The Effects of 5-Fluorouracil on Ocular Tissues In Vitro and In Vivo after Controlled Release from a Multifunctional Implant

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PURPOSE. To evaluate the effects of 5-fluorouracil (5-FU) on ocular cells in vitro and the effects of degradable 5-FU–loaded poly(DL-lactide-co-glycolide; PDLGA) implant 50:50 implant in the rabbit eye in vivo.

METHODS. Cytotoxicity was assessed with a tetrazolium salt WST-1 cell proliferation/viability test and a lactate dehydrogenase (LDH) leakage test in rabbit corneal stromal fibroblasts (SIRCs), bovine corneal endothelial cells (BCECs), human conjunctival epithelial cells (IOBA-NHCs), human retinal pigment epithelial cells (ARPE-19), and human corneal epithelial cells (HCECs). The 5-FU–loaded PDLGA implants were surgically placed in rabbit eyes with a deep sclerectomy technique and the histopathology of the eyes was examined.

RESULTS. In vitro, 5-FU affected cell proliferation and survival in a time- and dose-dependent manner. In the WST-1 test, adverse effects in serum-free conditions started from 0.0005 mg/mL 5-FU in SIRCs and HCECs, whereas in other cell types, 0.005 mg/mL 5-FU hindered cell proliferation. In serum-free conditions 72-hour 5 mg/mL 5-FU treatment decreased cell viability to 40% in BCECs and to 10% to 15% in other cell types. 5-FU had no or very minor effects on LDH leakage. In vivo, the 5-FU implant showed no signs of toxicity in cornea and retina, whereas in the conjunctival stroma near the implantation site, some inflammatory cells and a marked subepithelial condensation of stromal connective tissue was observed during the postoperative period of 4 weeks.

CONCLUSIONS. 5-FU had a broad therapeutic range, and the 5-FU implant showed only minor tissue reactions in conjunctiva at the surgical site. 5-FU is a possible candidate for controlled drug release. (Invest Ophthalmol Vis Sci. 2009;50:2216–2223) DOI:10.1167/iovs.08-3016

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5-Fluorouracil (5-FU) is a pyrimidine analogue originally known for its widespread use in cancer treatment. The ability of 5-FU to reduce fibroblast proliferation and subsequent scarring has made it one of the most often used antimetabolites in ophthalmology. Administration of 5-FU by serial subconjunctival injections or by single intraoperative applications is used for adjunctive treatment in filtration surgery in glaucomatous eyes. 5-FU is used in primary glaucoma filtering surgeries and in reviving failing filtering blebs, in dacryocystorrhinosomy, pterygium surgery, and vitreoretinal surgery to prevent proliferative vitreoretinopathy. After glaucoma surgery, scarring of filtering blebs is mainly caused by proliferation of subconjunctival fibroblasts. Fibroblast proliferation from the episclera and Tenon’s capsule has an important role in the scarring process. Preferably, an antimetabolite should limit the proliferation of scleral and episcleral fibroblasts, but not interfere in the replication of other ocular cells. Conventional antimetabolite treatment has been found to cause complications in various types of ocular cells. Therefore, the controlled slow release of an antimetabolite like 5-FU from a biodegradable device that releases small doses of 5-FU at the ocular surgical site has been an attractive topic for the further development of new ocular delivery techniques. A degradable 5-FU–loaded implant that releases small doses of 5-FU at the surgical site is likely to cause less local and systemic adverse effects than traditional 5-FU treatments used in ophthalmology.

For optimizing the biomaterial properties and release kinetics, the cytotoxicity profile of 5-FU on ocular tissues was evaluated in vitro in five cell line cultures from various ocular tissues, on rabbit corneal stromal fibroblasts (SIRCs), bovine corneal endothelial cells (BCECs), human conjunctival epithelial cells (IOBA-NHCs), human retinal pigment epithelial cells (ARPE-19), and human corneal epithelial cells (HCECs) as reference cells based on our previous 5-FU study. Furthermore, 5-FU release from the 5-FU–loaded biodegradable implants made of a 50:50 molar ratio of poly(DL-lactide-co-glycolide) (PDLGA) was evaluated. The in vivo effects of the intrascleral implants surgically placed with deep sclerectomy technique in the rabbit eyes as described in our previous study were examined.

