

# Mitogen-Activated Protein Kinase Inhibitor, PD98059, Inhibits Rat Retinal Pigment Epithelial Cell Replication by Cell Cycle Arrest

Katsuhiko Yamaguchi, Hiroshi Tomita, Eriko Sugano,  
Toru Nakazawa and Makoto Tamai

*Department of Ophthalmology, Tohoku University School of Medicine, Sendai, Japan*

**Purpose:** To investigate the effect of PD98059, a mitogen-activated protein kinase (MAPK) inhibitor, on the replication of rat cultured retinal pigment epithelial (RPE) cells.

**Methods:** Growth-phase rat RPE cells were exposed to various concentrations of PD98059 in serum-free F12 medium containing 0.1% dimethyl sulfoxide. Cell proliferation was assessed by cell counts using a hemocytometer. Cell viability was tested by CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay at 24 hours after PD98059 application. Hoechst 33258 and propidium iodide staining were used to assess nuclear morphology. Immunostaining with Ki67 antibody was used for cell cycle analysis because the staining patterns produced on cells are characteristic depending on their position within the cell cycle.

**Results:** PD98059 inhibited cellular proliferation of cultured rat RPE cells in a dose-dependent manner but did not induce cell death. Twenty-four hours after the application of PD98059, cultured RPE cells were not immunopositive for Ki67, indicating that their cell cycle was arrested in the G0/G1 phase.

**Conclusion:** These results demonstrated that MAPK inhibition arrested cell cycle progression of rat cultured RPE cells at the G0/G1 phase. The pharmacological induction of cell cycle arrest could be a new approach to inhibit cellular proliferation in such conditions as proliferative vitreoretinopathy. **Jpn J Ophthalmol 2002;46:634–639** © 2002 Japanese Ophthalmological Society

**Key Words:** Cell cycle, Ki67, mitogen-activated protein kinase inhibitor, PD98059, retinal pigment epithelium.

## Introduction

Retinal detachment, the physical separation of the neural retina from the retinal pigment epithelium (RPE), can induce a rapid activation of the cell cycle in RPE cells.<sup>1,2</sup> These activated cells can then migrate to the vitreoretinal intersurface and contribute significantly to the epiretinal membranes found in

eyes with retinal detachment.<sup>3</sup> The resultant membranes can distort the retina and exert tractional forces that may lead to proliferative vitreoretinopathy (PVR).<sup>4</sup> Thus, the surgical treatment of retinal detachment that is complicated by an advanced degree of PVR is difficult, and may require vitrectomy in addition to the conventional scleral buckling.

Pharmacological intervention to stop cell cycling and thus inhibit cellular proliferation has been used therapeutically to prevent PVR.<sup>5</sup> For example, drugs such as dexamethasone,<sup>6</sup> triamcinolone,<sup>7</sup> and colchicine,<sup>8</sup> which inhibit cellular proliferation and contraction, have been employed to control PVR. Earlier studies have shown that antiproliferative agents

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Correspondence and reprint requests to: Katsuhiko YAMAGUCHI, MD, PhD, Department of Ophthalmology, Tohoku University School of Medicine, 1-1 Seiryomachi, Sendai 980-8574, Japan

such as taxol,<sup>5</sup> 5-fluorouracil,<sup>9</sup> daunomycin,<sup>10</sup> and mitomycin C<sup>11</sup> were effective in controlling the development of PVR. Although these drugs were useful in inhibiting cellular proliferation, they are cytotoxic.

We have investigated the effects of PD98059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one], a mitogen-activated protein kinase (MAPK) inhibitor, on the cell cycling of cultured RPE cells. PD98059 was discovered by screening a chemical library for inhibitors of the MAPK cascade and has been used to determine the role of MAPKs in a variety of biological situations.<sup>12,13</sup> MAPKs (sometimes called extracellular signal-regulated kinases, or ERKs) are thought to be activated by the dual specificity kinase, MAPK/ERK kinase (MEK). PD98059 selectively blocks the activity of MEK, by inhibiting phosphorylation and activation.<sup>14</sup> To study the mechanism of PD98059 action on cultured RPE cells, we used the Ki67 antibody that induces characteristic staining patterns in cells depending on their position within the cell cycle.<sup>15-18</sup> The purpose of this study was to show that PD98059 inhibits the replication of RPE cells by arresting the RPE cells at the G0/G1 phase without subsequent cell death.

