Journal of Cellular Biochemistry 71:400–415 (1998)

Role of Biotin-Containing Membranes and Nuclear Distribution in Differentiating Human Endometrial Cells

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Abstract Human Ishikawa endometrial cells form domes when confluent monolayers are stimulated with fresh fetal bovine serum. Extensive structural and biochemical changes have been detected during the approximately 30 h differentiation period. The earliest detectable change involves the formation of multinucleated structures and the appearance of ''granules'' that stain for biotin within those structures. Nuclei become associated with each other and are ultimately enclosed within a biotin-containing membrane. Aggregated membrane-sheathed nuclei and the cells containing them begin to elevate from the dish as biotin staining becomes apparent in apical membranes. The elevated structures are called predomes and consist of one or more very large cells containing the sheathed nuclei. Apical membranes of these unusual cells extend far out into the medium in structures that resemble endometrial pinopods. A lumen under the elevated cells fills with transcytosed fluid. As differentiation proceeds, highly concentrated chromatin material that was flattened against apical and lateral membranes of the predome cells begins to disperse. Small mononuclear cells evolve from larger predome cells. Apical membranes of predome and dome cells continue to stain for biotin. Gel electrophoresis of SDS-solubilized biotin-containing membranes, followed by Western blot analysis using avidin-linked peroxidase, resulted in three stained bands with molecular weights similar to those of the mitochondrial carboxylases: propionyl carboxylase, methylmalonyl carboxylase, and pyruvate carboxylase. J. Cell. Biochem. 71:400–415, 1998.

Key words: Ishikawa cells; endometrium; biotin; multinucleated cells; predomes; domes; pinopods

Cultured human endometrial cells differentiate into three-dimensional structures such as domes and tubules in response to serum. Domes of epithelial cells, created when regions of the monolayer become elevated over fluid-filled lumen, were first observed in Madin-Darby Kidney cells [Lever, 1979] and subsequently in other epithelial cell lines, including mammary [Pourreau-Schneider et al., 1984] and endometrial [Fleming, 1995] lines. The literature on dome formation suggests three-dimensional structures arise when cells of the monolayer become polarized [Rodriguez-Boulan and Nelson, 1989]. The resulting differential distribution of proteins to the apical and basal membranes accompanies changes in adhesion properties, elevation of cells, and overall transport of fluid into the lumen under dome cells. Polarization has, in fact, been demonstrated in primary cultures of human endometrial epithelia [Schatz et al., 1990].

In the Ishikawa human endometrial cell line [Nishida et al., 1985], dome formation involves complex changes in cell morphology [Fleming, 1995] that may also reflect the process of polarization. At least three stages of differentiation can be distinguished within approximately 30 h following addition of the stimulus to differentiate. The initial stage, involving 5–10% of the cells, is characterized by the rapid development of multinuclear cells. During the second stage,

Contract grant sponsor: NSF RUI; Contract grant number: 9306492.

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Received 3 March 1998; Accepted 15 June 1998

multinuclear cells give rise to one or more than one enlarged cell containing aggregated nuclear material and elevated above the monolayer. Fluid accumulates under the basal membranes of these cells. By 24–30 h after the initiation of differentiation, the predome cells have evolved into smaller mononuclear dome cells that resemble cells in the surrounding monolayer except that they have lost their attachment to the dish. Furthermore, long, thin cytoplasmic processes are sometimes observed to extend from these cells into the developing lumen.

Differentiation occurs in response to the addition of excess serum or high molecular weight factors from serum [Fleming, 1995]. Following the initial differentiation, some domes collapse, while others persist for days, growing in size and becoming filled with secretions. When the differentiated state persists for long periods, more than 50% of the cells in a monolayer can become elevated in huge dome structures. One profound biochemical change accompanying dome formation is the appearance of biotin in the membranes of Ishikawa dome cells. The presence of biotin was first detected in controls of immunocytochemical experiments, when the addition of avidin-linked peroxidase alone resulted in stained membranes in dome cells. In the experiments done for this paper, cultures were fixed and stained as a function of time after the initiation of differentiation to determine when and where the biotin first appears. Prior to the start of differentiation, cells in the monolayer barely stain for biotin. Within 10 h it becomes possible to detect biotin in multinucleated structures. Hemotoxylin allows simultaneous visualization of chromatin material. Together, these two staining procedures reveal interesting changes in distribution of biotin as well as nuclei during successive stages of dome differentiation in Ishikawa cells.

MATERIALS AND METHODS

Ishikawa cells were cultured [Fleming, 1995] in phenol red–free, Minimum Essential Medium (MEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and .25 mg amphotericin B (Gibco, Grand Island, NY). The cells, obtained from Dr. Erlio Gurpide at Mt. Sinai Hospital in New York, 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. Cells seeded at an approximate density of 5×10^5 cells/cm^2, were grown for 1–2 weeks in MEM containing 5% calf serum (CS) and then transferred to medium containing 1% CS. Cultures left in MEM with 1% CS could survive for an additional 3–5 days with little proliferation. Assays for dome formation were done in confluent cultures, although differentiation has been observed to occur, to a limited extent, in nonconfluent cultures.

Differentiation was initiated with the addition of 10–15% fetal bovine serum (FBS). Multiple dishes were fixed and stained for biotin and/or for chromatin at different times during differentiation. Structures were viewed using an Olympus inverted stage microscope at powers of 40, 100, 200, and 400X.

Cells were fixed by adding 4% paraformaldehye in phosphate buffered saline (PBS) to the culture dish. After 10 min, the cells were washed gently four times with 5–10 ml PBS. A solution of 1% Triton X-100 was added to the cells to permeabilize the membrane. Again after 5 min, the culture was washed with successive changes of PBS. After washing, cells were exposed to a 1:200 dilution of Extravidin-conjugated horseradish peroxidase (HRP) (Sigma) for 30 min. After further washing, a solution of 3-amino-9-ethylcarbazole (AEC), prepared by dissolving 20 mg of AEC in 2.5 ml of dimethylformamide and diluting with 47.5 ml of 50 mM potassium acetate adjusted to pH 5.0, was added to the cells together with .25% H_2O_2. This solution was incubated at 37°C for 45 min to allow color to develop. The AEC solution was removed, and the cultures were examined and then stored in the presence of PBS at 4°C. If avidin linked to peroxidase is not added to the cultures, there is no reaction. If avidin without peroxidase is added first to the cultures, followed by avidin linked to peroxidase, staining is not observed. Staining does not occur if avidin-HRP is not added to the cultures prior to AEC indicating that an endogenous peroxidase is not responsible for the staining.

To ensure that avidin was reacting with biotin, we stained domes using streptavidin linked to horseradish peroxidase as well as primary antibody to biotin and secondary antibody linked to horseradish peroxidase. Staining occurred under all circumstances, indicating that avidin does indeed react with biotin.

Testing for Solute Endocytosis and Micropinocytosis

Two fluorescent dyes were tested for their ability to pass into the luminal fluid. One dye was Lucifer Yellow CH (LY) which has a molecular weight of 450 Da; the other was fluorescein isothiocyanate–conjugated dextrans (FD) with molecular weights of approximately 20,000 Da. LY was incubated with doming and nondoming cells at a concentration of 250 mg/ml at 37°C for times ranging between 5 min and 1 h. FD was added to cultures stimulated to form domes in the presence of 15% fetal bovine serum. Each of these dyes was washed out after 30 min using several changes of fresh medium. An Olympus BH-RFL fluorescent microscope with excitation filter BG12 and barrier filter 0530 and dichroic mirror B were used to detect fluorescence.

Biotin-Linked Proteins

Differentiated cultures, incubated with avidin linked to horseradish peroxidase and stained with AEC and H_2O_2, were scraped from the dish in 2 ml of hypotonic buffer containing 10 mM HEPES, pH 7.4, 1 mM EDTA, 0.1 mM $MgCl_2$, and 1.25 mM PMSF. The cells were homogenized using a glass/glass homogenizer, and the homogenates were fractionated on sucrose gradients to determine where on the gradient biotin-stained membrane fragments would appear. A 5 ml discontinuous gradient, made up of 20–60% sucrose prepared in a buffer containing 20 mM Tris Cl, pH 8.0, 1 M NaCl, and 5 mM EDTA, was layered in a Beckman (Fullerton, CA) Polyallomer (14 × 89 mm) ultracentrifuge tube. Two milliliters of cell homogenate was layered on the surface of the gradient and centrifuged at 180,000 × g for 1.5 h at 4°C in a Ti 41 rotor. Two pink bands formed in the gradient. AEC-stained material at the top of the gradient was not associated with any proteins and is likely to be free biotin. Two additional bands were found lower in the gradient. Stained material was collected, and membranes were pelleted by dilution with buffer followed by centrifugation at 200,000 × g for 45 min.