MATERIALS AND METHODS

Treatment of Ocular Cell Cultures with 5-FU

The 5-FU preparation (Flurapsilin, containing 50 mg 5-FU/mL, 5% [wt/vol]) was obtained from Pharmacia Italy S.p.A (Nerviano, Italy). Cytotoxicity evaluations were performed in five cell line cultures from various ocular tissues, on rabbit corneal stromal fibroblasts (SIRCs), bovine corneal endothelial cells (BCECs), human conjunctival epithelial cells (IOBA-NHCs), human retinal pigment epithelial cells (ARPE-19) cells, and human corneal epithelial cells (HCECs). 20 HCECs were used as reference cells. Cell cultures were maintained in conditions similar to those described in our previous studies. The cells were

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seeded at the density of $1 \times 10^4$, $1.5 \times 10^4$, and $2 \times 10^4$ cells/well into 96-well microplates for the exposure times of 72, 48, and 24 hours, respectively. Twenty-four hours after plating, medium was discarded and the preconfluent cell cultures were replaced with normal growth medium or with a test solution containing 0.00005 to 5 mg/mL 5-FU ($5 \times 10^{-6}$ to 0.5% [wt/vol]) in normal medium containing serum or without serum testing four wells per concentration. The cells were treated in a humidified atmosphere at 37°C in 5% CO₂/95% air for 24 to 72 hours.

**Cytotoxicity Tests**

Cytotoxicity was evaluated by two cytotoxicity tests: WST-1 test and LDH test. The WST-1 test reagent and the LDH kit were purchased from Roche (Basel, Switzerland). The WST-1 test is based on the cleavage of the tetrazolium salt WST-1 ($4\{3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-l,3-benzene disulfonate, slightly red\}$) to formazan (dark red) by various mitochondrial dehydrogenase enzymes. LDH assay as an index of plasma membrane integrity measures the leakage of the cytosolic enzyme lactate dehydrogenase into culture medium. The WST-1 and LDH tests were performed as previously described.21,22 The assays were conducted in three separate experiments by testing four wells per concentration. In the WST-1 and LDH tests, the mean optical density values corresponding to the nontreated control cells were taken as 100%. The results are expressed as a percentage of the optical density of the treated versus the untreated control cells. Dose–response curves were drawn from the results expressed as mean ± SE of mean (SEM; GraphPad Prism software; GraphPad, San Diego, CA) using nonlinear regression analysis. The EC₅₀ values, the concentrations of 5-FU that decreased the WST-1 reduction values to 50% of the controls, were determined when possible from the dose–response curves. The statistical significance of the differences between the cultures exposed to 5-FU without serum or with serum was determined with the Student’s two-tailed t-test (GraphPad). Differences were considered significant at $P < 0.05$.

**Preparation of the 5-FU–Loaded PDLGA Implant**

The biodegradable 5-FU-loaded implant was prepared by cutting an appropriate implant shape from a solvent-cast drug containing polymer film. The biodegradable copolymer used in this study was a 50:50 molar ratio of poly(ε-caprolactone-glycolide; PDLGA, Resomer 150; 50; Boehringer Ingelheim, Ingelheim, Germany). Typically 600 mg of PDLGA and 60 mg of 5-fluorouracil (>99%, Fluka, Buchs, Switzerland) was dissolved in 6 mL of dimethyl sulfoxide (DMSO, Honeywell Specialty Chemicals Seelze GmbH, Riedel-de-Haën, Germany) in a Teflon-coated Petri dish, diameter 48 mm. The solution was mixed for 2 hours with a magnetic stirrer before the dish was placed into an oven for drying (60°C, 4 days). After the polymer film achieved constant weight, it was carefully detached from the mold. The drug containing film was cut into implants with a specially designed cutting tool. An 0.8-mm hole was drilled through the implants with an electric power drill. The implants were then inserted into a mold and the drug containing film was stripped from the template. The polymer precipitate was separated by centrifugation (20 minutes, 70°C before preparation at 4°C). The resultant supernatant was then analyzed by free 5-FU by HPLC. Suitability of the above-mentioned pretreatment was tested with a 0.22-mm filter unit before injection.

