**OPC-12759 Increases Proliferation of Cultured Rat Conjunctival Goblet Cells**

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**Purpose:** To determine if the gastroprotective drug OPC-12759 increased proliferation of rat conjunctival goblet cells in culture.

**Methods:** Cultured goblet cells were incubated with 10−12 to 10−8 M OPC-12759 for 1 to 7 days. Fetal bovine serum (FBS) was used as a positive control. Cell proliferation was determined by a MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay and by immunohistochemical staining with anti–Ki-67, a marker of cell division. Goblet cells were identified by double-labeling with anti–Ki-67, a marker of cell division, and *Ulex europaeus* agglutinin I lectin, anti-MUC5AC and anticytokeratin 7. Stratified squamous cells were identified by using *Griffonia (Bandeiraea) simplicifolia* lectin and anticytokeratin 4 antibody.

**Results:** As determined by MTT conversion to formazan, OPC-12759 at 10−11 M induced an almost 2-fold increase in goblet cell proliferation on Days 1 and 3 of incubation but not on Days 5 and 7. The FBS at 10% increased cell proliferation by 2- to 3-fold at each time point. Daily replenishment of OPC-12759 for 3 consecutive days induced cell proliferation at all concentrations. Proliferation as determined by the number of Ki-67 positive cells increased by 4- and 3-fold at Days 1 and 3, respectively, with addition of 10−11 M OPC-12759. The FBS at 10% induced a 10-fold increase in goblet cell proliferation on Days 1, 3, and 5. Colocalization of *Ulex europaeus agglutinin* I, MUC5AC and anticytokeratin 7 with Ki-67 indicated that proliferating cells were goblet cells. Proliferating cells were negative for the nongoblet cell markers *Bandeiraea* lectin and anticytokeratin 4.

**Conclusions:** The OPC-12759 stimulates proliferation of conjunctival goblet cells in primary culture.

**Key Words:** Rebamipide, proliferation, conjunctiva, goblet cell

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Conjunctival goblet cells are the primary source for soluble mucin components of the tear film mucus layer. Mucin provides a physical and chemical barrier that shields the cornea and conjunctiva from harmful agents and aids in maintaining a smooth refractive surface essential for clear vision. In this regard, a decrease in the population of goblet cells or an inability of goblet cells to synthesize, store, and secrete normal levels of mucins can lead to an unstable tear film and drying of the ocular surface. These changes can culminate in a spectrum of ocular surface diseases known as dry eye syndromes (DES).

Attempts to develop therapeutic treatments for DES have been difficult because of the limited understanding of the underlying pathophysiological mechanisms. The traditional approach to treating DES is palliative, consisting primarily of lubricating eyedrops to supplement a patient’s natural tears or punctal occlusion to improve the residence time of the patient’s own tears. Recently, anti-inflammatory agents such as cyclosporin or autologous serum have been proposed as new treatment options for severe ocular surface disease. To date, however, there are no effective treatments for the majority of individuals with DES.

The experimental drug OPC-12759, 2-(4chlorobenzoylamino)-3-[2(1H)-quinolinon-4yl]-propionic acid known as rebamipide (Mucosta), has been used as a gastroprotective drug in the treatment of acute and chronic gastritis mucosal lesions, including erosion, bleeding, redness, and edema. This drug induces mucosal protection and healing of ulcers primarily by enhancing synthesis of prostaglandin E2 in the gastric mucosa and by increasing the expression of epidermal growth factor (EGF) and EGF receptor (EGFR) in normal and ulcerated gastric mucosa. Finally, this drug stimulates other functions in the stomach such as gastric mucus secretion, migration and proliferation of epithelial cell monolayers in wounds, and scavenging of active oxygen radicals. The OPC-12759 also attenuates the production of proinflammatory cytokines stimulated by nonsteroidal anti-inflammatory drugs and *Helicobacter pylori* that cause stomach ulcers. By this mechanism, the OPC-12759 functions to protect the stomach.

Preliminary pharmacological studies in the eye with topical OPC-12759 indicated that this drug increased the number of Alcian blue-positive goblet cells in the conjunctiva as measured by impression cytology, and reduced rose bengal scores after ocular surface dryness induced by air blast. Treatment with 1.0% OPC-12759 for 14 days improved rose bengal scores in corneas and conjunctivae of rabbits in which the mucus layer of the tear film was removed by the N-acetylcysteine treatment. Because of the ability of OPC-12759 to stimulate gastric mucus secretion, heal wounded...
ulcers, and improve the health of conjunctiva, we hypothesized that OPC-12759 would stimulate conjunctival goblet cell proliferation.

