# A Human Retinal Pigment Epithelial Cell Line That Retains Epithelial Characteristics After Prolonged Culture

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***Purpose.*** A spontaneously arising, apparently transformed, cell line has been cloned from a primary culture of human retinal pigment epithelial (RPE) cells and has been subcultured more than 200 times. The similarities of these cells to human RPE cells in vivo have been determined.

***Methods.*** The structure of the transformed cells has been determined by light and electron microscopy and by immunocytochemistry using antibodies that detect cytoskeletal and other proteins. The ability of the cell line to bind and phagocytose photoreceptor material has also been assessed by fluorescence and electron microscopy. The metabolism of all-*trans*-retinol has been investigated by incubation of the cells with $^{3}$H-all-*trans*-retinol and analysis of the metabolic products by high-performance liquid chromatography.

***Results.*** The transformed cells possess an epithelial cobblestone morphology with intercellular junctional complexes containing N-cadherin. The cytoskeleton of these cells comprises cytokeratins that are characteristic of epithelial cells, together with actin, spectrin, and vimentin. The keratins expressed are those typical of RPE cells. The cells also express cellular retinaldehyde binding protein and retinol dehydrogenase activity but do not express retinoid isomerase or lecithin retinol acyl transferase activities. These cells also exhibit phagocytic activity.

***Conclusions.*** This cell line retains many of the metabolic and morphologic characteristics of RPE cells in vivo although there are some differences, particularly the loss of some enzymatic activities and cytoskeletal polarization. These cells should be useful in further studies of RPE cell metabolism and other functions. Invest Ophthalmol Vis Sci. 1995;36:955–964.

The retinal pigment epithelial (RPE) cells of the human eye constitute one aspect of the blood–retinal barrier.[1–3] As such, they control access of blood-borne substances, including nutrients, growth factors, and hormones, to the neural retina. Retinal pigment epithelial cells participate in the visual cycle as the location of synthesis and storage of retinyl esters, isomerization of all-*trans*-retinoids to 11-*cis*-retinoids, and the conversion of retinol to retinal.[4–9] In addition, these and the Müller cells synthesize cellular retinaldehyde binding protein (CRALBP) involved in retinoid transport in the retina.[10] Retinal pigment epithelial cells also carry out phagocytosis and digestion of membranes shed from photoreceptor cell outer segments.[11,12]

Extensive investigations of human RPE cell structure and function have been limited by the necessary use of primary cultures that rarely survive more than eight to ten passages and that, moreover, may be contaminated by other cells. In addition, primary cultures of RPE cells frequently take on an altered morphology and, in some instances, lose their characteristic keratin-containing intermediate filaments, synthesizing instead intermediate filaments that are more characteristic of cells of mesenchymal origin.[13,14]

In view of the disadvantages of short-term cultures and the shortage of human donor eyes, a continuous cell line would be most useful for studies of RPE cell

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*Supported by National Institutes of Health grants EY06164 (RH), EY00444 and EY00331 (DB), and EY04377 (JT), and by the Retinitis Pigmentosa Foundation Fighting Blindness (DB). DB is the Dolly Green Professor of Ophthalmology at UCLA.*
*Submitted for publication September 14, 1994; revised October 5, 1994; accepted October 16, 1994.*
*Proprietary interest category: N.*
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function and the purification of enzymes unique to these cells. In this article, we report the cloning of such a cell line, D407 cells, that has now been maintained in culture for more than 200 passages. These cells retain their epithelial morphology, forming a hexagonal cobblestone layer with intercellular junctions, and possess a keratin-containing cytoskeleton although they also synthesize vimentin. Moreover, these cells make CRALBP and have retinol dehydrogenase activity, both of which are found in RPE cells in vivo.[10,15] However, they do not exhibit the extreme polarity of RPE cells as judged by their organization of spectrin, a submembranous skeletal protein associated with the apical surface of RPE cells in vivo,[16] and they fail to make some other enzymes found in these cells in vivo.

## MATERIALS AND METHODS

### Cloning and Growth of D407 RPE Cells

Cells were trypsinized from the globe of the eye of a 12-year-old white male child within 12 hours of death and cultured as described.[17] After nine passages, foci of rapidly growing cells arose in this culture. These cells had a cuboidal morphology at the center of the mass of cells. They were cylinder cloned and maintained in high-glucose Dulbecco's minimal essential medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine. The cells were subcultured at a ratio of 1:5 every 4 days, and the serum was reduced to 3%, in which they are maintained. All medium constituents were obtained from Gibco/BRL (Grand Island, NY).

The tenets of the Declaration of Helsinki were followed, and institutional human experimentation committee approval was granted for these studies.

