Development and Maintenance of Outer Segments by Isolated Chick Embryo Photoreceptor Cells in Culture

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Purpose. To investigate the capacity of isolated chick embryo photoreceptors to develop and maintain outer-segment processes in dissociated cell cultures, in the absence of pigment epithelial and glial cells.

Methods. Cells were obtained from the retinas of embryonic day (ED) 17 chick embryos, after the onset of outer-segment formation in vivo. After a 5- to 12-minute incubation in Ca ++ - and Mg ++ -free Hanks' balanced salt solution, neural retinas were freed from other optical tissues, including the pigment epithelium. Retinal cell suspensions were prepared by repeated pipeting after mild trypsinization and were grown in serum-containing medium on a polyornithine-coated substratum. Cell differentiation was evaluated using phase-contrast and transmission electron microscopes and by autoradiographic analysis of the uptake of putative amino acid neurotransmitters, lectin cytochemical analysis, and immunocytochemical analysis with rod- and cone-specific antibodies. Cells isolated from ED 8 retinas, before the onset of outer-segment formation in vivo, were also studied.

Results. At culture onset, ED 17 cells appeared morphologically undifferentiated and devoid of processes; differentiated features could be detected after 24 to 48 hours in vitro. Photoreceptor cells were the most abundant cell type after 6 days in vitro, followed by nonphotoreceptor multipolar neurons and morphologically undifferentiated cells. Autoradiographic analysis showed extensive Na+-dependent uptake of [2,3,4-3H]7-aminobutyric acid in nonphotoreceptor neurons, whereas photoreceptors were labeled predominantly with 3H-glutamate. Most of the photoreceptors were labeled with fluorescent peanut lectin and with a sheep polyclonal antibody against bovine rhodopsin. Subsets of photoreceptors, on the other hand, were immunoreactive with cone- or rod-specific monoclonal antibodies 50-1B11, or Rho4D2. Approximately 50% to 65% of the photoreceptors positive with these monoclonal antibodies showed a remarkable polarization of immunoreactive materials, which accumulated predominantly, or even exclusively, in an outer-segment-like apical process. When viewed on the transmission electron microscope, these outer-segment-like processes appeared as distal expansions of the photoreceptor cilium and contained disc-like membranous profiles. Outer-segment-like processes also could be detected using the electron microscope and by immunocytochemical analysis of cultures of ED 8 retinal cells.

Conclusions. After undergoing morphologic dedifferentiation as a result of tissue dissociation, isolated retinal photoreceptors, grown in the absence of contact-mediated cell interactions and of pigment epithelial and glial cells, can regenerate and maintain a highly polarized pattern of structural and molecular organization, including the formation of outer-segment-like processes. The cultures provide an experimental system for the investigation of cellular and molecular mechanisms regulating further development and maturation of these photoreceptor structures.


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Retinal photoreceptors are elongated, polarized, compartmentalized cells that can transduce light into electrochemical signals. Phototransduction requires the coordinated activity of a series of photoreceptor-specific molecules, such as visual pigments, second messengers, and ion channels and transporters (reviewed in Hargrave and McDowell1 and Yau2). These molecules are concentrated predominantly, if not exclusively, in the photoreceptor outer segment, a highly complex
specialized structure containing stacks of visual pigment-rich membranous discs. Outer-segment morphogenesis has been well described (for reviews, see Papermaster and Schneider 3 and Beharse 4), but the regulatory mechanisms through which developing photoreceptors acquire the unique capacity to form and maintain outer segments remain poorly understood. Organ culture experiments in rana, 5 Xenopus, 6 and mouse 7 and experimental retinal detachment in vivo 8 have shown that outer-segment development and maintenance requires the presence of the retinal pigment epithelium (RPE); stimulatory effects have been reported for RPE-conditioned medium 9 and for sugars such as lactose. 10 Outer-segment formation and maintenance, on the other hand, can be disrupted by protein glycosylation inhibitors, 11 phospholipid synthesis inhibitors, 12 cytoskeletal inhibitors, 13 vitamin A deficiency, 14 and genetic mutations 15; in many of these cases, outer-segment abnormalities eventually are followed by photoreceptor death and blindness.