## Materials and Methods

### *Cell Preparation and Culture*

All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. RPE cells were isolated as described in detail earlier.<sup>19</sup> In brief, the eyes of Long-Evans rats (6-8 days old) were enucleated, and the whole eyes were incubated at 37°C in two enzyme-containing solutions. The first incubation was for 40 minutes in Hanks balanced salt solution (HBSS, pH 7.0) containing 78 U/mL of collagenase (Wako, Osaka) and 38 U/mL of hyaluronidase (Wako). The second incubation was for 50 minutes in HBSS containing 0.1% trypsin (Sigma, St. Louis, MO, USA; 1:250). Eyes were then placed in a growth medium containing Ham F12 medium (BRL, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) and antibiotics (100 units penicillin, 0.1 mg streptomycin, and 0.25 µg amphotericin B per mL of medium). The anterior segment, lens, and vitreous were removed, and the retina with the attached RPE was peeled from the posterior eye segment. The retina with adherent RPE was incubated in growth medium at 37°C. After 1 hour, the RPE was collected into a conical centrifuge tube, centrifuged at 1,000 g, and washed with growth medium. The RPE cells were dissociated into a single-cell suspension by tritura-

tion using a Pasteur pipette and inoculated into Ham F12 medium supplemented with 20% FBS. The medium was changed every 3 days. The cells were passaged by 0.125% trypsin/0.01% ethylenediaminetetraacetic acid digestion. Cells of three to five passages were used in the present study.

One day before starting the experiments, the cultured RPE cells were resuspended in serum-free F12 medium and seeded into a 96-well plate at a viable cell density of  $5 \times 10^3$  cells/100 µL per well. Growth phase cells were washed with serum-free F12 medium and exposed to various concentrations (0.2 µM, 2 µM, and 20 µM) of PD98059 (BIOMOL, Plymouth Meeting, PA, USA). PD98059 was first dissolved in dimethyl sulfoxide (DMSO), and then this solution was added to serum-free F12 medium to contain 0.1% DMSO in the final concentration. During this procedure, PD98059 was still soluble in the serum-free F12 medium. For the control, serum-free F12 medium containing 0.1% DMSO without PD98059 was used. We used the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), which uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) as the reagent, to determine the number of viable cells. At 24 hours after the PD98059 application, 20 µL of reagent solution was added to each well of the 96-well plate. After incubation at 37°C in humidified 5% CO<sub>2</sub> for 1 hour, we recorded the absorbance at 490 nm using a 96-well plate reader. In addition, cell proliferation was assessed by trypsinized cell counting with a hemocytometer at 24 and 48 hours after PD98059 application. Cell counts are expressed as mean ± SD. Statistical analysis was performed using StatView 4.11J software for Macintosh. The Bonferroni/Dunnett multiple range test was used for planned comparisons among groups with various concentrations of PD98059 application at 24 or 48 hours.  $P < .05$  was considered statistically significant.

### *Viable Cell Staining*

The Hoechst 33552 (Bisbenzimidazole-H33342-fluorochrome trihydrochloride; Calbiochem, San Diego, CA, USA) and propidium iodide (PI) stains were used to visualize nuclear morphology. In brief, at 24 hours after 20 µM of PD98059 application, RPE cells were washed with phosphate-buffered saline (PBS), and 200 µL of Dulbecco PBS containing 1 µg of Hoechst 33552 and 1 µg of PI were added. The RPE cells ( $5 \times 10^3$  cells/well) were incubated at room temperature for 15 minutes in the dark, and the wells

were washed. The cells were fixed in 2% paraformaldehyde/0.1 M PBS and examined under a fluorescence microscope with a DAPI rhodamine filter.

