Effect of Diclofenac Sodium and Dexamethasone on Cultured Human Tenon’s Capsule Fibroblasts

Ran Sun, MD; Howard V. Gimbel, MD; Sue Liu, MD; Dianlin Guo, PhD; Morley D. Hollenberg, DPhil, MD

■ BACKGROUND AND OBJECTIVE: To investigate the effect of diclofenac sodium and dexamethasone on cultured human Tenon’s capsule fibroblasts.

■ MATERIALS AND METHODS: Two experiments were conducted. In the first experiment, fibroblasts were treated with either diclofenac sodium or dexamethasone at different concentrations, and the cell growth was quantified by using Coulter counter and hexosaminidase methods at 1, 3, 5, and 7 days after adding the drugs. In the second experiment, the cells were treated with each drug for 24 hours and then the cultures were switched to a drug-free medium. The cell growth was quantified at day 7 after removing the drugs from the medium.

■ RESULTS: In the first experiment, inhibition of fibroblast growth in a dose-dependent manner was observed from days 1 to 7 in the cultures treated with each drug. The inhibitory was more pronounced in the diclofenac treated cultures. The typical spindle-shaped fibroblasts treated with higher concentrations of the drugs became spherical cells. In the second experiment, inhibition was not observed when the cultures were switched to a drug-free medium. The spherical cells recovered to spindle-shaped cells and proliferated as normal cells.

■ CONCLUSION: Our results have shown that diclofenac sodium and dexamethasone can significantly inhibit human Tenon’s capsule fibroblast growth in a cell culture model. The inhibitory effect was not observed when the cultures were switched after 24 hours to a drug-free culture medium.


INTRODUCTION

Excimer laser photorefractive keratectomy (PRK) has been performed worldwide with consistent satisfactory results. However, postoperative corneal haze, particularly for high myopic correction, still is a concern. Previous studies suggested that corneal haze formation may be associated with fibroblast proliferation. Therefore, inhibiting fibroblast proliferation in corneal tissue using pharmaceutical agents may reduce postoperative corneal haze. Both corticosteroids and nonsteroidal anti-inflammatory agents (NSAIDs) have been reported to have an inhibitory effect on fibroblast proliferation in vitro. This inhibitory effect may be a result of the blocking of arachidonic acid (AA) metabolism to the lipoygenase (LO) pathway with consequent overproduction of leukotrienes. In addition, the LO pathway may produce superoxide radicals which can cause cell damage, consequently inhibiting cell proliferation. Therefore, these drugs
may be useful for preventing bleb failure after glaucoma filtration surgery. Diclofenac sodium is a new NSAID drug, therefore, the antiproliferative effect of this drug on human Tenon's capsule fibroblasts has not been established. To investigate this issue we conducted a cell culture study to evaluate the effect of diclofenac sodium and dexamethasone on cultured human Tenon's capsule fibroblasts.

**METHOD**

**Establishing Fibroblast Cell Lines**

The fibroblast cell line was established following an accepted technique. Tissue specimens of human Tenon's capsule were removed from cadaver eyes provided by a local eye bank. The specimens were processed within 10 hours of patient death. The blood from the donor was tested using standard testing methods and found to be serologically negative of HIV and hepatitis B.

Under sterile technique, the tissue was rinsed thoroughly with phosphate buffered saline (PBS) 3 times, cut into 1-mm cubes, then placed in 30-mm diameter tissue culture dishes. Five mL of Minimal Essential Medium (MEM, GIBCO/BRL), supplemented with 15% fetal bovine serum, (FBS) 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B were added into the culture dishes followed by a cover slip. The tissue was incubated at 37°C in 5% CO₂.

When the fibroblasts achieved a confluent monolayer in the culture within 2 weeks, the culture medium was removed and cells were washed with PBS, then incubated with 0.05% trypsin in PBS for 10 minutes. The action of trypsin was stopped by adding a culture medium containing 10% FBS. The cells were washed with PBS and resuspended following centrifugation. The suspension was separately seeded in different 80-cm² flasks for subsequent cultures. The fibroblasts were passage every other week and no cell was used after 10 passages.

**Drug Preparation**

Diclofenac sodium and dexamethasone (Sigma, Gaithersburg, MD) were diluted with the original MEM (without preservative) at 25, 50, 100, and 200 μg/ml for diclofenac sodium and 125, 250, 500, 1000 μg/ml for dexamethasone. Samples of both drug solutions at different concentrations were examined under the microscope to rule out precipitate.

