Development of Morphological and Functional Polarity in Primary Cultures of Immature Rat Uterine Epithelial Cells

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Abstract. The present study describes a culture environment in which luminal epithelial cells isolated from immature rat uteri and cultured on a matrix-coated permeable surface, with separate apical and basal secretory compartments, proliferate to confluence. Subsequently the cells undergo a process of differentiation accompanied by progressive development of functional polarity. Ultrastructural and immunocytochemical evidence verifies the ability of these primary cultures to regain polar organization, separate membrane domains, and form functional tight junctions as demonstrated by the development of transepithelial resistance. The appearance of uvomorulin is restricted to the lateral cell surface. Coordinated indices of functional polarity that develop progressively in postconfluent cultures include the preferential uptake of [^{35}S]methionine from the basal surface and a rise in uterine epithelial cell secretory activity characterized by a progressive preference for apical secretion. The time dependent development of polarity was characterized by differences in the protein profiles of the apical and basolateral secretory compartments. The maintenance of hormone responsiveness by the cultured cells was validated by the secretion of two proteins identified as secretory markers of estrogen response in the intact uterus. The technique of culturing the cells on a matrix-coated permeable surface with separate secretory compartments produces a uterine epithelial cell that morphologically and functionally resembles its in situ equivalent. The culture method and analytical approach used in this present study may be applied to primary cultures of a variety of natural epithelia, which have hitherto proven resistant to more conventional culture methodologies.

In vivo the apical and basal surfaces of uterine epithelial (UE)[1] cells express different, hormonally dependent functions involving cell–cell interactions as well as protein secretion. In particular, UE cells must alter the composition of their apical surface in response to interactions of progesterone and estrogen (1, 17, 41) to allow attachment of the embryo. At the same time the relationship of the UE cells with their basal lamina is altered (34). Moreover, apical secretions also must support development of the preimplantation embryo (33, 38). Coincident with embryo attachment, signals are transduced via the basal surface of the UE cell to induce differentiation of uterine stromal cells; i.e., the decidual cell response (19). This process is an initial step in placentation (12). These changes nominally represent some of the specialized functions of the UE cell.

The ability of absorptive secretory and transporting epithelia to carry out special vectorial functions depends upon polarization of their plasma membranes into functionally and compositionally distinct apical and basolateral domains (5, 22, 31, 32, 36). Establishment and maintenance of these polarized functions depends, to a large degree, on the integrity of the intercellular junctions of the lateral paracellular space and on the responses of the epithelial cell itself to environmental signals, including those originating in the cell substratum (6, 9, 15, 28).

Our knowledge of the relationships between morphological and functional cell polarity and the expression of specialized epithelial cell functions (absorption, secretion, vectorial ion transport) is based mainly on studies of cell lines derived from normal and malignant epithelia (32, 36). Studies of polarization in these cell lines demonstrate that normal epithelial phenotypic expression depends on their ability to establish tight junctions and distinct membrane domains and is highly dependent upon culture environment. Important contributions to normal epithelial cell function can be attributed to a permeable culture surface (5, 23, 36) and the proper extracellular matrix (9, 20). Very few studies have investigated the establishment and maintenance of polarity in primary cultures of epithelial cells (6, 9, 21). None have been reported with the UE cell.

There are no UE cell lines to facilitate the study of the biogenesis of polarity or to identify the mechanisms by which estrogen and/or progesterone affect the distinctions between apical and basal surface domains or secretions. Attempts to use whole uteri, explants of whole uteri, or endometrium

1. *Abbreviations used in this paper*: EHS, Engelbreth Holm Swarm; UE, uterine epithelial.

(UE plus uterine stromal) or primary cultures of endometrial cells for this purpose have been unrewarding (16, 33, 38, 39). Standard in vitro culture conditions provide a highly artificial environment that does not sustain morphological and functional polarity and results in the rapid disappearance of differentiated functions (14).

If specialized functions regulated by the direct action of hormones on the UE cell are to be distinguished from those controlled indirectly by the action of hormones and regulatory agents on other uterine cell types or delivered via the uterine vasculature, the UE cells must be studied as a isolated population of cells. Continued expression of specialized UE cell functions requires experimental conditions that maintain their polar organization. In this work we study UE cells from immature rats as an homogeneous population isolated from other endometrial cell types (11, 25). These cells were cultured on an EHS-tumor extracellular matrix applied on the semipermeable support of a Millicell apparatus under conditions that enhance their proliferation and growth and allow them to establish polar organization. As a consequence, these UE cells exhibit many of the same properties displayed in vivo. Under these conditions UE cells develop morphological and functional polarity. These culture conditions also allow accessibility to their basal as well as their apical surfaces for biochemical analysis and experimental manipulation.

Materials and Methods

Immature (21-d-old) female Sprague-Dawley rats were either purchased from Harlan Industries (Houston, TX) or raised in our own facilities. Millicell HA and CM apparati were obtained from Millipore Continental Water Systems (Bedford, MA). Nu-Serum and Matrigel (EHS-tumor matrix) may be purchased from Collaborative Research (Lexington, MA). Tissue culture media and supplements were obtained from either Gibco Laboratories (Grand Island, NY) or Microbiological Associates (Walkersville, MD). Tissue culture plates and other supplies were from Corning Glass Works (Corning, NY) and Nalge Co. (Rochester, NY). [^{35}S]methionine was obtained from Amersham Corp. (Arlington Heights, IL). SDS and urea were purchased from Bio-Rad Laboratories (Richmond, CA). Sigma Chemical Co. (St. Louis, MO) provided Tris buffer and phenylmethylsulfonyl fluoride (PMSF). All chemicals used were reagent grade or better.

Separation of UE Cells

Luminal epithelial cells were isolated from uteri of immature rats by the method developed in this laboratory (25), used with only minor modification. Briefly, uteri were removed from immature (21–25-d) Sprague-Dawley rats, stripped, and cut above the cervices and below the oviducts. The horns were separated, slit lengthwise, and collected in sterile Hank's balanced salt solution (HBBS; $-Ca^{++}$ $-Mg^{++}$). The horns were transferred to a sterile solution (10 uteri per 6.25 ml) containing trypsin (0.5%; type III bovine pancreas) and pancreatin, 2.5% in PBS $-Ca^{++}$ $-Mg^{++}$, for 60 min at 4°C and 60 min at 20°C. The enzyme soution was removed and the uteri transferred to sterile 15-ml conical tubes containing 5 ml HBSS ($-Ca^{++}$ $-Mg^{++}$) at room temperature. After vortexing, 10 s at No. 5 setting, the supernate was removed to a sterile 15-ml centrifuge tube. 5 ml HBSS ($-Ca^{++}$ $-Mg^{++}$) was added to the tissue and the procedure was repeated.

The pooled supernates were centrifuged (for 5 min at ~500 *g*). The cell pellet was resuspended in 3 ml HBSS ($-Ca^{++}$ $-Mg^{++}$) by gentle aspiration. The cell plaques were allowed to settle by gravity (5–10 min). The supernate was removed, and the process was repeated until the supernate was clear. The cell preparations were homogeneous with respect to luminal epithelial cells and were free of glandular epithelium, stroma, vascular cells, and myometrial cells. The epithelial cells were then pelleted by centrifugation, resuspended in medium, and plated.

Preparation of Matrix-covered Filters or Wells

Engelbreth Holm Swarm (EHS) matrix, extracted from EHS mouse tumor was a generous gift of Dr. George Martin (National Institute of Dental Research). EHS tumor matrix solution was used to coat filter or well surfaces. Dry Millicell HA filter inserts (.45 μm pore size, 10 mm inner diam) were transferred to a sterile multiwell dish (24 wells, 16 mm diam). EHS matrix solution sufficient to coat the entire filter or well surface was added rapidly, the dish was tilted, and excess EHS matrix solution was removed by aspiration. Because excessive thickness of the matrix layer may interfere with cells attaining confluence, the concentration of matrix to be used must be determined empirically. If necessary matrix may be diluted with basic culture medium. Coated wells or filters were incubated at least 1 h at 37°C in a humidified atmosphere and then rinsed with 0.5 ml HBSS before UE cells were plated.

Culture Medium

Basic culture medium was composed of DME high glucose, mixed 1:1 with Ham's F12, to which 0.1% BSA, 15 mm Hepes (pH 7.2), penicillin (100 U/ml), and streptomycin (100 mcg/ml) had been added. Complete medium was prepared by supplementing basic medium with 2.5% heat inactivated FCS and 2.5% Nu Serum. The final concentration of FCS in the complete medium was 3.125%.

Culture Conditions

Cells were suspended in complete medium at 1/4–1/2 uterine equivalents per 0.25 ml. Complete medium (0.5 ml) was added to the well containing the rinsed filter insert. Cell suspension (0.25 ml) was added to the upper surface of the filter insert. Cultures were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2. Unattached cells were removed at 24 h and fresh medium was added to both the apical and basal chambers. Medium was changed at 24-h intervals for the first four culture days and, unless otherwise noted, at 48-h intervals thereafter.

Cell Counts

Cells on EHS-matrix-coated wells or filters were detached with trypsin/EDTA (Gibco Laboratories) and counted on a hemacytometer. To determine the extent of filter coverage and the time at which proliferating cells became confluent, UE cells cultured on filters were stained and inspected microscopically. Cultures were designated as confluent when cells were observed to completely cover the filter surface. Filters in holders were rinsed briefly in PBS, fixed 10 min in 100% ethanol, rehydrated (10 min in PBS), and stained 1 min in Mayer's hematoxylin. After rinsing in PBS, filters were dried, removed from their holders, cleared by dipping in xylene, and mounted in Permount (Fisher Scientific Co., Pittsburg, PA).

