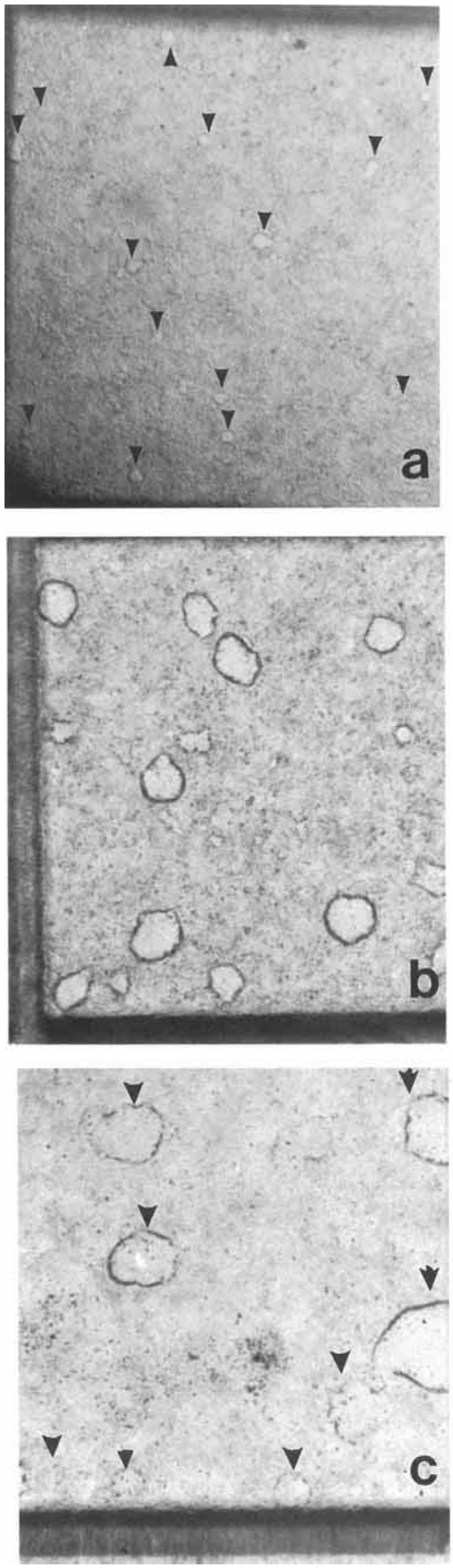


**Fig. 1.** Stages in differentiation of Ishikawa cells as seen under low magnification. A field of cells in a 35-mm culture dish (**a**), 8 h after the addition of 15% FBS to cells being maintained in 1% FBS, appears to contain numerous "gaps" (arrows). By 24 h, the gaps have enlarged and developed into dome structures that can clearly be detected by the ring of cells surrounding the lumen (**b**). After another 24 h, domes enlarge or fade away (**c**). ×40.