The photoreceptors that develop in cultures of cells dissociated from ED 5–8 chick retina express many cell-specific properties in a coordinated manner, including synthesis of visual pigments and other photoreceptor-specific macromolecules; structural and molecular polarity; mechanisms for the synthesis, release, and uptake of neuromodulators; and light responsiveness. 16 Researchers have hypothesized, therefore, that many aspects of photoreceptor development are regulated by a master plan acquired by photoreceptor precursors in early developmental stages. 16 Outer-segment–like processes sometimes have been observed in these cultures; however, the capacity of isolated photoreceptor cells to develop and maintain these highly differentiated processes has never been investigated in detail. Because outer-segment formation is a late event in photoreceptor differentiation, starting only after ED 15–17 in chick embryos, 17 we decided first to investigate the capacity of photoreceptors isolated from ED 17 retinas to grow outer segments in vitro. Even when grown in the absence of pigment epithelial and glial cells, isolated cone- and rodlike cells can develop and maintain outer-segment processes. These processes accumulate visual pigment immunoreactive materials and contain irregularly arranged membranous discs reminiscent of those observed at early stages of outer-segment development in vivo. Outer-segment–like processes can also be formed in vitro by photoreceptors isolated for culture as long as 1 week before the onset of outer segment formation in ovo.

METHODS

Reagents

The following reagents were used: 199 and Eagle’s basal media (Gibco, Grand Island, NY); Hanks’ balanced salt solution (HBSS), Ca++- and Mg++-free HBSS, and 0.25% trypsin (Irvine Scientific, Santa Ana, CA); Fetal calf serum (lot #11112086, Hyclone, Logan, UT); type IV DNase, bovine serum albumin, linoleic acid–albumin complex, penicillin, poly-L-ornithine, paraformaldehyde, glutaraldehyde, phosphate-buffered saline (PBS), Triton X-100, D(+) galactose, and 5-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO); ABC kit, rhodamine-labeled peanut lectin and secondary antibodies (Vector Laboratories, Burlingame, CA); hydrogen peroxide (Baker, Philipsburg, NJ); Hepes buffer (Calbiochem-Boehringer Diagnostics, La Jolla, CA); Aquamount (Polysciences, Warrington, PA); and nuclear track emulsion NTB-2, D-170 developer (Eastman Kodak, Rochester, NY).

Tissue Culture Techniques

White Leghorn chick embryos were used in all these studies, which adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. Eyes enucleated from 17-day-old embryos were placed in HBSS, and an eye cup preparation was generated by excising the anterior segment and gently removing the vitreous body with watchmaker’s forceps. The eye cups were transferred to a dish with Ca++- and Mg++-free HBSS and incubated with the retina side down at room temperature, until retinas began to separate from the pigment epithelium (usually after 5 to 12 minutes). This separation was extended using watchmaker’s forceps, and the isolated retinas were transferred to fresh Ca++- and Mg++-free HBSS. Some retinas appeared completely free of RPE when viewed under the dissecting microscope at this stage, whereas others showed some areas with residual RPE, frequently (but not exclusively) near the ora serrata and choroid fissure. These areas were removed using tungsten needles.

Dissociation and culture protocols were as previously described for retinal cells from 8-day-old chicks. 18 Briefly, retinal fragments were incubated at 37°C, first in Ca++- and Mg++-free HBSS for 10 minutes and then in 0.25% trypsin and 1.85 U/ml DNase for an additional 20 minutes. Tissue fragments were rinsed gently in Eagle’s medium supplemented with 1% bovine serum albumin and dissociated into a single-cell suspension by mechanical trituration through a small-bore Pasteur pipet. Cell suspensions were diluted to 4 × 10^5 cells/ml in medium 199 supplemented with 10% heat-inactivated fetal calf serum, linoleic acid–albumin (110 µg/ml), penicillin (100 U/ml), and glutamine (2 mM). Two-milliliter aliquots were seeded in polyornithine-coated, 35-mm tissue culture plastic dishes and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.
TABLE 1. Quantitative Analysis of Cell Survival in ED 17 Chick Retinal Cultures