Having determined that biotin-associated membranes ran in fractions 4 and 5, a similar experiment was done using cultures that had not been stained. Fractions 4 and 5, collected from gradients of homogenized differentiated and nondifferentiated cells, were collected, diluted, pelleted, resuspended in HEPES buffer, and incubated with constant agitation for 1–2 h with streptavidin-linked magnetic beads (Sigma, St. Louis, MO). After incubation, beads were captured with a magnet or pelleted in a Beckman tabletop centrifuge, and the supernatant was discarded. Beads were rinsed twice with buffer. The beads were heated with 1% SDS in boiling water for 10 min to dissociate biotin-linked proteins from the beads. Beads were separated using a magnet or centrifugation, and the supernatant was ready to run on an SDS gel.

Collecting Material From Under the Dome

The material from under the dome was collected by mechanically collapsing domes as follows. Medium was taken off the cells. The cultures were rinsed several times using MEM without serum and then incubated in PBS and agitated on a shaker until the domes collapsed. Large cell debris was pelleted from the lumen fluid by low speed centrifugation. The PBS solution containing dome fluid was removed from the dish and centrifuged with several washes in a Filtron (Northborough, MA) 10K microsep micro concentrator at 6,500*g* to remove salt and concentrate the fluid to 0.5 ml. Following isolation of luminal fluid, cells from the treated dish were scraped, homogenized, and treated with streptavidin beads. After washing several times with buffer, the beads were incubated with SDS at an elevated temperature to strip proteins for analysis on an SDS gel.

SDS–Polyacrylamide Gel Electrophoresis

Proteins from under the dome, from isolated membranes, or from whole cell homogenates treated with streptavidin magnetic beads were diluted 1:4 with an SDS containing buffer and heated in a boiling water bath for 10 min to solubilize the protein. Proteins were run on a 10% polyacrylamide gel containing 0.2% N,N-methylene-bis-acrylamide.

Proteins run on SDS-PAGE were transferred electrophoretically to a PVDF membrane (Millipore, Bedford, MA) by passing a current of 400 mA through the gel in a buffer of 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol for 8 h. Proteins were successfully transferred out of the gels, as checked by attempting to stain gels with Coomasie Brilliant Blue.

Following this procedure, membrane was immersed in 100% methanol for 10 sec and then allowed to dry. The membrane was blocked by incubating it in 5% nonfat dried milk solution

for 2 h, rinsed four times with PBS, and incubated for 1 h in a solution containing .05 mg/ml of avidin linked to horseradish peroxidase. The membrane was then incubated with AEC to develop stain where avidin-linked horseradish peroxidase was bound to biotin. Background staining was very low.

RESULTS

The formation of multinucleated structures, involving 5–10% of the cells in confluent monolayers, represents the first discrete morphological change in endometrial epithelial cells stimulated to differentiate by adding fresh medium and 10% fetal bovine serum to confluent monolayers. Initially, cell membranes, previously undetectable, become refractive and visible under phase contrast. Subsequently, gaps appear as some of these refractive membranes disappear. Close examination reveals that the gaps are greatly enlarged multinucleated cells, 2–50 times the size of typical cells that must result from fusion.

Examples of enlarged multinuclear cells, stained for biotin and for nuclei, are shown in Figure 1 approximately 10 h after initiation of differentiation. The monolayer was fixed with paraformaldehyde, permeabilized with Triton X-100, stained for biotin using peroxidase-linked avidin and AEC/H_2O_2, and additionally stained with hematoxylin. Biotin-staining material becomes apparent only, but not necessarily immediately, after the cells have fused. Hematoxylin staining reveals multiple pale nuclei in the center and small, dark nuclei on the periphery of the multinucleated structure in Figure 1A, with no apparent biotin staining. Biotin-containing granules that stain with AEC are visible in the multinucleated structure in Figure 1B. Granules can be detected on the outer edges of the structure as well as in the center. Higher magnification of a third multinucleated cell (Fig. 1C) reveals a spherical structure containing a cluster of hematoxylin-stained nuclei sheathed in a biotin-containing membrane. Nearby nuclei, not sheathed in membranes, have unusual shapes that suggest nuclear overlap or even fusion.

Four spheres staining for biotin are apparent in the multinucleated cell in Figure 1D. Inappropriate color balance in the film makes everything in the cell appear yellow, with biotin-containing structures staining a darker pink-orange. The spheres that stained for biotin also stain with hematoxylin (Fig. 1E), indicating the presence of nuclei within the spheres. The two stains result in a violet color similar to that associated with the sheathed nuclei in Figure 1C. Taking Figure 1C as a reference, four sheathed aggregates could enclose more than 20 nuclei. Doughnut shaped organelles only slightly smaller than the spheres (Fig. 1D,E), are apparently devoid of chromatin material, staining only for biotin. Additionally, a couple of biotin-stained filament-like structures are observed apart from the spheres or doughnuts. Biotin stain outside of the nucleus has become more pronounced in the structure shown in Figure 1F. Biotin- and hematoxylin-stained spherical structures (Fig. 1F), together with stained membrane, are slightly elevated out of the plane of the monolayer, suggesting the start of transition from multinucleated monolayer cell to the elevated predome structure.

In Figure 2A, aggregated nuclear material appears to be flattened against apical and lateral membranes defining the borders of a cell-like structure that appears to rise out of (Fig. 2B) but be continuous with the larger multinuclear structure. Hematoxylin staining of aggregated nuclear material is so dark that it is difficult to detect staining due to biotin. At least two other regions of concentrated nuclear material are apparent in this structure (arrows).

Figure 3 shows multiple finger-like projections similar to the single projection in Figure 2. Found approximately 16 h after the addition of serum, the structure, shown at two levels of focus (Figure 3A, B), contains at least five projections emerging from the center of the structure. Comparison of Figure 3A with Figure 3B illustrates that membrane projections of at least one structure extend further out into the medium than others. Unstained nuclei, usually apparent even in monolayers, are hard to detect in these projections. Fixation and hematoxylin staining of the predome in Figure 3 reveals the formerly undetectable nuclei, flattened against the membranes (arrows in Fig. 3C).

Bulging apical membranes are a distinguishing morphological characteristic of predomes, visible in unfixed cultures. Panels A and B in Figure 4 are phase contrast micrographs of an unfixed predome at 100 focusing on the surrounding monolayer and the developing lumen of a predome (Fig. 4A) and on the apical membranes (Fig. 4B) of the enlarged predome cells. Figure 4C is a phase micrograph of the same

apical membranes under higher magnification (400), and Figure 4D is a light micrograph at the same magnification taken with a blue filter. Some dark material that may be nuclear, seen in Figure 4B, becomes even more evident in Figure 4C (the orientation of the predome has been rotated to the right by 90). Figure 4D shows that membranes from two out of the three predome cells are clearly extending above the surface of the predome in structures that look like pinopods, as described for endometrium in vivo [Parr and Parr, 1972]. The stippling apparent in Figure 4C is frequently observed on the surface of bulging apical membranes in unfixed material. Probable involvement of membranes in endocytosis is suggested by their appearance at the time when fluid is rapidly accumulating under the basal membranes. Experiments with fluorescent materials indicate that dome cells are capable of both solute endocytosis and micropinocytosis. FITC linked to dextrans (MW 20,000 Da) will pass from the apical region to the basal region during the 24 h period of dome formation as determined by fluorescent microscopy. Lucifer Yellow, with a molecular weight of less than 500

