In Vivo Effects of 5-FU on Ocular Surface Epithelium Following Corneal Wounding

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We evaluated the dose relationship between the antiproliferative and toxic effects of 5-fluorouracil (5-FU) on the ocular surface epithelium following experimental corneal epithelial wounding in rabbits. Central corneal epithelial defects 8 mm in diameter were made using n-heptanol. 5-FU (0.05 mg, 0.5 mg, or 5.0 mg per day in divided doses) or saline was applied topically for up to 18 days beginning on the day of wounding. The animals were sacrificed at 1, 7 or 14 days after wound closure. The effect on the ocular surface epithelium was assessed by observation of the clinical and histological appearance, and determination of the rate of corneal epithelial defect closure, corneal epithelial mitotic rate and conjunctival goblet cell frequency. A daily dose of 0.05 mg of topical 5-FU for 18 days had no discernable clinical or histopathological effect compared to wounded, saline treated controls. Treatment with 0.5 mg daily prevented the high mitotic rate typically noted 1 day immediately following defect closure, yet had no significant effect on clinical appearance, histological appearance, or healing rate. Daily topical application of 5.0 mg of 5-FU reduced the corneal epithelial mitotic rate to approximately 1% of the wounded controls, with persistent epithelial defects occurring in 22% of the eyes in this group. In those eyes which did heal, the corneal epithelium was markedly thinner than controls 1 day after defect closure. Fourteen days after healing, epithelial thickness in this group varied from 2 to 13 cells across each cornea, with the thickest area occurring centrally and tapering gradually to the limbus. Cellular morphology was also abnormal, characterized by pleomorphism, loss of normal cell polarity, increased nucleocytoplasmic ratio and the presence of bizarre forms. Topically applied 5-FU, 0.5 mg daily, inhibited corneal epithelial mitotic activity without causing histological, clinical or functional corneal abnormalities. These findings suggest that the corneal complications encountered with fluoropyridimine treatment of ocular cicatricial disease can be minimized or eliminated by careful titration of dose.

There has been a great deal of recent interest in the use of antimetabolites for the treatment of several ocular disorders characterized by proliferation of non-neoplastic cells within or about the eye. The pyrimidine analog 5-fluorouracil (5-FU), with its potent mitotic inhibitory effect and high therapeutic index relative to other available antimetabolites, has been one of the most intensively studied of these agents. The pharmacokinetics after topical, subconjunctival and intravitreal administration have been investigated in rabbits. In addition, it has been administered intravitreally and subconjunctivally to rabbits and humans for the treatment of massive periretinal proliferation, and topically and subconjunctivally to prevent scarring of filtering blebs following glaucoma surgery in humans and primates. While retinal toxicity has been described, clinical utility has been limited primarily by corneal toxicity. The relationship between dose and antiproliferative effect of 5-FU on rabbit corneal epithelial cells has been investigated in vitro, but not in vivo.

In a previous study, we demonstrated that 9 mg per day of topical 5-FU had serious toxic effects on the ocular surface epithelium. The purpose of this study was to determine the dose of 5-FU which would reduce ocular surface mitotic activity without producing corneal toxicity. To this end, we evaluated the effect of several topically applied doses of 5-FU on clinical appearance, corneal epithelial healing rate, histology, mitotic rate and goblet cell frequency following experimental corneal epithelial wounding in rabbits.

Materials and Methods

Preparation of Medication

5-FU (Roche Laboratories, Nutley, NJ) at a concentration of 50 mg/ml and pH 9 was diluted with
phosphate buffered saline (PBS) to a concentration of 20 mg/ml. The osmolarity was brought to physiologic range by the addition of sterile distilled water. Following two further 1:10 dilutions with phosphate buffered saline, the pH was titrated to 7.6 with concentrated hydrochloric acid. All three preparations were sterilized by microfibre filtration. The final concentrations of 5-FU in the three solutions were 0.15 mg/ml, 1.5 mg/ml and 15.0 mg/ml. Final osmolarities ranged from 280–327 mOsm/l. The control solution consisted of the PBS vehicle alone. The preparations were coded to render the investigators masked to the dose administered to each treatment group. The concentration of the preparations was assessed by UV spectrophotometry and mass spectrometry at the beginning and end of the experiment and noted to be constant.

Animal Preparation

All investigations described in this manuscript conform to the ARVO Resolution on the Use of Animals in Research.