<table>
<thead>
<tr>
<th>In Vitro Stage</th>
<th>Total</th>
<th>Morphologically Undifferentiated</th>
<th>Nonphotoreceptor Neurons</th>
<th>Photoreceptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hours</td>
<td>291 ± 80</td>
<td>291 ± 80</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2 days</td>
<td>174 ± 29</td>
<td>30 ± 10</td>
<td>64 ± 22</td>
<td>81 ± 15</td>
</tr>
<tr>
<td>4 days</td>
<td>119 ± 30</td>
<td>17 ± 7</td>
<td>43 ± 25</td>
<td>59 ± 12</td>
</tr>
<tr>
<td>6 days</td>
<td>106 ± 26</td>
<td>13 ± 6</td>
<td>39 ± 20</td>
<td>58 ± 11</td>
</tr>
</tbody>
</table>

Values are the average from at least three dishes from two separate experiments ± standard deviation. Criteria for cell identification and sampling of the cultures were as discussed in Methods.

Retinal cells from ED 8 chick retinas were dissociated and cultured as previously described.18

Immunocytochemical Analysis

Cultures were rinsed in HBSS and fixed for 1 hour in 4% paraformaldehyde in PBS, which in some cases also contained 0.1% glutaraldehyde. After additional PBS rinses, cells were permeated with 0.25% Triton X-100 in PBS and blocked overnight in 10% serum (from the same species as the secondary antibody to be used). Primary antibodies were a sheep polyclonal antiserum against bovine rhodopsin (1:150), 3 monoclonal antibodies COS-1 (1:100 to 1:3000) and OS-2 (1:300), 21-22 Rho-4D2 (1:2500), 21,22 and 50-1B11 (undiluted culture supernatant, or 1:1000 ascitic fluid). 23 Rhodamine-labeled secondary antibodies were used for the polyclonal antibody, and the avidin-biotin “ABC” method was used for the monoclonal antibodies. Antibody specificities are indicated in the Results section and in the figure legends.

Lectin Cytochemical Analysis

Cultures were reacted with rhodamine-labeled peanut lectin as described previously. 24 Briefly, unfixed cultures were washed in Heps-buffered HBSS and incubated for 30 minutes at room temperature with 50 μg/ml lectin in Heps-buffered HBSS. In some cases, this incubation was carried out in the presence of 0.1 M D(+)-galactose. Cultures were then rinsed twice in Heps-buffered HBSS, and either given coverslips for microscopic observation or permeated with 0.25% Triton X-100 and processed for immunocytochemical analysis using the Rho-4D2 monoclonal antibody and a fluorescein-conjugated secondary antibody.

Examination With the Transmission Electron Microscope

Cells to be studied using the electron microscope were grown on Thermox coverslips pretreated with 50 μg/ml polyornithine. 18 The cells were fixed in 0.87% glutaraldehyde in PBSII (0.067 M PBS containing 3% sucrose) for 30 minutes at room temperature. They were then rinsed twice in the same buffer and post-fixed with a mixture of 0.15% osmium tetroxide, 0.5% K2Fe(CN)6, and 0.04% tannic acid in PBSII on ice for 15 minutes. After consecutive washes in PBSII, PBS (0.067 M), and distilled water, cultures were stained with 1% aqueous uranyl acetate for 90 minutes under aluminum foil, rinsed twice with distilled water, and dehydrated in an increasing series of acetones. The cultures were embedded in Epon, sectioned with a diamond knife, stained with uranyl acetate and Sato’s mixed lead solution, 25 and observed with a Hitachi (Woodbury, NJ) H-800 electron microscope.

Neurotransmitter Uptake and Autoradiographic Evaluation

The radioactive precursors used were [3,4-3H]L-glutamic acid (specific activity, 56 Ci/mmole) and [2,3,4-3H]γ-aminobutyric acid (specific activity, 89 Ci/mmole) from Amersham (Arlington Heights, IL). The cultures were rinsed in THM (40 mM Tris-Hepes, 1 mM MgCl2, 0.1 mM CaCl2, 5 mM KCl, and 140 mM NaCl, pH 7.4), incubated with the appropriate isotope in THM at 37°C for 15 minutes, rinsed in THM, and fixed in glutaraldehyde. After dehydration with an increasing ethanol series, cultures were coated with Kodak nuclear track emulsion NTB2 for autoradiography. 26 After 5- to 7-day exposures in the dark, autoradiograms were developed in Kodak D170 developer, washed in tap water, and fixed for 3 minutes in 5% sodium thiosulfate.