**In Vivo Release of 5-FU**

y-Sterilized implants were placed in 2 mL vials (Eppendorf, Fremont, CA) with 2 mL phosphate buffer (0.1 M, pH 7.4). The vials were placed in a shaking water bath (OLS 200; Grant Instruments, Ltd., Cambridge, UK) at 37°C. A 600-μL sample was taken after 2, 6, and 24 hours and 2, 3, 4, and 7 days. After that, a sample was withdrawn once a week up to 8 weeks. The entire buffer was replaced with a fresh buffer during the sampling. The released 5-FU was quantified with HPLC method developed by Chiang et al.3 with some minor changes. The HPLC system used included a pump (510 HPLC pump; Waters, Milford, MA), injector (717 Autosampler; Waters), UV detector (484 Tunable Absorbance Detector; Waters), and 3.9 × 300-mm column (μBondapak C18; Waters). The samples were detected at 266 nm. Eluent was a 95:5 mixture of 0.1 M (pH 4) sodium acetate buffer and methanol, respectively. Flow rate was adjusted to 1 mL/min. HPLC method was validated according to the guideline of Shah et al.23 at the concentration range of 0.1 to 100 μg/mL. Repeatability and accuracy of the method were evaluated with quality control samples during the measurements. Quality control samples were at three concentrations: 0.1 μg/mL, 10 μg/mL, and 100 μg/mL. The samples were filtered with a 0.22-μm filter unit before injection.

**In Vivo Release of 5-FU**

Rabbits were killed at 1 ($n = 2$), 2 ($n = 1$), and 4 ($n = 3$) weeks. The eyes were fixed in 10% formalin (J.T. Baker, Deventer, Holland), embedded in paraffin (Algowax; Algolab, Espoo, Finland), cut into 5-μm sections, and stained with hematoxylin and eosin (Merck, Darmstadt, Germany). The samples were photographed with an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan) equipped with a digital camera (model E995; Nikon). Images were processed with image management software (Photoshop 8.0; Adobe Systems Inc., San Jose, CA).

**Histopathology**

Rabbits were killed at 1, 2, and 4 weeks ($n = 2, 3, 3$, respectively). Before enucleation samples were collected from aqueous humor and the vitreous body. Samples were stored at −70°C before preparation for HPLC analysis. The samples were prepared as described by Schmidt Laugesen et al.,21 with minor changes. The samples were deproteinized by mixing with a 10-fold 2% zinc sulfate solution (Sigma-Aldrich, Poole, UK), and subsequently by centrifuging at 10,000 g for 8 minutes at 4°C. The resultant supernatant was then analyzed for free 5-FU by HPLC. Suitability of the above-mentioned pretreatment was tested with similar zinc sulfate treatment in samples of aqueous humor or vitreous body with a known amount of added 5-FU. The treatment did not have any influence on 5-FU concentration.

**Determination of 5-FU Loading**

Samples of 5-FU–loaded polymer film (5–7 mg) were dissolved in 200 μL of DMSO at 37°C. After the film was entirely dissolved, the polymer was precipitated by adding 1.5 mL of 99.5% ethanol to the solution. The polymer precipitate was separated by centrifugation (20 minutes, 13 000 rpm) and the supernatant was filtered. Samples of 200 μL of supernatant were evaporated (60°C, overnight) and the residual was dissolved in 2 mL of phosphate-buffered saline (PBS). The 5-FU content was determined with HPLC to be 8.1% ± 0.7%.

**RESULTS**

**In Vivo Ocular Cytotoxicity of 5-FU**

Treatment of cell cultures from various ocular tissues with 0.0005 to 5 mg/mL 5-FU affected cell proliferation and survival in a time- and dose-dependent manner, as measured with the WST-1 cell proliferation/viability test (Figs. 1A–F). In SIRCs and HCECs, treatment with as little as 0.00005 mg/mL 5-FU hindered cell proliferation in serum-free conditions after 48- and 72-hour
exposure times (Figs. 1D–F). In other cell types, antiproliferative effects in serum-free conditions were noted starting from 0.005 mg/mL concentration. In all the cell cultures, with the exception of BCECs, the presence of FBS had a slight protective effect on the cells. BCECs were more resistant to the effects of 5-FU in serum-free cultures. After a 72-hour treatment with 5 mg/mL 5-FU in serum-free conditions, cell viability decreased to approximately 40% in BCECs, whereas in other cell types, cell viability decreased to 10% to 15% (Fig. 1F). Despite the slight protective effect of FBS, the presence of FBS had no statistically significant effects, except after 48-hour exposure time (Figs. 1C, 1D) in SIRCs (*P* < 0.036) and IOBA-NHCs (*P* < 0.012). The EC50 values of 5-FU in various ocular cell types are presented in Table 1. According to the EC50 values, the decreasing order of vitality in the different ocular cell cultures was ARPE-19 > IOBA-NHCs > SIRCs > HCECs > BCECs. Based on the LDH test, 5-FU had no or very minor effects on the cell membrane (Figs. 2A–D). Only SIRCs and ARPE-19 cells showed some slight increase in LDH leakage in serum-free conditions after 48- and 72-hour exposure times. In the LDH test, the presence of serum had a statistically significant effect only after 24-hour exposure time in BCECs (*P* = 0.025) and ARPE-19 cells (*P* = 0.015) and after 72-hour exposure time in HCECs (*P* = 0.015).