### Immunohistochemical Analysis

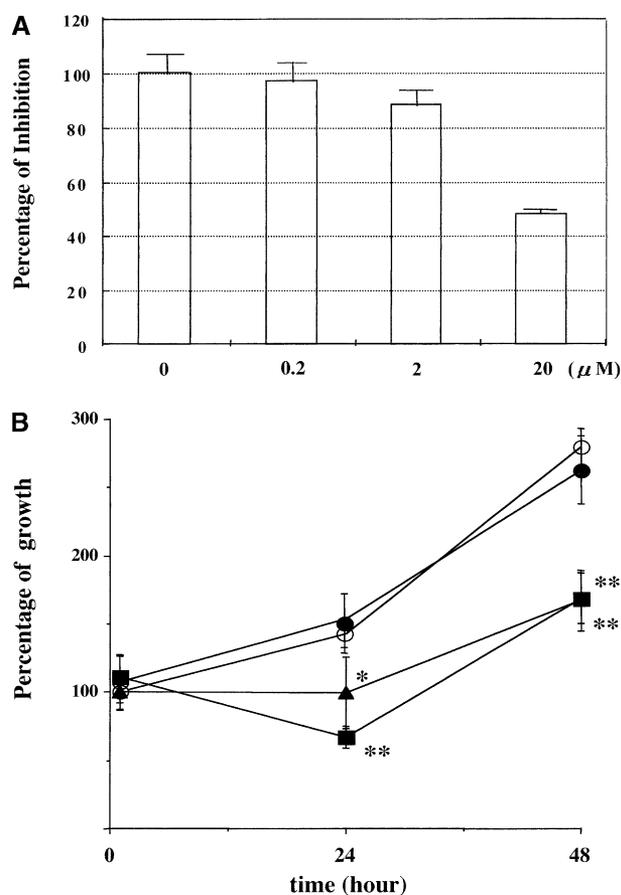
The cells were treated with 4% paraformaldehyde for 1 hour at 4°C and then rinsed with 0.5% Triton-X 100 in PBS for 5 minutes. The cells were inoculated with diluted anti-Ki67 antibody (Novocastra Laboratories, Newcastle Upon Tyne, UK; 1:200) and incubated for 1 hour and then rinsed with PBS three

times for 5 minutes each time. Next, rabbit anti-mouse IgG labeled with rhodamine (Rockland, Gilbertsville, PA, USA; 1:400) was added to each well and the cells were incubated at room temperature for 1 hour. The wells were then rinsed three times with PBS for 5 minutes each time, and examined by fluorescence microscopy with a rhodamine filter. Images were recorded by digital photomicrography.

## Results

### Cell Preparation and PD98059 Application

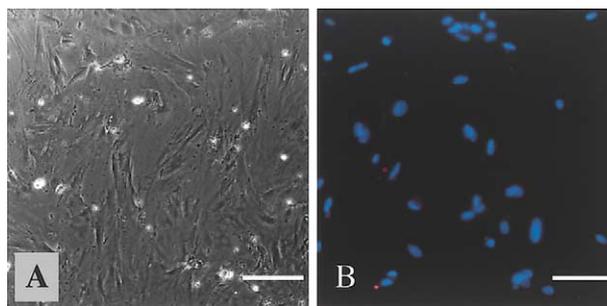
RPE cells from Long-Evans rats proliferated well in 20% FBS/F12 culture conditions. Using these proliferating cells, we examined the effects of PD98059 at concentrations of 0.2  $\mu$ M, 2  $\mu$ M, and 20  $\mu$ M, and control. CellTiter 96 AQueous One Solution Cell Proliferation Assay, performed at 24 hours after PD98059 application, demonstrated an inhibition of cellular proliferation in a dose-dependent manner (Figure 1A). Also, cell counts obtained by hemocytometer showed that application of 2 or 20  $\mu$ M of PD98059 resulted in inhibition of the proliferation of cultured RPE cells at 24 and 48 hours (Figure 1B).



**Figure 1.** Inhibition of retinal pigment epithelial (RPE) cell proliferation by PD98059. (A) CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, performed 24 hours after PD98059 application, demonstrates an inhibition of cellular proliferation in a dose-dependent manner. (B) Time course of RPE cell proliferation after application of PD98059. Application of 2 or 20  $\mu$ M of PD98059 results in inhibition of cultured RPE cell proliferation, compared to the control or 0.2  $\mu$ M of PD98059 application, respectively. Data are the mean  $\pm$  SD of six samples. ●: control, ○: PD98059—0.2  $\mu$ M, ▲: PD98059—2  $\mu$ M, ■: PD98059—20  $\mu$ M. \* $P$  < .05, \*\* $P$  < .01, Bonferroni/Dunnett multiple range test.