Electron Microscopy

UE cells cultured on matrix-coated filters or 1-mm segments of intact immature rat uteri were fixed 2 h in ice-cold 3% glutaraldehyde (wt/vol) in 0.1 M sodium phosphate, pH 7.4, and subsequently bathed in 0.2 M sucrose in 0.1 M sodium phosphate, pH 7.4, for a total of 1 h. In some cases, after a brief rinse in the sucrose–phosphate buffer to remove primary fixative, samples were incubated 60 min in 1% tannic acid in 0.2 M sucrose in 0.1 M sodium phosphate, pH 7.4, at 0°C. After a final rinse in the sucrose–phosphate buffer the specimens were routinely postfixed for 1 h in 0.4% osmium tetroxide (vol/vol) in 0.1 M sodium phosphate, pH 7.4. Filters were removed from their supports and dehydrated in a graded series of ethanol preparatory to embedding in Spurr's resin (37). The samples were polymerized 12 h at 60°C. Sections were cut on a diamond knife, mounted on grids, and stained 4 min in 4% uranyl acetate in 50% ethanol. After a brief rinse in distilled water, sections were poststained in saturated lead citrate according to the method of Reynolds (29). Micrography was performed on a Phillips 410 Transmission Electron Microscope operated at 60 KV.

Processing for Immunocytochemistry

Cultures on filters were rinsed briefly in complete HBSS at room temperature, fixed in 3% formaldehyde in HBSS for 20 min, rinsed twice in PBS, soaked 30 min in 50 mM NH_4Cl_2 in PBS, and rinsed three times in PBS. When UE cell cultures were lysed, the filter was treated 10 min in 0.5% Triton X-100, 1 mM $MgCl_2$, 1 mM EGTA, 80 mM Pipes, pH 6.9, followed

by two PBS rinses. All filters received two final rinses in 0.2% gelatin in PBS. Primary antibody was applied to the cell side of the filter and 0.2% gelatin/PBS was applied to the opposite surface to prevent drying (100 μl each side). Filters were incubated 1 h at 37°C in a humidified atmosphere and rinsed 3 times in PBS for 5 min. Second antibody was applied as described for the primary antibody and incubated 40 min at 37°C. Filters were rinsed three times in PBS (10 min each rinse) and air dried. Dry filters were removed from the holders, dipped in xylene, and mounted in Permount under coverslips. Cells grown on matrix-coated coverslips were processed as described previously (11). Samples were photographed on Kodak Tri-X-Pan film at an effective film speed of 3200 ASA using a Leitz Fluorescence Microscope.

Cytokeratins 7 and 8 (human numbering system) were recognized by mouse mAb B9 and cytokeratin 19 by mouse mAb E_3. Both were gifts of Dr. Warren Schmidt of Vanderbilt Medical School (Nashville, TN). The monospecific polyclonal rabbit antibody that recognized cytokeratin 18, i.e., anti-ENDO B, was a generous gift from Dr. Robert Oshima of the La Jolla Cancer Research Foundation (La Jolla, CA). The desmoplakins I and II were recognized by the mouse mAb DI and II 2.15 which was kindly provided by Dr. Pamela Cowan of the German Cancer Research Center (Heidelberg, FRG). Rabbit anti-uvomorulin was provided by Dr. Rolf Kemler of the Max Planck Institute (Freiburg, FRG). FITC-conjugated donkey anti-rabbit IgG and Texas red-conjugated sheep anti-mouse IgG were purchased from Amersham Corp.

Basal vs. Apical Uptake of [^{35}S]Methionine

The selective uptake of [^{35}S]methionine via the basal surface of UE cells grown on matrix-coated filters in complete medium was determined in preconfluent, early and late postconfluent cultures. The protocol was used as described (2) with the exception that 100 μCi/ml [^{35}S]methionine (1,000 Ci/mM) in methionine-free MEM was used.

Transepithelial Resistance Measurements

Electrical resistance across UE cell filter cultures was measured by methods modified from Matlin and Simons (23). Electrodes were constructed from two Pasteur pipettes filled with 3.5% agar in 2 M NaCl. Sintered Ag-AgCl pellets were inserted into the 2 M NaCl filling the shafts of the pipettes. The Ag-AgCl pellets were connected to a VΩ meter (model 8020A; John Fluke Mfg. Co., Inc., Everett, WA) which delivered a 20-μA current to measure resistance. This avoided electrode polarization during resistance measurement. The tips of these electrodes were briefly inserted into the medium bathing the two sides of the membrane on which the epithelia were grown and the resistance reading allowed to stabilize. The transepithelial-specific resistance was calculated using the relation:

$$R_{total} + R_{electrode} + RC/(1 + RC/RS),$$

where RC = (epithelial resistivity) × (surface area); and RS = (shunt resistivity of uncovered membrane) × (surface area).

pH Measurements

Media from respective apical and basal compartments of parallel filter cultures was pooled and the pH was immediately determined using a pH meter. Measurements were made when medium was changed so the data represent measurements made after a minimum of 24 h exposure of medium to the cells.

Analysis of Secreted Proteins

To label cells grown on filters their apical and basal compartments were rinsed twice with complete HBSS before the addition of 0.25 ml (apical) and 0.5 ml (basal) labeling medium. Cells on matrix covered wells received 0.5 ml labeling medium. The labeling medium consisted of methionine-free MEM containing 100 μCi/ml [^{35}S]methionine (1,000 Ci/mMol). Incubation of UE cells was performed at 37°C as described in the section on culture conditions.

At a selected time point medium was removed separately from the apical and the basal secretory compartments and precipitated with acetone at 4°C. Precipitated secretory products were rinsed twice with cold 10% TCA and twice with acetone. The precipitates were air dried and dissolved in extraction buffer (8 M urea, 1% SDS, 0.01% PMSF, 1% β-mercaptoethanol, 0.05 M Tris, pH 7.4) for analysis by electrophoresis.

Filters were rinsed in HBSS, removed from the Millicell holder, and extracted by placing the filter, cell side up, in a 1.5-ml microfuge tube and adding 250 μl extraction buffer. After vortexing for 1 min, aliquots of filter-extracted material were precipitated in 10% TCA at 4°C and processed in the same manner as described for the secretory proteins. Aliquots of redissolved secreted or filter-associated proteins were removed for determination of TCA-precipitable radioactivity. Isotope incorporation was linear over the 9-h period of metabolic labeling.

For pulse-chase experiments, polarized UE cell filter cultures were labeled under the same conditions. At the end of the pulse (9.5 h) the cultures were rinsed several times with prewarmed (37°C) MEM medium and the incubation continued in this chase medium. At 0, 2, 4, 6, and 8 h of the chase period analyses were made of the apical and basal secretory compartments and the cell-associated proteins.

Uterine strips were prepared from immature rats that had received either three daily subcutaneous injections of 1 μg estradiol-17-β in 0.1 ml 80% ethanol 20% saline or vehicle alone. 24 h after the last injection, the uterus was removed and the lumen was exposed by slitting the uterine horn longitudinally and the uterus was rinsed in HBSS. The conditions for metabolic labeling and media analysis were the same as those used to label the primary UE cell cultures.

Electrophoretic Techniques

One-dimensional PAGE on 10% acrylamide slabs was performed according to Porzio and Pearson (27). Samples redissolved in extraction buffer were prepared for one-dimensional PAGE by combining with an equal volume of Laemmli sample buffer (18) and heating 5 min at 90°C. For two-dimensional electrophoresis, samples were redissolved in lysis buffer and separated in the first dimension by nonequilibrium pH gradient electrophoresis (26), using an ampholyte range of pH 3.5 to 10.0. Separation in the second dimension was performed on 10% acrylamide slabs prepared according to Porzio and Pearson (27).

Results

Proliferation on Matrix-coated Surfaces

Freshly isolated immature rat UE cells were cultured in complete medium on EHS-matrix-coated filters (semi-permeable supports) or on matrix-covered tissue culture wells (solid supports). If cell density at attachment (24 h) exceeded 250 cells/mm^2, cultures reached confluence within 72–96 h. Once cells on matrix-coated filters became confluent proliferation either ceased or dropped to a maintenance level (Fig. 1 *a*). Cell density remained at the confluency level and the integrity of the monolayer was maintained without dis-

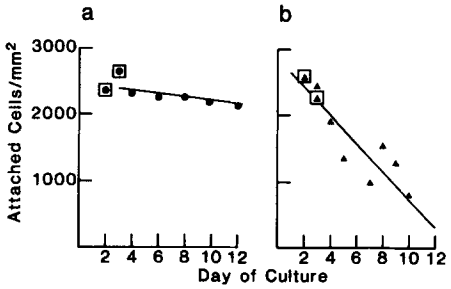


Figure 1. Postconfluent maintenance of attached UE cells: effect of culture surface. (*a*) Cells cultured on matrix-coated filters reach confluence between 48 and 72 h and maintain their confluent density throughout the course of study. (*b*) Cells cultured on matrix-coated wells attain confluence within the same time period but begin to detach after achieving confluence and are effectively gone by days 12–14. These data have been averaged from four culture series on filters and two series on wells from six individual UE cell preparations.

ruption for at least 22 d. Examination of the apical medium of postconfluent cultures showed little detachment of cells if they were cultured on matrix-coated filters. However, UE cells in confluent culture on matrix-coated wells began to detach from the matrix. Detachment started at the center of the culture and extended peripherally until all cells were detached by 12–14 d (Fig. 1 *b*).

Maintenance of Epithelial Cell Character

After 72 h of culture on matrix-covered coverslips, indirect immunofluorescent staining with antibodies specific for simple epithelial cytokeratins showed that immature rat UE cells continued to express the simple epithelial cytokeratins equivalent to human cytokeratins No. 7, 8, 18, and 19 (Fig. 2, *a–c*) in culture. Furthermore the cytokeratin intermediate filaments were associated with desmosomes in these cells (Fig. 2, *d* and *e*). After 22 d of culture on EHS-coated filters UE cells continued to express cytokeratins. They were uniformly recognized throughout the monolayer by their positive response to the antibody (Endo B) recognizing cytokeratin No. 18 (data not shown). No cytokeratin negative cells were detected at any stage of the experiment attesting to the effectiveness of the cell separation and the homogeneity of the cultures.

