Vitamin A Utilization in Human Retinal Pigment Epithelial Cells In Vitro

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Vitamin A (vit A) metabolism was studied in freshly isolated and cultured human retinal pigment epithelial (RPE) cells obtained from postmortem donor eyes. Fluorometric determination of vit A in human RPE cells demonstrated that freshly isolated cells contained approximately 1.0 to 4.0 pg vit A/cell which decreased with increasing time in culture; after 48 hrs in culture cellular vit A was reduced 80%. High performance liquid chromatography (hplc) profiles of the retinyl esters in freshly isolated RPE cells showed the presence of 11-cis retinyl stearate and palmitate and all-trans retinyl stearate, palmitate and oleate; all-trans palmitate was the major ester. Hplc analyses of cell cultures supplemented with all-trans retinol, using fatty acid-free bovine serum albumin as a carrier, showed that the cells in primary and subcultures took up all-trans retinol and esterified it to form palmitate, stearate, and oleate. Palmitate was the major ester synthesized by the cells in primary cultures. In the subcultures the esters synthesized differed from that found in freshly isolated cells and in the cells in primary culture; in the subcultures, the overall synthesis of ester was reduced and oleate was more prominent. The esters that were synthesized in culture were all-trans; the formation of 11-cis isomers was not observed in human RPE cells in culture. Electron microscopy of retinol-supplemented cultures indicated that vit A doses up to 1.0 μg/ml had no obvious effects on the cells; at higher doses the cells no longer adhered to the culture surface. Invest Ophthalmol Vis Sci 24:1227–1235, 1983

The retinal pigment epithelium (RPE) serves an essential role in the storage, metabolism, and transport of vitamin A used in the visual cycle. In some way, vitamin A is transported between the retinal epithelium and the photoreceptor cells depending upon the latter's need.1-3 Exactly what the role of the RPE cells is in the vitamin A cycle and whether it is similar in the retinas of different animals is not yet clear. There is some evidence, however, that this operation is quantitatively and even qualitatively different in different vertebrate species.1,2,4-11 For example, frog RPE contains relatively large quantities of vitamin A in both the light- and dark-adapted states; the rat, in contrast, stores large amounts of vitamin A in the RPE in the light-adapted state and almost none in the dark-adapted state. Most species store vitamin A in the ester form.1-3,7,10,12

Because of the difficulty to obtain viable human retinal tissue for biochemical studies, very little is known about vitamin A use and metabolism in the human retina. The postmortem viability of human RPE cells, and the ability to maintain these cells in culture13-16 have provided an opportunity to study the cell biology and biochemistry of human RPE cells under controlled conditions. This paper provides basic information on the way human retinal epithelial cells maintained in culture use and metabolize vitamin A.

Materials and Methods

Cell Cultures

Postmortem eyes of varying chronologic ages were received through the New York Eye Bank for Sight Restoration, the Retinitis Pigmentosa Donor Eye Program, and the National Diabetes Research Interchange. In most cases the eyes were received after the corneas were removed for corneal transplants. The cells were obtained by trypsin-dissociation and primary cultures were established and maintained according to methods previously described.15 Cells removed from postmortem eyes by trypsin-dissociation and processed immediately for biochemical studies are referred to as freshly isolated cells. In vitro time was calculated as postmortem time plus time in culture.

Subculturing was done when the primary cultures became confluent, generally 21 to 30 days after the...
primary culture was established. A solution of 0.05% trypsin and Versene (1:1000) was added to the culture dishes for 5 min at 37 C to release the cells. The number of cells in the suspension was determined with a hemocytometer and cultures were plated out at 1.5−2.0 × 10^5 cells/ml. Experiments on the subcultures were done on the seventh to tenth day when the subcultures were confluent, as determined by daily microscopic observations.