### Viable Cell Staining

In our experiment, the exposure of RPE cells to PD98059 did not cause cellular detachment from the well (Figure 2A). The adherent RPE cells demonstrated blue nuclei without any chromatin condensation. With PI staining, the nuclei of cultured RPE cells did not show any red staining from chromatin condensation (Figure 2B). In general, Hoechst 33552 stains the nuclei of all cells, while PI enters only late apoptotic and necrotic cells. These dyes fluoresce blue and red, respectively. Accordingly, viable cells



**Figure 2.** Viable and apoptotic cell staining. (A) Photomicrograph of the cultured retinal pigment epithelial (RPE) cells. (B) The adherent RPE cells exhibit no chromatin condensation on propidium iodide staining. Hoechst 33552 staining demonstrates no red chromatin condensation in the cultured RPE cells. Bars = 100  $\mu$ m.

demonstrate blue nuclei, early apoptotic cells show blue nuclei with chromatin condensation, late apoptotic cells demonstrate red nuclei with chromatin condensation, and necrotic cells shows red nuclei. Therefore, our results indicated that the cultured RPE cells remained viable after the treatment with PD98059. Based on these results, PD98059 was considered to stop the proliferation of cultured RPE cells without causing cellular mortality.

### *Immunohistochemical Analysis with Ki67*

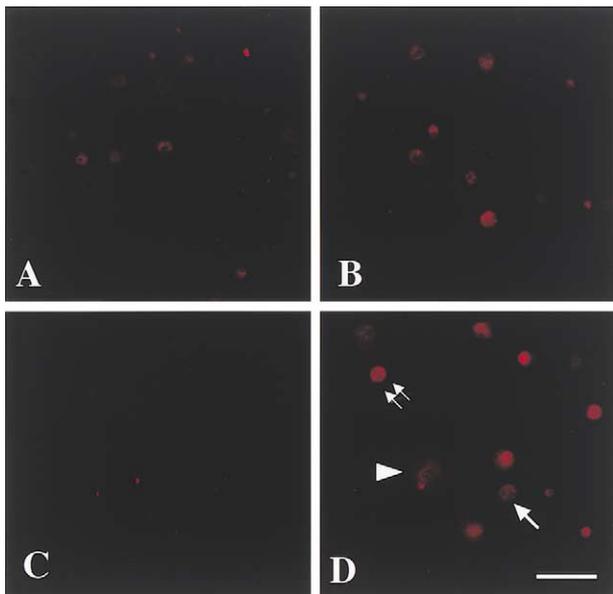
In the control, the Ki67 antibody produced several staining patterns in the cells, such as diffuse granular staining within the nucleus, a more distinct, large, dot-like staining in the nucleus, or reticulate staining that surrounded the condensed chromosomes. These staining patterns are considered to depend on their position within the cell cycle. Representative photomicrographs of typical Ki67 staining patterns of the cultured RPE cells are shown in Figure 3A. One

hour after application of 20  $\mu$ M of PD98059, no obvious change was seen in the nuclear staining of the RPE cells (Figure 3B). Typical Ki67 staining patterns were seen in the control at 24 hours (Figure 3D). Unstained cells were rarely seen. Diffuse granular staining within the nucleus was considered to represent cells in the G1 phase (Figure 3D, arrow). A more distinct, large, dot-like pattern was representative of the S/G2 phase (Figure 3D, double arrow). The M phase was characterized by mitotic figures stained with the Ki67. For example, Ki67 was distributed in reticulate structures surrounding the condensed chromosomes (Figure 3D, arrowhead). These results demonstrated that cultured proliferating RPE cells were in evidence in the late G1 phase through the M phase of the cell cycle. In contrast, at 24 hours after the application of 20  $\mu$ M of PD98059, there were no RPE cells immunopositive for Ki67 (Figure 3C). This is considered to indicate that these cells were in the G0/G1 arrest stage.

### **Discussion**

Adult RPE cells do not replicate normally but do so under some pathologic conditions such as PVR, and a large number of pharmacologic agents have been used to inhibit cellular proliferation in experimental models. An ideal drug suitable for the treatment of PVR should inhibit cellular proliferation without intolerable complications, and should also be effective when it is injected into the vitreous, as this will result in the highest concentration at the target site, and the avoidance, or at least the reduction, of systemic toxicity. The RPE cells form one component of the blood—retinal barrier and play an essential role in the function and survival of photoreceptors, including the phagocytosis of shed outer segments of rods and cones, and the synthesis and transportation of many substances.<sup>20</sup> Because of the importance of the RPE cells, it is preferable that the RPE cells on Bruch's membrane under the detached retina should not be killed by the antiproliferative agents. Among the antiproliferative agents studied, some induced multiple cellular responses, including apoptosis.<sup>21,22</sup> We have found that PD98059 inhibited RPE cell proliferation without cell death.

Ki67 antigen, a large basic protein having two forms with molecular weights of 345 and 395 kD, is the phenotype cell cycle-related nuclear protein expressed in proliferating cells in the G1, S, G2, and M phases, but not in the early G1 and G0 phases.<sup>23–25</sup> We detected every Ki67 staining pattern in the cell cycle in the nuclei of the proliferating cultured RPE



**Figure 3.** Immunohistochemical analysis with Ki67. (A) Photomicrograph of Ki67 staining of the cultured retinal pigment epithelial (RPE) cells demonstrates several patterns. (B) At 1 hour after application of 20  $\mu$ M of PD98059, no obvious change is seen. (C) At 24 hours after application of 20  $\mu$ M of PD98059, cultured RPE cells show no immunostaining, indicating these cells are in the cell cycle of G0/G1 arrest. (D) Ki67 staining patterns in the control at 24 hours. Diffuse granular staining within the nucleus represents cells in the G1-phase (arrow). More distinct, large dot-like staining is representative of the S/G2-phase (double arrow). Ki67 is distributed in reticulate structures surrounding the condensed chromosomes (arrowhead). Bars = 100  $\mu$ m.

cells, which indicates that the cells were in all active phases of the cell cycle from the late G1 phase through the M phase. In contrast, after treatment with PD98059, Ki67 staining was absent in the cultured RPE cells. Because Ki67 immunostaining is not evident in cells in the G0 and early G1 phases, unstained RPE cells in our experiment were considered to be in these phases. Thus, MAPK inhibition by PD98059 caused growth inhibition of RPE cells by arresting cells in the G0/G1 phase without subsequent cell death.

In our experiments, it was speculated that PD98059 exerted its inhibitory activity as a selective inhibitor of these MAPK-activating enzymes, MEK, and consequently, of the MAPK cascade. Geller et al<sup>26</sup> reported multiple reactive signaling responses after experimental retinal detachment, including phosphorylation of fibroblast growth factor receptor-1, and extended activated protein-1 expression, and ERK signaling. PD98059 may also be effective in controlling PVR in another way as it has been shown in experiments with human RPE cells that PD98059 is a potent inhibitor of fibronectin-induced haptotaxis and platelet-derived growth factor (PDGF)-induced chemotaxis.<sup>27</sup> These reports indicate that MAPK is critically involved in multiple components of RPE proliferation in vitro and suggests that PD98059 can also be a potential inhibitor of RPE proliferation by the inhibition of MAPK in vivo. Dudley et al<sup>14</sup> showed that the PDGF-stimulated tyrosine phosphorylation of immunoprecipitated MAPK was inhibited by preincubation with PD98059. Because PDGF is a major growth factor for RPE cells, we could speculate that PD98059 readily crosses the cellular membrane of RPE cells to exert its inhibitory activity.

In rheumatoid arthritis, a chronic inflammatory disease that involves multiple joints, synovial tissue hyperplasia results from a rapid cell cycle progression. Taniguchi et al demonstrated that cell cycle inhibition by p16INK4, a senescence gene, was an effective treatment for rheumatoid arthritis.<sup>28</sup> In a similar way, controlling the cell cycling of RPE cells is a possible way to treat cellular proliferative diseases of the eye. The capability of PD98059 to inhibit proliferation without cell death is a safer alternative to treat PVR than antineoplastic drugs. We believe further experiments on cell cycling will provide more clues on the mechanism of RPE cell proliferation in RPE-related disorders.

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