Indices of Morphological Polarity

Ultrastructure of uterine epithelial cells cultured on matrix-coated filters. Transmission electron microscopy was used to determine the extent to which filter-cultured UE cells displayed ultrastructural features characteristic of UE cells in situ (compare Fig. 3 with Fig. 4). The constituent columnar epithelial cells of the immature rat uterus in situ exhibited several structural features that are known to be involved in the establishment of polarized, confluent cell layers. As shown in Fig. 3 *a* these included distinct apical and basal cell surface domains that, respectively, marked the luminal and stromal (basal lamina) aspects of the epithelial cell. In addition, extensive membrane interdigitations, as well as junctional complexes (Fig. 3 *b*) were characteristic of the lateral aspects of the cell surface. Intracellularly, the Golgi complexes of the UE cell showed an apical position with respect to the nucleus.

In vitro UE cells displayed several distinct features indicative of progressive structural differentiation (Fig. 4). Compared to cells in situ preconfluent cells become extremely flattened (Fig. 4 *a*). Nevertheless these cells exhibited interdigitated lateral cell junctions (*arrowheads*) as well as rudimentary microvilli (*arrows*) representative of the elementary formation of basolateral and apical surface domains. An apical surface coat could be noted on many of these cells (Fig. 4 *a*) even at this relatively early stage of in vitro development.

In striking contrast confluent cultures were comprised of cells of relative columnar morphology (Fig. 4 *b*). In all cases examined, the cells were tightly adherent to each other. At this stage of development (Fig. 4, *b* and *c*) the ultrastructural evidence indicated that distinct cell surface domains had been established. Several additional structures distinguished the confluent UE cell. These included: (*a*) extensive lateral membrane interdigitations and numerous junctional complexes (Fig. 4 *b*) including desmosomes (Fig. 4, *b* and *c*); (*b*) elongated microvilli at the apical surface (Fig. 4, *b–d*); (*c*) apically oriented Golgi complexes (Fig. 4 *e*); and (*d*) relatively flattened basal surfaces associated with a thin extracellular lamina (Fig. 4, *f* and *g*).

Uvomorulin as a Marker of Morphological Polarity

Uvomorulin (E-cadherin) is a plasma membrane-associated protein that is exclusive to epithelial cells (3, 13). In situ its distribution is restricted to the basolateral domain and appears concentrated in the apical junctional complex (4) and in some epithelia the protein may be restricted to the zonae adherens (42). Apical application of anti-uvomorulin to formalin fixed, nonpermeabilized UE cells failed to generate a positive signal (Fig. 5 *b*) except in preconfluent cultures. Permeabilization (Triton X-100) of formalin-fixed cells provided access of the antibody to uvomorulin in the basolateral membrane (Fig. 5 *a*). Tight junctions were opened by 15 min treatment with 2.5 mM EGTA before formalin fixation to confirm that the absence of antibody recognition on the apical surface of nonpermeabilized cells was not due to failure of the antibody to recognize externally oriented uvomorulin epitopes on that surface. Under these conditions the antibody was able to interact with uvomorulin in the basolateral surface of the nonpermeabilized UE cell (Fig. 5 *c*).

Indices of Functional Polarity

pH Change. A pH differential between apical and basal media compartments was established by postconfluent cultures on matrix-coated filters. While cultures were in their preconfluent phase, the pH of both media compartments was similar to the pH of the medium alone (pH 7.2). During the early confluent period (days 4–5) the pH of both compartments became acidic; the change was more marked in the apical media (pH 6.2–6.3) than in the basal (pH 6.8). By day 6 or 7 the pH of the apical media began a progressive shift to basic pH so that, by day 10 of culture, the pH of the apical medium apeared uniformly basic (pH 7.6) and was maintained throughout the duration of culture. The medium in the basal compartment remained slightly acidic (pH 6.9). The cause of the pH shift remains to be identified. However, the consistency of the shift allowed it be used to distinguish between early and late confluent cultures.

Electrical Resistance

The development of transepithelial resistance resulting from the presence of functional tight junctions has been used as one type of evidence of cell polarity (5). UE cells cultured on EHS-matrix-coated filters also developed measurable electrical resistance (Fig. 6). Transepithelial resistance was first recorded after confluence and increased progressively, peaking at days 7–9 of culture.

Basal vs. Apical Uptake of [^{35}S]Methionine

In many epithelia, methionine transport systems are largely localized on the basolateral cell surface (36). Consequently establishment of preferential uptake of methionine by epithelial cells in culture has been used as another criterion of epithelial cell polarity (23).

In preconfluent UE cells, cultured on EHS impregnated filters, the uptake of [^{35}S]methionine via the apical surface was equal to uptake via the basal surface (Fig. 7 *a*). As cells

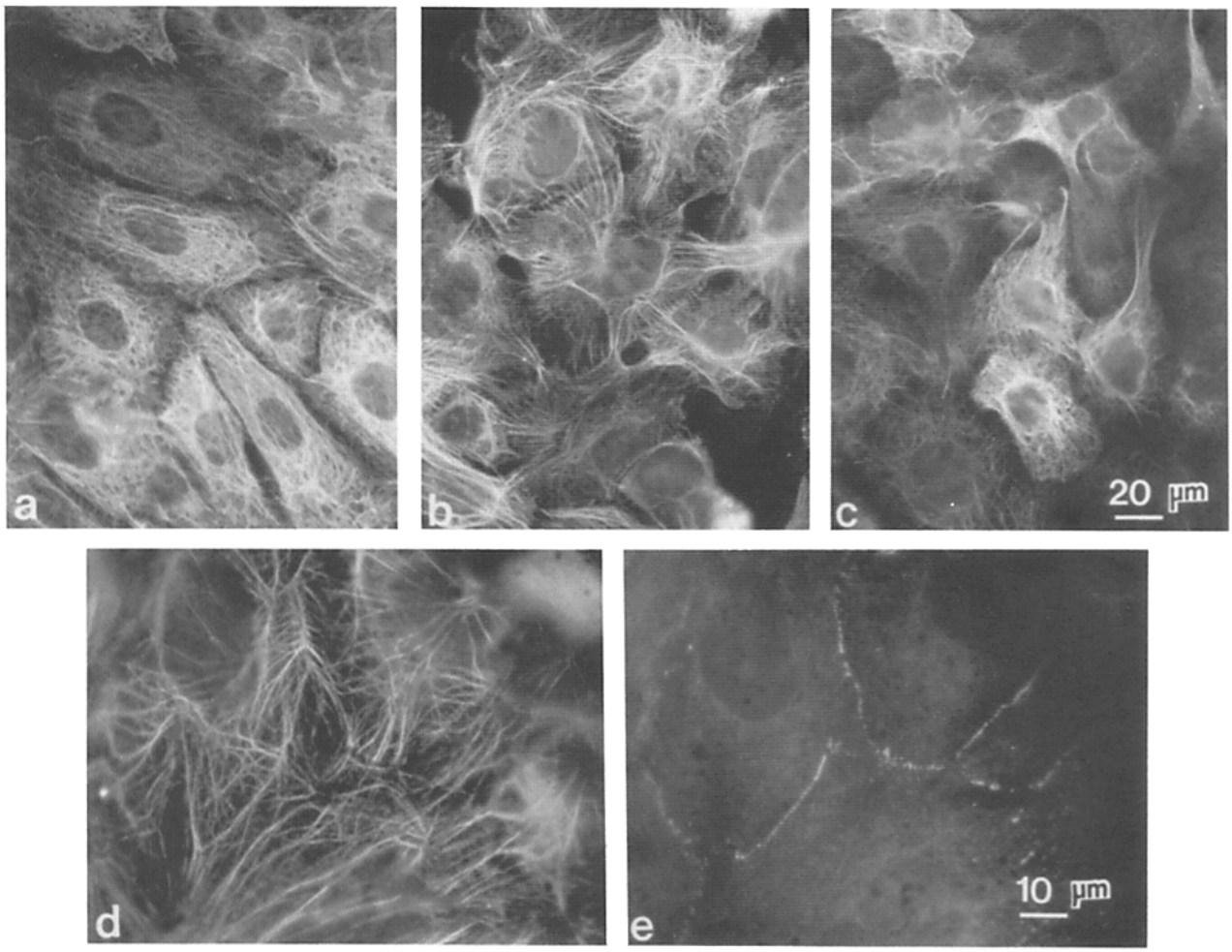


Figure 2. Indirect immunofluorescent staining for cytokeratins and desmosomes expressed in cultured rat UE cells. Immature rat UE cells cultured on matrix-coated glass coverslips for 72 h were processed for indirect immunofluorescent staining as described in Materials and Methods. (*a*) Cytokeratins 7 and 8 were recognized by mAb B9; (*b*) anti-Endo B recognized the expression of cytokeratin 18; (*c*) cytokeratin 19 expression was recognized by mAb E3; or (*d*) double labeling with anti-Endo B plus mAb DI and II 2.15 recognized the association of intermediate filaments containing cytokeratin 18 with desmosomes; (*e*) mAb DI and II 2.15 recognized desmoplakins I and II. Recognition of intermediate filaments by antibodies specific for simple epithelial cytokeratins and association of the cytokeratin tonofilaments with desmosomes demonstrated that the cultured UE cells maintain expression of these epithelial characteristics in vitro.

achieved confluence (day 4), the uptake of [^{35}S]methionine shifted preferentially to the basal surface. The basal/apical ratio of [^{35}S]methionine uptake during the early postconfluent stages (5–7 d) was approximately six, increasing to 10 in late postconfluent cultures (day 12).

Differential Protein Secretion to Apical and Basal Compartments

The measurement of [^{35}S]methionine incorporation into protein by UE cells and the proportion of newly synthesized protein secreted into the respective secretory compartments provided additional evidence for continuing development of polarity in filter-cultured UE cells. As a function of time in culture, total UE protein secretion, expressed as a percent of total incorporation, increased more than 10-fold (Fig. 7 *b*). The development of this increased secretory activity was accompanied by an increasing preference for apical secretion (Fig. 7 *c*), indicating the development of functional secretory differences that serve to distinguish between the apical and basolateral membrane domains.