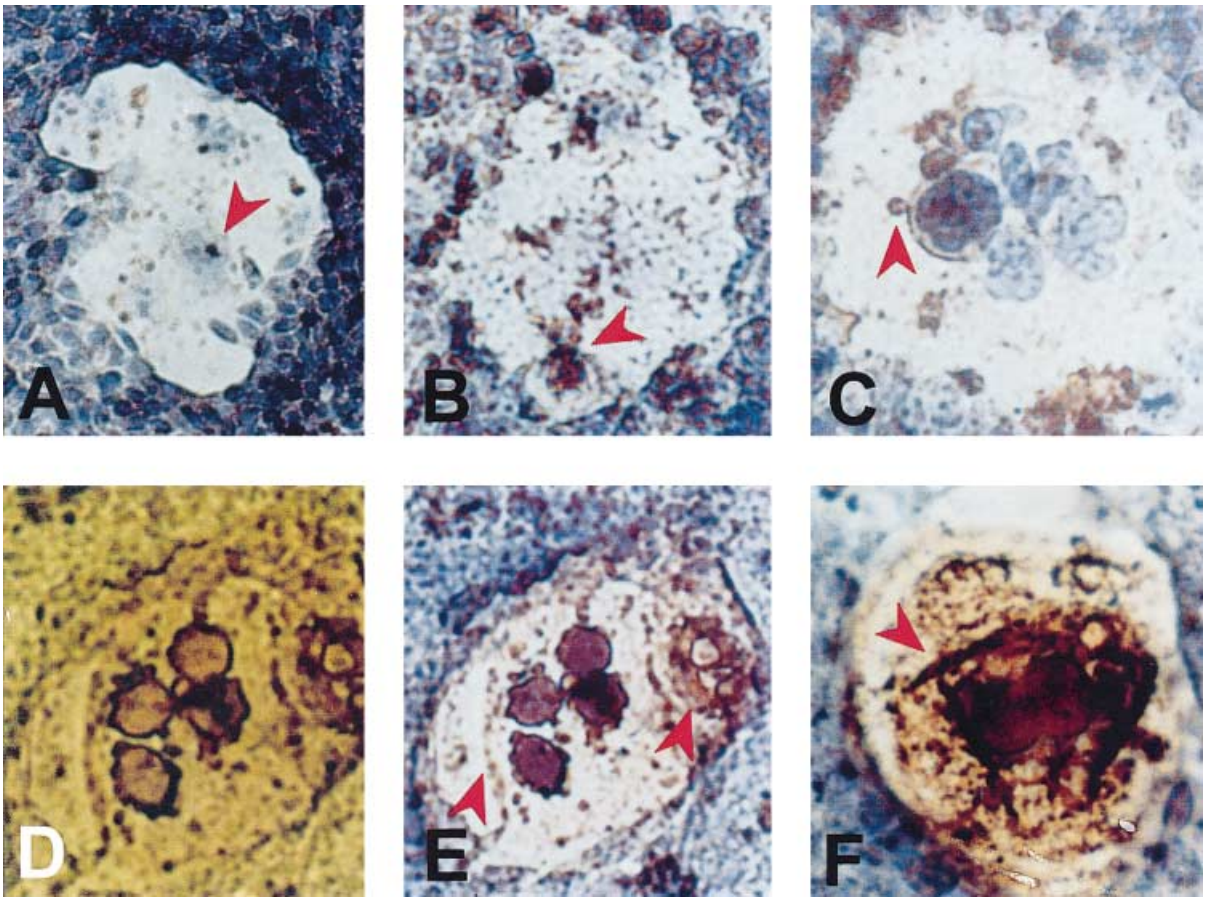

Fig. 1. Multinucleated cells were found in Ishikawa cultures 10 h after addition of excess serum to confluent cells; all of the structures were stained for biotin and with hematoxylin. **A:** Clustered pale nuclei (arrowhead) appear in the center of this multinucleated structure with smaller, more intensely staining nuclei around the edges. No biotin is found in this multinucleated cell. 200. **B:** Biotin-stained granular material extends from a biotin-containing sphere on the edge of the multinucleated structure (arrowhead) through to the center of the cell. Granular material is also to be found on the edges of the cell. Pale nuclei are clustered in the center of the cell. 200. **C:** At least four nuclei are associated with each other and enclosed by a membrane staining for biotin. Biotin granules are apparent outside of this cluster, including one granule that appears to be attached to the membrane-sheathed nuclei (arrowhead). The unusual shapes of additional nuclei suggest that nuclei are overlapping perhaps even fusing. 400. **D:** Four spheres stain for biotin. Two doughnut-shaped structures on the extreme right edge of the structure also stain for biotin. What looks like filaments or a linear array of granules are also stained in the cytoplasm. 200. **E:** The biotin-staining spheres in 1D take on a violet stain after the culture is also stained with hematoxylin but the doughnut-shaped structures (arrowhead) and the filaments (arrowhead) do not, indicating the absence of chromatin material in these structures. 200. **F:** Three or more biotin- and hematoxylin-stained spheres come into focus only above the monolayer. Biotin is associated with membranes in the stained filamentous material that surrounds the aggregate nuclei. (arrowhead) 400.

Da, passes into the lumen of preformed domes within a period of 50 min.

Figure 5 shows at least four different predomes stained with hematoxylin, whose characteristics are similar to those demonstrated by the projections in Figures 2 and 3. Nuclear material is flattened against the lateral membranes, sometimes forming a crescent around the edges of an enlarged and otherwise clear cell. Boundaries between cell-like projections sometimes appear to be incomplete. When the boundary is complete, the ratio of non-nuclear to nuclear material is significantly higher than what is found in mature dome cells. In the largest of these structures in Figure 5A, predome cells coexist with cells whose lower cytoplasmic to nuclear ratios are characteristic of mature dome cells, suggesting a structure in transition from predome to dome.

Figures 3, 4, and 5 contain examples of predomes consisting of multiple cells. Predomes are also found that appear to be a single, greatly enlarged elevated cell. Figure 6A,B shows one such predome, unfixed and unstained. When the microscope is focused on the monolayer, a region at the base of the predome (at 3 o'clock) appears to contain a very large nucleus (Fig. 6A). When the microscope is focused on the apical membrane, stippling of the membrane can be observed (Fig. 6B). A similar predome, shown in Figure 6C, was fixed and stained with hematoxylin, revealing aggregated chromatin tightly focused at the edge of the predome. As Figures 2 and 3 indicate, nuclear material is frequently associated with the apical membrane that is elevating, but, as the lumen fills under elevating predome cells, nuclear material may be displaced to the lateral edge of the structure.

As the predome evolves into a dome, the aggregated nuclear material begins to redistribute up under the apical membrane. Examples of predome structures, stained for biotin and with hematoxylin after 20 h, are shown in Figure 7A–C. Hematoxylin-staining material takes several forms in these transition structures. There are regions that are readily identifiable as aggregates of nuclei up under the predome membrane. Additionally, there are intensely stained chromatin foci on the edges of the predome. Finally, there are linear structures that look like membranes but stain as chromatin. Nuclear material in the process of redistribution might be expected to look like this as it moves within the structure.

Following redistribution, the final step in development of the mature dome from the predome involves the formation of cell membranes around the nuclei that have dissociated from aggregates. Mature domes first become observ-

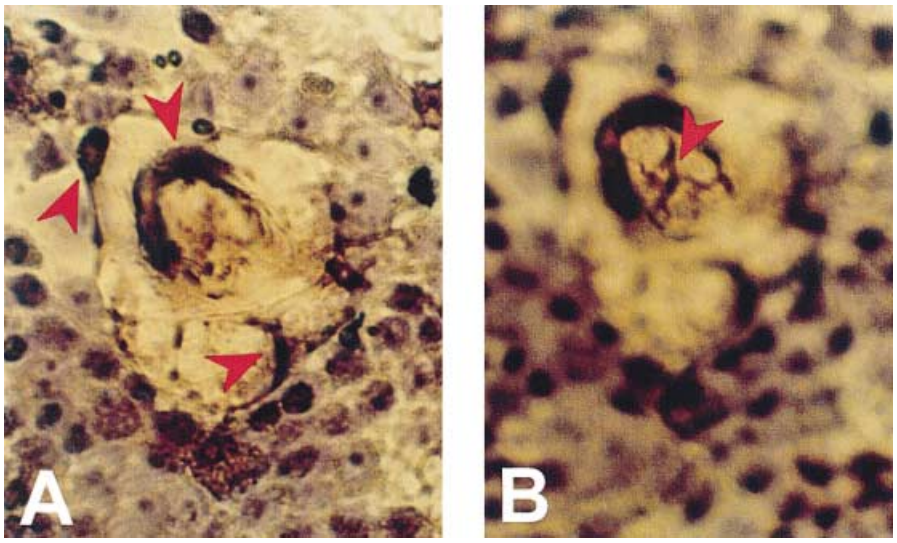

Fig. 2. Chromatin pushing up against the apical membrane of a multinucleated cell. 400. **A:** The nuclear aggregate appears to be at the tip of a finger-like projection pushing up out of the multinucleated structure (arrowhead). The nuclear material stains darkly with hematoxylin. At least two other regions of aggregated nuclear material are apparent in this structure (arrowheads). **B:** When the microscope is focused on the tip of the projection, the surrounding monolayer is out of focus. Biotin staining filaments (arrowhead) are observed in the membrane that stretches down into the multinucleated cell. An additional cell like projection may be forming below the central projection.

able 24–30 h after the start of differentiation. In the days that follow, some domes collapse, while others remain elevated and even enlarge. Apical membranes of mature domes continue to stain for biotin. A typical example of a dome 30 h after the start of differentiation is shown in Figure 8. When the microscope is focused on the monolayer, the biotin stain is faint (Fig. 8A). Biotin staining is much more pronounced when the microscope is focused on the surface of the dome (Fig. 8B). Additional staining with hematoxylin (Fig. 8C) reveals that almost all of the cells in this dome are small and have high nuclear to cytoplasmic ratios, very different from predome cells but quite characteristic of mature domes.