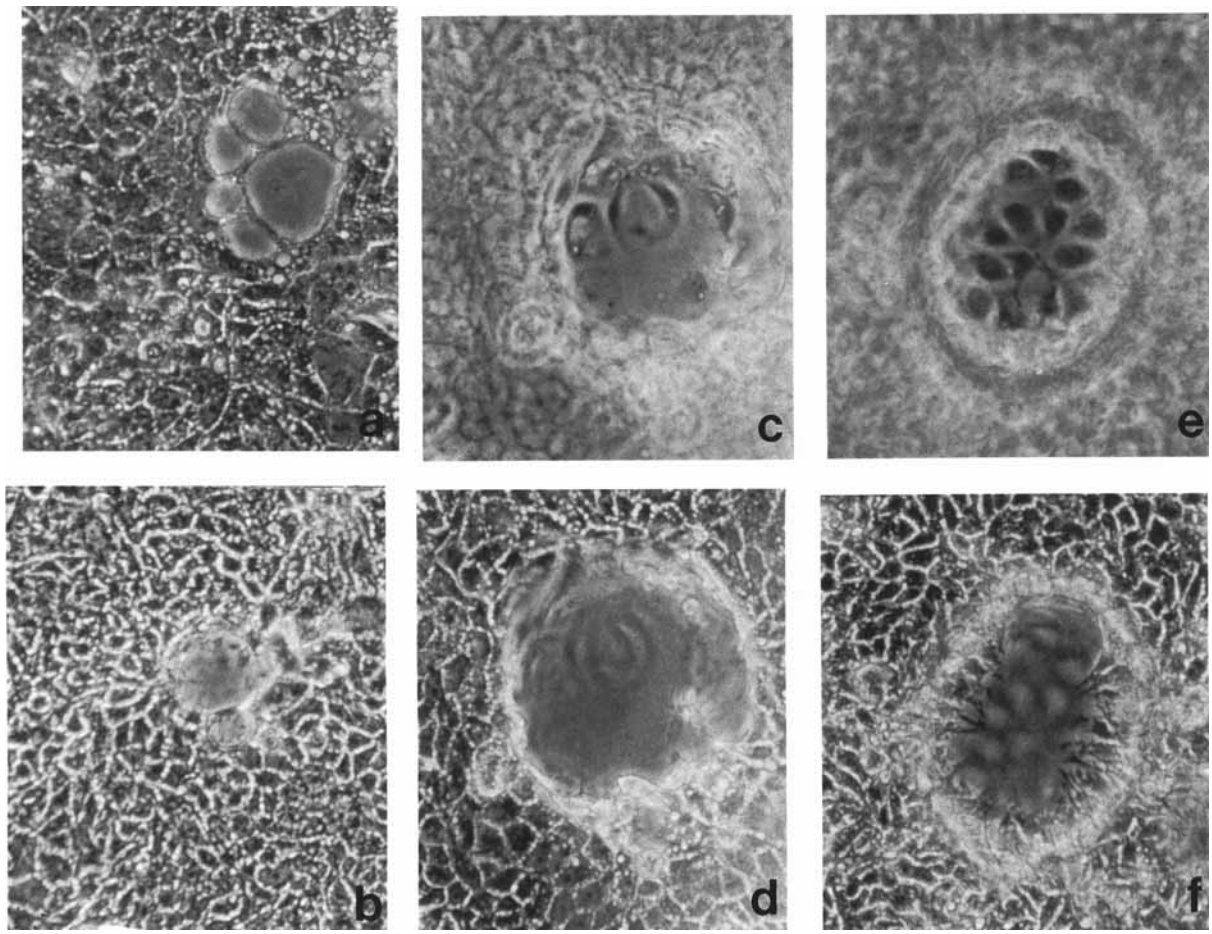


**Fig. 2.** Cellular changes accompanying differentiation in Ishikawa cells. Within 8 h of adding 15% FBS, clusters of cells (**a**) and single cells (**b**) become enlarged and lose the granular appearance of surrounding monolayer. By 16 h, enlarged differentiated cells have lifted off the surface of the dish (**c**), creating a lumen in the monolayer (**d**). By 24 h, the structures take on the appearance of mature domes. Focusing on the dome, cells are more numerous, smaller, and more granular (**e**) than the enlarged differentiated cells that initially lifted off the dish (**c**). Focusing on the monolayer (**f**), it is possible to see a lumen surrounded by a ring of cells. Processes extend from the cells into the lumen. ×200.

These differentiated structures can be quantified as a function of time after adding fetal calf serum to cultured cells. In the experiment illustrated in Figure 3, the number of differentiated structures resembling mature domes increased steadily 24–72 h after the addition of 25% FBS. Mature domes were counted in this experiment. A few were detected as early as 18 h after adding serum, with sharply increasing numbers found 24–72 h after the addition of FBS. Domes did not persist for more than 2 or 3 days. In this experiment, domes had begun to disappear by 96 h.

Figure 4 illustrates the dependency of dome differentiation on FBS. The number of mature domes was counted as a function of adding increasing concentrations of FBS, ranging from 5% up to 40%, to confluent wells otherwise maintained in 1% FBS. Increasing levels of FBS result in increasing numbers of differentiated structures, with an apparent limit reached at FBS concentrations of 30% or more. No dome formation was observed when only 1% FBS was present in the medium making this condition suitable for the controls.