**In Vitro Release of 5-FU in the 5-FU–Loaded PDLGA Implant**

5-FU–loaded PDLGA 50:50 implants were successfully prepared by cutting the proper shapes from solvent-cast drug-containing polymer film (Fig. 3). The implants were comparable to nonloaded PDLGA implants, in that both were rigid and transparent. The implants did not contain any detectable residual DMSO based on 1H-NMR measurements. 5-FU content in the implants was close to the feed ratio (i.e., 0.7 wt%). The gamma irradiation did not influence the 5-FU loading or the structure of 5-FU according to preliminary mass spectrometry studies (data not shown). In vitro release of 5-FU was relatively fast (93% within 2 weeks; Fig. 4A). During the first week, the cumulative release was directly proportional to the square root of time (i.e., the release was diffusion controlled; Fig. 4B). Degradation time for the polymer is 6 to 8 weeks.

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**Table 1. EC50 of 5-FU in Ocular Cell Line Cultures**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>24 h +FBS</th>
<th>24 h −FBS</th>
<th>48 h +FBS</th>
<th>48 h −FBS</th>
<th>72 h +FBS</th>
<th>72 h −FBS</th>
</tr>
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<tbody>
<tr>
<td>SIRC</td>
<td>*</td>
<td>*</td>
<td>5.5 × 10−2</td>
<td>*</td>
<td>2.1 × 10−2</td>
<td>*</td>
</tr>
<tr>
<td>BCEC</td>
<td>*</td>
<td>*</td>
<td>5.8 × 10−3</td>
<td>1.4 × 10−3</td>
<td>3.5 × 10−3</td>
<td>1.7 × 10−3</td>
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<tr>
<td>IOBA-NHC</td>
<td>3.5 × 10−1</td>
<td>8.1 × 10−1</td>
<td>1.5 × 10−1</td>
<td>8.2 × 10−2</td>
<td>7.5 × 10−2</td>
<td>7.4 × 10−3</td>
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<tr>
<td>ARPE-19</td>
<td>*</td>
<td>*</td>
<td>5.8 × 10−1</td>
<td>*</td>
<td>1.8 × 10−1</td>
<td>1.5 × 10−2</td>
</tr>
<tr>
<td>HCEC</td>
<td>1.0 × 10−2</td>
<td>*</td>
<td>5.7 × 10−3</td>
<td>*</td>
<td>6.2 × 10−3</td>
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</tr>
</tbody>
</table>

Data are milligrams per milliliter. The cytotoxicity evaluation was based on the WST-1 test.

* Could not be quantified.
Based on the diffusional release kinetics, the degradation of the PDLGA matrix did not significantly influence the release profile. The drug was released before the implant structure degraded.

In Vivo Ocular Tissue Response to the 5-FU PDLGA Implant

We investigated the biocompatibility of the 5-FU–loaded PDLGA 50:50 implant in the WHHL rabbit eye. The possible adverse effects of the implant near the implantation site in various ocular tissues, including cornea, retina, and conjunctiva, were examined. In general, there were no significant differences in responses at the three time points (1, 2, or 4 weeks after implantation) for a single ocular tissue. However, there were intertissue variations in the responses. The cornea (Fig. 5A) and retina (Fig. 5B) near the implantation site showed no abnormalities. In contrast, the 5-FU implant induced a mild-to-moderate tissue reaction in conjunctiva near the implantation site (Fig. 5C). In the conjunctival epithelium, the superficial cells were elongated. In the conjunctival stroma, we observed some inflammatory cells and a marked subepithelial condensation of stromal connective tissue.

In Vivo Release of 5-FU in the Rabbit Eye

Samples withdrawn from aqueous humor or vitreous body at 1, 2, and 4 weeks did not contain any detectable amount of 5-FU (i.e., the concentration in the samples was well below 0.1 µg/mL). According to the in vitro release tests, the release of 5-FU from the implant was fairly fast; 80% of 5-FU was released during the first week (Fig. 4B). Therefore, it is very likely that most of the drug had also diffused from the aqueous humor and vitreous body before the samples were taken for HPLC analysis.

DISCUSSION

5-FU is used in ophthalmic practice in various applications, in primary trabeculectomy, in combined trabeculectomy and cataract surgery, in congenital glaucoma surgery, in glaucoma drainage devices, in the revision of failed filtering blebs, in pterygium surgery, in dacryocystorhinostomy, and in vitrectomy for the prevention of proliferative vitreoretinopathy. In addition, 5-FU is gaining recognition in the treatment and surgical management of ocular surface malignancies like ocular surface squamous neoplasia. However, the specific action of the drug on highly proliferating cells limits its use in primary acquired melanosis of the conjunctiva. In ophthalmology, the most frequently used application is the administration of 5-FU by serial subconjunctival injections or by single intraoperative applications for adjunctive treatment in filtration surgery.
in glaucomatous eyes to reduce fibroblast proliferation and subsequent scarring, especially in those patients in whom the natural healing process might result in a poor surgical outcome.

In our previous studies, we evaluated the adverse effects of 5-FU on the human corneal epithelial cell line HCE,\textsuperscript{14} and the human retinal pigment epithelial cell line D407 and porcine retinal pigment epithelial cells.\textsuperscript{27} In both of these studies, we concluded that 5-FU has antiproliferative effects rather than acute toxicity effects. In the present study, we evaluated the adverse effects of 5-FU in vitro in five ocular cell line cultures. HCECs were used as the reference cell type, based on our previous study.\textsuperscript{14} In vitro 5-FU has a relatively broad therapeutic range from the local inhibition of cell growth to cytotoxicity (0.0005–5 mg/mL). In all the cell types studied, 5-FU affected cell proliferation in a time- and dose-dependent manner. Although the EC\textsubscript{50} showed the order of decreasing cell growth as ARPE-19 > IOBA-NHCs > SIRCs > HCECs > BCECs, it can be noted from the growth curves that the most sensitive cell type was SIRCs, after IOBA-NHCs and ARPE-19 cells. BCECs survived the treatments best, especially the highest concentrations, and unlike in the other cell types, the presence of FBS had an unfavorable effect on BCEC viability. In accordance with our previous studies with the HCECs, 5-FU had very minor effects on LDH leakage overall.

Previous in vitro studies have shown the antiproliferative effect of 5-FU on rabbit conjunctival epithelial cells,\textsuperscript{28–31} human scleral fibroblasts,\textsuperscript{32} and human Tenon’s capsule fibroblasts.\textsuperscript{33} Antiproliferative effects have also been shown on rabbit corneal epithelial cells,\textsuperscript{29,32,34} bovine corneal endothelial cells,\textsuperscript{35} rabbit lens epithelial cells,\textsuperscript{36} and human retinal pigment epithelial cells.\textsuperscript{37} However, these studies have never been conducted in the same experimental conditions. Therefore, the comparison of in vitro studies (Table 2) is difficult because of the difference in cell culture conditions, growth times, and evaluation methods used. In most of the published in vitro studies, the cells were grown to confluence in serum-containing medium in the presence of 5-FU, after which cell numbers were evaluated by a hemocytometer or a Coulter counter. Growth times varied in most of the studies from 2 to 3 days to 5 to 6 days. The estimated EC\textsubscript{50} is in the same concentration range—approximately $5 \times 10^{-3}$ mg/mL. The EC\textsubscript{50} in the present study was higher ($\sim 5 \times 10^{-2}$ mg/mL) most likely due to shorter exposure times. In a recent study of a human conjunctival cell line (Wong-Kilbourne derivate of Chang conjunctiva), 5-FU induced an apoptotic cell death with an EC\textsubscript{50} of $7 \times 10^{-3}$ mg/mL after 72 hours of exposure.\textsuperscript{38} In the same study with the MTT cytotoxicity test, 5-FU was shown to induce significant inhibition in proliferating rabbit corneal epithelial cells.
Tenon’s fibroblasts at the concentrations of 1 × 10⁻² mg/mL or greater after 24-hour exposure.³⁸

Although 5-FU injection favorably prevents scarring, experimental studies in rabbits have also shown its ocular toxicity. Subconjunctival administration of 5-FU causes ocular surface problems⁷ and retinal toxicity.⁵⁹ 5-FU treatment has been found to associate with various types of ocular toxicity, including corneal epithelium and endothelium, and the epithelium of the ciliary body.⁵⁻⁷,⁴⁰ In contradiction, several studies have reported no toxic effect of 5-FU on retina with the dosages used.⁴¹⁻⁴⁴ Despite all, the antiproliferative effect of 5-FU has been detected in retinal pigment epithelial cells.⁴⁵ Furthermore, marked inhibition of protein synthesis in photoreceptors and ganglion cells of rabbit retina have been reported.⁴⁶

To overcome the disadvantages of 5-FU, biodegradable implants have been prepared and tested at the site of glaucoma filtration surgery. 5-FU is a good candidate for the further development of controlled delivery techniques.⁸⁻¹³ A biodegradable drug delivery device that releases small doses of 5-FU at the surgical site is likely to cause less local and systemic adverse effects than traditional 5-FU treatments used in ophthalmology. In the present study, we used the PDLGA 50:50 implant loaded with 5-FU. In previous in vitro biocompatibility studies using the same cell lines as used in the present study, we showed that ocular cells can successfully be cultured in vitro on PDLGA 50:50 biopolymer.²¹ The following order for cell growth was observed: ARPE-19 > IOBA-NHCs > SIRCs > BCECs. Our previous biocompatibility studies with the PDLGA 50:50 degradation extract showed that HCECs, IOBA-NHCs, and BCECs appeared to be the most sensitive cell types, whereas SIRCs and ARPE-19 cells were more resistant to the PDLGA 50:50 degradation extract in cell culture.²⁵ Both our present in vitro studies with 5-FU and our previous bio-compatibility studies with the PDLGA 50:50 showed that conjunctival epithelial cells were among the most sensitive cell types. The PDLGA 50:50 implant was also found to be biocompatible in the rabbit eye.¹³ However, a thin capsule formation was present around the implant and it was not seen after total swelling of the PDLGA 50:50 implant. Furthermore, all the studied tissue reactions (formation of drainage vessels, encapsulation, eye irritation and macrophage accumulation around the implant) were comparable to those of the reference collagen glaucoma drainage device (AquaFlow, STAAR Surgical Co., Monrovia, CA). Thus, in this respect, PDLGA 50:50 could be considered for use as a material for the development of an ocular drainage device.

In this study, we used a 5-FU–loaded PDLGA 50:50 implant and evaluated its biocompatibility in the rabbit eye. Our 5-FU studies showed that the in vitro release was relatively fast (93% within 2 weeks), and the release was diffusion controlled. The 5-FU PDLGA implant showed no significant differences in responses after 1, 2, or 4 weeks of implantation for a single ocular tissue in the rabbit eye. The cornea and retina near implantation site showed no abnormalities whereas a mild to moderate tissue reaction in conjunctiva near the implantation site was observed.

In comparison, alternative degradable release materials have been prepared as a a drug delivery system for 5-FU in glaucoma filtration surgery. Ocular toxicity of these formulations has been tested in rabbit eyes. The apparently contradicting results in biocompatibility tests have been obtained with distinct biomaterials. A collagen shield implant containing 5-FU caused inflammatory reaction and capsule formation around the implant.⁴⁶ A hydrophobic poly(ortho ester) and 5-FU sys-

<table>
<thead>
<tr>
<th>Study</th>
<th>Cell Type</th>
<th>EC₅₀ Value (mg/mL)</th>
<th>Exposure Time</th>
<th>Evaluation Method</th>
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<tr>
<td>Mallick et al.²⁹</td>
<td>Rabbit corneal epithelial cells</td>
<td>6 × 10⁻⁴</td>
<td>5 days</td>
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<td>30/60 min</td>
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<td>2–3 days</td>
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<td>5 min; 24 h recovery</td>
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<td>24 hours</td>
<td>MTT cytotoxicity test</td>
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<tr>
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<td>Human retinal pigment epithelial cells</td>
<td>3.9 × 10⁻⁴</td>
<td>3 days</td>
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</table>

