Cytoskeletal Redifferentiation of Feline, Monkey, and Human RPE Cells in Culture

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Retinal pigment epithelial (RPE) cells in vivo have a polarized structure with specialized apical and basal faces. Isolated RPE cells lose but eventually regain their epithelial morphology under appropriate culture conditions. We evaluated the ability of isolated feline, primate, and human RPE cells to regain this morphology in culture with scanning electron, transmission electron, phase contrast, and immunofluorescence microscopy. In culture, isolated RPE cells lose their cuboidal shape, their apical microvilli, and their in vivo cytoskeletal organization. Stress fibers form in these cells; microtubules radiate from the cells’ center to their periphery; and vimentin filaments radiate from the cells’ nucleus to their periphery. As cultures become confluent, RPE cells aggregate into small groups, gradually regaining a cuboidal shape and acquiring microvilli on their apical surface. Filamentous actin redistributes to the apical face where it presumably forms the cytoskeletal core normally present in RPE microvilli. Stress fibers disappear and are replaced by a circumferential microfilament bundle (CMB). Confluent cells surrounding the colonies of differentiated RPE attain a cuboidal shape but do not show complete cytoskeletal redifferentiation. Such cells, while appearing to be differentiated by phase contrast microscopy, fail to develop a compacted CMB. In these cells, f-actin is organized as a loose peripheral band within the cell cytoplasm. Our observations indicate that confluency cannot be equated with the end stage of morphologic differentiation, and that cytoskeletal organization provides a more accurate gauge of RPE maturation in culture. Invest Ophthalmol Vis Sci 31:879-889, 1990

The retinal pigment epithelium (RPE), like most simple epithelia, is organized into a sheet of tightly packed cells that separates adjacent tissue compartments. The lateral, apical, and basal cell surfaces are functionally and structurally specialized. Neighboring cells are linked at their lateral margins by junctional complexes which act as a selective permeability barrier. The plasma membrane on the basal surface is highly infolded and lies immediately adjacent to a basal lamina (Bruch’s membrane). The apical surface consists of an extensive array of microvilli and microplicae that interdigitate with the photoreceptor outer segments.1-3

The cytoskeleton is intimately involved in the organization and maintenance of these structural domains. Numerous studies have established the presence of microtubules,1,2 microfilaments,3,4 and intermediate filaments5,6 in the RPE of numerous species. Our understanding of RPE cytoskeletal organization has come almost exclusively from chick RPE. These studies have shown that microfilaments, intermediate filaments, and microtubules are partitioned into distinct cellular domains. Actin-containing microfilaments are located primarily at the lateral margins7-11 and within the apical microvilli.12,13 Tubulin, on the other hand, is distributed throughout the cell cytoplasm.10 Vimentin, an intermediate filament protein, is concentrated at the lateral cell margins like actin.14-16 It has been reported that the specific set of cytoskeletal RPE proteins can vary considerably from species to species.17 In the current study, we show that the pattern of expression in cultured mammalian RPE cells (feline, monkey, and human) is similar to that identified in the chick RPE.

We evaluated the changing patterns of cytoskeletal organization in feline, primate, and human RPE cells as they differentiated in culture. Microfilaments, intermediate filaments, and microtubules were distributed throughout the cell cytoplasm in undifferentiated, flattened cells. However, as the cells became polarized and acquired the morphologic characteristics of a mature epithelium, they gradually reacquired their in vivo cytoskeletal organization. The expression of these cytoskeletal domains under in vitro conditions provides an opportunity to study the morphologic events which accompany polarization and the functions associated with it.
Materials and Methods

Feline RPE Cell Culture

Primary cultures of cells were obtained by a modification of the procedures described in Stramm et al. Young domestic cats were euthanized by intravenous injection of sodium pentobarbitol, and their eyes removed. All animals were maintained and used in accordance with the ARVO Resolution on the Use of Animals in Research. Eyes were briefly immersed in 70% ethanol to remove surface contamination, and the posterior chamber was exposed by dissecting the anterior chamber just behind the ora serrata. The eyecup was filled with calcium- and magnesium-free Hank’s balanced salt solution (BSS; pH 7.4) at 37°C. The retinas were gently detached and discarded; eyecups with adherent RPE were then filled with 1× trypsin-EDTA (Flow Laboratories, McLean, VA). Eyecups were maintained in 95% air, 5% CO2 at 37°C for 30–60 min. RPE cells were collected by gentle aspiration, and the eyecup was refilled with BSS. Collection and refilling of the eyecup was repeated up to five more times. The aspirate was placed in a 15-ml centrifuge tube, and the cells were concentrated by brief centrifugation at 200 g for 3 min. The supernatant was discarded, and the pelleted cells were placed in a medium containing 50% Dulbecco’s modified Eagle’s medium (DMEM), 50% Ham’s F-12, bovine serum albumin (BSA; 10–20%), fungizone, gentamycin, and nonessential amino acids. Cells were plated in multiwell tissue culture plates at a concentration of $1.0-6.0 \times 10^4$ cells/ml medium. A coverslip that had been coated with laminin (Collaborative Research, Bedford, MA) by incubating it for 1 hr in either DMEM or phosphate buffered saline (PBS) containing 5 \( \mu \)g/ml laminin (Collaborative Research) was placed in each well prior to seeding with cells.

Human and Monkey RPE Cell Cultures

Human RPE cells, obtained from 3- and 8-yr-old donor eyes, were grown using the method of Pfeffer et al. Primate RPE cells were obtained from adult cynomolgous monkeys. Sheets of RPE were harvested from the eyecup, after removal of the sclera and exposure of the choroidal side of Bruch’s membrane to dispase (Boehringer-Mannheim, Indianapolis, IN). Cells were seeded directly onto the surface of tissue culture flasks. Viable floating cells derived from primary cultures were removed from the flask...
and subcultured onto 24-mm glass coverslips that had been precoated with laminin as described for the cat cultures. Cells were seeded at a density of \(6 \times 10^4\) cells/ml. Cells were grown in Ham's F-12 supplemented with 1% fetal bovine serum (Flow), 2 mM glutamine (Sigma, St. Louis, MO), 45 \(\mu\)g/ml ascorbate (Sigma), 100 units/ml penicillin (Sigma), 100 \(\mu\)g/ml streptomycin (Sigma), 1% bovine retinal extract, 300 \(\mu\)g/ml putrescine hydrochloride (Collaborative Research), 5 \(\mu\)g/ml insulin (Collaborative Research), 5 \(\mu\)g/ml transferrin (Collaborative Research), 175 ng/ml selenious acid (Collaborative Research), 10 ng/ml hydrocortisone, 6.51 pg/ml triiodothyronine (Sigma), 10 ng/ml glycyl hystidyl lysine, and 90 \(\mu\)g/ml linoleic acid (Collaborative Research). Lyophilized reagents were dissolved in BSS. Cultures were confluent in 4-5 days when incubated at 37°C with 5% CO₂.

**Immunofluorescence Microscopy**

Coverslips were briefly washed with Hank's BSS at 37°C and fixed by gradually adding 3% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4). Culture medium was withdrawn as fixative was added until the medium had been completely

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**Fig. 2.** The distribution of tubulin and actin in confluent primate RPE cells. These cells have an intermediate morphology whose characteristics are halfway between fully dedifferentiated and undifferentiated cells. (A) Microtubules occupy the cytoplasm of the RPE, and their distribution reveals the cells' rounded configuration. This preparation had been extracted with the detergent Triton-X to provide a clearer view of the organization of the microtubules. Individual microtubules cannot be distinguished in cells which have not been extracted with detergent (×560). (B) The same group of cells, stained with phalloidin. Stress fibers have disappeared, and actin containing microfilaments are concentrated in the peripheral cytoplasm (×560).
replaced by fixative. Cultures remained in fixative for 30–60 min.

Cells were stained for actin with either fluorescein or rhodamine phalloidin (Molecular Probes, Eugene, OR); for tubulin with a monoclonal antibody directed to beta-tubulin; and for vimentin with a monoclonal antibody (Mab) obtained commercially (Dako, Carpinteria, CA). Cells were permeabilized by supplementing all antibody solutions and wash buffers with 0.03% Triton X-100. Cells were initially incubated in normal goat serum or 3% BSA in PBS for 30 min to block nonspecific protein binding.

Cells were stained overnight at 4°C in either fluorescent phalloidin or the primary antibody solutions, diluted 1:1000. Coverslips stained with primary antibodies were rinsed 4 times for 5 min each in PBS supplemented with 0.5% BSA. Affinity purified secondary antibodies (Cappell, Malvern, PA), which had been conjugated to either fluorescein or to rhodamine, were then applied to the coverslips for overnight staining at 4°C. Cells received four 5-min washes with PBS/0.5% BSA.

Cells were photographed with Ektachrome 800/1600 (Kodak), or with Tmax 400 film push-processed for an exposure index of 1600 with a Zeiss Photomicroscope III equipped with Olympus objectives 40× and 100×. Microscopy was also performed on an Olympus IMT-2 and Dage 66 SIT camera.
Fig. 4. Scanning electron micrograph of monkey RPE cell viewed en face. Its apical surface is covered with microvilli (×30,000).

Images were recorded on videotape or directly ported to a MegaVision computer for image processing.

Electron Microscopy

Confluent monkey RPE cultures were examined by transmission and scanning electron microscopy. Cells used for transmission electron microscopy were grown on plastic coverslips coated with laminin as described previously. Cells were fixed in 0.1% glutaraldehyde and 3% paraformaldehyde in sodium cacodylate buffer (pH 7.1). After 30 min fixation at room temperature, samples were washed in cacodylate buffer, dehydrated in a graded ethanol series, and embedded in Araldite. Cells used for scanning electron microscopy were grown on laminin-coated coverslips. These coverslips were postfixed in osmium tetroxide (1% in 0.1 M cacodylate buffer, pH 7.4), critical-point dried, coated with gold-palladium, and examined by scanning electron microscopy.

Results

Undifferentiated Cells

RPE cells plated onto glass coverslips attached rapidly to the laminin substrate and then appeared as irregularly shaped, flattened cells with scalloped borders (Figs. 1A–C). Immunofluorescence microscopy confirmed that these cells were definitely of RPE origin because they showed diffuse cytoplasmic labeling using antibodies to cellular retinaldehyde binding protein, a retinoid binding protein specific to RPE and Müller cells. These cells were not derived from Müller cells since the retina was removed from eyecups prior to trypsinization and harvesting of the RPE cells.

In undifferentiated cells, immunolabeling using anti-beta-tubulin revealed an extensive array of microtubules that radiated outward from the cell nucleus to the peripheral cytoplasm (Fig. 1A). Rhodamine phalloidin stained a series of stress fibers that spanned the cell cytoplasm and ran parallel to the cell's long axis (Fig. 1B). No apical surface staining was present, and scanning electron microscopy indicated that microvilli were absent at this stage. Both anti-beta-tubulin and anti-vimentin labeling consisted of a series of cables radiating centripetally from the nucleus. These cables did not colocalize with each other or with the f-actin distribution (Fig. 1C).

As the cultures became confluent (1–4 weeks after seeding) and the lateral edges of adjacent cells came into contact, the thin scalloped borders of the cells became smoother and the cells increased in height...
Mature Differentiated RPE Cells

After several weeks in culture the human and monkey cells showed an epithelioid phenotype and were uniform in appearance. In contrast to the feline cells, they were not organized into colonies of epithelioid cells surrounded by areas of less differentiated cells. In epithelioid cells from all three species, the apical surface was elevated 8–10 μm above the substrate and was covered by a dense carpet of microvilli (Fig. 4). The cells were much smaller in diameter, had a polygonal shape, and were delineated by a phase dense line at their lateral borders (Fig. 5A). Phalloidin staining revealed two distinct patterns of fluorescence: a dense, uninterrupted line at the cells' circum-
Fig. 6. A confluent colony of differentiated cat RPE labeled with anti-betatubulin and phalloidin. Cells have been extracted with the detergent Triton X-100 to reveal the microtubule network. (A) F-actin is heavily concentrated in the CMB. Some of the cell profiles are polygonal (×560). Punctate-labeling actin is evident in dark cells in the center of the micrograph. (B) Tubulin is highly concentrated in the cell body of differentiated RPE as a random network (×560).

ference that coincided with the density observed by phase contrast (Figs. 5B, 6A), and a dense punctate fluorescence distributed randomly over the apical surface (Fig. 6A). When the cells were viewed en face, the punctate pattern was most prominent; however, when the plane of focus paralleled the apical surface, a dense fringe of fluorescence was clearly apparent (Fig. 5B). There was no phalloidin staining in the
Fig. 7. A transmission electron micrograph of the basal surface of a human RPE cell grown using the procedures outlined in the text. There are no basal infoldings. There is an extracellular matrix (arrow) between the cell and plastic substrate (asterisk) (X23,000).

interior or the basal portion of the RPE cell. Scanning and transmission electron microscopy showed that the apical surface of these cells was populated by microvilli; however, there was no evidence for basal infoldings characteristic of the in vivo cell (Fig. 7).

In slides that were detergent-extracted prior to fixation, anti-beta-tubulin labeling was resolved as a dense random meshwork within the cytoplasm (Fig. 6B). No differences between central and peripheral cytoplasm were apparent. Anti-vimentin labeling, however, was concentrated near the cell border slightly proximal to the α-actin domain (Fig. 8A). After image enhancement, this labeling could be resolved into two parallel lines at the border of neighboring cells (Fig. 8B). In contrast, α-actin appeared as a tight band that could not be resolved into separate components after image enhancement. Cytoplasmic anti-vimentin labeling was generally diffuse, and only occasionally were individual filaments resolved. The apical or basal cell surfaces were not labeled with either anti-beta-tubulin or anti-vimentin.

Discussion

The RPE is engaged in a number of different functions. It regulates the movement and transport of ions, metabolites, and water between the photoreceptors and the choriocapillaris. It is responsible for the phagocytosis of effete photoreceptor disc membranes; it may act to limit light scattering in the subretinal space; and it almost certainly plays an integral role in maintaining retinal adhesion. RPE cytoskeletal organization and the other morphologic specializations associated with the apical, basal, or lateral surfaces are structural manifestations of these functional requirements. Clearly, the study of RPE cell function has been enhanced by the use of cultured cells. Our studies suggest that the cells' state of differentiation in vitro is an important variable that needs to be taken into consideration when studying cellular events that depend on the presence of specialized structures and domains present in vivo.

Under our experimental conditions, confluent mammalian RPE cell cultures contain cells at several different maturational stages. We observed three different phenotypes in confluent cultures: 1) fibroblastic (ie, flattened, spread configuration); 2) differentiated cells that have the surface polarity and cytoskeletal organization of RPE cells in vivo; and 3) cells whose general morphology and cytoskeletal organization was intermediate between these two configurations. These are essentially the same phenotypes observed in chick primary cultures. Therefore, the evidence indicates that the sequence of cytoskeletal redifferentiation in culture is the same in a variety of vertebrates ranging from chick to human. Furthermore, the end point of cytoskeletal redifferentiation in culture approximates the in vivo pattern.
microvilli. This is in contrast to the apical surface in vivo, which consists of a mixture of concentrically arrayed microvilli and microvilli—the former ensheathing cone outer segments, and the latter interdigitating with rod outer segments.\(^{23-25}\) It is likely that the formation of the cone sheath must be induced by the proximity of a cone outer segment, since retinal detachment results in their disappearance\(^{30}\) and reattachment stimulates their reappearance.\(^{27}\) Similarly, in the current study, the infoldings along the basal surface were not as extensive as they normally are, and there was no indication of an associated basal lamina. Presumably, this is because the cells were grown on an impermeable substrate (laminin-coated glass), which limits the access of metabolites, whereas the basal surface in vivo is adjacent to a capillary bed.

Under in vivo conditions, microfilaments are associated with the lateral junctional complexes\(^{7,8}\) and the core of apical microvilli.\(^{9,12,22,25,28-30}\) A virtually identical distribution is reported in chick RPE explants,\(^{4,5,10,12}\) and in the current study, in cultured mammalian RPE cells. The distribution of other cytoskeletal proteins, tubulin and vimentin, do not appear to be useful markers for differentiation in culture. Microtubules are ubiquitous cytoskeletal components important in RPE function. Drug disassem-
bly of microtubules inhibits the digestion of phagosomes.31,32 However, microtubules lack a distinctive pattern that can be correlated with RPE differentiation in culture. The function of vimentin, an intermediate filament present in the RPE of some vertebrate species, is unknown.17,33 Docherty14 suggested that vimentin functions like a cytokeratin and mediates the adhesion between adjacent RPE; however, this hypothesis has not been confirmed.17 Vimentin can be expressed in primate RPE cells in vivo but apparently, only under pathologic conditions.34 Other epithelial cells which normally do not express vimentin can do so in culture.35,36

The finding that filamentous actin and microtubules are distributed appropriately in these cultured mammalian RPE cells suggests that they can be used successfully to study their functional roles in vivo. On the other hand, there is an apparent discrepancy between vimentin distribution in culture and under in vivo conditions. The significance of vimentin distribution and the distribution of other intermediate filaments in mammalian RPE cells remains for future study.

Key words: retinal pigmented epithelium, cytoskeleton, actin, vimentin, tubulin, cell culture

References


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