Paclitaxel-Releasing Thin Biodegradable Film for Prevention of Bleb Avascularity Without Compromising Filtration in Rabbits

Tetsuhiko Okuda¹, Tomomi Higashide¹, Mayumi Sakurai¹, Yukako Fukuhira², Hiroaki Kaneko², Masatsugu Shimomura³, and Kazuhisa Sugiyama¹

¹ Department of Ophthalmology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan

² Research Laboratories III, Integrative Technology Research Institute, New Business Development Group, Teijin Limited, Tokyo, Japan

³ Department of Bio- and Material Photonics, Faculty of Photonics Science and Technology, Chitose Institute of Science and Technology, Chitose, Japan

Correspondence: Tetsuhiko Okuda, Department of Ophthalmology, Kanazawa University Graduate School of Medical Science, 13-1, Kanazawa, Japan; e-mail: okuda@med.kanazawa-u.ac.jp

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Purpose: A honeycomb-patterned film (HPF) prevents bleb scarring and mitomycin C (MMC)-related bleb avascularity in a rabbit model of filtration surgery. In this study, we examined whether a HPF-releasing paclitaxel (PTX) can prevent bleb avascularity without compromising filtration.

Methods: Filtration surgery was performed in one eye of rabbits. A 14- μ m thick HPF made from poly(L-lactide-co- ϵ -caprolactone) was placed subconjunctivally over the filtration site with the honeycomb surface turned toward the subconjunctival Tenon tissue. The rabbits were divided into four groups ($n = 5$ each): 1, HPF with no drug; 2, HPF + PTX 50 μ g; 3, HPF + 5 μ g; 4, HPF + 0.5 μ g. Intraocular pressure (IOP) measurements and bleb evaluations using ultrasound biomicroscopy were performed periodically for 4 weeks followed by histological examination. A longer follow-up study (12 weeks) was performed for group 4 (experiment 2; $n = 8$).

Results: Among all groups at the 4-week follow up, two blebs failed in group 1. The postoperative IOP decrease was significantly greater in PTX-treated eyes than in group 1. The bleb avascular area persisted for 4 weeks in groups 2 and 3. However, no avascular area was observed in groups 1 and 4 at 4 weeks postoperatively. Histology showed minimal fibrosis at the filtration site in all the PTX groups. In experiment 2, some blebs became flatter starting at 10 weeks after surgery.

Conclusions: PTX released from HPF promoted bleb survival and IOP decrease. The lowest dose of PTX (0.5 μ g) was effective at preventing bleb avascularity without compromising filtration.

Introduction

Trabeculectomy is the most effective method for intraocular pressure (IOP) reduction for glaucoma patients. The most common reason for the failure of this procedure is postoperative scarring of the filtration site. To prevent postoperative scarring, mitomycin C (MMC) has commonly been used for filtration surgery. However, avascular bleb formation due to local application of MMC is associated with undesirable side effects such as bleb infection and late-onset bleb leak.^{1–5} Bleb-related endophthalmitis is the most serious complication.^{6–8} Late-onset bleb

leak, which is closely associated with bleb avascularity, is a common risk factor associated with bleb infection among multiple studies.^{6–8} One study showed that bleb-related infection resulted exclusively from an avascular or hypovascular bleb.⁶ Therefore, preventing avascular bleb formation is considered to be very important to avoid the development of bleb-related endophthalmitis.

To address these risks, our group has proposed to develop a local, slow release drug delivery system. Intraoperative application of sponge-soaked MMC (0.2–0.4 mg/mL, 2–5 minutes) to the surgical site is a current standard procedure for preventing the scarring in trabeculectomy. Sustained local release and

application of low doses of antifibrotic drugs at the filtration site for the first 2 weeks postoperatively seems to have more antiproliferative effects and less possibility of conjunctival damage compared with the transient application of high doses of antifibrotic drugs during surgery. Furthermore, a system consisting of a biodegradable material with slow drug release is advantageous because nonabsorbable materials may cause mechanical damage to the overlying conjunctiva or erode during a long follow-up period. Biodegradable polymers such as polylactic acid (PLA), poly(lactic-co-glycolic acid; PLGA), and polycaprolactone (PLC), which is used as an absorbable suture,^{9–16} have been used for experimental filtration surgery.^{9–16} A honeycomb-patterned film (HPF), which was used in our previous reports,^{17,18} is a biodegradable film made from poly(L-lactide-co-ε-caprolactone) and has a long biodegradation time; longer than 10 months when placed in the anterior chamber of a rabbit eye.