**MATERIALS AND METHODS**

**Materials**

The RPMI 1640 culture medium, l-glutamine, and penicillin/streptomycin were obtained from Bio Whittaker (Walkerville, III); the fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, Utah). The falcon tissue culture plastic wear was obtained from Becton Dickson Labwear (BD, Franklin lakes, NJ). The monoclonal antibody against Ki-67 was purchased from Novocastra laboratories (Newcastle upon Tyne, UK). The polyclonal antibodies against Ki-67 and the secretory mucin MUC5AC were purchased from NeoMarkers (Fremont, Calif). The polyclonal antibodies against cytokeratin 4 (CK4) and cytokeratin 7 (CK7) were purchased from ICN (Aurora, Ohio). The fluorescein isothiocyanate (FITC)-conjugated lectin *Ulex europaeus agglutinin I* (UEA-I) was obtained from Eugene Tech International (Ridgefield Park, NJ). The FITC-conjugated lectin *Griffonia (Bandeiraea) simplicifolia* was purchased from Vector Laboratories (Burlingame, Calif). Rhodamine-conjugated secondary antibodies donkey antimouse and antirabbit IgG were purchased from Jackson Laboratories (West Grove, Pa). The FITC-conjugated donkey antimouse- or antirabbit-IgG secondary antibodies were purchased from Jackson Laboratories (West Grove, Pa). The vectashield mounting medium containing DAPI was purchased from Vector Laboratories (Burlingame, Calif). The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and other chemicals were from Sigma (St. Louis, Mo). The OPC-12759 was provided by Otsuka Pharmaceutical (Hyogo, Japan).

**Animals**

Male Sprague Dawley rats (150 g, approximately 3 weeks of age) were purchased from Taconic (Germantown, NY). The rats were anesthetized for 1 min in CO2 and killed by decapitation. All experiments were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Schepens Eye Research Institute Animal Care and Use Committee.

**Isolation and Culture of Conjunctival Goblet Cells**

Conjunctival goblet cells were isolated and cultured according to methods described previously by Shatos et al.19 Briefly, small pieces of approximately 1 mm3 from the inferior conjunctiva and nictitating membrane were dissected and anchored onto either a 24-well culture plate or glass coverslips placed within 6-well culture plates. The tissue plugs were incubated in RPMI 1640 supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO2-atm incubator. Cells derived from the explants were permitted to grow from the tissue plug for 72 h. After the third day of culture, the tissue plug was removed to prevent the growth of connective tissue cells that could overgrow the culture. Cultures displaying exclusively connective tissue morphology were discarded. Goblet cells, identified by the presence of numerous secretory granules, were further isolated from epithelial cells by scraping away contaminating nongoblet cells with a rubber policeman.19 The remaining cells were trypsinized and seeded at 500 cells per well in a 24 Primaria cell culture plate. This low density ensured that growth in all cultures proceeded to approximately same degree of subconfluence.

**Experimental Paradigm**

After serum starvation for 48 h, cells were exposed to RPMI supplemented with increasing concentrations of 0.3%, 1.0%, 3.0%, and 10.0% FBS for 1, 3, 5, and 7 days to determine the optimal concentration that induced conjunctival goblet cell proliferation. To determine the effect of OPC-12759 on goblet cell proliferation, cells were incubated with or without increasing concentrations of 10−12 to 10−8 M OPC-12759 in serum-free RPMI supplemented with 0.1% bovine serum albumin (BSA) as a protein source for 1, 3, 5, and 7 days. The BSA was also added to the 10% FBS medium that was used as the positive control for the OPC-12759 experiments. Addition of 0.1% BSA to the culture media did not alter goblet cell proliferation compared with the absence of BSA (data not shown). In separate experiments, cells were daily replenished with or without increasing concentrations of 10−12 to 10−8 M OPC-12759 or 10% FBS for 3 consecutive days.