Over time, biotin becomes detectable in the lumen of the dome in addition to apical membranes. Figure 9 shows a 1-week-old dome under low power (40) stained for biotin. In an older differentiated culture, the combination of biotin stain in dome cell membranes and under the dome can be so intense that spherical pink regions, corresponding to large domes, can be

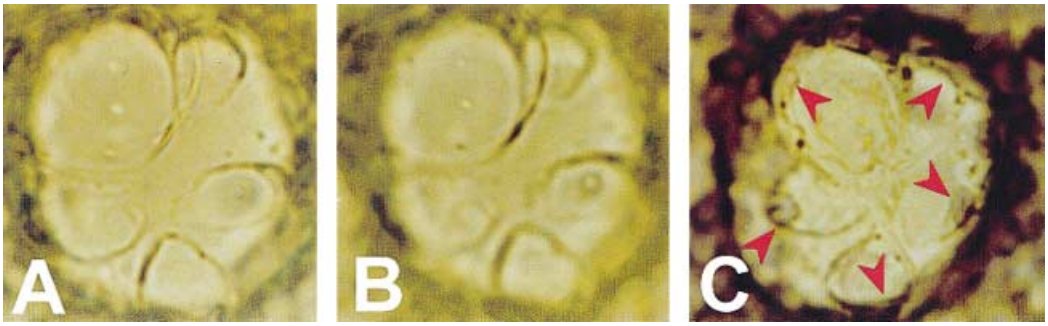

Fig. 3. Multiple cell-like projections with protruding apical membranes rising out of a multinucleated structure. 400. **A:** Unfixed and unstained predome containing at least five cell-like projections clearly elevated above the monolayer. **B:** Predome from panel A with focus on the bulging apical membrane for the largest predome cell (top left). **C:** Fixed and stained predome reveals nuclear material pushed laterally against membranes (arrowheads).

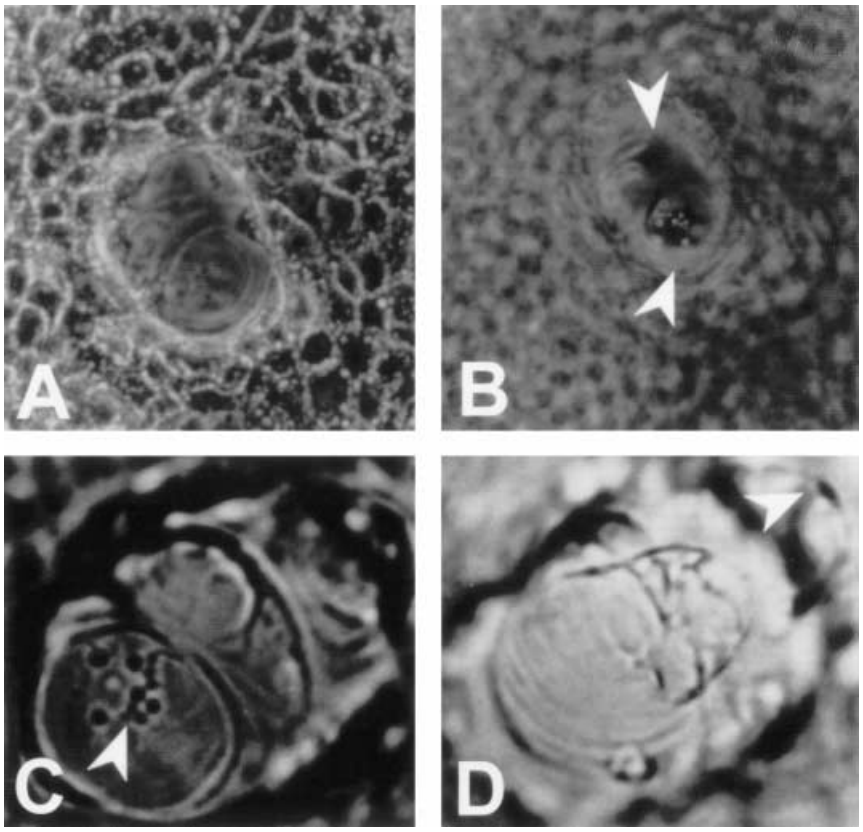

Fig. 4. Apical membranes of unfixed, unstained predomes. **A:** Phase contrast micrographs of a predome focusing on the monolayer and the developing lumen. 100. **B:** Phase contrast micrograph of the same predome focusing on the apical membranes of the three predome cells. Darkened regions (arrowheads) may correspond to nuclear material apposed to the membrane. 100. **C:** Light micrograph of apical membranes. Pore-like structures, highlighted by the arrowhead, are sometimes observed in membranes involved in endocytosis. 400. **D:** Light micrograph taken with blue filter. Apical membranes of two of three predome cells extend out into the medium in structures that look like pinopods. The darkened region of membrane extension may be nuclear material (arrowhead). 400.

detected with the naked eye in stained culture dishes. Examining material under the dome at higher powers reveals darkly staining vesicular material under the dome. At least some but not all of the membrane-bound vesicles exocytosed into the lumen under the dome stain for biotin.

Proteins Associated With Stainable Biotin

Little stainable biotin is detected in proliferating undifferentiated monolayer cells, even though all cells contain biotin-linked mitochondrial and cytoplasmic carboxylases. Stainable biotin in differentiating Ishikawa cells appears progressively in granular form, in membrane sheaths encasing multiple nuclei, associated with membranes of predome and dome cells and on vesicles under the dome. Stainable biotin in differentiated cells could be free or associated with proteins. Cell material from a differentiated stained culture was scraped from a dish, homogenized, and fractionated on sucrose gradients. While some stained material remained on the top of the gradient, possibly indicative of free biotin or a soluble, biotin-containing protein, most of the stained material sedimented approximately halfway down the gradient in two bands. The sucrose containing bands, coming off the gradient as fractions 5 and 4, were diluted, and membrane pellets were collected following centrifugation.

Membranes from differentiated and undifferentiated cultures that had not been stained were similarly fractionated from whole cell homogenates. The membrane pellet was collected and solubilized in a solution of SDS in buffer. This material was run in a 10% polyacrylamide gel, blotted onto PVDF membrane, and stained for biotin using avidin or streptavidin linked to HRP and incubated with AEC and H_2O_2. Proteins in fractions 4 and 5 for both the nondifferentiated and differentiated cultures appeared to contain the same three biotin-binding proteins: a band at 127 kDa and bands at 74 kDa and 69 kDa (Fig. 9). The masses of these proteins are similar to the molecular weights of the three carboxylases found in animal mitochondria: pyruvate, propionyl, and methyl-crotonyl carboxylases [Lau et al., 1979]. The same proteins were observed in nondifferentiated and differentiated cultures but corresponding bands might be more intense in differentiated cells even though cell homogenates were prepared from approximately the same number of cells for nondifferentiated and for differentiated cells.

Luminal fluid under the dome, which stains intensely for biotin, afforded an opportunity to analyze staining material whose appearance is

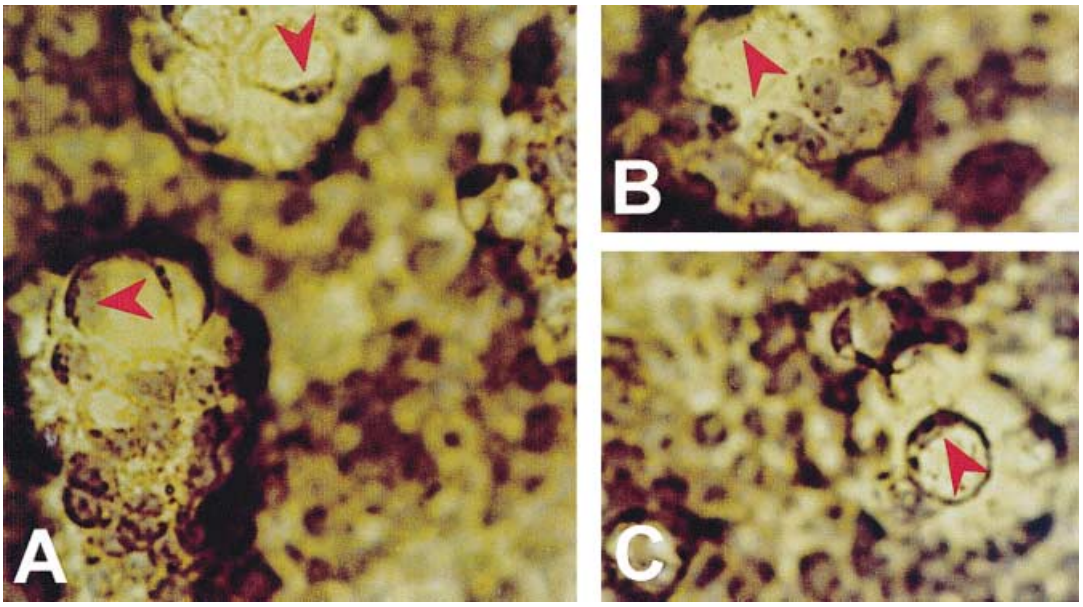

Fig. 5. Multiple predome cells with marginalized chromatin. **A:** Parts of three predomes containing cell-like projections stained with hematoxylin. Predome cells are characterized by marginalized chromatin and a high ratio of cytoplasm to nuclear material (arrowheads). The bottom half of the largest predome contains some cells more characteristic of mature domes, with high ratios of nuclear to cytoplasmic material. 400. **B,C:** More examples of predome cells containing marginalized chromatin (arrowheads). 400.

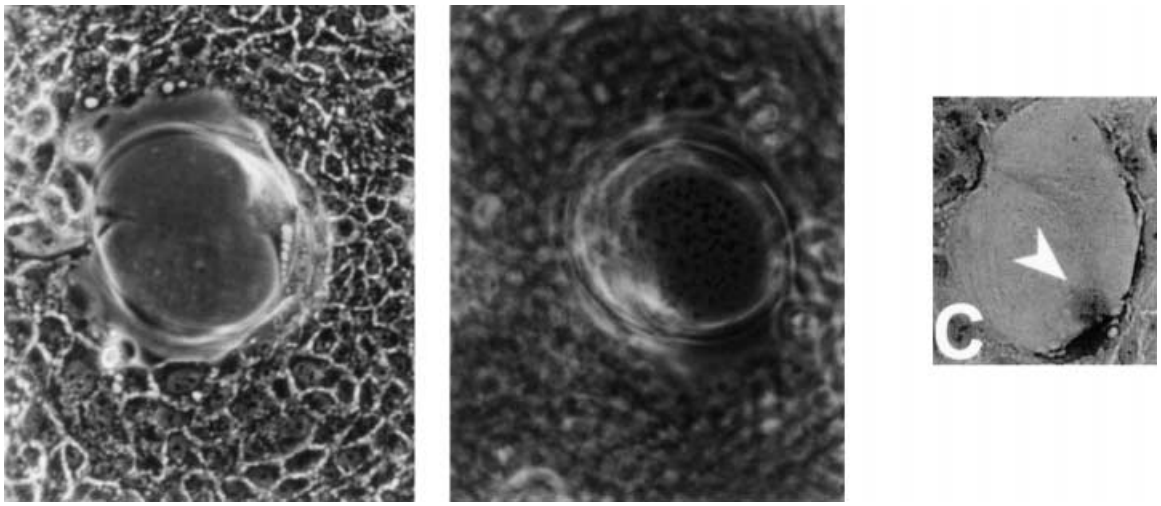

Fig. 6. Predome with a single greatly enlarged cell-like structure. **A:** Some predome structures appear as a single elevated structure that can be as large as ten or more cells. At the level of the monolayer, a region at the base of the predome appears to contain a single large nucleus (at 3 o'clock). **B:** When the microscope is focused on the apical membrane, stippling of the membrane can be observed. 200. **C:** A predome fixed and stained with hematoxylin demonstrates that nuclear material is tightly focused in a single region (arrowhead). 200.

completely dependent on differentiation. Fluid was collected from under domes by agitating petri dishes containing differentiated cultures in the presence of a balanced salt solution until most of the domes had collapsed. Any significant fragments of intact cells were removed from approximately 1 ml of luminal fluid by low speed centrifugation. The luminal fluid was concentrated and desalted by centrifuging the luminal fluid through a Filtron 10K filter with successive washes with 10 mM tris buffer, pH 7. The concentrated material was treated with SDS and run simultaneously on the two 7.5% polyacrylamide gels in Figure 11 (the b lanes). Two milliliters of medium removed from the cells prior to the extraction of fluid from the domes was similarly concentrated, desalted, and run on two gels (Fig. 11, c lanes). Finally, cell material from the same dish was homogenized and incubated with avidin linked to magnetic beads. Biotin binding proteins were washed from the avidin magnetic beads using 1% SDS at 80C and run simultaneously on two gels (Fig. 11, a lanes). Three of the biotin binding proteins found in the cell membranes (Fig. 10) were identical to those found in whole cell homogenates, with an additional fourth band at 84 kDa (Fig. 11). Approximately 60 µg of proteins was run in the c lanes, 10 µg of protein in the b lanes, and less than 2 µg of protein in the a lanes. Much of the protein in the c lanes is serum protein that was added to the medium; no biotin binding proteins are evident. With lower overall amounts of protein added to the b lanes, biotin binding proteins can be detected at 74 kD Da; traces of the second band at 69 kDa and a third diffuse band, somewhat larger than 127 kDa, also can be detected. The comparison of lane b and lane c indicates that biotin-staining material from under the dome does not have its origins in the medium, a conclusion reinforced by the biotin-stained vesicles under the dome (Fig. 8). Material secreted under the dome and observed to stain for biotin (Fig. 8) contains at least two and perhaps a third biotin binding protein.

DISCUSSION

Does the in vitro morphological differentiation of endometrial epithelial cells mimic any aspects of in vivo differentiation of uterine endometrium? With allowances for the different perspective afforded by microscopy of monolayers when compared with sectioned tissue, differentiating Ishikawa cells resemble differentiating endometrial tissue in two ways. First, cross-sections of endometrial glandular epithelia are found to contain nuclei associated with biotin [Sickel and diSant'Agnese, 1994; Yokoyama et al., 1993; Cooper et al., 1997]. Similarly, biotin-staining granules closely associated with nuclei as well as biotin-staining membranes that sheath aggregated nuclei (Fig. 1) are observed in differentiating Ishikawa cells. Second, marginalized chromatin initially observed by Ma-

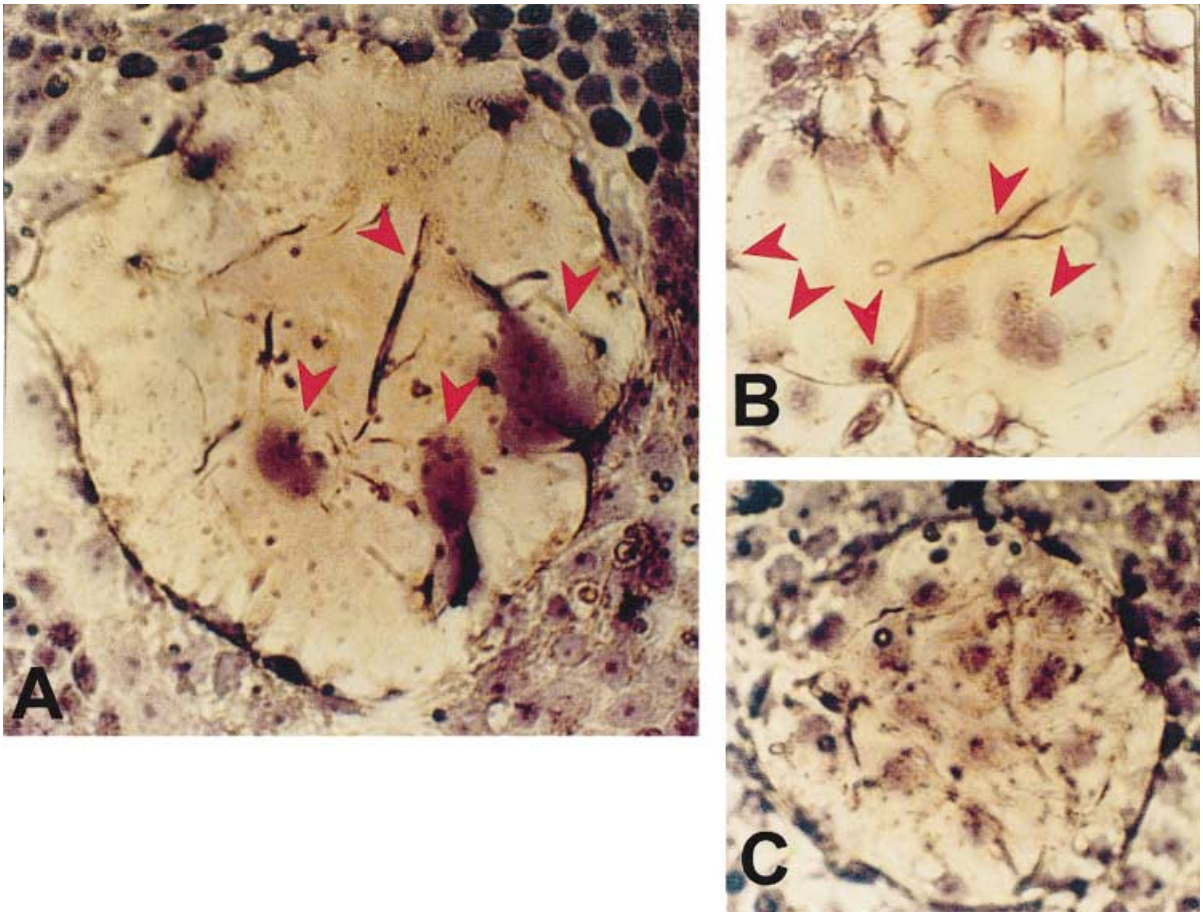

Fig. 7. Redistribution of nuclear material in predome stained for biotin and with hematoxylin. **A:** Hematoxylin stain of a predome reveals three forms of chromatin material: compact, darkly staining regions on the edges of the predome; hematoxylin staining of nuclei distributed under the apical membrane (arrowheads). Linear structures also stained with hematoxylin 400. **B:** A predome in which nuclear material has become more dispersed but still contains hematoxylin staining regions on edges (three arrowheads on left), clusters of stained nuclei (arrowhead on right) and linear structures that stain with hematoxylin (arrowhead on top). 400. **C:** A predome characterized by an array of nuclei distributed throughout a biotin-stained apical membrane. 400.

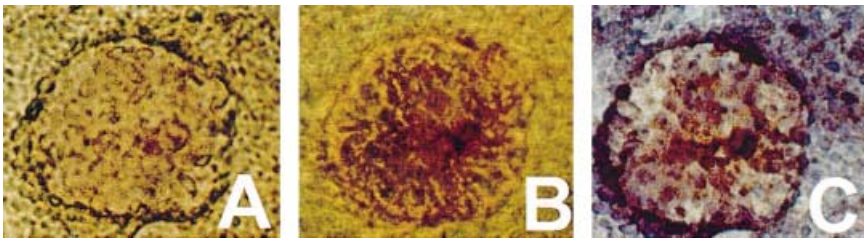

Fig. 8. Mature dome membranes stain brightly for biotin. **A:** Focusing on the monolayer and the lumen under the stained dome. 100. **B:** Focusing on the cells in the dome, biotin staining clearly differentiates the dome from the surrounding monolayer. 100. **C:** Staining with hematoxylin reveals small, regular nuclei in cells in a mature dome.

zur and colleagues [1983] in cross-sections of endometrial tissue are also observed in differentiating Ishikawa cells (Fig. 4A–C) when aggregated nuclei become flattened against apical and lateral membranes as predome cells elevate (Figs. 2, 3, 4). Researchers have been puzzled for more than a decade by the phenomenon of marginalized chromatin in vivo and the intimate association of biotin with chromatin. Our results suggest that these structures may

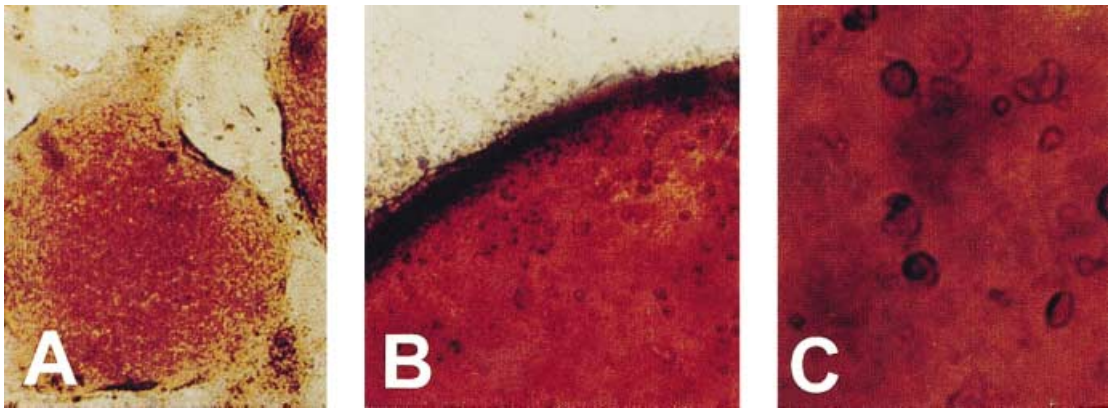

Fig. 9. Week-old domes contain lumen material that stains for biotin under a mature dome that also stains for biotin. **A:** After 7 days, domes were fixed and stained. The biotin stain associated with the dome is intense, with no evidence of biotin staining in the surrounding monolayer. 40. **B:** The biotin stain under the dome is particularly intense. 100. **C:** Some of the membrane fragments in the lumen stain for biotin. Not all of the fragments stain for biotin, and not all of the stain is associated with vesicles large enough to detect. 400.

be a transitory manifestation of epithelial differentiation.

Yokoyama and his colleagues [1993] examined more than 200 endometrial samples. Biotin-associated marginalized nuclei were found in many of the samples of gestational endometrium, obviously a tissue that is undergoing extensive differentiation as maternal cells interact with fetal cells. Biotin-associated nuclei were not found in the 147 samples of normal endometrium. At face value, this result suggests that the kind of epithelial differentiation represented by marginalized chromatin and biotin associated with nuclei only occurs following implantation. On the other hand, even a sample of normal tissues as large as 147 might not be sufficient to find transitory differentiating cells that might be restricted to certain regions of endometrial epithelia during the few hours when the tissue is becoming competent for implantation.

Whether or not nuclear-associated biotin can be found in pregestational endometrium, it is important to note that biotin-containing nuclei are not exclusively endometrial. Marginalized nuclei associated with biotin have been found in pulmonary blastoma [Yang et al., 1995] and in thyroid carcinoma–containing morules [Okamoto et al., 1995]. Biotin-containing intranuclear inclusions have also been found in endometrioid adenocarcinoma of the ovary [Tsujimoto et al., 1991] and in pulmonary endodermal tumors (Nakatani et al., 1994].

Origins of the Biotin

Undifferentiated Ishikawa cells in monolayer do not stain or stain only faintly for biotin. It is well known that the cells contain biotin linked to carboxylases. It is assumed that this biotin does not stain because the carboxylases are present in small amounts in the cytoplasm and in numerous, discrete mitochondria. A distinct biotin stain appears in differentiating cells within approximately 8 h. It is seen first as granular material in multinucleated cells, then in a sheath that encases clusters of nuclei and in filamentous structures in the cytoplasm, next in overall staining of apical membranes, and finally in vesicles exocytosed into lumen under the domes.