### Fluorescence Microscopy

**Cytoskeletal Elements and N-cadherin.** D407 cells were grown on glass coverslips, fixed for 5 minutes in ice-cold methanol–acetone (1:1), and rehydrated in Dulbecco's phosphate-buffered saline (PBS). They were stained for keratin using AE1 and AE3 (Boehringer–Mannheim, Indianapolis, IN) monoclonal antibodies, for vimentin using anti-vimentin monoclonal antibody (ICN, Irvine, CA), for glial fibrillary acidic protein (GFAP) using monoclonal anti-GFAP (Boehringer), for actin using fluorescein isothiocyanate-phalloidin (Sigma, St. Louis, MO), for spectrin using a polyclonal anti-spectrin antibody (R. Hunt, unpublished data, 1982) and for N-cadherin using a polyclonal anti-N-cadherin antibody (kindly donated by T. Borg).

**CRALBP.** Cells were fixed in freshly made 4% paraformaldehyde in PBS and washed in PBS containing 50 mg bovine serum albumin (BSA) per milliliter. The cells were then incubated with 0.5% Triton X-100 in PBS–BSA. CRALBP was detected using a polyclonal antibody (kindly donated by Dr. J. Saari).

**Factor VIII.** Cells were fixed in freshly made 4% paraformaldehyde in PBS and washed in PBS–BSA. They were then incubated with monoclonal anti-Factor VIII antibody (Boehringer).

**SV40 Large T-antigen.** Cells were fixed in absolute methanol at −20° for 5 minutes and rehydrated in PBS. They were then incubated for 1 hour with monoclonal anti-SV40 T-antigen antibody (culture medium from PA 101 hybridoma; ATCC, Rockville, MD).

In all cases except for the detection of SV40 T antigen, in which the culture medium was used undiluted, primary antibodies were diluted 1:100 in PBS–BSA and incubated with cells at 37°C for 30 minutes. After incubation with these antibodies, the cells were washed in PBS–BSA three times, and bound antibodies were detected by incubation with the appropriate fluorescent second antibody (diluted 1:250 in PBS–BSA) for 30 minutes at 37°C. The cells were washed in PBS–BSA and observed using a Biorad (Richmond, CA) MRC600 confocal microscope system or a Nikon (Melville, NY) Microphot FXA fluorescence microscope.

### Binding of Retinal Rod Outer Segments

Rod outer segments were prepared from pooled human neural retinae using gradient centrifugation, as described.[18] The second band from the top of the gradient that contained rod outer segments was washed free of sucrose using PBS and was labeled overnight at 4°C in 1 ml PBS containing 500 μg 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (Sigma) per milliliter (added as a ×10 stock solution in dimethyl sulfoxide). The labeled rod outer segments were washed three times in PBS and resuspended in 1 ml PBS. D407 RPE cells were grown on glass coverslips and incubated with 100 μl rod outer segment suspension per milliliter PBS at 37°C for 10 minutes. The coverslips were again washed in PBS at 4°C and were observed with a Meridian (Okemos, MI) Instruments ACAS 570 adherent cell cytometer in both the confocal fluorescence and phase-contrast modes.

### Electron Microscopy

D407 cells were grown on laminin-coated (Collaborative Research, Bedford, MA) culture well inserts (Costar, Cambridge, MA) with 0.45 μm pores or on laminin-coated culture ware (Costar). In one experiment, cells on culture ware were also incubated with a crude retinal homogenate at 37°C. The cells were fixed with 2% glutaraldehyde in Sorrensen's buffer and processed for electron microscopy as described.[17]

### Processing of $^3$H-all-*trans*-retinol

Confluent cultures were rinsed in serum-free medium and scraped into 50 mM sodium phosphate buffer (pH 7.3). The cells were sonicated (10 seconds, 100 watts) with a probe sonicator, and cellular debris was removed by centrifugation at 600*g* for 10 minutes at 4°C. The extract was then centrifuged at 100,000*g* for 1 hour at 4°C. The supernatant liquid was discarded and the pellet rinsed three times in phosphate buffer. The pellet was resuspended in phosphate buffer with brief sonication, and this membrane preparation was either used immediately or stored frozen. The protein concentration was 3 mg/ml to 5 mg/ml.

One hundred microliters of membrane preparation were incubated at 37°C for 2 hours in the dark with 100 μl 1% BSA (fatty acid free; Sigma) and 0.5 μCi $^3$H-[11,12]-all-*trans*-retinol in 0.5 μl ethanol (NEN, 46.2 Ci/mMol, confirmed to be >99% $^3$H-all-*trans*-retinol by high-performance liquid chromatography (HPLC)). In some experiments, a ×20 concentrate of $NAD^+$ or $NADP^+$ was added to a final concentration of 1 mM. The reaction was terminated by the addition of 600 μl 67% methanol. Radiolabeled retinoids were extracted into hexane containing 0.1 mg butylated hydroxytoluene per milliliter to inhibit oxidation and isomerization. The extract was supplemented with an isomeric mixture of retinol, retinal, and retinyl palmitate standards.