Several factors could affect the validity of the qualitative and quantative analyses of the apical and basal secretory profiles; i.e., sequestering of basally secreted protein by the matrix and/or filter, nonlinear secretion from respective cell surfaces, and a possible contribution due to cell detachment and/or lysis during the incubation period. The first of these possibilities was tested by applying [^{35}S]methionine-labeled secretions to matrix-coated HA and CM filters in the absence of cells to determine whether the secretory products could diffuse freely across the filter. Filters were preincubated 24 h in medium with or without 3% FCS, rinsed, and exposed to labeled secretions in serum-free medium applied to either

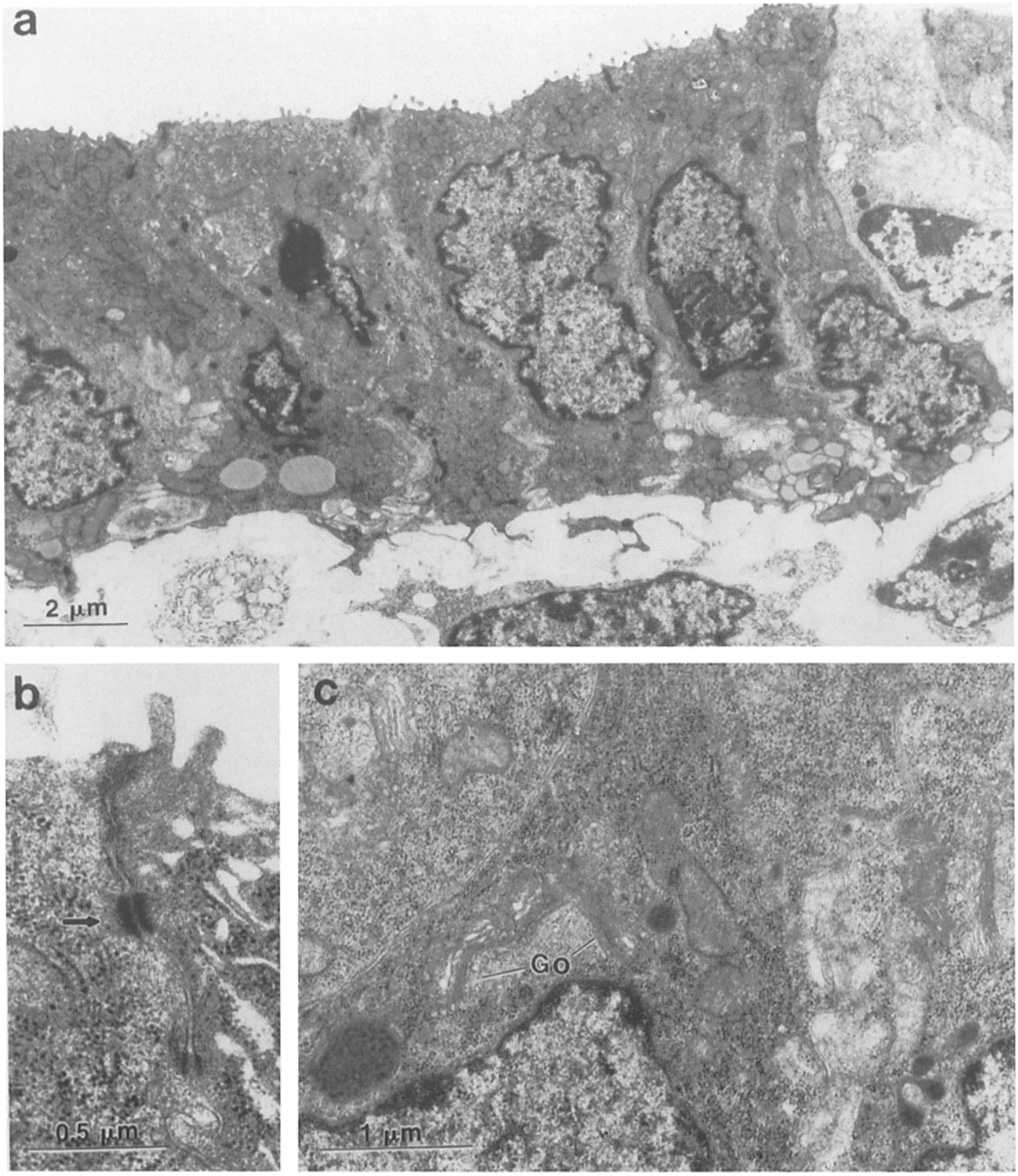
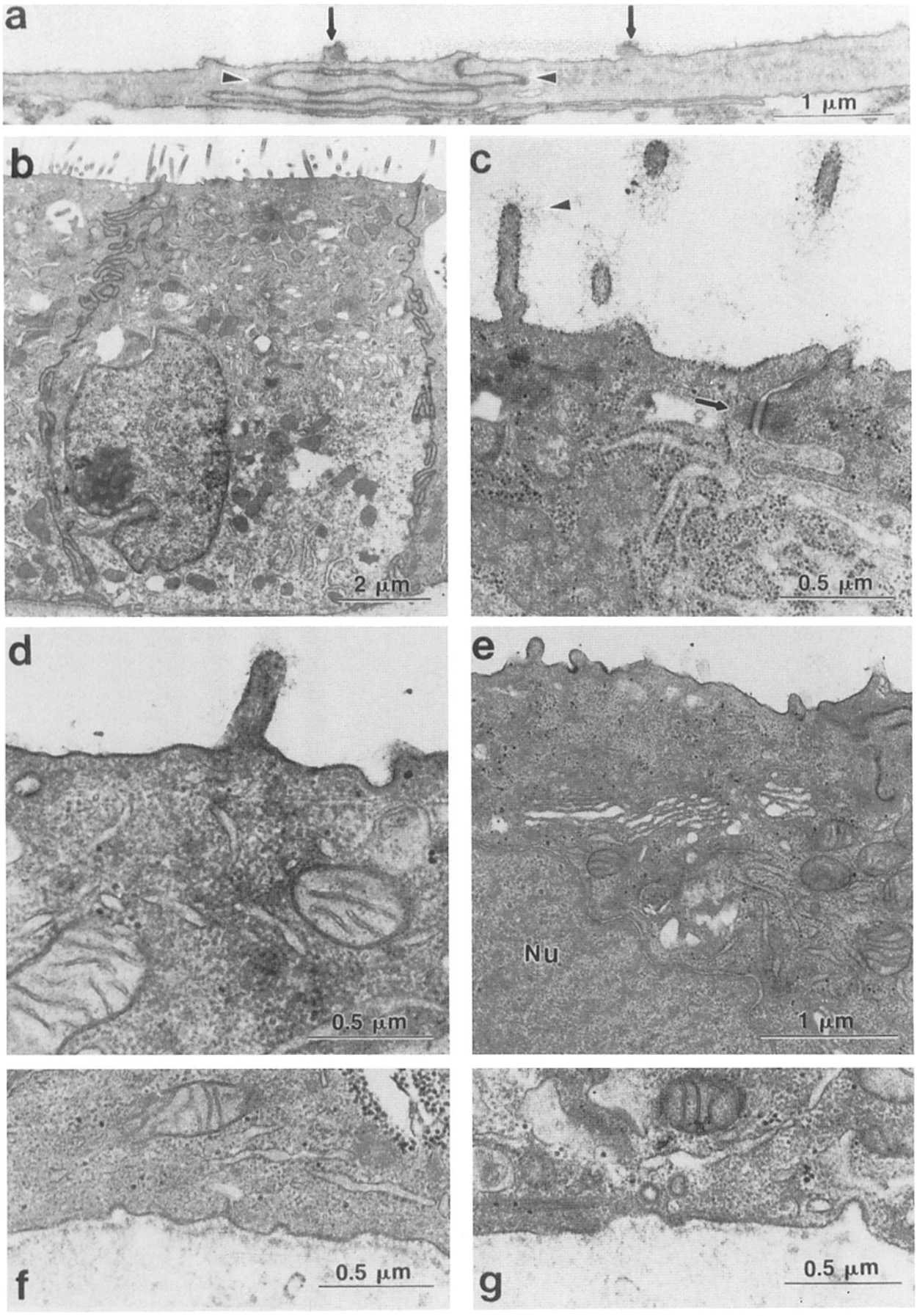


Figure 3. Ultrastructure of immature rat luminal UE cells in situ. Transmission electron micrographs of luminal epithelial cells in the intact uterus of the immature rat demonstrates the ultrastructure characteristic of a polarized epithelial cell: microvilli associated with the apical surface (*a* and *b*), tightly apposed lateral membranes with numerous interdigitations (*a*), desmosomes with attached tonofilaments in the lateral membrane (*arrow in b*), a relatively flat basal surface associated with a basement membrane (*a*), and the Golgi complex situated in a supranuclear position (*c*). *Go*, Golgi complex.