Membranes staining for biotin can be harvested from a dish of mature domes by collecting luminal fluid of domes or by isolating membranes from whole cell homogenate. When a differentiated culture is stained and homogenized, pink membranes can be isolated in two discrete bands on sucrose gradients. These membranes contain biotin binding proteins with approximate masses of 74 kDa, 69 kDa, and 127 kDa. Biotin stain associated with nuclei in ovarian endometroid adenocarcinoma was also found to be conjugated with a protein of molecular weight 69 to 72 kDa (Tsujimoto 1991). But the fact that these biotin binding proteins are also observed in membranes prepared from undifferentiated cells suggests that differentiation does not result in the synthesis of any unique biotin binding protein. The masses of these three membrane-associated biotin binding proteins corresponds to the approximate molecular masses of biotin-containing subunits for the mitochondrial carboxylases. The absence of any evidence for a unique biotin binding protein in differentiated cells suggests that

Fig. 10. Western blot of biotin-containing proteins from membranes. One dish each of nondifferentiated and differentiated cultures were scraped from 10 cm dishes, homogenized, and run on sucrose gradients. Membranes from fractions 5 and 4 from nondifferentiated and differentiated cultures were solubilized in SDS and run on a 10% polyacylamide gel. Bands at 127 kDa, 74 kDa, and 69 kDa were found in both fractions, in nondifferentiated as well as differentiated. Fraction 4 from the differentiated culture appears to contain more 74 kDa protein than the comparable fraction in the nondifferentiated dish. Most importantly, no unique biotin binding proteins were detected in membranes from differentiated culture.

biotin-staining granules and biotin-staining membranes may result from changes in the quantity or distribution of biotin binding proteins already present in the cells. This result could be explained if differentiation is accompanied by increases in the size or number of mitochondria or by extensive elaboration of mitochondrial membranes.

Increased biotin staining, especially the appearance of particulate-stained material, could also reflect changes in the distribution of mitochondria. It has been suggested that mitochondria are semiautonomous organelles that can change shape and location inside a living cell. Mitochondria have been reported capable of elongating, shortening, branching, buckling, swelling, fusing, and dividing. [Bereiter-Hahn and Voth, 1994]. Evidence is mounting that cellular differentiation can lead to profound changes in mitochondria and increased association of this organelle with nuclei. In the dividing fertilized egg, mitochondria aggregate with and almost surround nuclei. In chicken embryo fibroblasts, elongated tubular and dynamic mitochondria move to the perinuclear region and form a tight ring of short, swollen, and sometimes fused organelles [Collier et al., 1993]. Changes in mitochondrial structure and function have been reported for cytotrophoblasts fusing to form syncytiotrophoblasts [Martinez et al., 1997]. In yeast, mitochondria have been described not only as fusing but also as forming a continuous filamentous structure at certain times during development [Smith et al., 1995].

The manifestation of biotin staining in our cultures that is most difficult to explain by mitochondrial rearrangement is the appearance of a transparent biotin-staining membrane around a cluster of nuclei (Fig. 1C). It is hard to imagine that fused mitochondria would not be more dense than this membrane appears to be. One possible explanation is that carboxylases, normally shuttled to the mitochondria, are being incorporated into alternative membranes, first into a unique membrane that is elaborated around multiple nuclei (Fig. 1D) and ultimately into apical membranes. The appearance of biotin-staining membranes in the lumen under the domes could result from transcytosis of biotin-containing endocytotic apical membranes and their ultimate expulsion on the basal side of the cell. Analysis of biotin-containing proteins under the dome clearly shows a 74 kDa protein. The 69 kDa protein can also be detected but is fainter. The third protein, at 127 kDa in whole cell homogenate, appears to be larger and more diffuse in dome material. Perhaps secreted biotin binding proteins are altered in some way.

If the appearance of stainable biotin reflects increasing synthesis of carboxylases and/or increasing numbers of mitochondria, the obvious next question is whether these changes play a role in the differentiation process that prepares epithelia for blastocyst implantation or sustains implantation once it has occurred. Since mitochondria are essential in the generation of energy, the increased energy requirements of gestation might benefit from an increase in the number or size of mitochondria. Furthermore, increased carboxylases in mitochondria or in membranes outside of mitochondria would be better able to deal with CO_2, the most significant waste product of the developing blastocyst, especially if present on the membranes of epithelial cells that may come in contact the blastocyst. In this regard, perhaps it is relevant that the formation of giant branched mitochondria has long been known to be a characteristic of differentiating endometrium during the early

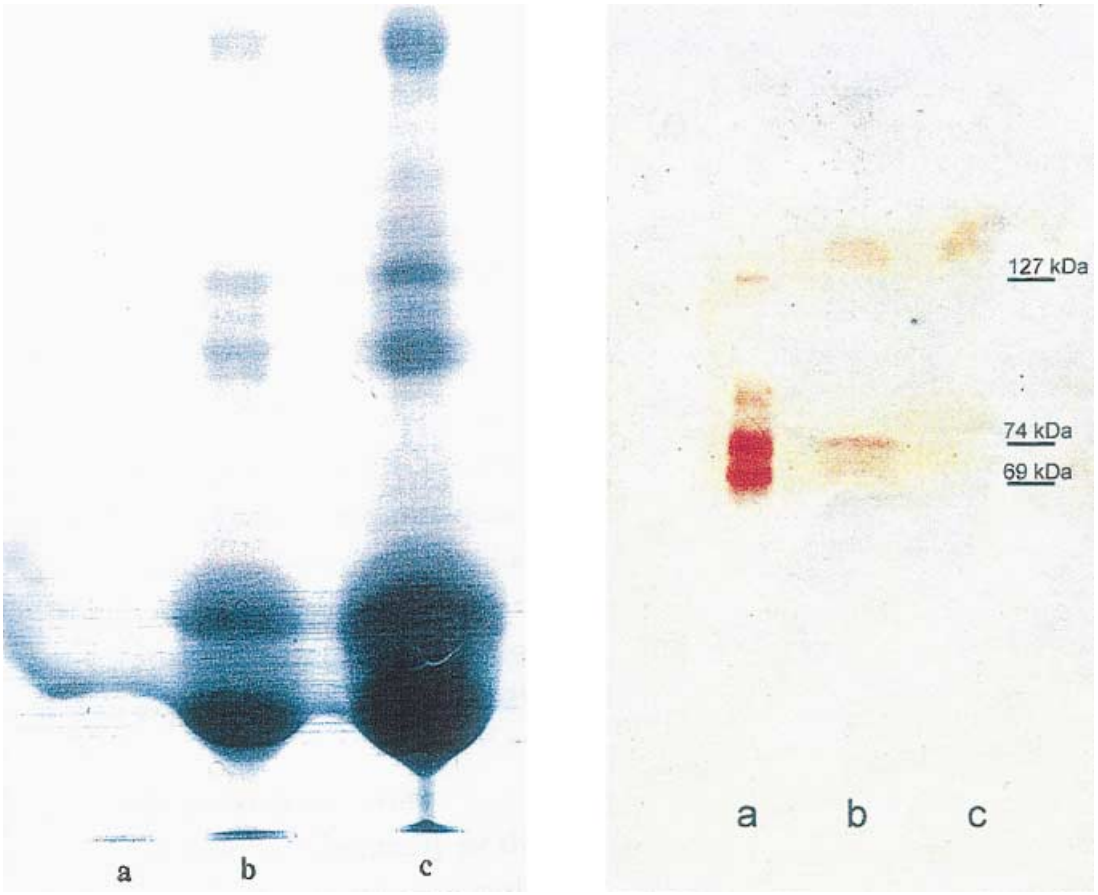

Fig. 11. Protein stain and Western blot of proteins from cells, (**a**) µg from fluid under the domes (**b**) and from used media (**c**). lane b. Approximately 60 ug of protein was applied to lane c; 10 ug of protein was applied to lane b, and less than 2 ug of protein was applied to lane a. The gel on the left was stained using Coomasie Brilliant Blue; the gel on the right was used for a Western blot using avidin linked to HRP and AECL H_2O_2 for staining. Protein staining on the left reveals that lane c had much more protein than lane b. Nevertheless, the 74 kDa and 69 kDa biotin containing proteins are found only in the luminal fluid. Cells from the same dish were homogenized and incubated with avidin linked to magnetic beads so that biotin-containing proteins could be compared to luminal fluid. Despite the presence of little detectable protein in lane a, the biotin binding proteins show up as they did in Fig. 10, with one additional protein appearing at approximately 84 kDa.

luteal phase prior to implantation [Li et al., 1994].