Quantitative Analysis of ED 17 Cultures

All studies were done on a minimum of three replicate dishes from each of two separate experiments, with the data representing means ± standard deviations. In all cases, the investigator was unaware of the identity of the sample being studied. The total number and relative frequency of each cell type in cultures fixed at 3 hours and at 2, 4, and 6 days after seeding was determined by counting the cells in 25 randomly selected fields under 400X magnification using an inverted phase-contrast microscope. Cells were identified as either morphologically undifferentiated round cells,
FIGURE 1. Phase-contrast photomicrographs of dissociated ED 17 chick embryo retinal cells grown in vitro for 3 hours (A,E), 2 days (B), 4 days (C), and 6 days (D,F). Note that at culture onset cells appear morphologically undifferentiated, with a circular outline and no cell processes. Photoreceptors (long arrows) and nonphotoreceptor neurons (short arrows) already can be recognized after 2 days in vitro. The presence of outer-segment-like processes, suggested by phase-contrast microscopic examination (F, arrowhead), was verified by immunocytochemical and electron microscopic analysis (see Figs. 3 and 4). Magnification bar corresponds to 20 μm (A to D) and to 13 μm (E,F).

photoreceptors, or nonphotoreceptor neurons according to previously described criteria. The 25 random fields correspond to 1/8 of the dish area. For cytochemical and immunocytochemical experiments, the relative frequency of stained cells was determined by counting 100 to 200 randomly selected photoreceptors under 400X magnification.

RESULTS

ED 17 Neural Retina Isolation and Dissociation
Isolation of the neural retina from the RPE, readily accomplished until ED 14, becomes difficult after outer segments begin to form (ED 15 to 17). Pilot studies showed that the tight adhesion between RPE and neural retina observed at ED 17 could be loosened by incubation in Ca++- and Mg++-free HBSS, allowing the separation of most, or even all of the retina from the RPE. Absence of RPE from neural retina tissue used for dissociation was verified by visual inspection under the dissecting microscope, and by the absence of RPE cells from the cultures. After mild trypsinization, RPE-free retinas yielded well-dispersed cell suspensions, with an average of 26 ± 6 x 10^6 cells per retina (n = 15). More than 85% of the cells excluded dye in a trypan blue assay (n = 5).

Overall Development of ED 17 Cultures
Table 1 summarizes the results and Figure 1 illustrates them. Cell attachment was essentially complete 3 hours after seeding (the earliest time examined), when cultures showed an average of 291 ± 80 x 10^3 cells per dish area.
cells/35 mm dish. At this stage, nearly all cells showed a circular outline and lacked cell processes (Figs. 1A–E). After 2 days in vitro (div) (Fig. 1B), approximately 46% of the cells could be identified as photoreceptors based on previously described morphologic criteria (elongated cell body; a single, short neurite; presence of a lipid droplet; see Figure 1F and Adler.16,28 The remaining cells appeared either as morphologically undifferentiated, process-free round cells (17%) or showed a larger, polyhedral cell body and several processes, characteristics of nonphotoreceptor, multipolar neurons (37%; see Adler et al.31). Photoreceptors continued to be the most abundant cell type after 4 to 6 div (Figs. 1C, D), but the total number of cells per culture decreased by 6 div to an average of \(10^6 \pm 26 \times 10^3\) cells/dish. Analysis of the cultures by propidium iodide staining showed frequent pyknotic images, suggesting the involvement of apoptotic mechanisms in the death of the cultured cells (data not shown).