**Drug Treatment**

Two experiments were conducted. In the first experiment, fibroblasts were trypsinized from the feeder flask and transferred to 50-mL centrifuge tubes and centrifuged at 1,200 rpm for 10 minutes after washing. Centrifuging and washing were done twice. The fibroblasts (1.0 × 10³ cells) in 200 μl of medium containing 10% FBS were placed in each well of 96-well culture plates. After 24 hours of incubation at 37° in 5% CO₂, 100 μl of the medium in each well was replaced with 100 μl of different concentrations of diclofenac sodium (25 to 200 μg) or dexamethasone (125 to 1000 μg/ml). Each drug concentration was tested in quadruplicate cell monolayers. The final concentration of FBS in the medium was 5%.

Cell growth was measured at days 1, 3, 5, and 7 after adding the drugs using a Coulter counter, and colorimetric endogenous hexosaminidase methods.

In the second experiment, fibroblasts (1.0 × 10³ cells) in 200 μl of medium were placed in each well for 24 hours and 100 μl of the medium was replaced with 100 μl of the drugs (concentrations were the same as in the first experiment) for a further 24-hour incubation period. The culture was then switched to a drug-free medium (5% FBS) and cell growth was measured after 7 more days.

**Determination of Cell Growth**

**Coulter-counter**

Each plate was inverted to remove the culture medium which was washed with PBS to remove unattached or dead cells. The cells were incubated with trypsin for 10 minutes and the reaction was stopped by adding MEM containing 10% FBS. The cells were transferred to the Coulter counter vials (Model ZF, Coulter Electronics Inc. Hialeah, FL) containing 9.40 mL of Isotone (VWR Canlab Co., Mississauga, Ontario, Canada). The cell numbers in four wells were measured, and the average was considered as the cell count value for that drug's concentration.

**Hexosaminidase**

The culture medium was removed by inverting the plates which were washed with PBS. The p-nitrophenol reagent (Sigma) was prepared for a 7.5 mM solution in 0.1 M citrate buffer (pH 5). This solution was mixed 1:1 with 0.5% Triton X-100 in distilled water and 50 μl of the solution added into each well for 2 hours incubation. The reaction was stopped by adding 100 μl of 150 mM glycine/5 mM ethylene
diaminotetra acetic acid (EDTA) disodium salt buffer at pH 10.4 (Sigma) to each well. The optical density of each well was read at 405 nm by EL 340 Bio-kinetics Reader (Bio-TEK Instrument Inc. Winooski, VT). Four wells for each drug concentration were read and the average was considered as cell count value for that drug concentration.

Data Analysis

Percentage of cell growth control was determined by using the cell count value in each drug concentration divided by the cell count value in the medium without drugs (control group) and multiplied by 100%. The relative potencies of each drug at different concentrations were compared based on curve shifts or changes in dose effects and increased time of incubation. Multiple regression (S-plus 3.3 1995) was used for all the statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

In the first experiment, inhibition of fibroblast growth was observed in a dose-dependent manner, especially at the highest concentrations and longer time exposures in the cultures treated with each drug (Figures 1, 2, and 3). The effect of dexamethasone on the fibroblast growth is shown in Figures 1A and 1B, based on hexosaminidase and Coulter counter assays respectively. The initial inhibitory effect on fibroblast
growth for dexamethasone was observed at a concentration of 125 μg/ml (P < 0.001) but became more pronounced with relative higher concentrations (P < 0.001). Also, the inhibition seemed to correlate to the incubation time. However, our day 5 culture data is inconsistent which may be due to experimental variability or other unknown factors (Figures 1A and 1B).

In a similar manner, the initial inhibitory effects of diclofenac sodium were observed at concentrations of 25 μg/ml (P < 0.05), and the inhibitory effect became more pronounced in the cultures treated with higher concentrations of drug and with relative longer time of incubation (P < 0.001) as seen in Figures 2A and 2B. The inhibition of the fibroblast growth was also shown in Figures 3A, 3B, and 3C in which the numbers of cells in the cultures treated with both drugs were significantly less than those in the control group. In addition, morphologic changes were observed in the cultures treated with higher concentration of the drugs in which typical spindle-shaped fibroblasts gradually became spherical cells which may indicate cell damage (Figures 4A and 4B).

In the second experiment, inhibition was not observed when the cultures were switched to a drug-free medium. The spherical cells recovered quickly to the spindle-shaped cells, and proliferated as normal cells (Figures 5 and 6).

**DISCUSSION**

The mechanism of corneal haze formation following PRK surgery has been well studied.\(^9,11\) It has been...
suggested that corneal haze may be associated with fibroblast proliferation and the growing of new connective tissue such as Type III, VII collagen in the corneal stroma. A number of animal studies have found that after excimer laser ablation the keratocytes are converted to fibroblasts. By approximately 3 to 6 weeks after ablation the subepithelial zone is filled with fibroblasts. In addition, Type III and VII collagen appear in the stroma, an amount of keratan sulfate proteoglycan is also increased. During this time clinical subepithelial haze is observed. From approximately 3 months to 1 year after surgery, fibroblasts and Type III and VII collagen gradually disappeared. Corneal stroma returned to relatively normal lamellar structure occupied by scattered keratocytes. Clinically, this corresponds to decreased corneal haze.

Another study has examined human corneal haze following PRK surgery and found oxytharen fibers and elastic fibers in the stroma which may present corneal haze. Since new collagen fibrils are thicker than normal and do not run in parallel bundles to the surface they may cause light scattering. More recently, a study has shown that there was a clear correlation between the corneal haze density and fibroblast cell count in the stroma. There was a poor correlation between the haze density measurement and the thickness of the new connective tissue layer in the stroma. These results suggest that the scattering of light is directly related to fibroblast density in the stroma. Therefore, fibroblast proliferation may play a major role in corneal haze.
role in forming corneal haze after PRK surgery. Consequently, inhibiting fibroblast proliferation in corneal tissue with pharmaceutical agents may reduce corneal haze. Some clinical studies have shown that steroid and nonsteroid agents could reduce corneal haze after PRK, but this result has been contradicted by other studies.11-13

To further investigate the management of corneal haze with pharmaceutical agents, we conducted a cell culture study to evaluate the inhibitory effect of steroid and nonsteroid agents on cultured human Tenon’s capsule fibroblasts. Both Coulter counter assay and hexosaminidase assay were used to determine the numbers of fibroblasts. The Coulter counter assay is a direct reflection of actual cell numbers, while the hexosaminidase assay reflects the amount of intracellular enzyme hexosaminidase present in the treated cell population. Therefore, the hexosaminidase assay is an indicator of cell numbers provided that the enzyme activity is unaltered by experimental drugs, and is equally distributed throughout the entire cell population.

Our results show that dexamethasone and diclofenac sodium can significantly inhibit human ocular fibroblast growth in a cell culture model. The inhibitory effect was more pronounced in the cultures treated with diclofenac sodium. In addition, cells treated with higher concentrations of drugs lost their typical spindle-shape and became rounded cells which indicated cell damage. However, when the cultures were switched to a drug-free medium after 24-hours of exposure, the spherical cells reverted to spindle shape and proliferated as normal cells. These results indicate that the inhibitory effect did not persist when the drugs were removed from the cultures.

Wound healing is a complex and multifactorial process which cannot be completely and accurately reproduced in an in vitro environment. In addition, Tenon’s capsule fibroblasts may not be a perfect model in which to study activated keratocytes in the cornea. However, the experiments provide important data which demonstrated that steroid and nonsteroid agents can inhibit fibroblast growth in a cell culture model. These results may have a clinical potential to modulate corneal haze following excimer laser surgery by inhibiting fibroblast proliferation and production of new collagen, but when the drugs were discontinued the inhibitory effect no longer existed and fibroblasts grew as quickly as normal cells. This finding may explain the clinical situation where corneal haze may reappear several months after PRK when topical steroid agents are discontinued. Currently there is disagreement regarding the use of steroid or nonsteroid anti-inflammatory agents to manage corneal haze after PRK surgery. The benefit of using steroid and nonsteroid anti-inflammatory agents is that they may reduce or delay corneal haze following PRK but the haze may return when the drugs are stopped. Therefore, the real benefit of using steroids and nonsteroids to manage corneal haze after PRK surgery still needs further investigation.

In conclusion, our results have shown that diclofenac sodium and dexamethasone can significantly inhibit human Tenon’s capsule fibroblast growth in a cell culture model. The inhibitory effect was more pronounced in the cultures treated with diclofenac sodium. Inhibition was not observed when the cultures were switched to a drug-free culture medium after 24 hours of treatment. These results indicate that diclofenac sodium and dexamethasone may have clinical potential to modulate corneal haze following PRK surgery by inhibiting fibroblast growth and production of new collagen. However, this effect may not exist after discontinuing these drugs.

REFERENCES