As suggested by the previous experiment, domes do form when cells remain in growth medium containing 5% FBS beyond 3–4 days. In the presence of lower levels of FBS, fewer domes differentiate, but the domes are usually larger and can persist for longer periods of time. It is under these conditions that some domes are observed to send a budlike projection of cells out from the dome. An example of a large dome that

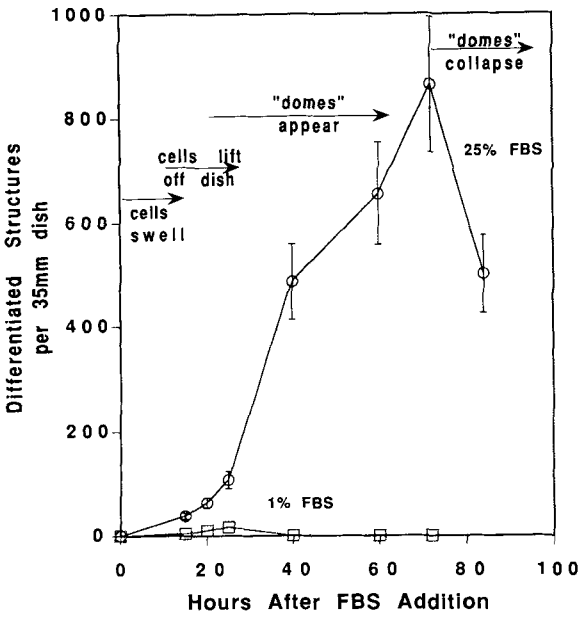


Fig. 3. Fully differentiated structures or domes were quantified in monolayer cultures over a period of 100 h in response to adding 25% FBS. Three different stages of differentiation could be detected in the cultures. Swollen differentiating cells could be detected as early as 6 h after the addition of serum. Differentiated cells began to lift off the dish approximately 12 h after serum was added. The first mature domes were detected at 18 h after adding serum. Steadily increasing numbers of domes were counted from 24 h up to 72 h; by 96 h it was clear that the number of domes was decreasing. For each time point, three separate dishes were counted. The average for the counts is shown, together with the standard error for the count. Control cells were exposed to 1% FBS.

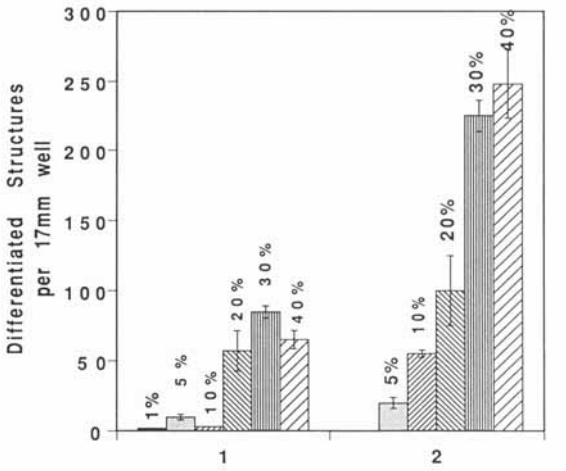


Fig. 4. Domes were counted as a function of increasing concentrations of fetal calf serum. The cells were grown for 3 days in 5% FBS and transferred to 1% FBS for this experiment. At 1% FBS, a concentration sufficient to maintain cells, there was no evidence of dome formation. As increasing percentages of serum were added, increasing numbers of domes could be counted at 24 and 48 h.

developed under these conditions is shown in Fig. 5a,b. The epithelia surrounding the lumen at the base of the dome take on a columnar shape and have projections into the lumen. Granules appear in the lumen (Fig. 5b). A bulge of cells (Fig. 5a,b) projects from the side of the dome.

We were curious to test the effects of steroids on dome differentiation since steroids have been implicated in dome formation in other cell lines [Gierthy et al., 1991; Cereijido et al., 1981; Oberleithner et al., 1990]. While 10 nM progesterone in the presence of 1% fetal calf serum did not bring about differentiation, the same concentration of progesterone seemed to enhance the differentiation activity of 10% FBS (Fig. 6). On the other hand, estradiol, tested under similar conditions, did not have any effect on the number of domes formed in response to the addition of FBS (data not shown). The results from the previous two experiments indicate that a factor in FBS is absolutely necessary for dome formation. Progesterone can enhance activity of this factor, but the steroid has no effect in the presence of only 1% FBS.

To obtain some information on the size of the factor responsible for differentiation, FBS was fractionated through a series of filters with cutoffs ranging from 10,000 daltons up to 300,000 daltons. When the material that passed through filters up to and including the 300,000-dalton filter was tested for differentiation, no activity was found. Data are shown in Figure 7 for serum material passing through, and material retained by, the 300,000-dalton filter. Approximately one-half of the protein, but none of the dome-forming activity, passes through the 300,000-dalton filter. The activity retained by the 300,000-dalton cutoff filter was inactivated by incubation with trypsin (data not shown), indicating that the differentiation factor is either a very large protein or is associated with large proteins. These results make it less likely that free progesterone is essential for the differentiation, as progesterone would be expected to pass through the exclusion filters.