Figure 4. Ultrastructure of immature rat UE cultured on matrix-coated filters. UE cells in proliferating preconfluent cultures (*a*) have a flattened appearance with sparse, stubby microvilli at the apical surface (*arrows*) and elongated lateral membrane interdigitations (*arrowheads*). Postconfluent UE cell cultures (*b–g*) resemble their in situ equivalents. Cell height increases. Numerous elongated microvilli with an associated glycocalyx (*c, arrowhead*) appear on the apical surface of the columnar UE cells (*b* and *c*). Lateral membranes are tightly apposed with complex interdigitations (*b*) and frequent desmosomes (*c, arrow*). The basal membrane is relatively flat and associated with material resembling basement membrane (*f*). Nuclei assume a basal position in the cell (*b*) and the golgi complex is generally in a supranuclear location (*e*).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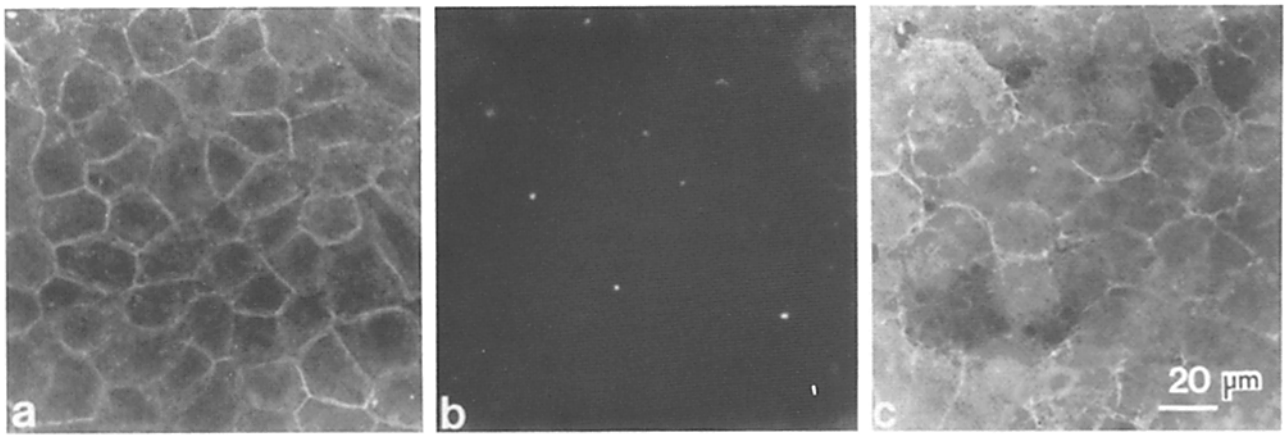


Figure 5. Recognition of uvomorulin in UE cells whose apical junctions were opened by EGTA treatment. Cells cultured on matrix-coated filters for 14 d were left untreated (*a* and *b*) or exposed to 2 mM EGTA for 20 min at 37°C (*c*) before fixation in 3% formalin with (*a*) or without (*b* and *c*) permeabilization after fixation. Anti-uvomorulin (1:30 dilution) was applied to the apical surface.

the apical or basal surface. For free diffusion, the expected apical/basal ratio at equilibrium would be 0.5. By 8 h the apical/basal ratio was 2.2, 0.55, 0.71, and 0.53 for apical application or 0.08, 0.33, 0.30, and 0.29 for basal application to matrix-coated HA(−FCS), HA(+FCS), CM(−FCS), and CM(+FCS) filters, respectively. Precipitable cpm in filter extracts exceeded 10% only when matrix-coated HA filters were not previously exposed to serum. Thus, exposure to serum-containing medium appeared to effectively block HA filter binding sites. This was confirmed by one-dimensional PAGE analysis of the filter extracts. Unlabeled serum proteins were detected in extracts of matrix-coated HA filters but not in extracts of matrix-coated CM filters. The low levels of labeled secretions present in extracts of matrix-coated HA or CM filters exposed to serum protein and unexposed CM filters were equivalent and were probably in the fluid phase of the filters. Since all UE cell cultures on matrix-coated HA filters in the present study were performed in medium containing 3.125% FCS, binding of secretory products by the filter or matrix should not have affected the results.

Linearity of both incorporation and secretion was established by labeling cells from various culture series with [^{35}S]methionine at various days in culture for 2, 4, 6, 8, and 10 h. Conditions of incubation were not rate limiting and total incorporation was linear through 10 h of incubation. Total

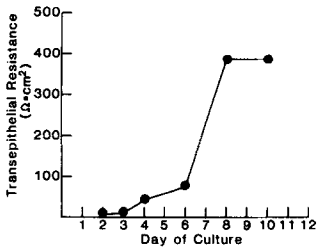


Figure 6. Development of transepithelial resistance in UE cells cultured on matrix-coated filters. Measurable resistance was first detected when cells achieved confluence at day 4 of culture. A modest increase between days 4 and 6 was followed by a marked rise in epithelial resistance between days 6 and 8. This level of resistance was maintained for the remaining period of the culture.

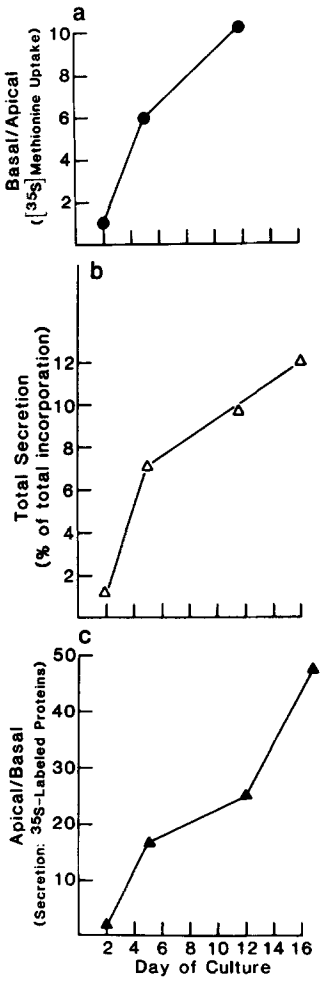


Figure 7. (*a*) Development of basolateral preference for the uptake of [^{35}S]methionine in primary cultures of UE on matrix-coated filters. Uptake (10 min) was measured from either the apical or basal side of the filter. The data is expressed as the ratio of basal/apical TCA-precipitable cpm incorporated by filter-cultured cells on day 2 (preconfluent), day 5 (early postconfluent), and day 12 (late postconfluent). All values represent averaged duplicate samples from two separate culture series. (*b*) Development of increased secretory activity in postconfluent UE cells cultured on matrix-coated filters. Total secretion (TCA-precipitable cpm in apical plus basal secretions) expressed as the percentage of total TCA precipitable cpm incorporated into UE cells plus their secretions. Samples were collected from duplicate filters labeled 8 h with [^{35}S]methionine on the day of culture indicated. Data was averaged from six culture series. (*c*) Development of apical preference for secretion. UE cells cultured on matrix-coated filters were labeled with [^{35}S]-methionine for 8 h on the day of culture indicated. The ratio represents total precipitable cpm secreted into the apical relative to that in the basal secretory compartment. Data are averaged duplicates from six culture series.

secretion, as a percent of total incorporation, was also linear throughout the 10-h incubation. Secretion from each respective cell surface was likewise linear through the 10-h incubation with a constant apical/basal ratio maintained for all the time points of the incubation period (Fig. 8). These observations proved to be unaffected by the day of culture on which they were performed.

Neither cell detachment or lysis contributed to the protein present in apical or basal medium. The profile of secreted proteins in each secretory compartment (Fig. 8) was similar for all incubation periods. The cumulative increases with time, in secretion to each compartment, reflected quantitative rather than qualitative changes in secretory profiles. When pulse–chase studies were done after a prolonged pulse (Fig. 9) >70% of the total amount of labeled protein secreted during the chase period appeared in the first 2 h. The profile of secretions from the chase period was similar to that of secretions accumulated during the pulse incubation but distinct from the profile of cell proteins extracted from the filters (compare Fig. 9, *a* and *b* with *c*). Further, the profile of cell-(filter) associated labeled proteins remained constant during the chase period. This pattern reflected the absence of relevant cell detachment as well as the low level of newly synthesized secretory products present in UE cells at the end of the pulse period. The distinction between cell- (filter) associated labeled proteins and secreted proteins was evident when each fraction was separated on two-dimensional PAGE (Fig. 10). Although secreted proteins and their cell-associated precursors cannot be positively identified in the absence of antibodies specific for the core protein, each secreted protein could be matched to a cell-associated protein with a similar migration coefficient but fewer charge variants. None of these secretory proteins were among the more prominently labeled UE cell proteins. Actin, present in high concentrations in cell (filter) extracts (Fig. 10 *a*), was not detectable in secreted proteins (Fig. 10, *b* and *c*).

The increased secretory activity of postconfluent cultures could be attributed to one parameter; i.e., an increased rate of secretion that was independent of cell number. Since cell number would determine the absolute amount of cpm incorporated, expression of secretion as a percent of incorporation allowed comparison of samples collected in the same culture series, at various times in culture, when cell numbers could be significantly different, and it normalized results from the same day in culture for two or more culture series in which cell densities may vary slightly.

Analysis of Secretory Products Labeled with [^{35}S]Methionine

The profiles of apical and basal secretory products were initially examined for proteins that might have been uniquely delivered to the apical or basal secretory compartment. A conservative analysis identified only one band at 130 kD, in low relative concentration, which appeared only in apical secretions (Fig. 8). A second band, at 88 kD, exhibited a