Thirty-six New Zealand albino white rabbits of both sexes weighing 2–3 kg each were randomly assigned to one of four treatment categories, each to receive either PBS or one of the daily doses of 5-FU described below. Following appropriate intramuscular anaesthesia consisting of 1 cc (25 mg) of promazine hydrochloride followed by 2 cc (200 mg) of ketamine hydrochloride, topical proparacaine was applied to each eye and proptosis was induced. Eight millimeter central epithelial defects were created using heptanol-soaked filter paper discs, as described previously.19 The resultant circular defect was stained with fluorescein to confirm complete epithelial removal, and erythromycin ophthalmic ointment was applied. After wounding on day 1, between noon and 2 PM, medication was begun and continued at 2 hr intervals seven times daily until sacrifice. Treatment was administered by instillation of 50 μl of the appropriate coded solution to both eyes of each animal to yield total daily doses of 0.0 mg (PBS vehicle alone), 0.05 mg, 0.5 mg or 5.0 mg of fluorouracil. Three animals from each dosage group were sacrificed 5, 11 and 18 days after wounding.

Determination of Healing Rates

All eyes were examined and photographed immediately after wounding and daily thereafter until defect closure or the day of sacrifice. Photographs of the eye and a standard 10 mm reticule were taken at a fixed magnification. Images were then digitized and healing rates were determined using the IS2000 digital image processing system (PAR Microsystems, New Hartford, NY).20

Histology and Autoradiography

All animals were sacrificed between the hours of 11 AM and 1 PM Corneal-scleral-conjunctival preparations were incubated in 2 ml of tissue culture medium (Dulbecco’s Modified Eagles Medium, or TC199) with 10 μCi/ml of tritiated thymidine (New England Nuclear, Boston, MA; 20 μCi/mmol) at 37°C in an air:CO₂ (95%:5%) water-jacketed incubator for 2.5 hr, followed by 30 min incubation in isotope-free medium. Samples were then fixed in 10% buffered formalin and 7 μm histological sections were prepared. Sections were stained with the periodic acid Schiff (PAS) reaction or hematoxylin and eosin (H&E). Other sections used for autoradiography were dipped in periodic acid, rinsed, dipped in Kodak NTB2 emulsion and then stored for 14 days at −20°C. They were developed with Kodak D-19 developer, fixed with Kodak fixer and stained with Schiff’s reagent and hematoxylin.21

Morphological Analysis

Histological sections were evaluated in a masked fashion for overall corneal thickness, morphology and adhesion to underlying basement membrane. The mitotic rate (MR) was determined by counting the number of epithelial cells across the cornea which incorporated tritiated thymidine (S-phase cells) in 2.5 hr. The results are expressed as labeled cells per 100 basal corneal epithelial cells. Goblet cells, which stained with PAS, were counted in the conjunctiva. Goblet cell frequency (GCF) is expressed as goblet cells per 100 basal conjunctival epithelial cells. Two histologic sections from each eye were counted in a masked fashion by the same investigator, and averaged to yield a single value. Data from both eyes of each animal were also averaged. Final results are expressed as averages bracketed by the standard errors of the mean, with n = 3 animals for all MR and GCF data. The P values were calculated using analysis of variance, with a posteriori multiple comparisons among treatment means by Tukey’s Least Significant Difference Test.

Results

Clinical Appearance

Fluorescein staining immediately after heptanol de-epithelialization revealed intense central corneal epithelial defects with sharply demarcated edges. The eyes in the PBS, 0.05 mg/day and 0.5 mg/day groups revealed no evidence of inflammation or discharge at
any point in the study. The lids of animals in the 5.0 mg/day group became thickened along their margins after 11 days of treatment. After 18 days of treatment, inward puckering of the upper lids was noted uniformly in this group (Fig. 1), and proptosis was difficult to induce. Eyes with persistent defects were white and quiet. Multiple iris hemorrhages were noted in one eye at sacrifice on day 18.