Vitamin A Studies

Total vitamin A determinations: To determine the total vitamin A content of freshly isolated and cultured human RPE cells, the vitamin A was extracted from the cells with hexane after saponification, concentrated under nitrogen, and separated chromatographically on an alumina column as described by Harrison et al.17 Each fraction was assayed fluorometrically according to the method of Thompson and his colleagues18 with the modification of Muto et al.19

In order to determine the amount of vitamin A in the fetal calf serum (Sterile Systems) used to supplement the culture medium, an equal volume of 100% ethanol was added to 2 ml of serum and the mixture was extracted with n-hexane as previously described.20 The extract was analyzed by high performance liquid chromatography (hplc) to identify its isomer content.

Retinol supplementation: RPE cell cultures were supplemented with all-trans retinol (Hoffman-LaRoche) for varying intervals of time from 30 min to 24 hrs. Retinol, in concentrations of 0.1 to 50.0 µg/ml, was added to Eagle's minimum essential medium (MEM) (Flow Laboratories) which contained 500 µg/ml fatty acid free bovine serum albumin (BSA) (Sigma) as the retinol carrier. In these studies the fetal calf serum was removed from the incubation medium 18 hrs before retinol was added. After the desired incubation time, the cultures were washed with Hank's balanced salt solution, Ca ++ and Mg ++ free, and removed from the culture-plate surface by mild trypsinization. Vitamin A content per cell in the retinol-supplemented cultures was determined with respect to incubation time with retinol. The optimum supplementation concentration of retinol was determined.

Twelve additional pairs of eyes were used to study the rate of retinol uptake in RPE cell homogenates. To obtain the RPE cell homogenates, the eyes were dissected as described for cell culturing.21 After removal of the neural retina, the RPE was rinsed twice with Puck's saline at pH 7.4. A small aliquot of Puck's saline was then added to the eyecup and the RPE cells were gently brushed off Bruch's membrane. The cell suspension was homogenized in a glass/glass homogenizer and centrifuged at 100,000 × g for 1 hr. The supernatant was then removed. The pellet was resuspended in Puck's saline at pH 7.4 and divided into 0.5 ml aliquots each containing 204 µg protein (as measured by the method of Lowry22). To each aliquot was added 0.8 µg [11,12-3H]-retinol (either 11-cis or all-trans). The [11,12-3H]-retinol was prepared from the corresponding retinoic acid20 and labeled 11-cis and all-trans isomers were separated on a preparative scale by hplc. The retinol (1.8 × 10^6 dpm) was added in 20 µl of ethanol and incubations were made at 37 C. The reaction was stopped at various times by the addition of 10 ml chloroform-methanol (2:1 v/v).

Two milliliters of water were then added, the upper phase removed and discarded. The lower (chloroform) phase was then washed with Folch theoretical upper phase (methanol:H_2O:CHCl_3, approximately 48:48:1). The chloroform extract was then dried in a stream of prepurified nitrogen and the residue dissolved in 10% dioxane in n-hexane. This solution was then chromatographed on alumina as described previously.20 The eluent from the hplc columns was collected into 7-ml polypropylene scintillation vials at 0.2- to 1.0-min intervals. Six milliliters of Scintilene (Fisher) were then added to each vial and the radioactivity was counted in a Packard Tricarb Liquid Scintillation Spectrometer. The counts emerging in the peaks corresponding to retinyl stearate and palmitate were used to measure the amount of retinol substrate esterified.

High performance liquid chromatography analysis: Hplc was used to identify the specific retinyl esters present in freshly isolated and cultured human RPE cells and to identify the fatty acid moiety of the retinyl esters synthesized in retinol-supplemented cultures. In these studies the esters were extracted from the cells with 2 × 4 ml volumes of acetone. The extract was filtered through glass fiber, evaporated under a stream of nitrogen, and redissolved in 1 ml of n-hexane containing 10% v/v dioxane. The extract was purified on an alumina column (5% water deactivated) and eluted with 10% v/v dioxane in n-hexane. A portion of the eluent was prepared for total vitamin A measurement by the Carr-Price reaction as previously described20 or saponified and prepared for vitamin A determinations as described above in section on total vitamin A determinations. For normal phase chromatography, two 4.6 mm × 25 cm columns, an Ultrasphere Si 60 and a Spherisorb CN, packed with 5 µm adsorbents were used separately or in series. The mobile phase for retinyl ester analysis was 0.4% ether/n-hexane at 1 ml/min. All solvents were hplc grade (Burdick & Jackson). The equipment for hplc
analysis has been previously described.4,23 Standardization and preparation of authentic retinoids is described in reference.20