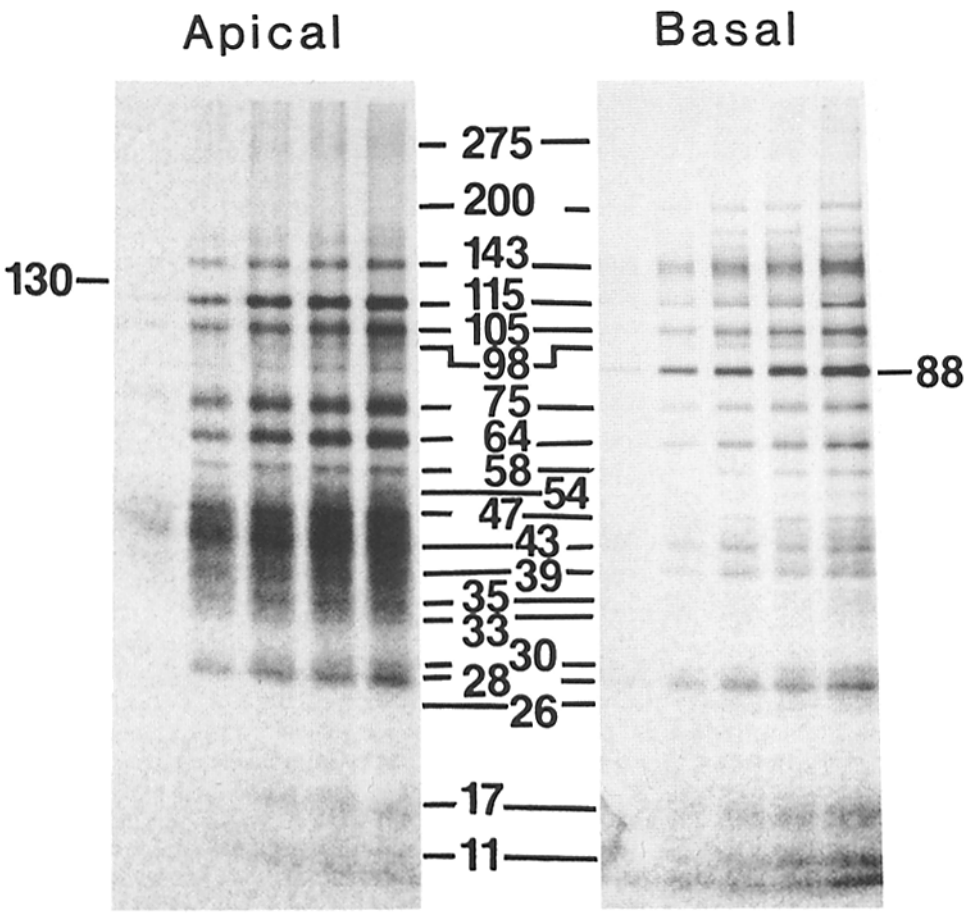


Figure 8. Time course of secretion. UE cells cultured on matrix-coated filters for 11 d were labeled with [^{35}S]methionine. Secretory products were collected from the respective compartments at 2, 4, 6, 8, and 10 h of incubation (from left to right). Autoradiographs were exposed for 18 h (*Apical*) and 7 days (*Basal*). Molecular mass ($\times 10^{-3}$) is designated by number. Components common to both compartments are indicated at center while components which are either uniquely (130 kD) or preferentially (88 kD) secreted to a particular compartment are indicated at the sides. Secretion into both compartments was linear with time through 10 h of incubation with an average apical/basal ratio of 26 for all time points.

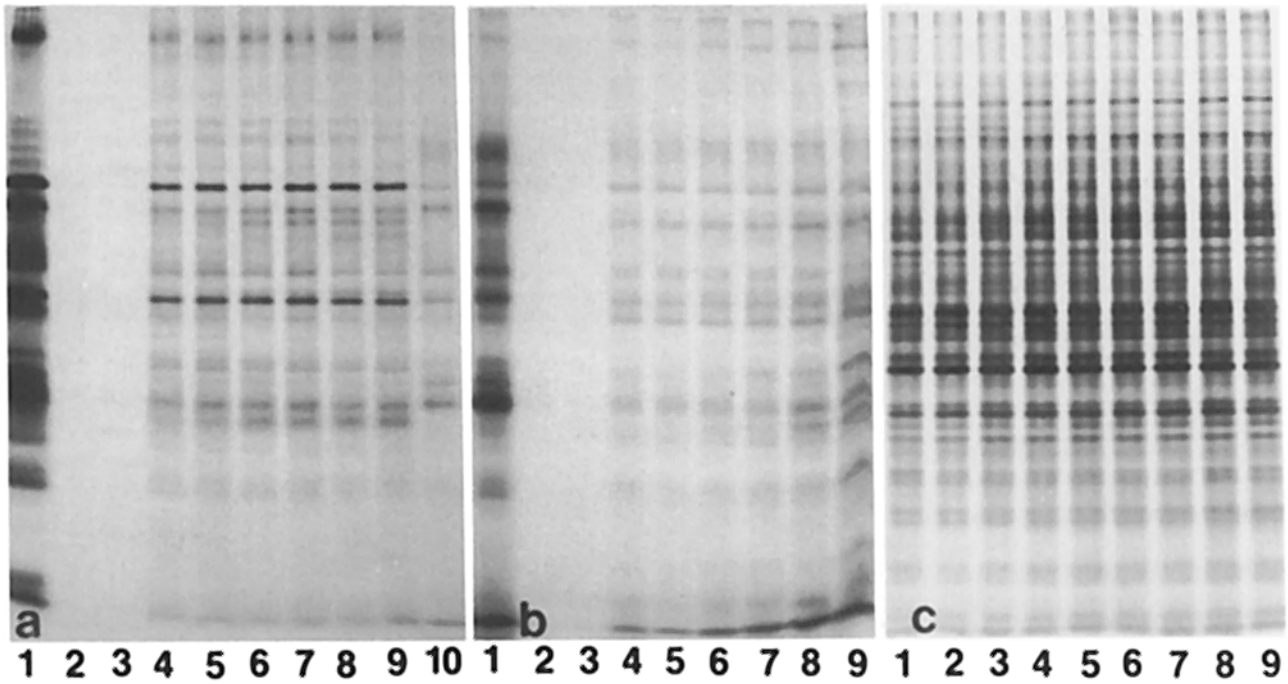


Figure 9. Pulse–chase incubation: rapid appearance of [^{35}S]methionine-labeled secretory products in apical and basal secretory compartments. 12-d filter cultures were labeled for 9.5 h with [^{35}S]methionine, rinsed with prewarmed basic medium, and chased into serum-free basic medium. The autoradiographs represent one-dimensional PAGE analysis of (*a*) 80% of total apical secretory products, 24-h exposure, (*b*) 80% of total basal secretory products, 7-d exposure, and (*c*) 4% of filter-associated products, 24-h exposure. Samples were collected at the end of the pulse (lanes *1*, *a*, *b*, and *c*, and lane *10 a*), after the rinse (lanes *2* and *3*, *a*, *b*, and *c*), or after chase intervals of 2 h (lanes *4* and *5*, *a*, *b*, and *c*), 4 h (lanes *6* and *7*, *a*, *b*, and *c*), and 6 h (lanes *8* and *9*, *a*, *b*, and *c*). Lane *1 b*, and *10 a* are the same sample. The two intensely labeled bands in the apical secretory compartment (lanes *4–9* in *a*) are the 115- and 64-kD secretory proteins.

preferential but not unique delivery to the basal compartment. While the remainder of the bands were common to both compartments it should be emphasized that in postconfluent cultures >90% of these common components were delivered to the apical compartment.

To determine whether the increased secretory activity of postconfluent UE cells represented synthesis and secretion of the same products synthesized by preconfluent cells or additional proteins, [^{35}S]methionine-labeled secretory products from different days of culture were analyzed by polyacrylamide gel electrophoresis. Bands migrating at 250 and 54 kD, which were prominent in secretions from UE cells cultured on matrix-coated wells, were also detected in secretions from cells in the same culture series cultured on matrix-coated filters (Fig. 11, lanes *1* and *2*). The 250 and 54 kD bands could not be detected in the secretions of UE cells cultured on filters after they achieved confluence. In contrast bands at 275, 105, 88, and 58 kD appeared or increased in concentration relative to the other secretory components in the early postconfluent cultures. The 88-kD protein was detectable only in secretions of confluent UE cells cultured on matrix-covered filters.

The apical secretions of cultured cells were also compared to the secretory products released by intact uterine horns into their incubation medium. Two proteins, considered as markers of estrogen action on the intact uterus (16, 43), appear at relatively elevated concentrations in the incubation media from uterine strips from estradiol-treated immature rats (Fig. 12, *b* and *b'*). These two proteins, which migrate at 115 and 64 kD, were two of the most prominent proteins in the apical secretions of UE cells (Fig. 12, *c* and *c'*). Furthermore, the appearance of these proteins in secretions of isolated UE affirm that these proteins are indeed products of UE cells and not merely passaged to the uterine lumen by the UE cell from another uterine cell type. Since estradiol was present in the medium at a physiological concentration, the appearance of two marker proteins of estrogen action in the secretory profile implies that these filter-cultured UE cells are capable of responding to estradiol in vitro.

The hormonal response does not appear to be a property of postproliferative differentiation of the UE cell since both the 115 and 64 kD species could be identified in the secretions of preconfluent cultures on both solid and porous surfaces.

Discussion

The unique nature of the epithelial cells of the uterine endometrium is defined by the multiplicity and complexity of the special functions they express. In the nonpregnant female, cyclic reproductive behavior can be characterized by morphological and functional alterations at the apical and basal surfaces of the UE cells. The changes occurring at the opposite surfaces may be coordinated but they are not necessarily quantitatively or qualitatively similar. The asymmetry of changes in the apical vs. basal plasma membrane domains