**Colorimetric Assay to Measure Proliferation**

The quantification of goblet cell proliferation was determined in triplicate by an MTT colorimetric assay.20,21 Actively proliferating cells convert MTT to a dark blue formazan product that was detected by a Dynatech MR 580 Microelisa reader using the ratio of absorbance at 570:630 nm, and calibrated to 1.99.

**Histochemistry**

After OPC-12759 stimulation for 1 to 5 days, goblet cells grown on cover slips were washed in phosphate buffered saline (PBS) (containing in mmol/L: 145 NaCl, 7.3 Na2HPO4, and 2.7 KH2PO4, pH 7.2), fixed in absolute methanol at 4°C for 10 minutes, washed in PBS, and blocked in PBS containing 1% BSA and 0.2% Triton X-100 for 1 h at room temperature. Proliferating cells were incubated with an antibody to Ki-67 (1:100 in PBS containing 0.2% Triton X-100) for 24 h at 4°C. They were washed in PBS followed by incubation with secondary antibody, rhodamine-conjugated donkey anti-mouse IgG (1:200 in PBS), for 1 h at room temperature. Primary antibody was omitted from the negative controls. To investigate if proliferating cells were goblet cells, the FITC-conjugated UEA-I lectin (1:800 in PBS), which recognizes the terminal carbohydrates of secretory product in the goblet cell, was added along with the secondary antibody. Mouse monoclonal antibodies for MUC5AC and CK7 were also used to identify goblet cells, whereas mouse monoclonal antibody for CK4 and the lectin *Bandeiraea* were used to identify stratified squamous cells.
Cover slips containing cells were mounted on microscope slides using DAPI-containing mounting medium to stain cell nuclei. Slides were masked, and the number of Ki-67 positive and total number of cells were counted. Five representative fields were photographed using a Nikon UFX II microscope equipped for epi-illumination microscopy. Results were expressed as the number of Ki-67-positive cells (proliferating cells) divided by the number of DAPI-positive cells (total cells) and multiplied by 100 to obtain a percentage.

Data Analysis
Data from multiple experiments are expressed as mean values ± SE. Statistical analysis was determined using Student t test. P ≤ 0.05 was considered significant.

RESULTS

Effect of FBS on Goblet Cell Proliferation
As measured by the MTT assay, FBS at 0.3% to 10% increased cell proliferation after 1, 3, 5, and 7 days of culture. A statistically significant increase in proliferation was obtained with 10% FBS and was independent of the duration of stimulation (Fig. 1). A maximum 4-fold increase in goblet cell proliferation was obtained with 10% FBS after a day of incubation. When stimulated with 0.3% to 10% FBS, goblet cells continued to proliferate for at least 7 days. The FBS at 10% was chosen for use as a positive control for subsequent experiments.

Effect of OPC-12759 on Goblet Cell Proliferation
The OPC-12759 at 10^{-10} and 10^{-11} M induced an almost 2-fold increase in goblet cell proliferation after a day of incubation (Fig. 2). The OPC-12759 at 10^{-11} M significantly increased cell proliferation by 1.9-fold after a day of incubation and 1.3-fold after 3 days of incubation. After 5 and 7 days of incubation, no statistically significant increase in proliferation was detected for this compound (Fig. 2). However, when 10^{-12} to 10^{-8} M OPC-12758 was re-added to the cultures for 3 consecutive days, each concentration significantly increased goblet cell proliferation when assayed on Day 3 of culture, reaching a 3-fold maximum at 10^{-10} M (Fig. 3). In similar experiments, when 10% FBS was added for 3 consecutive days, it caused a 10-fold increase in proliferation.

Histochemical Assays
Because 10^{-11} M OPC-12759 stimulated conjunctival goblet cell proliferation, we used this concentration to determine, by immunohistochemical assay, the characteristics of the cells that were produced. Very few mitotic cells positive for Ki-67 were present in cultures treated with media...
alone (Fig. 4A). Numerous Ki-67-positive cells were present in cultures treated with either $10^{-11}$ M OPC-12759 or 10% FBS (Fig. 4B, C). The percentage of Ki-67-positive cells in control culture medium ranged from 7.4% ± 2.9 on Day 1 to 4.4% ± 1.6 on Day 5. When treated with $10^{-11}$ M OPC-12759, 30.2% ± 3.0 of cells were Ki-67-positive on Day 1 (Fig. 5). This percentage fell to 17.7% ± 2.5 at Day 3 and 9.3% ± 0.8 at Day 5. This increase was significant at Days 1 and 3 but not at Day 5 when compared with controls. The positive control, 10% FBS, induced statistically significant

FIGURE 2. Effect of OPC-12759 on cell proliferation. Conjunctival goblet cells were serum-starved for 48 h and then treated with the indicated concentrations of OPC-12759 or 10% FBS. OD 570/630 nm indicates the amount of proliferating cells. Values are expressed as mean ± SEM, N - 3, *P ≤ 0.05 compared with control with no FBS.