Electron Microscopy

For ultrastructural studies, RPE cell cultures were supplemented for 18 hours with all-trans retinol (0.5 and 1.0 μg/ml) and then were fixed with 3% gluteraldehyde for 2 hrs at 4 C. The cultures were then washed with Earle’s buffer (pH 7.4) and postfixed in 1% osmium tetroxide for 1 hr. After ethanol dehydration, Epon was poured into the culture dish and polymerized. Following polymerization, the culture dish was cut into blocks and the sections were cut perpendicular to the culture surface to study the apical and basal cell surfaces. Thin sections were stained with uranyl acetate and lead citrate and studied on a Siemens electron microscope.

Results

Total Vitamin A in RPE Cells

In a previous paper24 we reported that the total amount of vitamin A in human RPE cells, freshly isolated from 24 pairs of donor eyes of varying chronologic ages ranged from approximately 1.0 to 4.0 pg/cell. Data from 14 additional pairs of eyes confirmed our initial report (Fig. 1).

Normal phase hplc analyses of the retinyl esters in freshly isolated RPE cells showed that these cells stored 11-cis retinyl palmitate and stearate and all-trans retinyl palmitate, stearate, and oleate (Fig. 2). Occasionally, 13-cis retinyl stearate and palmitate were present. However, there was great variability in the presence and the content of the 13-cis esters in these cells; the factors that control this variability are unknown at this time.

Loss of Vitamin A in RPE Cells in Vitro

Analysis of the vitamin A content in RPE cells in vitro demonstrated that vitamin A is rapidly lost from these cells in culture even though the cells are maintained in medium supplemented with fetal calf serum (FCS). Our data showed that within 2 to 4 days in vitro, there is an 80% decrease in the total amount of vitamin A per cell (Fig. 1; Table 1). Analysis of the FCS used to supplement the culture medium showed that the serum contained approximately 12 μg all-trans retinol per 100 ml serum. The amount of retinol varied slightly in different serum lots. Over 90% of this retinol was in the all-trans configuration.

Hplc analysis of the isomeric ester forms present in freshly isolated and cultured RPE cells from the same donor cell populations (Fig. 3) indicated that there is no preferential loss of specific isomeric forms of retinyl palmitate and stearate in vitro. Within 48 hours in culture there is a significant loss of the all-trans, 11-cis, and 13-cis palmitate and stearate in human RPE cells. After 48 hours in culture, all-trans palmitate and stearate had decreased by 85%; 11-cis palmitate and stearate by 75%, and 13-cis palmitate and stearate by 65%. However, the initial level of all-

![Fig. 1. Vitamin A content in human RPE cells in vitro. Closed circles (●) represent freshly isolated cells; open circles (O) represent cells in culture. Data points connected by a line indicate cell populations from the same donor.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933116/)

![Fig. 2. Normal phase hplc separation of retinyl esters extracted from freshly isolated human RPE cells. Mobile phase = 0.4% diethyl ether in n-hexane at 1.0 ml/min. Columns = Ultrasphere Si60 and Spherisorb CN in series. Peak identification: 1 = 11-cis retinyl stearate; 2 = 11-cis retinyl palmitate; 3 = unknown; 4 = all-trans retinyl stearate; 5 = all-trans retinyl palmitate; 6 = all-trans retinyl oleate.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933116/)
Table 1. Decrease in vitamin A content in retinal epithelial cells in vitro

<table>
<thead>
<tr>
<th>In vitro time* (days)</th>
<th>No. of donors</th>
<th>Vit. A content + SEM (pg/cell)</th>
<th>Percent decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly isolated</td>
<td>35</td>
<td>2.02 ± 0.16</td>
<td>—</td>
</tr>
<tr>
<td>2-4</td>
<td>9</td>
<td>0.41 ± 0.10</td>
<td>80%</td>
</tr>
<tr>
<td>8-12</td>
<td>14</td>
<td>0.36 ± 0.08</td>
<td>82%</td>
</tr>
</tbody>
</table>

* In vitro time = postmortem time + time in culture.

trans palmitate and stearate was significantly greater than the 11-cis and 13-cis forms. The ester profile from the cells that did not attach to the culture surface suggested that the loss of each isomeric form was slightly less in the unattached cells than in the attached cells. The difference in ester loss between the attached and unattached cells was minimal.

Retinol Supplementation

Supplementation of primary cell cultures for an 18-or 24-hr period with medium containing all-trans retinol in concentrations of 0.10 to 50.0 μg retinol per ml culture medium with BSA as the retinol carrier demonstrated that concentrations of 0.25 to 0.50 μg retinol per ml culture medium could raise the vitamin A content in the RPE cells in vitro to levels commensurate with those in freshly isolated cells (Fig. 4). Retinol supplementation doses greater than 2.0 μg/ml were found to affect cell attachment when presented to the cells in this way. At higher retinol concentrations the cells rounded up and detached from the culture surface.

Hplc analysis of human RPE cells in primary and subcultures showed that the cells were able to take up all-trans retinol and esterify it to the major esters found in these cells in vivo. Figure 5 is an hplc chromatogram of the esters extracted from cells maintained in primary culture for 11 days. It is clear that these cells contained negligible amounts of retinyl esters (Fig. 5A). After 18 hrs supplementation with all-trans retinol (Fig. 5B), however, the cells synthesized all-trans retinyl palmitate, stearate, and oleate; palmitate was the major ester synthesized. Similarly, there was no evidence of retinyl esters in subcultured cells after 50 days in culture (Fig. 6A). When supplemented with all-trans retinol for 24 hrs, the subcultured cells were also able to take up and esterify all-trans retinol, forming retinyl palmitate, stearate and oleate (Fig. 6B). Subcultured cells formed a higher proportion of oleate compared with that present in freshly isolated cells or that formed by cells in primary cultures. The total amount of retinyl esters synthesized in each cell population studied is summarized in Table 2. There was no evidence of 11-cis isomers in the cultured RPE cells.

The data also showed that in the retinol-supplemented cultures the amount of retinyl ester present in primary culture of RPE cells from the same donor increased gradually with increased supplementation time over a 24-hr period (Fig. 7). After 24 hrs of supplementation with all-trans retinol, the amount of retinyl esters extracted per μg protein for cells in primary cultures was equivalent to the range of retinyl esters present in freshly isolated normal RPE cells (Fig. 5A).
ester content in freshly isolated RPE cells of the various donors studied. In supplemented subcultures, the amount of retinyl esters extracted from the cells increased with time over seven hours for each donor cell population that was studied; however, the total amount of retinyl esters synthesized per μg protein was not as high as in RPE cells in primary cultures (Fig. 7).

The uptake and esterification of 11-cis, and all-trans retinol by the particulate fraction of RPE cell homogenates is illustrated in Figure 8. The esterification process appeared to be rapid for both isomers but the total amount esterified was 10^4-fold less (per μg protein) than observed with whole cells in primary or subculture. Part of this difference may be due to the different modes of delivery. When an ethanol solution of retinol is added to an aqueous buffer, the resulting dispersion is highly susceptible to destruction by oxidation. It is notable that both 13-cis and all-trans retinyl esters are formed in addition to 11-cis when the substrate is 11-cis retinol (Fig. 8). Thirteen-cis and all-trans esters are also formed when the substrate is all-trans retinol.