**Autoradiographic Analysis of ED 17 Cultures**

Previous studies using ED 8 cells showed that retinal cells develop energy- and Na\(^+\)-dependent, high-affinity uptake mechanisms for putative amino acid neurotransmitters, with \(\gamma\)-aminobutyric acid being taken up predominantly by nonphotoreceptor neurons, and glutamate being taken up by the photoreceptors; these uptake mechanisms were investigated in ED 17 cultures to evaluate the differentiation and integrity of neurons and photoreceptors. Autoradiographic

### TABLE 2. Outer Segment Frequency in ED 17 Photoreceptors After 6 Days In Vitro

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunoreactive PhR (% of total PhR)</th>
<th>PhR With Outer Segment (% of immunoreactive PhR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep antibovine rhodopsin</td>
<td>98 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>Rho-4D2</td>
<td>17 ± 9</td>
<td>51 ± 22</td>
</tr>
<tr>
<td>COS-1</td>
<td>56 ± 23</td>
<td>56 ± 22</td>
</tr>
<tr>
<td>OS-2</td>
<td>52 ± 25</td>
<td>51 ± 18</td>
</tr>
<tr>
<td>50-1B11</td>
<td>22 ± 9</td>
<td>65 ± 15</td>
</tr>
</tbody>
</table>

Values are the average of three dishes from each of three separate experiments \((n = 9)\), ± standard deviation. Specificity of each antibody, sampling of the cultures, and criteria for outer segment identification were as described in the text. PhR = photoreceptors; ND = not determined.
analysis showed heavily labeled cells in cultures incubated with $10^{-7} - 10^{-8}$ M $[2,3,4^{-3}H]\gamma$-aminobutyric acid or $^3$H-glutamate in the presence of Na$^+$ (Fig. 2) but not in its absence (not shown). With $10^{-8}$ M $[2,3,4^{-3}H]\gamma$-aminobutyric acid, heavy labeling was observed in multipolar neurons and in some morphologically undifferentiated cells, but photoreceptors appeared unlabeled (Figs. 2A, B). Higher $[2,3,4^{-3}H]\gamma$-aminobutyric acid concentrations yielded similar patterns, although some photoreceptors sometimes showed scattered grains (not shown). With $^3$H-glutamate, on the other hand, labeling was observed predominantly in photoreceptor cells, with nonphotoreceptor neurons showing only light labeling (Figs. 2C, D).

Cytochemical and Immunocytochemical Characterization of Cultured ED 17 Photoreceptor Cells

Table 2 and Figure 3 summarize the results of cytochemical and immunocytochemical characterization of cultured ED 17 photoreceptor cells. Most (96% to 100%) of the photoreceptors were immunoreactive with a sheep antiserum against bovine rhodopsin (Figs. 3A, B), which correlates with widespread photoreceptor immunostaining observed with this antibody in the chick embryo retina in vivo28 (data not shown). Photoreceptor subsets were immunoreactive with cone-specific antibodies 50-1B11,11 OS-2 and COS-1,19,20 or with the rod-specific Rho-4D2 monoclonal antibody.21,22 Their average relative frequency (from three separate experiments run in triplicate), was 22 ± 9% (50-1B11), 52 ± 25% (OS-2), 56 ± 23% (COS-1), and 17 ± 9% (Rho-4D2). There was much less variability between replicate dishes within individual experiments than between different experiments (not shown). Given that the immunoreactivity of some of the antibodies used in these studies is affected by light,20,22 the randomness of light conditions during the culture period may have contributed to this inter-experimental variability. The degree of polarity achieved by isolated photoreceptors in the distribution of immunoreactive materials was remarkable. In more than 50% of the cells (Table 2), immunoreactivity was concentrated either exclusively (Figs. 3E, F, Figs. 3J, I, Figs. 3Q, R) or predominantly (Figs. 3A, B, Figs. 3M, N; see below) in an outer-segment–like process. In photoreceptors without outer segments, immunoreactivity was found predominantly in the inner segment (Figs. 3E,F, Figs. 3I, J, and Figs. 3M, N) and was practically never seen in the nucleus-containing cell body or in the axonal process.

Approximately 90% of the cultured photoreceptors showed binding of rhodamine-labeled peanut lectin, usually polarized to the inner segment plasma membrane (Figs. 3C, D) and completely abolished in the presence of $\beta$-galactose (not shown). In some cases, peanut lectin-positive membranous materials could be seen projecting from the inner segment onto the culture substratum (Fig. 3). Double-labeling experiments with Rho-4D2 and peanut lectin were performed to determine whether rodlike cells also displayed peanut lectin binding; the experiments showed
The presence of outer-segment-like processes in vitro is suggested by sequential studies with the 50-1B11 antibody. Other outer-segment-like processes appeared elongated in some cells, but their length and extent of their peanut lectin fluorescence was usually weaker than in Rho-4D2-negative photoreceptors (see Figs. 3S, T).