**Histology**

Keratocyte depopulation of the anterior third of the stroma, resulting in a histologically detectable acellular band, was noted in all groups 1 day after healing. The 0.05 mg/day group was histologically indistinguishable from the wounded controls at all times. The corneal epithelium was somewhat thinner in the 0.5 mg/day group than the controls 1 day after defect closure (Fig. 2A,B), but indistinguishable from controls by 7 days after closure. In the 5.0 mg/day group, corneal histology was abnormal at 1, 7 and 14 days after defect closure. The corneal epithelium was markedly thinner than controls 1 day after healing (Fig. 2C), with poor adhesion and little morphological uniformity across each cornea. Seven days following wound closure, thickness varied from 2 to 11 cells across each cornea (Fig. 2D), with the thickest area noted centrally in a "pitcher's mound" configuration, tapering gradually out to the limbus. Goblet cells were present on the peripheral cornea in this group alone, noted in 9 of the 12 eyes which received 11 to 18 days of treatment. Anterior stromal keratocyte repopulation failed to occur within 14 days after defect closure, again in this group alone. Polymorphonuclear leukocytes were present in the stroma of those eyes with unhealed defects, despite the lack of clinical signs of inflammation.

**Number of Defects Healed and Healing Rates**

Ninety-eight percent (53/54) of all of the control, 0.5 mg/day and 0.05 mg/day treated eyes healed approximately 4 days after wounding. Seventy-eight percent (14/18) of the 5.0 mg/day treated eyes healed over the same time period (P < 0.03, Chi square). Corneal epithelial healing rates in the 5-FU treated groups with closed defects were not significantly different from controls. As shown in Figure 3, the average rate of healing in the persistent nonhealing 22% (4/18) of eyes in the high dose group paralleled that of the controls for 2 days (0.7 mm-sq/hr versus 0.8 mm-sq/hr, respectively), but was significantly different from the control rate by day 3 (P < 0.03, ANOVA). Eyes with defects which had not closed by the fifth day after wounding remained unhealed, and the defect size remained fixed for the duration of the study. One regression was observed in an eye from the high dose group which had closed within 4 days after wounding, only to demonstrate a recurrent defect 2 days later.

**Mitotic Rates and Goblet Cell Frequency**

As shown in Table 1, the corneal epithelial mitotic rate of 9.6 ± 1.6 tritiated thymidine-labeled cells per 100 basal cells per 2.5 hr incubation period noted in the controls 1 day after healing was more than triple the rate of 3.0 ± 0.3 found 2 weeks following defect closure (P < 0.05). The mitotic rates in eyes receiving the lowest dose of 5-FU daily were not significantly different from wounded controls at 1, 7 or 14 days following defect closure. A dose of 0.5 mg/day prevented the high mitotic rate normally detected on the first day after closure (P < 0.05). There was no significant difference between the MR of the control eyes and those receiving this dose on days 7 or 14. Five milligrams per day of 5-FU reduced the MR on day 1 to 4% of wounded controls (P < 0.01), and the MR on days 7 and 14 to approximately 1% of the corresponding control values (P < 0.01 and 0.05, respectively).

In contrast to the MR data, no significant difference in GCF was noted across or within dosage groups over any of the stated time intervals.

**Discussion**

The purpose of this study was to determine the dose of 5-FU capable of lowering ocular surface mitotic activity without producing toxic side effects. The healing rate, MR, and GCF data for the wounded, vehicle-treated controls are consistent with...
results of our previous studies. Treatment with 0.5 mg per day for up to 18 days caused inhibition of the high post-wounding corneal epithelial mitotic rate. Although subtle histological changes were noted 1 day after healing, there were no detectable effects on any observed parameter by 7 days after defect closure at this dose.

The 78% of eyes in the high dose group which healed did so at a rate similar to controls. This is consistent with in vitro demonstrations that neither stimulation of mitosis by EGF nor suppression of EGF-stimulated mitosis with 5-FU had any effect on rate of closure of 6 mm defects, which heal primarily by cell sliding. In this high-dose group, central corneal epithelial thickness was increased to up to 13 cells after 11 days of treatment. Since corneal epithelial mitotic activity was essentially nil in these eyes, superficial layers of corneal cells could not have arisen from the normal proliferation and differentiation of cells from the basal layer. They must, instead, have been drawn from the wound periphery, evidenced by the presence of goblet cells on the thinned peripheral cornea of 75% (9/12) of eyes from animals treated for 11 days or more. The 22% of eyes with persistent defects healed at a rate similar to controls for the first 48 hr after wounding, halting abruptly thereafter. Thus, daily topical treatment with the highest dose of 5-FU resulted in the following findings: an expected inhibition of mitotic activity, cessation of corneal epithelial migration in four eyes with
resultant persistent defects, and an apparent failure of contact inhibition in 14 eyes which healed successfully.