The synthesis of retinyl esters from exogenous retinol by whole cells was considered to be a true indication of retinol uptake since an artifactually high level of free retinol could occur in the cell suspensions because of adsorption to surface membranes.

Electron Microscopy

Electron microscopic studies of the retinol supplemented cells showed intact cells with ultrastructural features characteristic of RPE cells in vitro^5^,^16^ and similar to the control cells (Fig. 9A). The micrographs of the human RPE cells supplemented for 18 hrs with 0.5 and 1.0 μg retinol per ml culture medium (Figs. 9B, C) demonstrated that these retinol concentrations had no apparent effect on the cultured RPE cells. The cells showed an apical-basal polarity with numerous slender processes along the apical surface and dense infoldings of the basal surface. Mitochondria with well-defined cristae and dense concentrations of intramitochondrial granules in addition to channels of rough endoplasmic reticulum and numerous ribosomal granules were scattered among the lipofuscin granules in the cytoplasm of the cells. Although both the retinol-supplemented and control cells had ultrastructural features suggestive of much activity on the apical surface, there appeared to be a greater number of pinocytotic vesicles along the apical surface of the retinol-supplemented cells.

Discussion

Our data showed that human RPE cells contain approximately 1.0 to 4.0 pg vitamin A per cell as
determined through fluorometric analysis. The actual amount of vitamin A per cell in the freshly isolated RPE cells is probably slightly higher since 11-cis retinol has less fluorescence than the all-trans form.25

Vitamin A is stored in the RPE cells of most species. Even when there is a tremendous reduction in the liver store due to vitamin A deprivation, a large store of vitamin A is generally retained in the retina.2 This fact insures that the photoreceptors are supplied with vitamin A, which is essential for normal visual function. Within 48 hrs in culture, however, the amount of vitamin A in the human RPE cells is drastically reduced. This suggests that some factor that influences the storage and release of vitamin A is missing in human RPE cells in vitro. This factor may be an abnormal concentration gradient for vitamin A across the RPE cell membrane, drawing the intracellular vitamin A into the media. The concentration of vitamin A in calf serum is about 25% of that in normal human serum.26 The vitamin A remaining in these cells is much less than 1% of their normal levels, however, implying that it is not mass action alone that is responsible for the vitamin A depletion. It is possible that the extracellular vitamin A concentrations in the extracellular pool around the photoreceptors is higher than it is in serum. It cannot be too high, however, because vitamin A as suggested by both our results and those of others27 appears to be toxic in concentrations much higher than those in normal serum. Further experiments will be necessary to uncover the factors that cause this massive and relatively sudden loss of vitamin A from cultured RPE cells.

There is presently no evidence that the loss of photoreceptors will lead to a concomitant loss of vitamin A from retinal epithelium. It is interesting that detachment of the neural retina in cats28 and monkeys (our unpublished results) leads to proliferation of retinal epithelium. Normally retinal epithelial cells in mammals do not enter mitosis after birth. Under normal conditions, however, RPE cells have relatively high levels of vitamin A compared to other body cells except the liver cells. The proliferation of retinal epithelium after retinal detachment, as well as the proliferation observed in tissue culture, may be related to the loss of vitamin A from these cells.

Our studies showed that all-trans retinol added to culture medium containing fatty acid free bovine serum albumin as the retinol carrier was taken up by the cells in vitro and esterified to form the major esters found in freshly isolated human RPE cells.