One hundred microliters of hexane extract was injected onto an isocratic, normal-phase HPLC system consisting of an 8 μm silica gel column (Dynamax-60A; Rainin, Emeryville, CA) eluted with 8% dioxane in hexane at 1 ml/minute. Detection was by absorbance at 326 nm and liquid scintillation counting in which the eluate was mixed with an equal volume of betafluor scintillant (National Diagnostics, Atlanta, GA) and passed through an in-line β-RAM liquid scintillation counter with a 1-ml flow cell.

### Chromosome Analysis

Semiconfluent, logarithmically growing cells were incubated with 0.01 μg colcemid per milliliter (Gibco/BRL). The cells were detached with trypsin/ethylenediaminetetraacetic acid solution (Gibco/BRL), centrifuged, and resuspended in hypotonic medium (3 parts distilled water/1 part complete culture medium). After 30 minutes at 37°C, the suspension was centrifuged at 800 rpm and fixed in glacial acetic acid/absolute methanol (1:3 vol/vol) at 4°C. The fixative was changed five times. Chromosome preparations were obtained by dropping the cell suspension on wet glass microscope slides. The slides were dried and stained with 10% (vol/vol) Giemsa solution in water.

## RESULTS

### The Morphology of D407 Cells

Primary cultures of human RPE cells grown to confluence exhibit a "cobblestoned" epithelial morphology (Fig. 1A) similar to cells in the retina. Areas of the culture are devoid of pigmentation because cultured RPE cells, unless they are obtained from fetuses, generally fail to synthesize melanin, and the original pigment granules are divided among the daughter cells. The highly pigmented cells in Figure 1A are those that have not divided in culture.

D407 RPE cells were originally grown on laminin-coated plastic in 10% serum in which they form a contact-inhibited cobblestoned monolayer (Fig. 1B shows cells at passage 6). The serum was reduced to 3%, and laminin was omitted. The cells have continued to grow with the same morphology after being subcultured 200 times (Fig. 1C shows cells at passage 114). They have lost pigmentation but otherwise have a morphology similar to early-passage RPE cells.

### Electron Microscopy

Late-passage D407 cells were seeded onto filters containing 0.45 μM pores (Fig. 2A) or onto plastic culture dishes (Figs. 2B, 2C 2D). The cells at passage 80 formed monolayers with tightly apposed membranes linked by intercellular junctional complexes (arrowheads). Associated with these junctional complexes were cortical microfilaments running parallel to the plasma membrane (Fig. 2C, arrow). Numerous short microvilli were apparent on the apical surfaces but these were considerably shorter than those of RPE cells in vivo.

### The Cytoskeleton of D407 Cells

Most cells of epithelial origin contain keratin intermediate filaments, and these proteins are considered diagnostic of epithelial cells; in contrast, cells of mesenchymal origin, such as fibroblasts, contain vimentin intermediate filaments. When subconfluent D407 cells were permeabilized and stained with a mixture of two monoclonal antibodies that stain a range of epithelial keratins, a typical keratin intermediate filament network was observed (Fig. 3A). When the cells reached confluence, the keratin filaments formed a network throughout the cytoplasm (Figs. 3B, 3C, 3D).

The intermediate filament system of RPE cells in vivo contains keratins characteristic of simple epithelia, i.e., keratins 8 and 18.[13] When cultured in vitro, a more complex spectrum of keratins is observed, and keratins 7 and 19 are also found.[18] Western blot analysis (data not shown) indicated that D407 keratins are similar to those of RPE cells in primary culture, i.e., they contain keratins 7, 8, 18, and 19.

Actin forms a cortical web of microfilaments that

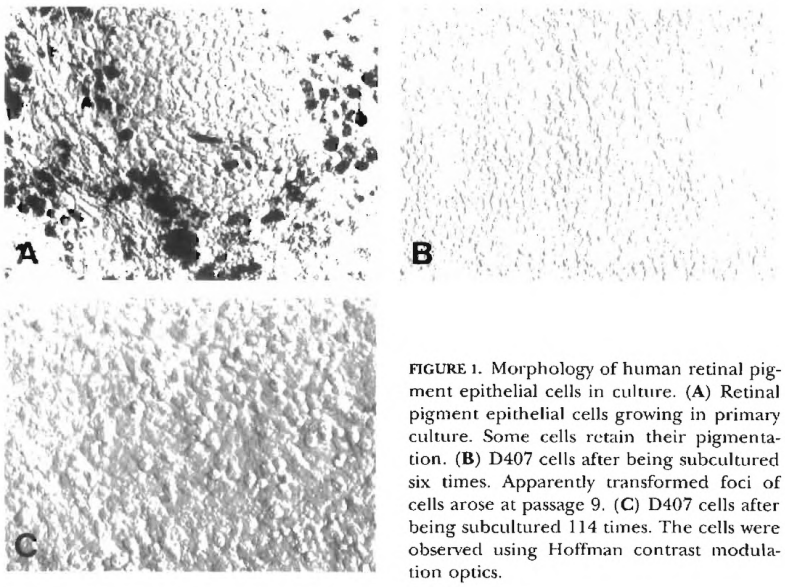


FIGURE 1. Morphology of human retinal pigment epithelial cells in culture. (**A**) Retinal pigment epithelial cells growing in primary culture. Some cells retain their pigmentation. (**B**) D407 cells after being subcultured six times. Apparently transformed foci of cells arose at passage 9. (**C**) D407 cells after being subcultured 114 times. The cells were observed using Hoffman contrast modulation optics.

interacts with junctional complexes near the apical region of the lateral membranes of many epithelial cells, including RPE. In addition, actin-containing microfilaments are found throughout the cytoplasm. Confocal microscopy showed that a tight cortical layer of actin filaments bounds the lateral surface of D407 cells (Fig. 3F). These filaments were much more closely apposed to the lateral membrane than the intermediate filaments (see Figs. 3C, 3F). In the apical cytoplasm, short actin aggregates rather than filaments were observed (Fig. 3E). These may be concentrated in the short microvilli of D407 cells, although RPE cells do not exhibit the paracrystalline array of filaments seen in the microvilli of some other epithelial cells.[19]

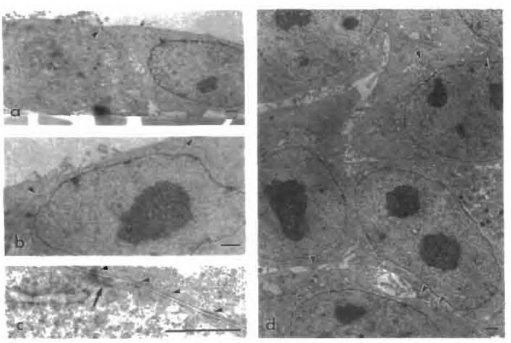


FIGURE 2. Morphology of D407 Cells. D407 cells (passage 80) were grown on membrane filters (**A**) or laminin-coated tissue culture ware (**B, C, D**). The fixed monolayer of cells was sectioned vertically (**A, B**) or en face (**C, D**). Arrowheads indicate junctional complexes close to the apical surface of the cells. These are associated with a layer of submembranous microfilaments (*arrow*). Original magnifications: (**A**) ×4320; (**B**) ×7700; (**C**) ×30,000; (**D**) ×6400. Bars = 1 μm.

D407 cells stained positively for vimentin (Fig. 3G), which is also found in cultured RPE cells of various species.[20–22] These cells did not contain GFAP (Fig. 3H).

Spectrin, a high molecular weight component of many cell cytoskeletons, exhibits a highly polarized distribution in human RPE cells in vivo and is highly concentrated under the apical surface.[16] This protein was found as a filamentous web throughout the cytoplasm of D407 cells and appeared to be more concentrated at the basal surface (Figs. 3I, 3J, 3K).

On the lateral membranes of D407 cells was a belt of N-cadherin (A-CAM)-containing structures (Fig. 3L) that, at higher magnification, had a punctate appearance (Fig. 3M). This suggests that the array of junctional complexes seen in electron micrographs of en face sections (Fig. 2C) extend as spot welds around the circumference of the cell.

## Expression of CRALBP by D407 Cells

CRALBP binds 11-*cis*-retinal in RPE and Müller cells of the retina[10] and was found in D407 cells using a specific polyclonal anti-CRALBP antibody (Fig. 4).

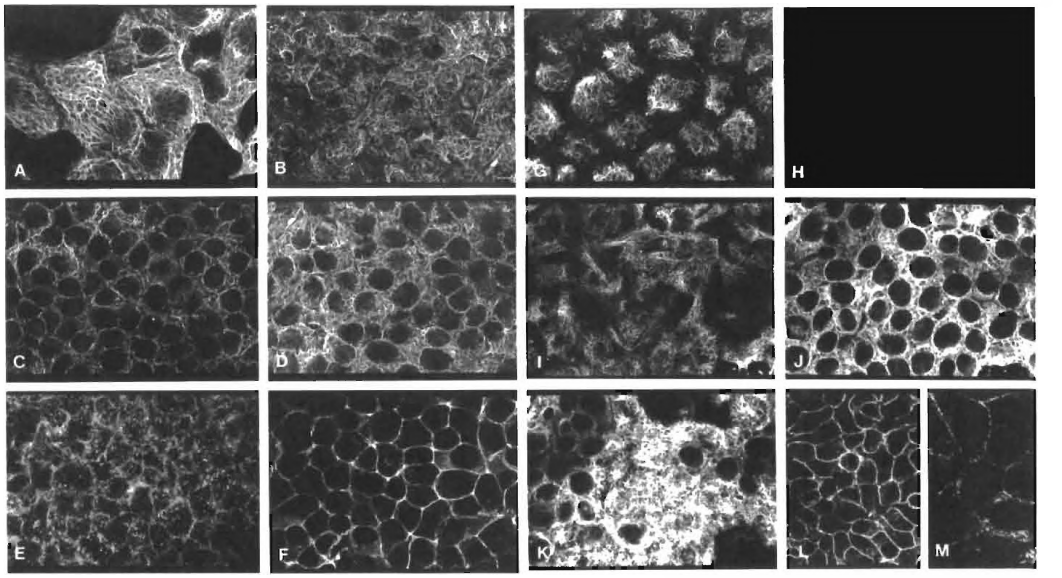


FIGURE 3. Confocal fluorescence microscopy of the cytoskeleton of D407 cells. (**A**) Subconfluent cells stained for keratins. (**B**) Confluent cells stained for keratins. Confocal optical section near the apical surface. (**C**) Confluent cells stained for keratins. Section near the middle of the cell. (**D**) Confluent cells stained for keratins. Section near the basal surface. (**E**) Confluent cells stained for actin. Section near the apical surface showing concentration of actin in short microfilaments. (**F**) Confluent cells stained for actin. Section near the middle of the cell showing cortical microfilaments. (**G**) Confluent cells stained for vimentin. Section near the apical surface. (**H**) Confluent cells stained for glial fibrillary acidic protein. (**I**) Confluent cell stained for spectrin. Section near the apical surface. (**J**) Confluent cells stained for spectrin. Section near the middle of the cell. (**K**) Confluent cells stained for spectrin. Section near the basal surface. (**L, M**) Confluent cells stained for N-cadherin. All cells shown in this figure were from passages 18 to 25. Cells at passage 89 were similar in appearance (data not shown).

## Binding of ROS by D407 Cells

A major function of RPE cells is the uptake and degradation of shed membranes from photoreceptor outer segments by what appears to be a specific receptor-mediated process.[11,23,24] To test whether D407 RPE cells are capable of rapid binding of photoreceptor fragments, the latter were fluorescently labeled and incubated with D407 cells at 37°C for 10 minutes, followed by extensive washing. Figure 5 shows confocal fluorescence (panel A) and phase-contrast images (panel B). In panel C, the phase-contrast image is merged with the fluorescence, which is shown in black for better contrast. It can be seen that within the 10-minute period, the D407 cells bound a considerable amount of the labeled material. Further incubation (data not shown) showed continued accumulation of fluorescent vesicles by the cells.

D407 cells were incubated with an homogenate of neural retina to investigate their phagocytic activity further. Retinal material was found to be associated with a thickening of the plasma membrane (Fig. 6A) or engulfed by microvilli after a 10-minute incubation (Fig. 6B). After a further period, numerous inclusions that were often darkly stained were observed (Figs. 6C, 6D).

## Processing of $^{3}$H-all-*trans*-retinol by D407 Cells

D407 cell membranes were incubated with $^{3}$H-all-*trans*-retinol, after which radiolabeled retinoids were extracted and analyzed by HPLC. This allows the identification of three enzymatic activities characteristic of RPE cells: (i) lecithin retinol acyl transferase (LRAT), which esterifies retinol with long chain fatty acids, typically palmitate; (ii) retinoid isomerase, which converts all-*trans*-retinoids to visually active 11-*cis*-retinoids; and (iii) retinol dehydrogenase (RDH) activity, which oxi-

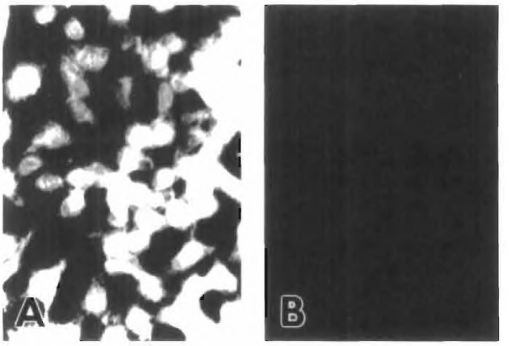


FIGURE 4. Detection of CRALBP in D407 cells. Nonconfluent D407 cells were stained with a polyclonal antibody against CRALBP (**A**) or with nonimmune rabbit serum (**B**). Cells were at passage 100. CRALBP = cellular retinaldehyde binding protein.

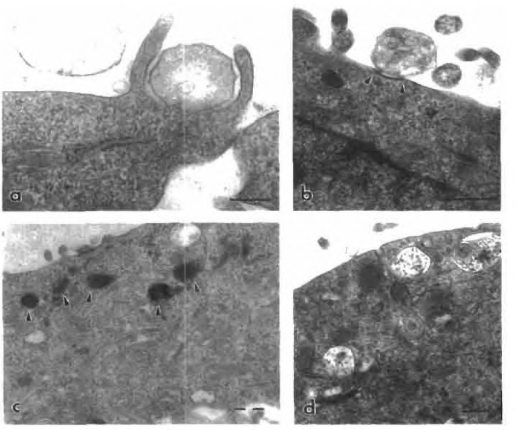


FIGURE 6. Binding and phagocytosis of retinal material by D407 cells. D407 cells (passage 191) were incubated for 10 (**a, b**) or 60 (**c, d**) minutes with a homogenate of human neural retina. The cells were then fixed for electron microscopy. (**a**) Engulfment of a retinal fragment by microvilli. (**b**) Thickening (*arrowheads*) of the plasma membrane before phagocytosis. (**c**) Darkly stained phagosomes (*arrowheads*). (**d**) Lightly stained phagosomes. Original magnifications: (**a**) ×30,000; (**b**) ×45,000; (**c**) ×22,500; (**d**) ×22,500. Bar = 0.5 $\mu$m.

dizes retinol to retinal. Figure 7 shows a typical HPLC chromatogram of the retinoids formed after 2 hours incubation of $^3$H-all-*trans*-retinol with D407 membranes. A major peak was observed at the position of all-*trans*-retinal, indicating RDH activity. Typically, approximately 50% of the substrate was oxidized to retinal in a 2-hour assay (Table 1). Some isomerization to 13-*cis*-retinol and 13-*cis*-retinal was observed, but this has been attributed to nonspecific thermal isomerization during the assay and extraction process,[25] and, indeed, 13-*cis*-retinol was found when the incubation was carried out in the absence of membranes (Fig. 7). Although 11-*cis*-retinal and 13-*cis*-retinal are not adequately separated on this HPLC system, analysis of the extracts in 4% dioxane in hexane, which separates the two isomers, revealed that the small peak is exclusively 13-*cis*-retinal (data not shown). RDH activity was significantly enhanced ($P < 0.05$) by the addition of 1 mM $NADP^+$, whereas $NAD^+$ had no significant effect (Table 1). In the absence of membranes, no all-*trans*-retinal was formed (Fig. 7).

No esterification of $^3$H-all-*trans*-retinol or formation of 11-*cis*-retinoids was observed (Fig. 7), showing that LRAT and retinoid isomerase activities are undetectable. The lack of these two enzyme activities was confirmed in an experiment in which D407 cells were

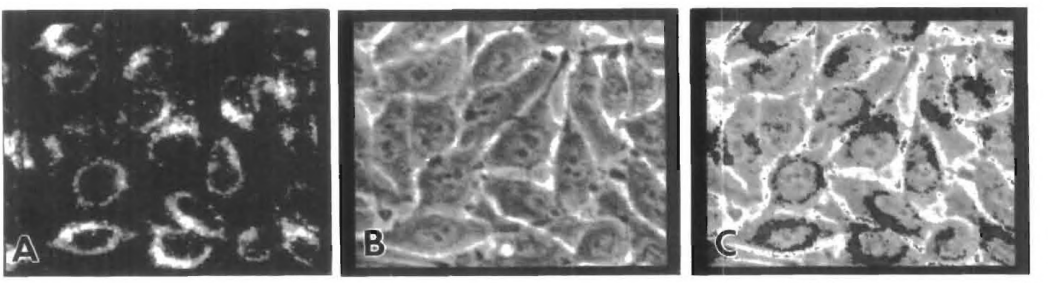


FIGURE 5. Binding of fluorescein-labeled rod outer segments by D407 cells. D407 cells (passage 89) were incubated for 10 minutes at 37°C with fluorescein-labeled rod outer segments and observed by confocal fluorescence microscopy (panel A) and phase-contrast microscopy (panel B). The fluorescence and phase-contrast images are merged in panel C, in which the fluorescence is shown in black.

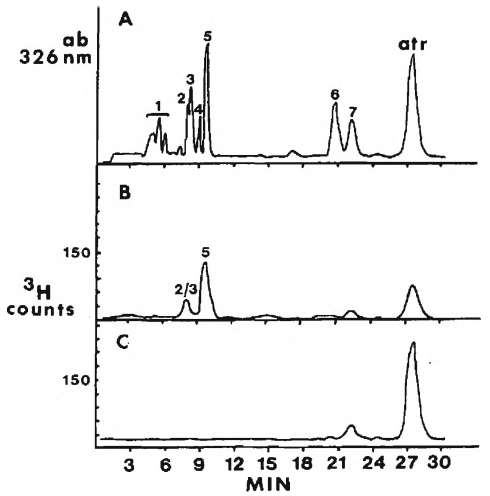


FIGURE 7. Metabolism of $^3$H-all-*trans*-retinol by D407 cells. Membranes from D407 cells at approximately passage 100 were incubated with $^3$H-labeled all-*trans*-retinol. The retinoids were extracted and analyzed by HPLC. (**A**) Absorbance (at 326 nm) profile of standard retinoids. 1 = retinyl esters; 2 = 13-*cis*-retinal; 3 = 11-*cis*-retinal; 4 = 9-*cis*-retinal; 5 = all-*trans*-retinal; 6 = 11-*cis*-retinol; 7 = 13-*cis*-retinol; atr = all-*trans*-retinol. (**B**) Radioactive retinoids produced after a 2-hour incubation of D407 cell membranes with $^3$H-all-*trans*-retinol. (**C**) Radioactive retinoids after a similar incubation in the absence of cell membranes.

grown on Millicell filter supports and $^3$H-all-*trans*-retinol was delivered to cells in association with retinol binding protein (data not shown). Additionally, experiments on D407 cell membranes using an 11-*cis*-retinal substrate demonstrated that these cells lack 11-*cis* specific RDH activity (data not shown).

### Karyotypic Analysis of D407 Cells

A karyotype analysis showed that D407 cells are of human origin and, therefore, are unlikely to be derived from contamination by other epithelial cells used in these laboratories. In early passages, the cells showed a modal chromosome number of 44 ± 2. The chromosomes were distributed according to the standard human karyotype (data not shown); however, by passage 52, the cells showed a near triploid modal chromosome number (70 ± 4). No diploid or near diploid metaphases were observed after screening over 50 metaphase spreads. Analysis of the metaphases showed that almost all the chromosomes could be assigned to the A to G groups of the human karyotype (Fig. 8); however, some chromosomes in each spread analyzed did not match the morphology of normal human chromosomes. An extra long submetacentric chromosome was observed in almost all metaphases examined and was classified as a marker chromosome.

TABLE 1. Retinol Dehydrogenase Activity in D407 Cell Membranes

| | *% Conversion to Retinal** |
|---|---|
| Control D407 membranes | 46.4 (±15.5) |
| D407 membranes + 1 mM $NAD^+$ | 53.5 (±6.9) |
| D407 membranes + 1 mM $NADP^+$ | 76.6 (±8.0)† |

* Mean ± standard deviation ($n$ = 3).
† Significantly different from control ($P < 0.05$).
Membranes were prepared from D407 cells and incubated with $^3$H-all-*trans*-retinol for 2 hours. The products were analyzed by high-performance liquid chromatography.


FIGURE 8. The karyotype of D407 cells. A representative karyotype of a Giemsa solid-stained D407 metaphase, including 71 chromosomes. Most chromosomes were distributed in the classical alphabetic groups of the standard human karyotype. Two chromosomes designated, m1 and m2, could not be assigned to any group and are considered marker chromosomes.

### Other Characteristics of D407 Cells

D407 cells did not bind antibodies raised against Factor VIII, indicating that they are not of endothelial origin[26,27] (data not shown). These cells synthesize S100 antigen (data not shown), which has, hitherto, been reported to be absent from RPE cells,[28] and they also secrete apo-lipoprotein AI into their surrounding medium. Because SV40 virus has been used in the authors' laboratories, the cells were stained for SV40 T antigen and found to be negative.

## DISCUSSION

Cultured human and other mammalian RPE cells have been the subject of many investigations of the blood–

retinal barrier, especially with regard to the transport of circulatory molecules into the neural retina. Such studies have necessarily been carried out on primary cultures or cells that have been subcultured a limited number of times. Large numbers of bovine or chick RPE cells may be obtained under such conditions, but human RPE cells pose the problem of limited availability and heterogeneity of human donor eyes. Moreover, human and other mammalian RPE cells, under conditions used in many laboratories, frequently lose many of their epithelial characteristics when subcultured. For example, melanin synthesis ceases under most culture conditions, and the polarized cobblestoned epithelial morphology with junctional complexes between monolayer cells is lost. The cells become fusiform and often form multilayers of cells with sporadic, disorganized junctions. In some cases, the cells lose their keratin intermediate filaments, characteristic of epithelial cells, and synthesize vimentin-containing intermediate filaments,[13] which are more characteristic of cells of mesenchymal origin.

For these reasons, continuous cultures of RPE cells would be useful, and several lines have now been established. Human[29] and rat[30] RPE cells have been transformed using SV40 T antigen, adenovirus E1A, or cellular oncogenes such as c-myc and Ha-ras, and some of these retain their epithelial characteristics, as do recently reported spontaneous rat transformants.[31] Human spontaneous transformants have also been reported, but they have received less characterization,[32-35] and, where their morphology has been shown, they appear to be flattened rather than cuboidal, with cellular extensions overlying other cells and rather sporadic intercellular junctions.[33] For studies of the metabolism of the human eye, an RPE cell line that maintains a high degree of epithelial morphology would be useful. We have attempted to transform RPE cell cultures with plasmids expressing the SV40 large T antigen and have obtained transformed cells expressing the T antigen, but they lost their characteristic morphology (Davis and Hunt, unpublished data, 1989). However, a clone of cells from an RPE cell culture has been obtained that appears to be spontaneously transformed in the absence of any viral transformation vector. The cells, D407, have now been subcultured more than 200 times. Such spontaneous transformation seems rare; we have only seen it on one other occasion during the culturing of cells from almost 2000 pairs of human donor eyes. By passage 52, almost all cells examined had approximately 70 chromosomes and at least one marker chromosome, suggesting that out of the initial diploid cells, a near-triploid clonal population has emerged and prevailed. It appears that the rearranged human genome meets the requirements for unlimited replication under the conditions provided by in vitro culture.