FIGURE 3. Effect of OPC-12759 replenishment on goblet cell proliferation. Conjunctival goblet cells were serum-starved for 48 h and then treated with the indicated concentrations of OPC-12759 or 10% FBS every day for 3 days. OD 570/630 nm indicates the amount of proliferating cells. Values are expressed as mean ± SEM, N = 3, *P ≤ 0.05 compared with control with no FBS.
increases in Ki-67-positive cells of 56.7% ± 7.0, 50.2% ± 1.2, and 34.0% ± 0.8 on Days 1, 3, and 5, respectively (Fig. 5).

Colocalization of Ki-67 and UEA-I, MUC5AC, or CK7 in Cultured Goblet Cells

To investigate if the proliferating cells had the characteristics of goblet cells, we determined if the Ki-67-positive cells also bound UEA-I lectin and had MUC5AC mucin and CK7 intermediate filaments, all of which are characteristic of goblet cells. To differentiate between proliferating goblet cells and proliferating stratified squamous cells, we stained Ki-67-positive cells for the conjunctival stratified squamous cell markers Bandeiraea lectin detectable carbohydrates or CK4.

Under control conditions, all cells contained nuclear DAPI staining and were reactive with UEA-I which indicates goblet cell secretory products (Fig. 6A). The cells were also positive for MUC5AC secretory mucin (Fig. 7A, left) and for CK7 (Fig. 7A, right). Few cells were proliferating and contained Ki-67. After addition of OPC-12759 (10⁻¹¹ M) for 1 day, a population of cells began proliferating and labeled with Ki-67 (Fig. 6B). These cells were also positive for the goblet cell markers UEA-1 (Fig. 6B), MUC5AC, and CK7 (Fig. 7B). As a control, all cells proliferating in response to FBS (10%) addition for 1 day were also positive for UEA-1 (Fig. 6C), MUC5AC, and CK7 (Fig. 7C). By contrast, no cells either proliferating or nonproliferating, stained for either Bandeiraea lectin or CK4 intermediate filaments, markers for stratified squamous cells (Fig. 8). Addition of
OPC-12759 (10^{−11}) or FBS (10%) stimulated cell proliferation, but not of cells stained with Bandeiraea or CK4. Thus, all proliferating cells could be characterized as goblet cells based on the presence of UEA-I, MUC5AC, and CK7 reactivity, and none could be characterized as stratified squamous cells.

FIGURE 5. Immunohistochemical quantification of proliferation. Conjunctival goblet cell cultures were serum starved for 48 h and then incubated with no additions, 10^{−11}M OPC-12759, or 10% FBS for 1, 3, and 5 days. The number of Ki-67–positive cells and total number of DAPI-staining cells were counted in 5 representative fields. Data were expressed as the number of Ki-67–positive cells per total number of DAPI-labeled cells × 100%. Values expressed as mean ± SEM, N = 3, *P ≤ 0.05 compared with 0.

FIGURE 6. Colocalization of anti-Ki-67 and UEA-I lectin binding. Representative sets of immunofluorescence micrographs show colocalization of Ki-67 and UEA-I lectin in cells after a day of culture with (A) no additions, (B) 10^{−11}M OPC-12759, or (C) 10% FBS. The Ki-67 antibody recognizes a nuclear antigen found only in proliferating cells (red). The lectin UEA-I recognizes carbohydrates at the l-fucose terminal of glycoconjugates of goblet cells secretory products (green) and indicates goblet cells. The DAPI stains all cell nuclei (blue). Pink indicates colocalization of Ki-67 and DAPI and thus indicates proliferating cells. Magnification ×200.
DISCUSSION

That FBS induced proliferation of goblet cells in a dose-dependent manner suggests that serum might be useful to support the clinical proliferation of conjunctival goblet cells. Serum is usually nonallergenic and has biomechanical and biochemical properties that are similar to those of normal tears. In fact, cell culture experiments show that corneal epithelial cell morphology and function are better maintained by serum than by pharmaceutical tear substitutes. Recently, the use of unpreserved autologous serum in the form of eyedrops was reported as a new treatment for severe ocular surface disorders. However, the protocols to prepare, store, and use autologous serum eyedrops varied considerably among the studies. These variables might influence the effect on goblet cell proliferation. Therefore, the role of serum eyedrops in the management of severe ocular surface disease has to be evaluated in more detail.