Table 2. Composition of esters in retinol-supplemented cultures

<table>
<thead>
<tr>
<th>Donor age (yrs)</th>
<th>Culture</th>
<th>In vitro time* (days)</th>
<th>Retinol supplementation (hrs)</th>
<th>All-trans retinyl esters ng/10⁶ cells</th>
<th>All-trans Stearate</th>
<th>Palmitate (%)</th>
<th>Oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>Freshly isolated</td>
<td>½</td>
<td>Unsupplemented</td>
<td>448.7</td>
<td>21.7</td>
<td>73.7</td>
<td>4.6</td>
</tr>
<tr>
<td>48</td>
<td>Primary</td>
<td>11</td>
<td>Unsupplemented</td>
<td>869.8</td>
<td>38.2</td>
<td>47.7</td>
<td>13.7</td>
</tr>
<tr>
<td>48</td>
<td>Primary</td>
<td>11</td>
<td>18</td>
<td>138.5</td>
<td>36.3</td>
<td>45.0</td>
<td>18.1</td>
</tr>
<tr>
<td>71</td>
<td>Primary</td>
<td>11</td>
<td>Unsupplemented</td>
<td>44.3</td>
<td>29.6</td>
<td>39.7</td>
<td>25.0</td>
</tr>
<tr>
<td>71</td>
<td>Primary</td>
<td>11</td>
<td>24</td>
<td>55.4</td>
<td>36.1</td>
<td>34.5</td>
<td>30.7</td>
</tr>
<tr>
<td>30</td>
<td>Subculture</td>
<td>50</td>
<td>Unsupplemented</td>
<td>44.3</td>
<td>33.0</td>
<td>38.2</td>
<td>29.1</td>
</tr>
</tbody>
</table>

* In vitro time = postmortem time + time in culture. ** Negligible amount = could not be determined.
Previous studies on human \textsuperscript{29,30} and bovine \textsuperscript{31} RPE that reported that RPE cells do not take up retinol without its being bound to serum retinol binding protein must be incorrect. In human RPE, the highest concentration of retinol that could be added to the culture medium without producing toxicity was about one-fifth that customarily used to supplement liver cell cultures with vitamin A. \textsuperscript{27} This implies that human RPE is relatively sensitive to free retinol. With the addition of SRBP, Saari et al \textsuperscript{32} demonstrated that the uptake of retinol by retinoblastoma cells was much slower compared with retinoblastoma cells that were supplemented with free retinol. Thus, SRBP may control the uptake of retinol by RPE cells through its interaction with membrane receptors and thus protect these cells from the toxic effect of free retinol.

The increased amount of oleate and the decreased amounts of palmitate and stearate esters synthesized by RPE cells in subculture is the first evidence of a biochemical alteration in human RPE cells in culture. This change may be occurring early in the in vitro life of human RPE cells since primary cells also show a slight increase in the amount of oleate synthesized by these cells when compared to freshly isolated cells. This difference is probably not due to a unique composition of acyl carriers provided to the cells by the fetal calf serum since both the primary and subcultures have been exposed to identical serum and show very different ester profiles. The time for turnover of endogenous pools, the size and stability of which are unknown, may also be a contributing factor to the differing ester profiles.

Human RPE cells in culture, under the conditions studied, do not isomerize all-trans retinol to 11-cis, a reaction that is critical to rhodopsin synthesis and photoreceptor function. This reaction has been thought to require the RPE, \textsuperscript{10,21} although there has been evidence that this may not be the case in rat retina. \textsuperscript{33} The 11-cis esters found in human RPE cells in vivo and in freshly isolated human RPE cells may be due to the interaction of RPE cells with photoreceptors as suggested for the frog. \textsuperscript{1} The isomerization
Fig. 9. A, Electron micrograph of human RPE cells from a 74-year-old donor maintained in primary culture for 14 days with MEM plus 20% FCS. The cells maintain an apical(a)-basal(b) polarity with microvilli projections (m) on the apical surface (magnification = 6,000×). B, and C, Electron micrographs of human RPE cells from the same donor as in A supplemented with 0.5 μg/ml all-trans retinol for 18 hrs. Note the number of microvilli projections (m) and pinocytotic vesicles (v) on the apical surface (B, magnification = 6,500×) (C, magnification = 12,500×).
of all-trans retinol to 11-cis in the human retina may depend upon the presence of the neural retina.

**Key words:** vitamin A, RPE cells, human, in vitro

References