Light Microscopic and Immunocytochemical Evidence of Outer-Segment Development in ED 17 Cultures

The presence of outer-segment-like processes in cultured photoreceptors was suggested by phase-contrast microscopy (Fig. 1F), and supported by observations in immunostained preparations (Fig. 3). Outer-segment-like processes are defined here as apical processes, emerging from the photoreceptor inner segment, with a three-dimensional appearance and marked accumulation of COS-1, OS-2, 50-1B11, or Rho-4D2 immunoreactivity (Fig. 3). Their three-dimensional appearance (sometimes more readily apparent by changing the focal plane of the microscope than in two-dimensional micrographs) is an important feature, because it allows outer-segment-like processes to be distinguished from thin, narrow, flat membranous inner-segment projections that, although usually undetectable using the phase-contrast microscope, are immunoreactive with visual pigment antibodies (Fig. 3B). Outer-segment-like processes appeared elongated in some cells, but their length varied (compare the short 50-1B11-positive process in Figures 3Q, R with the COS-1-positive process in Figures 3E, F). Other outer-segment-like processes had a more pear-shaped configuration and showed a nonuniform distribution of immunoreactive materials (Figs. 3I, J). Finally, in some cases outer-segment-like processes overlapped with the inner segment, requiring focal plane changes for proper microscopic identification (Figs. 3M, N). We could not correlate particular outer-segment shapes with any of the antibodies used.

Quantitative analysis of cultures grown in vitro for 6 days showed heavily stained outer-segment-like processes in 50% to 65% of the photoreceptors immunoreacted with either COS-1, OS-2, 50-1B11, or Rho-4D2 antibodies (Table 2). That these processes develop in vitro is suggested by sequential studies with the 50-1B11 and OS-2 antibodies, which showed that, 3 hours after culture onset, outer segments were either undetectable (50-1B11) or were present in only 5% of immunoreactive cells (OS-2) (Table 3).

Electron Microscopic Analysis of Outer-Segment-like Processes in ED 17 Cultures

The transmission electron microscope was used to further characterize the outer-segment-like processes. For 3-day cultures, 297 randomly selected cells were studied; for 6-day cultures, 62 cells were studied. Respectively, 64% and 42% of the cells showed a cilium in 3- and 6-day cultures, whereas oil droplets were observed in 54% of the cells at both in vitro stages. The cilia, which always emerged from the photoreceptor inner-segment region near the area occupied by mitochondria, the oil droplet, or both, showed no central microtubules (as is typical in photoreceptors) but in many cases had distal expansions (Figs. 4B, F) and vesicular elements (Figs. 4E, G). In addition, disclike profiles were observed in 35% and 54% of the processes in 3-day and 6-day cultures, respectively, with substantial variability from cell to cell in number and orientation of the discs (Fig. 4). This ultrastructural configuration is reminiscent of that previously described for early stages of outer-segment development in vivo (reviewed in Papamaster and Schneider and in Beharse). It is noteworthy that outer-segment-like processes were found in photoreceptors devoid of intercellular contacts and in those showing different degrees of contact with other cells.

Outer-Segment Development in Cultures of ED 8 Retinal Cells

As already noted, outer-segment formation cannot be detected during chick embryo retinal development in ovo until embryonic day 15. Therefore, we decided to investigate whether photoreceptor precursor cells isolated before this stage, and grown in the absence of pigment epithelium and glial cells, can also form outer segments in vitro. Cultures of embryonic day 8 were grown for 6 days in vitro and processed immunocytochemically with Rho-4D2 or 50-1B11 monoclonal antibodies. Quantitative analysis of triplicate cultures showed that 50 ± 7.5% of the photoreceptors were immunoreactive with the 50-1B11 antibody, with 22.8 ± 4.2% of the immunoreactive photoreceptors showing an outer-segment-like process. Only 7 ± 2% of the photoreceptors were immunoreactive with Rho-4D2, and 7.6 ± 2.3% of these showed an outer-segment-like process. Outer-segment formation has also been observed in cultures of retinal cells isolated between ED 9 and ED 15 (data not shown).