Bulging Apical Membranes Resemble Pinopods

One more significant adaptation in differentiating Ishikawa cells that mimics normal endometrial tissue is the transitory appearance of bulging apical membranes. Apical membranes protrude as predome cells become elevated. The elevation of predome cells indicates that significant amounts of fluid are being transported into a lumen under the basal membrane of the cell. Micropinocytosis or endocytosis are the two most likely mechanisms for such movement. Results with FITC-labeled material suggests the involvement of both mechanisms in the passage of fluid from apical to basal regions. Furthermore, the apical protrusions observed in differentiating Ishikawa cells resemble the pinopod structures that have been identified in the secretory endometrium for at least five different species, including humans [Parr and Parr, 1974]. Microscopy of the surface of uterine luminal epithelium excised during the luteal phase demonstrates the existence of membranous projections called pinopods. These structures, shown to be involved in endocytosis, are usually present for limited periods—24 h in humans.

Pinopods, or apical membrane extensions in luteal endometrium, have been of interest since their discovery in the 1950s. The structures were observed in human [Borell et al., 1959; Wynn and Wooley, 1967], rabbit [Meyer, 1970], rat [Warren and Enders, 1964; Psychoyos and Mandon, 1971; Nilsson, 1972], and mouse [Nilsson, 1962] endometrium. During the 1960s,

when the structures were initially studied, they were sometimes called domes, potentially confusing terminology for our purposes. It is important to note that these "domes" consisted of apical membrane protrusions from single cells. More recently these structures have been called pinopods, and the term *dome* has been used for multicellular structures resulting from differentiation of cultured monolayers.

When originally observed, pinopods were assumed to be involved in exocytosis of cell material into the uterine lumen. Subsequently, the structures were actually shown to mediate the uptake of fluid (pinocytosis) [Enders and Nelson, 1973] and macromolecules (endocytosis) [Parr and Parr, 1974] from the uterine cavity. It has been suggested that pinopod-facilitated endocytosis ensures better contact of a blastocyst with the endometrial wall by shrinking the cavity [Enders and Nelson, 1973]. Alternatively, Parr and Parr [1974] suggested that endocytosis may facilitate uptake of macromolecules signaling the arrival of a blastocyst in the endometrium. Since the pinopod extension projects far out of the cell surface, a third possibility is that these membranes are the first points of contact between a blastocyst and the endometrial wall. Despite agreement that uterine fluid is endocytosed by the pinopods, there is no clear evidence about what happens to the endocytosed fluid in vivo. The fluid could be transcytosed across the epithelial layer into the region between epithelium and basal lamina so that it can be taken up by insinuating trophoblasts when implantation occurs.

Denker and colleagues have suggested that profound changes in both apical and basal membranes in uterine epithelium are essential to facilitate implantation [Denker, 1993, 1994]. The formation of pinopods is one profound structural change that can be documented in vivo. In humans, the limited period when pinopods are observed coincides with that period of time when blastocyst implantation can occur [Martel et al., 1991]. Psychoyos and Martel [1990] have suggested that pinopods should be considered a marker of endometrial readiness for implantation [Nikas et al., 1995]. Our results clearly demonstrate structural (bulging apical membranes that look like pinopods) as well as biochemical (biotin) changes in the apical membrane of differentiating Ishikawa cells. The biochemical change includes the appearance of biotin, first in granules, then in membranes, and finally under the dome. Biotin itself is not expected to make apical membranes "sticky" since it only binds avidin or an avidin-like protein, but its appearance may signal other changes essential for blastocyst adhesion to endometrial epithelium.

Dynamic of Differentiation

Discussion thus far has focused on transitory structures that appear during dome formation: biotin-associated nuclei, marginalized chromatin, and apical membrane protrusions. In cell culture, these structures are readily observed as part of a dynamic process that begins when two, three, ten, or even more cells fuse. Cell fusion is a frequently observed process in endometrial tissue preceding and following implantation. But whereas it has been shown that rabbit endometrial epithelia becomes completely syncytial prior to implantation [Davies and Hoffman, 1976], multinucleation in luminal epithelium in humans is not so apparent. On the other hand, multinucleated cells are found throughout the placenta following implantation, resulting from fusion of maternal cells with each other and with decidual cells.

As an event in differentiation of the Ishikawa monolayer, cell fusion is followed by the appearance of stainable biotin, possibly indicative of changes in mitochondrial distribution. Subsequent changes in nuclear distribution are remarkable. Nuclei aggregate with each other and may even fuse in structures that become surrounded by biotin-containing membranes. A multinucleated cell containing 20–30 nuclei can become a structure with four or more nuclear aggregates. These aggregates are flattened against apical and lateral membranes as finger-like protrusions become elevated from multinucleated structures. That flattening may be due to pressures created as fluid is transported from the apical side of the cells to the basal side. The appearance of elaborate apical protruding membranes coincides with the passage of fluid into a lumen under the predome.

In the final stages of dome formation, enlarged predome cells with marginalized chromatin and low nuclear to cytoplasmic ratios become dome cells with high nuclear to cytoplasmic ratios. Nuclei that aggregated 6–10 h earlier become redistributed over a period of hours, giving rise to cuboidal, mononuclear epithelial cells in the mature dome. These cells are morphologically similar to surrounding monolayer cells but biochemically different in that they are no longer anchorage-dependent and

continue to contain stainable biotin. By what process is this final transition made?

While the phenomenon of syncytium formation has been described in a variety of cell systems, it is rarer to find descriptions of how multinucleated cells resolve back into mononuclear cells. Perhaps it is an event less frequently observed. One of the more extensively described examples, labeled cellularization, occurs in the drosophila blastocyst. The process involves simultaneous synchonous formation of membranes around nuclei in a syncytium formed by DNA replications without cell division during the early stages of embryo development [Loncar and Singer, 1995]. What has been observed in predomes may be similar. Aggregated nuclei pull apart and become distributed under the stretched elevated apical membrane, with subsequent formation of individual cell membranes.

Studying Differentiation in Cultured Endometrial Cells

Differentiation in a monolayer of endometrial epithelial cells is not expected to precisely duplicate differentiation observed in vivo since endometrial differentiation obviously involves interactions of different cell types. Furthermore, the environment of the dish favors two-dimensional association. Nevertheless, appropriately stimulated Ishikawa cells move into the third dimension forming domes and even tubules. In fact, there is evidence from other laboratories that endometrial cells can form gland-like structures in culture dishes [Rhinehart et al., 1988]. Staining differentiating Ishikawa cultures hour by hour has revealed that differentiation is a complex process involving the formation of multinuclear cells, nuclear aggregation, membrane enclosure of aggregated nuclei, elevation of cells containing these structures, and subsequent redistribution of aggregated nuclei within the elevated enlarged cells.

We are encouraged that, at least in nuclear association with biotin and in the formation of membrane protrusions resembling pinopods, the differentiation observed in vitro includes morphological and biochemical changes characteristic of differentiating human endometrium in vivo. These observations suggest that, to a limited but potentially useful extent, differentiation of endometrial epithelia can be studied in cell culture. Success along these lines has already been achieved in studying endometrial fibroblast differentiation that mimics decidualization.

Steroid hormones would be expected to play a role in the differentiation of endometrial cells. As is true of so many phenomena involving steroid hormone effects, the results in vitro are somewhat confusing. While progesterone has been observed to increase the number of differentiating structures, the steroid must be added together with large (greater than 300 kDa) factors from serum [Fleming, 1995]. The serum factor will elicit differentiation whether or not progesterone is added. When the serum is fractionated to some extent, it is the serum material larger than 300 kDa that brings about differentiation, suggesting that the factor is a complex of macromolecules that could even contain a progesterone binding protein [Fleming et al., 1995]. Charcoal stripping of serum is not observed to diminish differentiation, but complexed progesterone, even free progesterone, is not easily stripped from serum. It has not been possible to establish that progesterone is absolutely essential for the differentiation; neither can a role for progesterone be ruled out.

It is generally agreed that the differentiation of human endometrium proceeds through to the stage at which it can implant a blastocyst whether or not a blastocyst is present. Beyond that point, further differentiation of maternal epithelium is expected to be dependent on interaction with fetal cells. We would like to suggest that the process that leads to the formation of domes and the process that prepares luminal and glandular epithelium for implantation may have commonalties. The ability to manipulate and study stages in dome differentiation may allow us to learn more about the regulation of epithelial differentiation essential for implantation.

ACKNOWLEDGMENTS

The author thanks Donald Gruol for his helpful comments on the manuscript. This work was supported by NSF RUI award (9306492).

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