The common denominator for these latter two paradoxical findings may be the interruption of glycoprotein synthesis. Fluorouracil is converted to two active nucleotides: 5'-fluorodeoxyuridine monophosphate (5-FdUMP), which blocks DNA synthesis, and 5'-fluorouridine triphosphate (5-FUTP), which inhibits ribosomal maturation, RNA processing and RNA function during protein synthesis. Several interrelated and overlapping events are necessary for effective corneal epithelial defect closure: mobilization, migration, contact inhibition, proliferation and differentiation. Continued glycoprotein synthesis is known to be necessary for sustained corneal epithelial migration following wounding. Fluorouridine has been shown to inhibit membrane glycoprotein synthesis in leukemic L1210 cells. Cell membrane glycoproteins have recently also been shown to be responsible for contact inhibition in human diploid fibroblasts. Interference with cell membrane glycoprotein synthesis by 5-FUTP could therefore conceivably either inhibit migration or block atten-
The role of the peripheral ocular surface epithelium in corneal epithelial maintenance and repair. We previously described a decrease in GCF peripheral to conjunctival epithelial defects\textsuperscript{29} and to wounds that removed corneal, limbal and 1–2 mm of bulbar conjunctival epithelium in rabbits,\textsuperscript{29} indicating that the conjunctival epithelium plays an active role in cell sliding and wound closure of conjunctival defects and corneal epithelial defects of this size. In the present study we observed no reduction in the number of PAS-positive conjunctival goblet cells 4 days following the creation of 8 mm central corneal epithelial defects. We have noted previously that changes in conjunctival MR and GCF after creation of 5 mm or 10 mm central corneal epithelial defects are detectable only in the first 2 days after wounding, returning to normal by 4 days post-wounding.\textsuperscript{16,29} Any alteration in conjunctival GCF in response to central corneal epithelial wounding in this study is likely to have occurred prior to the earliest date of sacrifice, ie, 5 days after wounding. The absence of any significant difference in GCF even in the high dose group as compared to controls, despite profound inhibition of mitosis for up to 18 days, may be due to the slower turnover rate of the conjunctival goblet cell population\textsuperscript{30} relative to amucotic conjunctival epithelial cells,\textsuperscript{31} since fluorouracil exhibits relative specificity for cells which turnover rapidly.\textsuperscript{32,33}

These results have practical implications for the treatment of ocular disorders characterized by structural and functional damage as a consequence of fibrocellular proliferation. Recent clinical studies have shown that subconjunctival 5-FU following glaucoma filtering surgery enhances the success rate in eyes at high risk for failure. The most frequent complication associated with this treatment was the development of corneal epithelial defects.\textsuperscript{9,10} In this study, topical administration of 0.5 mg of 5-FU daily inhibited corneal epithelial mitotic activity without causing histological, clinical or functional corneal abnormalities. This suggests that the corneal complications encountered with fluoropyridimine treatment of ocular cicatricial disease can be minimized or eliminated by careful titration of dose. If feasible, topical administration of fluoropyrimidines would also eliminate the risks and discomfort associated with subconjunctival injections.

**Key words:** ocular surface epithelium, epithelial defect, healing rate, mitotic rate, 5-FU

### Table 1. Mitotic rate of corneal epithelium

<table>
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<tr>
<th>Days after closure</th>
<th>Dosage group (mg/day)</th>
<th>0.0</th>
<th>0.05</th>
<th>0.5</th>
<th>5.0</th>
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<tr>
<td>1</td>
<td>9.6 ± 1.6*</td>
<td>13.0 ± 1.7</td>
<td>3.3 ± 1.9</td>
<td>0.4 ± 0.2</td>
<td></td>
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<tr>
<td>7</td>
<td>3.3 ± 0.7</td>
<td>2.6 ± 0.1</td>
<td>4.3 ± 0.04</td>
<td>0.5 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>3.0 ± 0.3</td>
<td>3.9 ± 0.9</td>
<td>3.1 ± 1.0</td>
<td>0.5 ± 0.2</td>
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</table>

Corneal epithelial mitotic rate expressed as mitoses per 100 basal corneal epithelial cells per 2.5 hr in controls and in all three 5-FU-treated groups. Values are given as averages bracketed by the standard error of the mean (n = 3 rabbits; values from both eyes of each animal were averaged to yield a single value).

* Mitotic rate ± SEM.

### References

2. Fantes FE, Heuer DK, Parrish RK, Sossi N, and Gressel MG: