Retinal Pigment Epithelium Wound Closure in Vitro

Pharmacologic Inhibition

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Early-passage bovine retinal pigment epithelium (RPE) cells were grown to confluence in 24-well plates, and a central defect was created mechanically in the monolayer within quadruplicate wells, sequentially over 9 days. Closure of the wounded area occurred by single-cell migration of elongated RPE cells from the edge of the wound and subsequent cell proliferation. Ten days after wounding, the cultures were fixed, stained, and photographed, and the residual wound area was quantified by computerized planimetry. Cell counts of unfixed cultures were determined with a Coulter counter. Wound closure was complete after 10 days. Using this technique, we assessed the response of RPE to various concentrations of 5-fluorouracil (5-Fu), colchicine (COL), and cytochalasin-B (CYT-B). 5-Fu (10 μg/ml) and COL (0.1 and 1 μg/ml) inhibited migration and proliferation of RPE cells. CYT-B (5 μg/ml) inhibited migration. This model allows in vitro study of the response of RPE cells after loss of contact inhibition. The technique provides a quantitative model for assessing the dynamic capabilities of RPE cells in response to a localized mechanical defect and for assessing the pharmacologic modulation of these responses. Invest Ophthalmol Vis Sci 31:481-488, 1990

Migration and hyperplasia of retinal pigment epithelium (RPE) cells are known responses to a full-thickness retinal tear with or without retinal detachment.1,4 The intensity of these reactions varies from a benign demarcation line to widespread dispersion of RPE cells within the vitreous cavity.1,2,5 Human pathology and experimental studies in vivo suggest that RPE cells are intrinsically involved in the formation of periretinal membranes causing proliferative vitreoretinopathy (PVR).5,7 The determinants governing the extent of RPE involvement after retinal tear and detachment are little understood. In vivo, RPE responds to localized trauma with both migration and proliferation.4,6

An in vitro model of the RPE response to various conditions may be helpful in understanding the pathophysiology of PVR. Previous efforts to examine RPE in vitro under varying growth conditions have assessed migration, proliferation, and cell contractility,9,10 all of which seem to occur in vivo. These experiments differed from ours in that they utilized nonconfluent cell cultures with time courses of only several hours.

With the techniques and experiments described here, we quantitated cell migration and proliferation over a 10-day period after localized "wounding" of a confluent RPE monolayer, and monitored the cellular responses to potential therapeutic agents. We found that certain antimetabolites at low concentrations are capable of producing a differential effect on RPE migration and proliferation.

Materials and Methods

Isolation of RPE

Bovine RPE was isolated and cultured with methods modifying several techniques described previously.11-12 Calf eyes were obtained within 2 hr after death, rinsed in sterile phosphate-buffered saline (PBS) containing 200 IU/ml penicillin + 200 μg/ml streptomycin (pen-strep) (Gibco, Grand Island, NY), and opened under sterile conditions. After mechanical removal of vitreous and retina, 2 ml of 0.1% trypsin-EDTA (1:250; Gibco) was placed in the eyecup and incubated for 10 min at room temperature. RPE cells were isolated into suspension by repeated irrigation of trypsin along the inner wall of the eyecup. The cell suspension was added in equal proportion to
Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% heat-inactivated calf serum (Gibco) + 200 IU/ml penicillin and 200 µg/ml streptomycin (Gibco) at pH 7.4. Cells were centrifuged for 10 min at 2000 rpm and resuspended in fresh DMEM.

RPE cells then were cultured on 60-mm dishes (Corning, Corning, NY), precoated with 1% gelatin (USP-Fisher Scientific, Fair Lawn, NJ), and incubated at 37.5°C in 95% air/5% CO₂. Culture medium was changed every 48 hr. Cells were released from confluence with 0.1% trypsin and transferred to gelatin-coated, 16-mm culture wells (first passage) within a 24-well plate (Corning) at an initial density of 20,000 cells/well. Cultures were incubated as above for 8 days. Some experiments were conducted with primary cultures of RPE cells that had been transferred to 100-mm dishes (first passage) and grown to confluence before culture in 16-mm wells (second passage). Thus experiments were conducted with either first- or second-passage RPE cells.

**In Vitro Wound Closure and Proliferation**

Wound closure was determined by a slight modification of a previously published method for corneal epithelium.14 Disks 6 mm in diameter were cut in 0.45-µm filter paper (Millipore, Bedford, MA) with a corneal trephine, rinsed eight times in distilled water at 100°C, and autoclaved. Upon confluence within 16-mm wells, four cultures of RPE were wounded (day 0) by pressing a filter disk against the center of the monolayer. The disk with adherent cells was then discarded. Each well was rinsed with 1 ml Earle's balanced salt solution (EBSS; Gibco) to remove non-adherent cells suspended in the course of manipulation. Four additional cultures on the same 24-well plate were wounded with this method on days 2, 4, 7, and 9. The last column was kept as an unwounded control (Fig. 1). Cultures were photographed daily with phase-contrast microscopy.

To demonstrate wound closure, on day 10 all wells were rinsed with PBS, fixed with 10% buffered formalin, and stained with 1% toluidine blue in 1% sodium borate. The stained plates were photographed with high-contrast black-and-white film (Kodak, Rochester, NY) for analysis of the residual wound area by computer-assisted planimetry (Videoplan 2; Zeiss, Oberkochen, West Germany). To demonstrate proliferation, comparable wounded cultures were treated with 0.1% trypsin and cell counts determined with a Coulter counter (Model ZF; Coulter Electronic, Hialeah, FL).

Wound closure and proliferative responses of wounded RPE were monitored with continuous exposure to 5-fluorouracil (5-Fu, 0.1-100 µg/ml; Sigma, St Louis, MO), colchicine (COL, 0.01-10 µg/ml; Calbiochem, La Jolla, CA), or cytochalasin-B (CYT-B, 1-20 µg/ml; Calbiochem). Culture medium containing these agents was changed every 48 hr. Dimethyl sulfoxide (DMSO, 99.9% spectrophotometric grade; Aldrich Chemical, Milwaukee, WI) was used to solubilize CYT-B at a 0.5% concentration in the medium.

![Fig. 1. RPE monolayers. a, Control, unwounded; b, Day 0, immediately after wounding; c, day 2; d, day 4; e, day 7; and f, day 9, after wounding. Each column shows four duplicate cultures.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933577/)
Results

Cultures reached confluence in 24-well plates after 10 days, at which time there were 43,600 ± 3200 cells/well. Immediately after creation of the central defect, 29,300 ± 2100 cells remained on the plate, representing a 33% cell loss due to wounding. The area of the central defect was 29 ± 0.8 mm², corresponding to less than 15% of the 201-mm² monolayer surface.

Controls (DME)

Single-cell migration at the wound margin was first noted by phase-contrast microscopy 12 hr after wounding. In the course of migration, the cells lost their polygonal configuration and assumed a spindle-shaped, elongated appearance (Fig. 2). Clusters of cells were noted in the wounded or denuded zone within 24 hr, and thereafter until day 9; these cells were smaller than cells in the original monolayer and contained less pigment. After attaining confluence, cells within the original wounded area assumed a polygonal shape. Cells in the unwounded monolayer that were at least eight cell diameters from the wound margin showed no apparent change in size or pigmentation.

Wound closure occurred linearly for the first 4 days, after which the curve became hyperbolic (Fig. 3). The wounded area was closed completely after 10 days, as determined by gross inspection of the stained cultures and by planimetry (area = 0 mm²). Cell density increased linearly with time until wound closure was complete; final cell counts were 42% higher than before wounding (62,000 ± 1000 vs 43,600 ± 3200; Fig. 4).

5-Fluorouracil (5-Fu)

After 24 hr of exposure to 100 µg/ml 5-Fu, there was progressive shrinkage and detachment of cells. Detached cells remained viable and could be cultured in fresh medium without 5-Fu if total exposure to this dose of 5-Fu was less than 48 hr. At a dose of 10 µg/ml, 5-Fu inhibited wound closure (Fig. 5) by preventing proliferation and presumably by preventing migration. The remaining cells were polygonal but somewhat balloononed, and the wound area was free of spindle-shaped, elongated cells. By day 9, 1 µg/ml 5-Fu resulted in a decreased rate of wound closure and a 58% decrease in proliferation (Figs. 3A, 4A). At a dose of 0.1 µg/ml 5-Fu, a decreased wound closure rate and a 29% reduction in proliferation were noted (Figs. 3A, 4A); migration persisted, as evidenced by the presence of flattened and stellate cells with a fibrillar cytoplasm within the wounded area (Fig. 6).

Colchicine (COL)

After 24 hr in the presence of 10 µg/ml COL, cell toxicity was evidenced by polymorphism and cell death. The cells did not regain normal growth patterns even though COL was removed after 24 hr and cultures were refed with DME. COL at 0.1 and 1 µg/ml inhibited wound closure and cell proliferation (Figs. 3B, 4B). RPE cells became larger and flatter but...
Fig. 3. Calculated residual area within RPE monolayers on specified days after wounding. Cultures were exposed to varying doses of (A) 5-fluorouracil (5-Fu), (B) colchicine (Col.), and (C) cytochalasin-B (Cyt-B).

Fig. 4. Total cell density on specified days after wounding of the RPE monolayer. Cultures were exposed to varying doses of (A) 5-fluorouracil (5-Fu), (B) colchicine (Col.), and (C) cytochalasin-B (Cyt-B).

maintained their polygonal shape (Fig. 7). No spindle-shaped cells were seen in the wound area despite the overall apparent vitality of the remaining monolayer. COL at 0.01 μg/ml decreased wound closure rate and proliferation significantly (Figs. 3B, 4B); however, cells continued to engage in the normal se-
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Fig. 5. Absence of cells within the wound margin (arrows) after exposure to 5-Fu (10 μg/ml) for 9 days after wounding (phase-contrast, ×50).

Fig. 6. RPE cells within the denuded zone after exposure to 5-Fu (0.1 μg/ml) for 9 days after wounding (phase-contrast, ×50).

quence of morphologic transformation to spindle-shaped cells after wounding.

Cytochalasin-B (CYT-B)

RPE cells retracted and detached from the plate after 48 hr in medium containing 20 μg/ml CYT-B. At 5 μg/ml CYT-B, inhibition of migration was evidenced by the absence of cells within the wounded area and by widened intercellular spaces. The cells assumed a stellate configuration (Fig. 8). Wound closure was inhibited and cell density was reduced (Figs. 3C, 4C). This concentration of CYT-B was not toxic; cells regained their normal appearance when returned to DMEM after 48 hr, whereas cells exposed to 20 μg/ml did not recover. CYT-B at 1 μg/ml delayed wound closure with no significant effect on proliferation (Figs. 3C, 4C). DMEM containing 0.5%
DMSO, which was used to solubilize CYT-B, had no effect on wound closure, cell shape, or proliferation of RPE cells after wounding.

**Discussion**

We have demonstrated a sensitive assay that quantifies the response of an RPE monolayer to wounding in vitro. This model allows quantification of wound closure and proliferation as well as observation of migration of RPE cells, processes that participate in the reformation of a monolayer in vitro and are critical events during healing in vivo. The pattern of closure of a circular wound produced by this in vitro model generated reproducible, quantifiable data. Therapeutic agents at various concentrations affected proliferation or wound closure, or both, with consistent dose-response correlations; toxic effects on RPE cells such as severe swelling or atrophy were obvious.

The migration pattern displayed by the RPE cells by phase-contrast microscopy suggested movement of single cells within the wounded area rather than movement of cell sheets, as has been seen for other
types of epithelia.\textsuperscript{14-16} Although cells well behind the wound margin did not demonstrate changes in their confluent, apparently nonmotile morphology, there may have been some migration of cells as a sheet within this zone. In this model, we interpret the change in cell shape to a spindle-shaped, fibroblast-like cell as a form of cell migration. Therefore, the polygonal cell shape appeared to be related to immobility, as seen in the confluent, contact-inhibited state.

Previous studies have described methods for monitoring the RPE response to chemoattractants and antiproliferative and antimigratory agents. These techniques used either a modified Boyden chamber to assess migration\textsuperscript{9} or a three-dimensional collagen lattice to study cell proliferation and contractility.\textsuperscript{10} Because conditions such as proliferative vitreoretinopathy (PVR) involve complex mechanisms, an in vitro investigation may require several techniques. The wound closure model should prove complementary to assays of chemotaxis and directed migration in extrapolating to the in vivo state. The inability to monitor three-dimensional cell migration, such as that which occurs in PVR, is a shortcoming of this wound closure model.

As shown in other in vivo\textsuperscript{17} and in vitro\textsuperscript{10,18} studies, 5-Fu does not affect cell contractility or migration. Contractility was not assessed in this model; however, 5-Fu at 10 \(\mu g/ml\) inhibited RPE migration over 10 days. The inability to close the wound may have been due to inhibition of proliferation and a concomitant reduction in “back pressure” at the wound edge. The sensitivity of cells to 5-Fu may vary according to confluence status and cell-to-cell interaction. The absence of an effect of 5-Fu on migration observed in the modified Boyden chamber model over a course of several hours may have been the result of minimal contact inhibition within the nonconfluent culture.

5-Fu at a dose as low as 0.1 \(\mu g/ml\) was noted in our model to reduce significantly cell proliferation, as well as migration, similar to a dose of 0.5 \(\mu g/ml\) previously reported to inhibit fibroblast growth in vitro.\textsuperscript{10}

COL, a vinca alkaloid, binds cytoplasmic and nuclear tubulin, and therefore, in the range of 0.01–1 \(\mu g/ml\), is a potent inhibitor of cell migration and division.\textsuperscript{20} In other studies,\textsuperscript{10} the anticontractile effect of COL was present even at a dose of 0.05 \(\mu g/ml\) (\(10^{-8}-10^{-10} M\)) without an antiproliferative effect. Despite reports of in vivo toxicity\textsuperscript{21,22} involving irreversible electrophysiologic changes with intravitreal injections of 10–100 \(\mu g/ml\) (2.5–25 \(\mu M\)), other reports\textsuperscript{23,24} suggest that COL may be a potent drug against PVR. Pharmacokinetic assays must be performed to establish the washout of COL from the vitreous cavity, as has been done for other drugs.\textsuperscript{25,26}

CYT-B inhibits cell migration presumably through its depolymerizing effect on filamentous actin.\textsuperscript{27} At a dose of 1 \(\mu g/ml\), the change in cell shape to the stellate configuration still permitted migration and proliferation. In contrast, a higher but nontoxic dose of CYT-B (5 \(\mu g/ml\)) inhibited migration into the wounded area and significantly reduced cell counts compared with controls, perhaps as a result of continued contact inhibition among cells within the unwounded area. CYT-B alone or in combination with other agents may be of considerable clinical interest for its ability to prevent the initial migration of membrane-forming cells.

Prevention and optimal treatment of PVR require a detailed understanding of the kinetics and mechanisms of periretinal cell growth. In vitro models of cell proliferation and migration can screen potential therapeutic agents. Our findings suggest that 5-Fu, COL, and CYT-B may prove effective in treating PVR and merit further investigation. In addition, pharmacologic manipulation of tissues such as RPE require a more fundamental study of cellular response than that presented here, and should include the effect of these agents upon other vital cellular functions, such as phagocytosis, exocytosis, and complex membrane actions. Biochemical and biophysical analysis of the wounded monolayer, in addition to measurement of the secondary effects of migration and proliferation, will certainly enhance our understanding of a drug’s action and the chance of therapeutic success.

**Key words:** retinal pigment epithelium, 5-fluorouracil, colchicine, cytochalasin-B, proliferative vitreoretinopathy

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