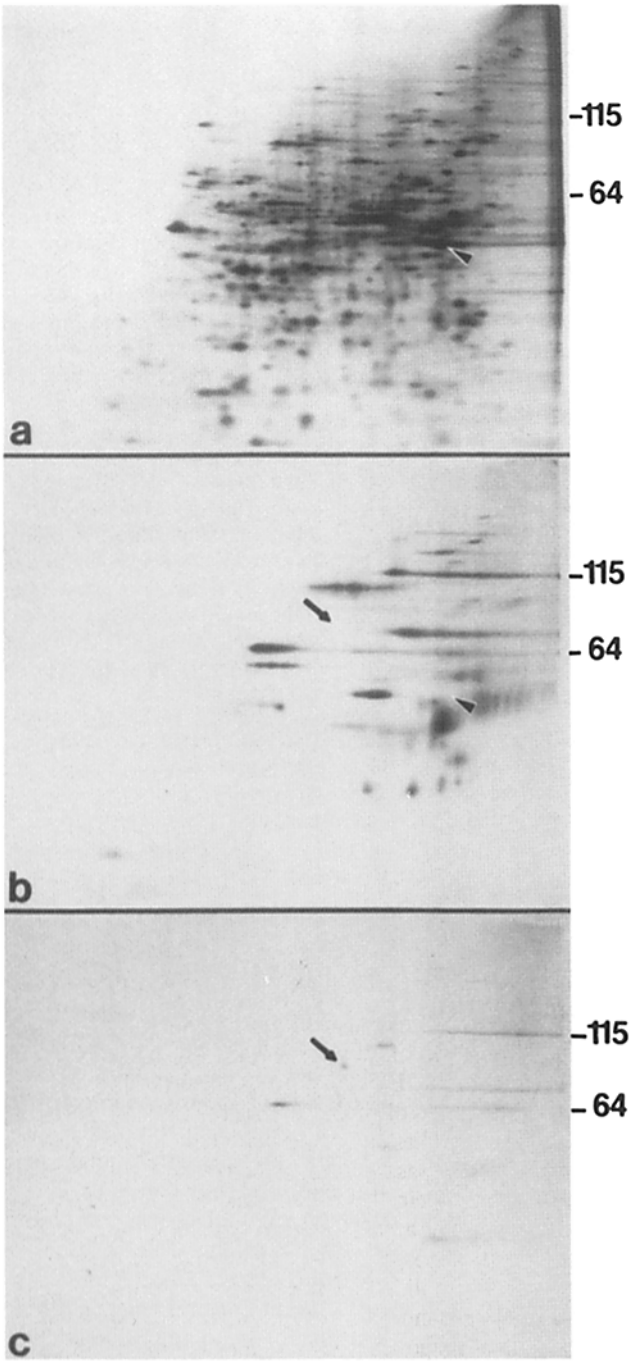


Figure 10. Comparison of UE cell- (filter) associated proteins (*a*) with proteins secreted into the apical (*b*) and basal (*c*) secretory compartments. Samples were collected after 8 h labeling with [^{35}S]methionine of UE cells cultured on filters for 21 d and processed for NEPHGE two-dimensional PAGE as described in Materials and Methods. (*a*) One-fifth of the extract from a single filter; (*b*) total secretion into the apical medium compartment from the same filter; (*c*) total secretion into the basal medium compartment. The autoradiographs were exposed for 6 (*a* and *b*) and 19 d (*c*). The arrow indicates the 88-kD secretory component present in basal secretion (*c*) which is absent in the apical secretion (*b*). The arrowheads designate actin present in the UE cell extract (*a*) but absent in the apical secretions (*b*), demonstrating that cell lysis does not contribute to proteins present in the secretory compartments. For reference, the molecular masses ($\times 10^{-3}$) of two major apical secretory components are indicated by numbers to the right of each autoradiograph.

of the UE cell is emphasized when reproductive cyclicity is interrupted by pregnancy. Those changes that take place at the apical surface and in the apical (luminal) secretory compartment have been implicated in providing the pathogen-free environment (44) required for sperm capacitation (35) and subsequent changes related to embryo metabolism and development (34, 39). Modifications at the apical surface of the UE cell are synchronized with alterations in the embryo and are necessary for the development of a transient period during which the blastocyst can attach to the apical surface of the UE cell (1, 12, 17).

Coordinate with the acquisition of apical receptivity for attachment are changes at the basal surface of the UE cell. These changes have been defined largely in morphological terms because of the inaccessibility of that surface for biochemical analysis. Observed changes infer that the basal lamina is undergoing degradative modifications (34). These alterations are compatible with the observation that, as the trophectoderm cells of the blastocyst differentiate, they move towards the basal lamina easily displacing the UE cells (34), which separate the embryo from the underlying decidualizing stroma. Finally differentiation of uterine stroma (decidualization) is initiated by signals transduced at the time of embryo attachment via the basal surface of the UE cells (19). Decidualization is critical to establishing the hemochorial placenta (12) and its induction stresses the point that the asymmetric structural and biochemical changes occurring at the apical and basal plasma membrane domains, which are rigorously regulated by hormonal signals, are critical to the normal functions of the UE.

The present studies describe the development of a culture system that resolves most of the problems that have limited the application of in vitro methods to the analysis of the specialized functions of UE cells (34). Culturing homogeneous populations of immature rat UE cells on porous filter supports impregnated with EHS-tumor matrix not only enhanced attachment, proliferation, and growth but fostered the expression, in vitro, of many characteristics of UE cells in situ. The availability of this cell culture system provides, for the first time, experimental access to both cell surface domains of viable, long-term cultures of hormonally responsive UE cells.

The conditions required for establishing and maintaining UE cell polarity in vitro are not satisfied simply by providing EHS-tumor matrix as a substratum for cell attachment. When cultured directly on tissue culture plastic only 15–20% of the immature UE cells attach and they are all detached by 72 h. Coating tissue culture wells with EHS matrix increases cell attachment at 24 h to 80–85%. These cells will proliferate, grow and become confluent within 48–72 h depending on their seeding densities. Within the first 72 h there are no notable differences in the behavior of UE cells cultured on either solid or porous supports if they are covered with EHS matrix. These similarities include identical rates of cell division, formation of tight junctions, and presence of desmosomes. However cells on matrix-covered solid surfaces do not maintain their confluent density. They soon begin to detach and all cells are gone by 12–14 d. When replated, the detached cells do not reattach.

As an initial index of functional polarity, confluent UE cells cultured on matrix-covered porous filters develop a preference for the uptake of [^{35}S]methionine via their basal