Table 2. Toxicity of 5-FU In Vitro
tem triggered conjunctival hyperemia and chemosis.\textsuperscript{57} In contrast, bis(p-carboxyphenoxy) propane and sebacic acid with 5-FU resulted in no postoperative complications such as corneal haze or pigmentation or endophthalmitis and the morphology of the ciliary body epithelium and trabecular meshwork was normal.\textsuperscript{58}

Recently, 5-FU–loaded poly(lactic acid) discs have been used for trabeculectomy in rabbit eyes.\textsuperscript{49} The results showed no evidence of adverse effects of 5-FU in the conjunctiva of New Zealand White rabbits. In contrast, we found inflammatory cells and a marked subepithelial condensation of stromal connective tissue. Our glaucoma filtration surgery experiments were performed in a WHHL rabbit model. Traditionally, New Zealand White rabbits or pigmented rabbits have been used in glaucoma filtration surgery. WHHL rabbit is a strain that exhibits hyperlipidemia, and therefore, can be considered as an animal model for studying the effect of hyperlipidemia on the success of glaucoma filtration surgery. Glaucoma is usually associated with comorbid diseases such as cardiovascular disorders and hyperlipidemia.\textsuperscript{59,60} It is widely accepted that these diseases are not risk factors for glaucoma, but virtually nothing is known about their effect on the success of filtration surgery. Fibrosis or scar formation plays an important role in filtration surgery and excessive subconjunctival scarring results in surgical failure.\textsuperscript{51} On the basis of our results, we speculate that 5-FU cannot prevent scar formation in the conjunctiva of the WHHL rabbit. Pathologic differences in the eye of the rabbit strains may provide an explanation. At present, it is known that foam cells and lipid depositions are accumulated in the ocular tissues of WHHL rabbits.\textsuperscript{52} Elevated levels of lipids and their metabolites in the presence of foam cells may alter the healing response in the conjunctiva of WHHL rabbits. It is known that lipid-mediated changes in tissues accelerate extracellular matrix accumulation and fibrosis.\textsuperscript{53,61} Furthermore, lipid metabolites can stimulate proliferation.\textsuperscript{54} Thus, both functions of lipids weaken the action of antiproliferative agent 5-FU. In glaucoma surgery, 5-FU has been applied to prevent scarring, but certain patient groups fail filtration surgery even after 5-FU treatment.\textsuperscript{55} At present, the reason for this is unknown. On the basis of these facts, we speculate that the accumulation of lipid depositions in the conjunctiva of these patients is a possible explanation for extensive postoperative scarring after administration of 5-FU.

Also of importance is the in vivo release profile of 5-FU from the implant, because it may have an impact on the biocompatibility parameters. We used a PDLGA 50:50 implant. It has been shown that the in vivo release rate of 5-FU from a PDLGA implant is fast (80% of drug dose is released within 12 hours), and a therapeutic concentration of 5-FU is observed up to 8 days after implantation.\textsuperscript{56} In contrast, 5-FU is released for a longer time from poly(lactic acid) discs. The aqueous humor showed detectable amounts of 5-FU for 10 weeks.\textsuperscript{49} Solid implants such as PDLGA 50:50 tend to release an initial burst of drug, followed by a slower continuous release phase, and then a final burst.\textsuperscript{50} It is possible that near the implantation site, these bursts may increase 5-FU concentrations above therapeutic level. Therefore, the uncontrolled release of 5-FU may cause undesirable side effects resulting in local foreign body reaction in the conjunctiva.

In conclusion, our present in vitro studies testing 5-FU cytotoxicity and our previous studies testing PDLGA 50:50 toxicity with ocular cell line cultures\textsuperscript{21,22} are in accordance with our present in vivo studies that showed that conjunctival cells were the most sensitive cell type to the studied implant. The biodegradable 5-FU–loaded PDLGA 50:50 implant is a potential candidate for use in the deep sclerectomy surgery in the treatment of glaucoma.

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