## DISCUSSION

A protein factor from fetal calf serum can effect differentiation in monolayers of human endometrial cells. Cells, and clusters of cells, acquire a distinct morphological appearance,

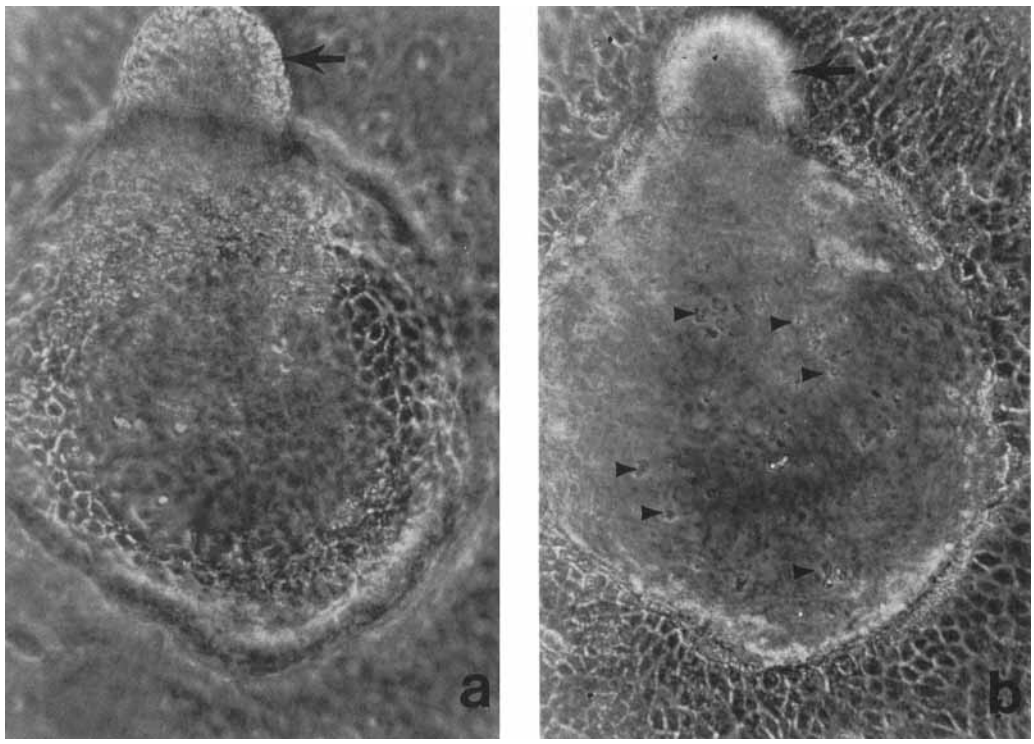


**Fig. 5.** In some domes epithelial cells bud out, forming a single projection. Fewer domes differentiate in cells maintained in 5% FBS, but the domes tend to be larger and sometimes a budlike extension of cells can be seen to push out from the dome. **a:** Focusing on the top of the dome. **b:** Lumen of that structure. Frequently, granules (arrows) can be detected in the within large mature domes. ×100.

sometimes as rapidly as 6 h after the addition of 10–20% FBS to confluent 5-day-old cultures being maintained in 1% FBS. After an additional 6–8 h, differentiated cells appear to lift off the dish in structures that prefigure domes. After 8–10 more hours, these predomes develop a ring of cuboidal or columnar cells bordering a lumen enclosed by a dome of cells. Differentiated dome cells become smaller and more polygonal as the domes mature.

Cellular differentiation leading up to dome formation for other cell lines has not been extensively described. Sometimes the process of dome formation in the kidney and intestinal cell lines is referred to as a "blistering" of epithelial patches [Cereijido et al., 1981; Oberleithner et al., 1990]. This term seems to imply that cells, otherwise indistinguishable from the surrounding monolayer cells, become detached from the dish as fluid accumulates under the cells. However, for the Ishikawa line, cells undergo differentiation within hours of the addition of FBS and prior to becoming detached. Whether these are significant differences in the process of dome

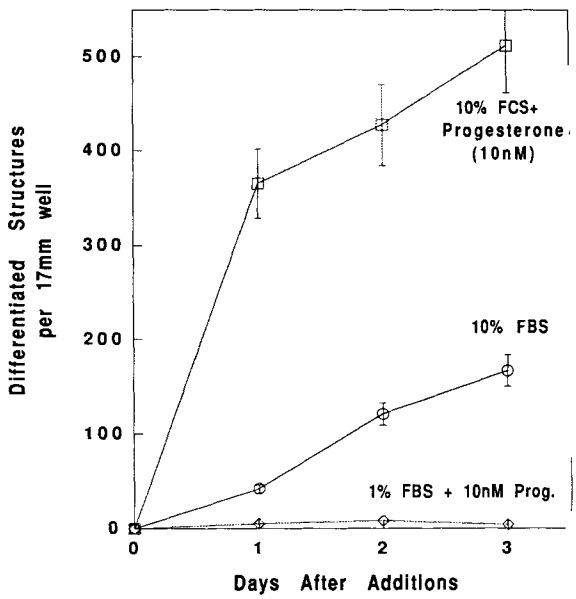


**Fig. 6.** Progesterone enhances dome formation. The addition of 10 nM progesterone together with 10% FBS substantially enhanced dome formation, whereas progesterone added to 1% FBS had no effect.

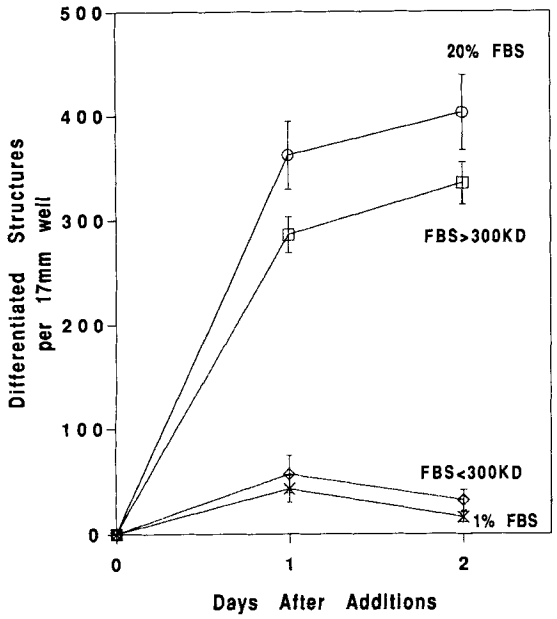


Fig. 7. Factor in fetal calf serum does not pass through a 300-kd exclusion filter. Cells were grown in 5% FBS for 4 days and then transferred to 1% FBS. To each of three wells, 100 μl of fractionated fetal calf serum was added: serum material that passed through the 300K filter, and serum material that was retained by the 300K filter.

formation is unclear. The final dome structures in Ishikawa cells do look very similar to those formed in other epithelial cell lines and it must be assumed that the process similarly involves at least the formation of tight junctions among the cells, cell polarization, and fluid accumulation.

An interesting feature of the Ishikawa domes is that they are able to bud out. Sometimes the buds elongate into structures that look unexpectedly like short glands, seeming to reverse a process that is observed when epithelia migrate out of cultured human endometrial glands and form monolayers of viable epithelial cells [Fleming et al., 1980]. The buds do not grow much beyond what is shown in Figure 5. Although budding structures have not been described for any other cell lines that form domes, three-dimensional structures more complicated than domes have been reported for epithelial cells growing in collagen [Montesano et al., 1992]. Normal human endometrial cells have been shown capable of forming single layers of polarized cuboidal and columnar cells in vitro on collagen [Rinehart et al., 1988; White et al., 1990]. At least one endometrial adenocarcinoma cell line, ECC-1, has been shown to form glandular structures and to secrete mucin in a collagen containing gel [Satyaswaroop and Tabibzadeh, 1992]. Hollow spheres on multicellular stalks have been observed in a mammary cell line [Gierthy et al., 1991].