Cultures of ED 8 retinal cells were also fixed for electron microscopic examination after 6 days in vitro. Eighteen of 101 photoreceptor cells with oil droplet (17.8%) showed a ciliary expansion suggestive of outer-segment development. Moreover, 9 of the 18 ciliary expansions showed membranous disclike structures. In the example shown in Figure 5, the membranous discs appeared remarkably well organized, but in other cases the disclike structures were more randomly distributed within the outer-segment-like process.

DISCUSSION

The studies reported here can be summarized as follows. Cells obtained by dissociation of ED 17 retinas...
TABLE 3. Developmental Changes in Outer Segment Frequency in ED 17 Photoreceptors (PhR) In Vitro

<table>
<thead>
<tr>
<th>Time In Vitro</th>
<th>Immunoreactive PhR (% of total PhR)</th>
<th>PhR With Outer Segment (% of immunoreactive PhR)</th>
<th>Immunoreactive PhR (% of total PhR)</th>
<th>PhR With Outer Segment (% of immunoreactive PhR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hours</td>
<td>6 ± 2*</td>
<td>6 ± 6*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 days</td>
<td>46 ± 29</td>
<td>42 ± 22</td>
<td>16 ± 8</td>
<td>76 ± 24</td>
</tr>
<tr>
<td>4 days</td>
<td>59 ± 28</td>
<td>36 ± 9</td>
<td>27 ± 15</td>
<td>61 ± 28</td>
</tr>
<tr>
<td>6 days</td>
<td>62 ± 27</td>
<td>44 ± 17</td>
<td>22 ± 11</td>
<td>65 ± 26</td>
</tr>
</tbody>
</table>

Values are the average of five dishes from two experiments. Specificity of the antibodies, sampling of the cultures, and criteria for outer segment identification were as described in the text.

* Cells appeared morphologically undifferentiated at this stage; therefore, the frequency of immunoreactive cells is expressed as percent of the total number of cells.

PhR = photoreceptors.

appear morphologically undifferentiated as culture begins. Between 2 and 6 div, approximately 50% of the ED 17 cells express photoreceptorlike phenotypic properties; 35% appear as nonphotoreceptor, multipolar neurons; and the remaining cells retain a morphologically undifferentiated appearance. Nearly 100% of the cultured ED 17 photoreceptors are immunoreactive with a sheep polyclonal antiserum against bovine rhodopsin; subsets of photoreceptors are immunoreactive with monoclonal antibodies specific for cones (OS-2, COS-1, or 50-1B11) or rods (Rho-4D2). Isolated photoreceptors develop and maintain a highly polarized phenotype in culture, detectable at the structural level by a compartmentalized organization, and at the molecular level by the asymmetric distribution of visual pigment immunoreactivity and peanut lectin binding. Outer-segment–like processes can be identified in the ED 17 cultured photoreceptors by the accumulation of immunoreactive materials and ultrastructurally by the presence of characteristic membranous discs. And outer-segment–like processes could also be identified by immunocytochemical analysis and using the electron microscope in cultures of retinal cells isolated as early as embryonic day 8; that is, several days before outer-segment formation begins in vivo.

Although mature central nervous system neurons are generally less amenable than their less-differentiated counterparts to growth in dissociated cell culture, adult retinal cell cultures from amphibians,29 rats,30 and humans31 have been reported. In humans, more than 98% of the neuronal elements were identified as rod photoreceptors,31 apparently caused by lower photoreceptor susceptibility, compared with other neuronal cells, to the trauma of tissue dissociation. A similar mechanism may explain the predominance of photoreceptors in cultures from ED 17 chick embryo, because the retina has a mature organization at this stage; nonphotoreceptor neurons were also present in these cultures but were less abundant than in preparations from ED 6–8 chick retinas.16,24

Based on the reported specificity of various antibodies, the subsets of photoreceptors immunoreactive with OS-2, COS-1, and 50-1B11 can be tentatively identified as conelike.10,20,25 On the other hand, rodlike photoreceptors, representing 10% to 20% of the total, could be identified based on their immunoreactivity with the Rho-4D2 antibody, which recognizes an N-terminus epitope in rhodopsin.21,22 No obvious morphologic differences between Rho-4D2 (+) and Rho-4D2 (−) photoreceptors were observed in cultures; it must be noted, however, that the presence or absence of the lipid droplets that are characteristic of avian cone cells could not be used as a criterion, because the use of detergents during the immunocytochemical procedure frequently results in their extraction. As many as 80% of the Rho-4D2 (+) cells showed some peanut lectin binding, usually confined to the inner segment plasma membrane; this is not necessarily inconsistent with their identification as rods because, although peanut lectin (+) matrix sheaths are usually associated with the outer segments of cones and not of rods,32,33 peanut lectin (+) materials have also been detected on the plasma membrane of other retinal cells.34

Like other retinal cells, ED 17 photoreceptors seem to undergo a process of morphologic dedifferentiation as a result of tissue dissociation, which is reflected in the initial appearance of the cultures as a homogeneous population of process-free, round cells. The low density of the cultures, in which cells develop in the absence of contact-mediated cell interactions, provided a test of the capacity of embryonic photoreceptors to regenerate their characteristically elongated, compartmentalized and polarized phenotype. That they do so is consistent with the hypothesis proposed on the basis of studies with ED 8 retinal cells, suggesting that the structural and molecular polarity...
outer-segment development in vitro

FIGURE 4. Electron micrograph of ED 17 photoreceptors grown in culture for 3 days (see the Methods section). (A) Outer-segment-like processes (arrow) in a photoreceptor cell devoid of a lipid droplet. (B,C) Photoreceptor cell containing a lipid droplet (asterisk) and an outer segment with several membranous discs (arrows). (D to G) Details of ciliary expansions from several photoreceptors. Elements found within these processes include small tubulovesicular structures (D, arrow), vesicles (E to G), and membranous, disc-like structures (E-G, arrowheads). Magnification bar corresponds to 0.5 μm.

of cultured photoreceptors is largely controlled by intracellular determinants, acting in a cell autonomous manner. It remains to be determined whether microtubule- and actin-dependent forces, similar to those described in previous studies, are also active in the more mature photoreceptors used in this study.

The cell autonomy of photoreceptor polarity also can be detected at the molecular level. In more than 50% of the ED 17 photoreceptors expressing visual pigment immunoreactive materials in vitro, the latter appeared predominantly or even exclusively concentrated in an outer-segment-like process. This suggests that mechanisms similar to those responsible for the vectorial transport or sequestration of visual pigment molecules in photoreceptor outer segments in vivo may be active in isolated photoreceptors devoid of intercellular contacts. Outer-segment processes rarely could be detected at culture onset; given that their development begins in the chick retina around ED 15, their apparent absence from freshly isolated ED 17 cells may be due to the traumatic effects of the enzymatic and mechanical treatments necessary for tissue dissociation. In any event, the increasing frequency of photoreceptors with outer segments observed during the culture period suggests that photoreceptors isolated after the onset of outer-segment formation can continue this process, at least to some degree, even in the absence of the pigment epithelium. Equally remarkable is the observation that even cells isolated on ED 8, several days before the onset of outer-segment formation in vivo, can form outer-segment-like processes in dissociated cultures of this type. However, the ultrastructural features of outer-segment processes in the cultured cells resemble those seen at early stages of outer-segment development in vivo, ranging from the presence of a few vesicular elements within the cilium to the presence of fairly large cilary expansions, containing abundant disc-like profiles. The fact that a fully mature configuration, with discs arranged in parallel stacks, generally was not observed in the cultures is consistent with the possibility that the pigment epithelium, materials from the interphotoreceptor matrix, or both may be necessary for further outer-segment maturation, as suggested by studies with other systems. This culture preparation thus offers an additional bioassay for investigating these and other mechanisms involved in the induction and maintenance of outer segments in photoreceptor cells.

Key Words

cell culture, cell differentiation, cell polarity, chick embryo, outer segment, photoreceptor, retina, visual pigments.

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