Naturally, the question arises of whether these cells are indeed of RPE origin or whether they arose from a small number of non-RPE cells that contaminated the original culture of highly pigmented cells. Clearly, the cells exhibit a typical cobblestoned epithelial morphology and, at the back of the eye, only the pigment epithelium contains such cells. D407 cells do not express GFAP, indicating that they are not of glial (Müller) cell origin and do not express Factor VIII, a marker of endothelial cells. They do express vimentin, a protein characteristic of intermediate filaments of mesenchymal cells, but many epithelial cells in culture,[36-39] including RPE cells,[13,21] also express this protein. Keratin is the only intermediate filament protein found in human RPE cells in vivo or in most other human epithelia, and this is expressed by D407 cells; indeed, D407 cells synthesize keratins 8 and 18, typical of simple human epithelia.[13,14]

Spectrin (fodrin) is a submembranous skeletal protein that is associated with actin and transmembrane proteins. In vivo this protein in RPE cells is highly polarized and is found on the cytoplasmic surface of the apical plasma membrane, where it may interact with the highly polarized $Na^+,K^+$-ATPase system. [16] In D407 cells, spectrin is found in association with apical and basolateral surfaces, indicating that the extreme polarization of this protein is not retained in these cultured RPE cells. Loss of polarization in cultured chick RPE cells also has been observed,[40,41] suggesting that polarization in vivo may reflect the interactions of RPE with surrounding cells and their extracellular matrices.

In addition to their epithelial morphology and their expression of keratins, D407 cells have other properties that suggest their RPE origin. At confluence, the cells form a belt of junctional complexes linking adjacent cells by N-cadherin (A-CAM), a protein characteristic of junctional complexes of a number of epithelial cell types.[42-44] In vivo RPE cells carry out phagocytosis of shed photoreceptor outer segment membranes,[11,12] probably when the latter bind to a specific receptor on the RPE cell surface,[23,24,45] and a similar process may occur in D407 cells. Moreover, CRALBP, which participates in the transport of retinoids from the circulation to the retina and is found in RPE and Müller cells of the retina in vivo,[10] also is synthesized by D407 cells.

The D407 RPE cell line exhibits the ability to metabolize vitamin A in the form of $^3$H-all-*trans*-retinol. However, only the RDH activity was observed, whereas freshly isolated RPE cells also express LRAT and retinoid isomerase activities,[8,9] and primary and secondary cultures express LRAT but not retinoid isomerase.[9] Nevertheless, this appears to be the first continuous RPE cell line for which any retinoid processing capacity has been reported. Although D407 cells possess

an RDH activity that can metabolize added all-*trans*-retinol, they lack a comparable activity that can use the 11-*cis* isomer. In vivo both the RPE and the rod outer segments possess the all-*trans*-specific RDH,[15] whereas the *cis* specific RDH is thought to be unique to the RPE.[46,47] The all-*trans* specific RDH in the rod outer segments is considered to be an integral enzyme in the visual cycle required for the conversion of all-*trans*-retinal to all-*trans*-retinol after bleaching of rhodopsin and before transport to the RPE. This enzyme's role in the RPE is not well defined because these cells are thought to be supplied with exogenous vitamin A exclusively in the form of all-*trans*-retinol. It is possible that the all-*trans*-retinal dehydrogenase activity may form part of the cells' pathway for generation of retinoic acid that occurs as two steps, during which retinol is converted to retinoic acid via retinal.

Other characteristics of D407 cells not addressed in this article seem to be those of RPE cells. Like RPE cells in vivo, they secrete basolateral extracellular matrix molecules and matrix-degrading metalloproteinases. In addition, they release iron that they have taken up from diferric transferrin in a low molecular weight form that has been shown to be characteristic of primary cultures of RPE[48] and other barrier cells.[49,50] These cells should prove to be useful in other studies of the biology of pigment epithelium.

### *Key Words*

human pigment epithelium, transformed cell, cytoskeleton, phagocytosis, cell culture

### *Acknowledgments*

The authors thank Dr. Robert Price for his generous help with the electron microscopy and Dr. Walter McConathy for the Western blot analysis of secreted lipoproteins. They also thank Drs. T. Borg and J. Saari for antibodies and Dr. William O'Day for culturing the cells for the retinoid experiments. Human donor eyes for retinal pigment epithelial cell culture were kindly donated by the South Carolina Lions' Eye Bank.

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