In the present study we demonstrated that OPC-12759 induced the proliferation of rat conjunctival goblet cells in vitro as measured by metabolic and histochemical methods. The effect of OPC-12759 was maximal at $10^{-11}$ M for a day of incubation or at $10^{-10}$ M when it was replenished for 3 consecutive days. In all cases, it induced less proliferation than the 10% FBS. These results are similar to those of

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FIGURE 7. Colocalization of anti–Ki-67 with anti-MUC5AC or anti-CK7 antibody binding. Representative sets of immunofluorescence micrographs show colocalization of Ki-67 with MUC5AC or CK7 in cells after a day of culture with (A) no additions, (B) $10^{-11}$ M OPC-12759, or (C) 10% FBS. The Ki-67 antibody recognizes a nuclear antigen found only in proliferating cells (red). The MUC5AC antibody recognizes goblet cells secretory products (green, left) and indicates goblet cells. The CK7 antibody (green, right) recognizes cytoplasmic filaments found only in differentiated conjunctival goblet cells. The DAPI stains all cell nuclei (blue). Pink indicates colocalization of Ki-67 and DAPI and thus indicates proliferating cells. Magnification ×200.
in vitro studies where $10^{-5}$ M OPC-12759 induced the proliferation of cultured rabbit corneal epithelial cells only when it was renewed every day (personal communication, 2004). Thus, OPC-12759 can be more effective in stimulating long-term goblet cell proliferation when it is applied daily.

The concentration of OPC-12759 that stimulated goblet cell proliferation in the present experiments was several orders of magnitude lower than that which was effective on cultured corneal epithelial cells. This suggests that the conjunctival goblet cells are more sensitive to OPC-12759 than are the corneal cells. Based on preliminary results, 1% OPC-12759 in ophthalmic suspension delivered 6 times/day for 14 days caused increases in corneal and conjunctival mucin content of NZW female rabbits. Our study indicates that the increase in conjunctival mucin content could be due to stimulation of goblet cell proliferation.

FIGURE 8. Colocalization of anti–Ki-67 with Bandeiraea lectin or anti-CK4 binding. Representative sets of immunofluorescence micrographs show colocalization of Ki-67 and Bandeiraea lectin (green, left) or CK4 (green, right) in cells after a day of culture with (A) no additions, (B) $10^{-11}$ M OPC-12759, or (C) 10% FBS. The Ki-67 antibody recognizes a nuclear antigen found only in proliferating cells (red). The DAPI stains all cell nuclei (blue). Pink indicates colocalization of Ki-67 and DAPI and thus indicates proliferating cells. Magnification ×200.

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The cellular mechanism by which OPC-12759 induced goblet cell proliferation remains to be elucidated. Recently, we demonstrated that the mitogen-activated protein kinase signaling pathway is transactivated through the EGFR during cholinergic stimulation of conjunctival goblet cells and that this leads to stimulated mucin secretion. The OPC-12759 also increases the expression of EGF and EGFR in normal gastric mucosa and in regenerating glands of the ulcer scar. Signaling through the EGFR induces cyclooxygenase-2 expression in some cell lines, including gastric epithelial cells. Cyclooxygenase-2–derived prostaglandin E2 transactivates the EGFR and stimulates mitogen-activated protein kinase to induce cell proliferation.
proliferation, migration, and invasion. Therefore, it is possible that the OPC-12759 induces conjunctival goblet cell proliferation through similar signaling pathways.

In summary, the present results indicate that OPC-12759 is capable of inducing proliferation of rat conjunctival goblet cells in vitro. This suggests that OPC-12759 may be a useful therapy in DES by increasing the number of goblet cells and healing the ocular surface.

REFERENCES