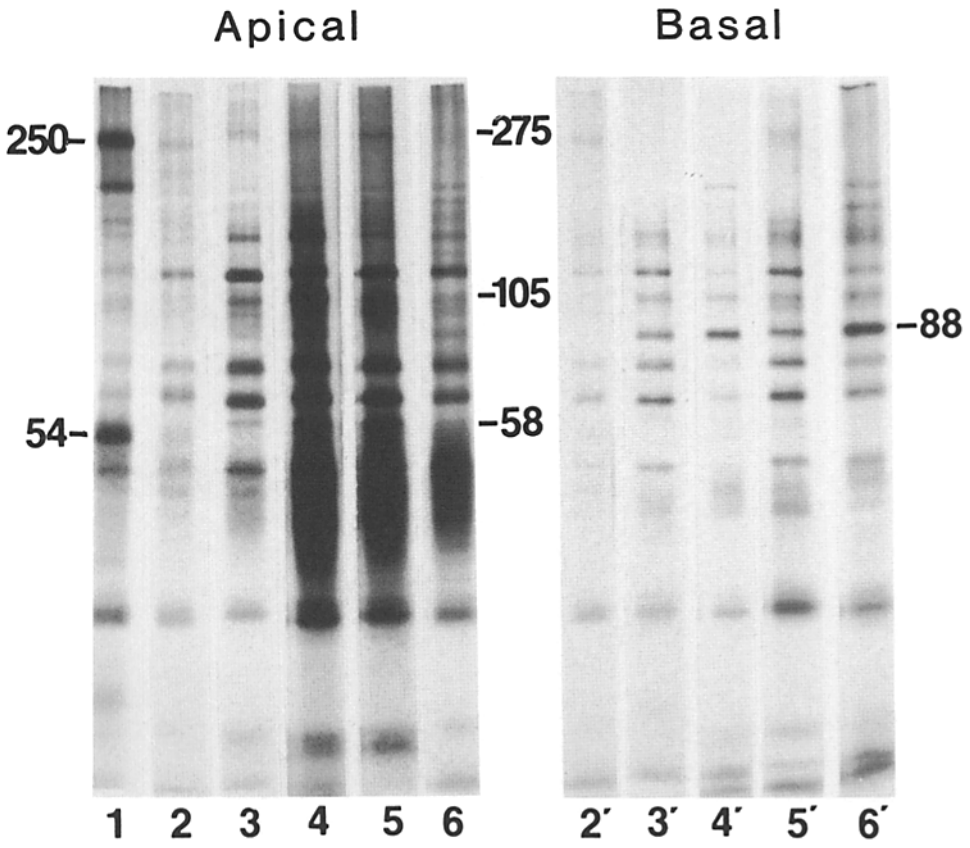


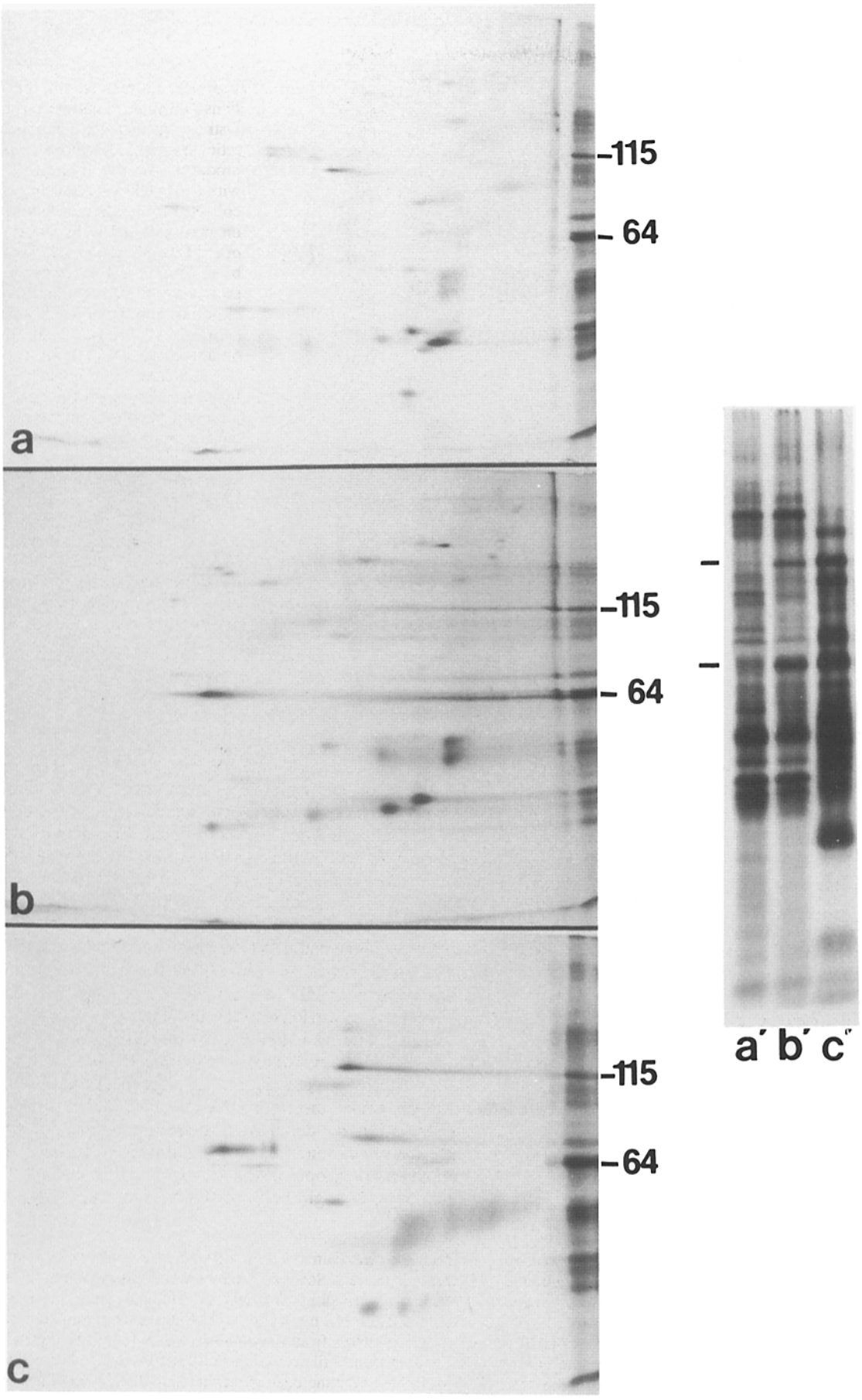
Figure 11. Effect of culture conditions and time in culture on the distribution and gel electrophoretic profiles of UE cell secretory products. After 8 h incubation with [^{35}S]methionine, media was collected from UE cells cultured on matrix-coated wells (lane *1*) or from the apical (lanes *2–6*) and basal (lanes *2′–6′*) secretory compartments of cells cultured on matrix-coated filters. Cells were cultured for 3 (lanes *1*, *2*, and *2′*), 6 (lanes *3* and *3′*), 10 (lanes *4* and *4′*), 12 (lanes *5* and *5′*), and 16 d (lanes *6* and *6′*). Lanes *1*, *2* and *2′* represent samples from the same culture series whereas samples in lanes *3–6* and *3′–6′* were from other individual culture series. Molecular masses ($\times 10^{-3}$) of those secreted proteins that varied as a function of culture conditions (250 and 54 kD) or time in culture (275, 105, 88, and 58 kD) are indicated. Autoradiographs were exposed for 18 h (lanes *3–6*) or 7 d (lanes *1*, *2*, and *2′–6′*).

surface. This difference between the basolateral and apical plasma membranes increases with time in culture. Synchronous with changes in uptake is an increase in total secretion (apical plus basal compartments) while total incorporation expressed on a per cell basis remains constant. As a function of time in culture, the progressive increase in secretory activity is characterized by a shift that establishes the apical surface as the preferred site for secretion.

During the development of polarity total secretion by UE cells increased ~10-fold. Moreover, during the same interval (12 d) the preference for apical secretion increased 20–30-fold. Consequently it appears that increased secretory activity is primarily attributable to enhanced development of apical secretory activity relative to similar changes at the basal surface. It is not known if this increase in apical secretory preference bears any relationship to the apical vs. basal pH differential observed in UE cells.

The criteria used to validate development of morphological and functional polarity have been established mainly by the study of established epithelial cell lines such as Madin-Darby canine kidney (MDCK) cells (32, 36). In applying these indices of polarity to the present study, both similarities and differences between primary cultures of UE cells and established cell lines have been noted. Common to both was the appearance of ultrastructural features characteristic of polarized epithelial cells; i.e., junctional complexes, desmosomes, increased cell height, and increased height and number of apical microvilli, as well as preferential basal uptake of methionine and development of electrical resistance. These characteristics are similar to the criteria used to confirm polarity of MDCK cells (22, 32, 36). Additionally, uvomorulin was restricted to the basolateral surface of the UE cell in culture as is the case for polarized MDCK cells (3, 13). While the restricted distribution of uvomorulin in UE cells does indicate plasma membrane reorganization during the transition from preconfluence to early post-confluence, it is not definitive evidence of complete plasma membrane separation. Functional distinctions between the two domains developed more slowly in UE cells than in MDCK cells although their initial appearance in both cases

Figure 12. Coidentification of the 115- and 64-kD secretory components from UE cells cultured on matrix-covered filters with luminal secretory products of intact uterine strips from control and estrogen primed immature rats. Secreted proteins were collected after an 8-h labeling with [^{35}S]methionine and processed for one- or two-dimensional PAGE as described in Materials and Methods. Intact uteri were preincubated 30 min in methionine-free medium before labeling. Secreted proteins from 100 mg wet weight of uterus from immature rats treated with vehicle only (*a* and *a′*) and secreted proteins from 100 mg wet weight of uterus from immature rats receiving three injections of 17β-estradiol at 24-h intervals (*b* and *b′*) were compared with apical secretions from a filter culture (33 d) of immature UE cells (*c* and *c′*). Samples of secreted protein from intact uteri of estrogen-treated immature rats were included as standards in the second dimension of two-dimensional PAGE to identify the position of the estrogen-stimulated secretory products at 115 and 64 kD.


a
b
c
115
64
115
64
115
64
a′ b′ c′

was dependent on confluence. For example the basal/apical ratio of 12 for methionine uptake was recorded within 48 h after MDCK cells became confluent whereas UE cells required 8 d to establish a similar ratio. Relative to the separation of the respective membrane domains additional apical and basolateral markers will have to be identified and validated for UE cells. Markers characteristic of domain segregation in one cell type (i.e., MDCK; reference 22) may not be applicable, equally or at all, to other cells.

Studies of functional polarity have depended on a commonly used analogue for vectorial secretion in MDCK cells; i.e., the polarized secretion of viral particles (10, 22, 23, 24). The use of viral infection to monitor polarized secretory processes is complicated by a multiplicity of events; i.e., infection, viral envelope protein localization, viral budding, each of which is not equally dependent on cellular polarization in MDCK cells (22). For these reasons additional biochemical indices were developed to characterize functional polarity of the UE cell. These included the acquisition of preferential apical secretion of endogenous proteins that paralleled the development of preferential basal uptake of [^{35}S]methionine. By studying the behavior of particular endogenous secretory components in the apical and basal secretion, it should be possible to determine to what extent the results of viral studies reflect normal transport processes versus those specific to viral infection.

As the preference for apical secretion becomes more pronounced in postconfluent UE cells the relative abundance of a number of proteins, found in both apical and basal secretions, necessarily increases in the apical secretory compartment. However some proteins are subject to polarized secretion. For example, we noted the explicit apical secretion of a 130-kD protein as well as the preferential basal secretion of an 88-kD protein in the secretions of postconfluent UE cells. These observations indicate that secretory proteins may serve as markers of functional polarity. Furthermore, the qualitative and quantitative differences in polarity-dependent secretion of UE endogenous proteoglycans, to be detailed in the accompanying paper, offer strong additional support for the use of secretory profiles as markers to track the establishment and development of functional polarity of primary cultures of UE cells. Sequestration of basally secreted proteins by the matrix and/or filter, nonlinearity of secretion at each respective cell surface, and possible contamination of the secretory profiles by detached and/or lysed cells have been experimentally discounted as factors that could affect the validity of these biochemical analyses. Thus the biochemical profiles reported in this paper are faithful representations of the differential development of UE cell secretory activity and can be used reliably to characterize the mechanisms that regulate the functional distinctions between apical and basal membrane domains of the differentiating UE cell.

Estrogen treatment of rats stimulates the secretion of proteins (16, 43) and glycoproteins (40) by uterine strips. The detection of these estrogen-stimulated marker proteins in the apical secretions of UE cells cultured on matrix-covered filters is evidence that the polarized UE cell monolayer is hormonally responsive. Furthermore this data indicated that the secretory response of the cultured cells to estrogen is similar to that evoked in UE cells in situ. The maintenance of hormone responsiveness and the ability to establish morphological and functional polarity are the salient characteristics of this culture system. It is not yet certain how, or to what degree, epithelial cell polarity and the ability to respond to steroid hormones are related. However, it appears from these studies that these processes must be coupled if the difficulties that have complicated definition of the mechanisms that regulate expression of UE cell special functions are to be resolved (14).

A prevailing concept of developmental biology of the epithelial cell holds that epithelial cells differentiate in response to instructive or permissive signals arising from the underlying mesenchymal (stromal) cells. These results were derived from experiments using long-term kidney capsule transplants of recombinants of epithelial and stromal tissues (8,30). The present study suggests that coculture of UE cells (applied to matrix side of the filter) with stromal cells from the uterus, at different stages of differentiation (11) or from different organs (applied to the noncoated or basal side of the filter), creates a novel cell–cell recombination model. In this case homogeneous populations of homotypic or heterotypic cells can be examined over short periods of time to analyze not only stromal–epithelial interactions but epithelial–stromal interactions, which may also play a pivotal role in cellular responsiveness. Comparing responses of the present UE model with those generated in cell–cell recombinants will indicate if the action of a hormone or growth factor on the UE cell is direct or is mediated through the stroma (7, 8, 30). Finally, since cellular and biochemical as well as morphological data can be obtained from cell–cell recombinants, the potential value of the model is increased.

We are indebted to Dr. Karl S. Matlin, presently of Harvard Medical School, for introducing us to and sharing his insights regarding epithelial cell polarity; Dr. John M. Caffrey, Department of Physiology and Molecular Biophysics, who was most generous with his time and counsel; and also we are most appreciative of the illustrative work by Mr. David Scarff, the photography of Ms. Debbie Delmore, and word processing by Ms. Georgietta Brown.

This work was supported by National Institutes of Health grants HD-22785 and HD-07495 (to S. R. Glasser). D. D. Carson was supported by American Cancer Society grant BC-508 and March of Dimes grants 1-958 and HD-18590.

Received for publication 6 June 1988, and in revised form 15 August 1988.

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