Predomes seen after 12–18 h of culture usually consist of fewer than 10 cells; after 24–48 h, it is possible to find domes containing tens or even hundreds of cells. Division of the dome cells may occur but could not account for the very large number of cells found in some domes, since the doubling time of Ishikawa cells is approximately 30 h [Holinka et al., 1986a]. Perhaps enlargement of the predomes occurs by a process analogous to the blistering effect already described or even by migration of cells up into the predome. Cell movement and cell rearrangement such as this is recognized as important in generating morphogenetic changes in embryonic tissues [Rodriguez-Nelson et al., 1989; Gumbiner, 1992; Schoenenberger and Matlin, 1991]. One other important feature of dome differentiation is the fact that vacuole formation in the cells seems to increase prior to differentiation. Vacuoles similarly observed in dome-forming MDCK cells have been shown to be involved in formation of a distinct apical membrane during polarization [Vega-Salas et al., 1987].

A protein factor(s) appear(s) to be responsible for these differentiation events in the Ishikawa cells. A variety of molecular species have been found to be responsible for polarization and dome formation in other cell types: nonphysiological inducers of differentiation such as dimethylsulfoxide (DMSO), dimethylformamide, and hexamethyl bis-acetamide will induce domes in MDCK cells; physiological agents such as prostaglandin $E_1$ and cyclic AMP have also been found to induce dome formation [Lever, 1979] as has the lack of glucose in cell medium [Taub, 1989].

This may be the first time that a serum protein factor has been implicated in this process. Fetal calf serum was found to induce differentiation of MCF-7 cells, but the activity was lost when FBS was stripped by incubation with dextran-coated charcoal, implicating a small molecule as the active agent. The small molecule appears to be estradiol [Gierthy et al., 1991]. Estradiol is not the only steroid hormone that can affect dome differentiation. MDCK cells have been reported to form domes in serum-free medium with the addition of the steroid hormone aldosterone [Kimoshita et al., 1986; Oberlei-

thner et al., 1990]. By contrast, although the female sex steroid progesterone can enhance differentiation in Ishikawa cells, it has no effect in the presence of only 1% FBS. A serum factor is essential.

Preliminary characterization of that factor indicates that it is associated with material having a molecular weight greater than 300,000. Macromolecules have been implicated in dome formation in other cell lines. A fibroblast factor has been shown to stimulate dome formation in mammary epithelial cells [Strange et al., 1991]. The cytokine known as hepatocyte growth factor (HGF) has recently been shown to stimulate epithelial tubulogenesis by MDCK cells in collagen gels [Montesano et al., 1992].

The capacity for polarization is probably characteristic of all forms of epithelia, especially in the developing embryo. Nevertheless, there is substantial variability in the retention, or at least expression, of the capacity to polarize when epithelial cells are cultured in vitro. In a recent survey of 20 human colon carcinoma cell lines, eight of the lines were able to form domes. Some of the lines that could not form domes were nevertheless able to achieve some degree of polarization [Chantret et al., 1988]. The capacity for polarization sometimes appears to be lost when cells become malignant [Schoenenberger and Matlin, 1991]. Similarly it has been found that some primary cultures started from a single mouse mammary tumors are able to form domes, whereas other cells from the same tumor are not [Bell et al., 1988]. It is not obvious why there is so much variability in expression of this capacity for differentiation.

Even among dome-forming cell lines, it is possible that there are subtle differences in the behavior of the cells due to the origin of the epithelial line. In vitro differentiation for an intestinal adenocarcinoma cell line, that ultimately involves dome formation, has been described in terms of stages of differentiation in intestinal embryonic development [Hekmati et al., 1990]. It is possible that the differentiation of epithelia that will form glands may not be the same as the differentiation of epithelia that will form tubules, even if the polarization accompanying both processes results in dome formation in monolayer cultures. In the neonatal mouse, uterine gland formation has been reported to begin with the invagination of the epithelial cell layer into the underlying connective tissue stroma [Wordinger et al., 1992]. Perhaps dome formation and budding of the Ishikawa endometrial cells is analogous to this kind of "invagination," with the dome cells extending up into the medium rather than into connective tissue. It is interesting that progesterone, which is certainly involved in endometrial differentiation in vivo, facilitates epithelial polarization and dome formation in vitro.

The morphological and biochemical changes accompanying differentiation of uterine endometrium create a microenvironment that will support the fertilized egg and facilitate the process of implantation. When implantation is unsuccessful, it is usually thought to be a problem with the endometrium, and not with the blastocyst. A simple monolayer system in which some of the endometrial differentiation process can be studied may allow us to learn more about these incredibly complex processes.

## ACKNOWLEDGMENTS

This investigation was supported by an NSF Career Advancement award (9210538) and by an NSF RUI award (9306492). The author wishes to thank Don and Donna Gruol for helpful discussions.

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