Recently, we reported that a 7-μm thick HPF prevented wound adhesion and prolonged bleb survival in a rabbit model of full-thickness glaucoma filtration surgery.¹⁸ Moreover, a 14-μm thick HPF, a laminated film made of original 7-μm thick HPF and 7-μm thick film without a honeycomb surface, which was placed subconjunctivally over the filtration site, reduced avascularity and conjunctival damage in the bleb without compromising filtration in the rabbit model using intraoperative MMC treatment in the traditional manner.¹⁷

There have been some reports of drug delivery systems using paclitaxel (PTX) in experimental eye surgery.^{19–21} PTX has been shown to inhibit the growth of fibroblasts at low concentrations in a fibrosis model.²² In cardiovascular intervention, the extended release of PTX from coated coronary stents led to decreased rates of restenosis of coronary arteries.²³ In addition, PTX has been used for various basic research studies in animal eyes.^{19–21,24–28} Koz et al.²⁷ reported that PTX provided MMC-like antifibrotic effects during conjunctival wound healing. In addition, Choritz et al.²⁸ reported that exposure of human Tenon's fibroblasts to PTX inhibited proliferation and migration of the cells. Furthermore, Jampel et al.²⁶ reported that PTX was effective in promoting the success of experimental filtration surgery.¹⁹ Thus, the purpose of this study was to examine whether HPF releasing PTX can be used as a drug delivery system to prevent bleb avascularity without compromising filtration after experimental glaucoma filtration surgery in rabbits.

Materials and Methods

Biodegradable Film

Details and an illustration of the HPF can be found in our previous report.^{17,18} Briefly, a HPF is a thin biodegradable film made from poly(L-lactide-co-ε-caprolactone).²⁹ The film consists of approximately 88% PLA and 12% PLC. It has a unique surface structure characterized by pores of a hexagonal shape interconnecting horizontally along one surface of the film. The opposite side of the film is smooth. The honeycomb surface allows tissue attachment, while the smooth surface prevents tissue adhesion.¹⁸ It took approximately 10 months before a comparable film (6 × 6 mm and 7-μm thick) inserted in the anterior chamber of the rabbit eye dissolved (unpublished data).

To prepare a 14-μm thick film, a 7-μm thick HPF was stacked onto a smooth 7-μm thick film of poly(L-lactide-co-ε-caprolactone). Poly(L-lactide-co-ε-caprolactone) was dissolved in chloroform at a concentration of 5 mg/mL at room temperature. A surfactant, dioleoylphosphatidylethanolamine (0.5% wt/vol), was added based on the weight of poly(L-lactide-co-ε-caprolactone). The honeycomb-patterned films were fabricated by casting under a blowing current of highly humid air followed by evaporation of the water drops.²⁹ The smooth film was made by drying the polymer solution without humidity. PTX was added to the polymer solution and mixed before drying. To allow release of a larger amount of PTX into the bleb space, PTX was loaded only into the 7-μm thick smooth film. As the same mechanism of drug release from other drug eluting stents, PTX was eluted from the HPF. The amount of PTX loaded in the film was based on a previous report.¹⁹

Filtration Surgery

Japanese white rabbits (weight range, 2.5–3.0 kg) were used. All procedures involving animals were performed according to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Experimental procedures were approved by the Committee on Animal Experimentation of Kanazawa University, Takara-machi campus, Japan. All surgeries were performed by the same surgeon (TO). All rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg) for the filtration

surgery, recording of bleb appearance, and ultrasound biomicroscopy (UBM). All of the images were recorded in a uniform manner regarding the sites, magnification and angle. Also, all images were analyzed without knowledge of the groups until after the completion of all experiments. The operator or examiner was blind to the experimental group regarding all of the filtration surgeries, examinations, and histological evaluations.

An experimental filtration surgery was performed in 20 rabbits, five of which were assigned to the control group and the other 15 received PTX treatment (Experiment 1). For the control group (1), HPF with no drug was used. For PTX treatment groups, a HPF loaded with different amounts of PTX (2, PTX 50 μ g; 3, 5 μ g; 4, 0.5 μ g; $n = 5$ for each) was used. For all groups, filtration surgery was performed in one eye. The eye for film placement was randomly assigned to minimize subjective bias regarding the surgical procedure. Also, the dimensions of the scleral flap and the sclerotomy were strictly checked by an assistant surgeon (TH) during the surgery. An additional pilot study with a longer follow-up was performed, where the rabbits ($n = 8$) underwent filtration surgery as above and were treated with a HPF loaded with 0.5 μ g PTX (Experiment 2).

Under general anesthesia, an eyelid speculum was inserted and the eye was fixed with a corneal traction suture (8-0 vicryl; Ethicon, Piscataway, NJ). A fornix-based conjunctival flap was prepared in the superior-nasal quadrant and a 3×3 mm partial-thickness scleral flap was then outlined. A sclerotomy (1×2 mm) was performed under the scleral flap using a Kelly Descemet's membrane punch. A piece of HPF (6×6 mm) was placed over the sclerotomy site. The film, with the honeycomb surface turned toward the subconjunctival Tenon tissue, was secured by suturing onto the sclera with 10-0 nylon at the limbal two corners and at the center of the two sutures. The remaining two corners of the film were loosely secured with 10-0 nylon onto the sclera. A peripheral iridectomy was not performed to avoid bleeding and the scleral flap was not sutured. The conjunctival incision was closed with 10-0 nylon sutures. After surgery, a subconjunctival injection of 1 mg triamcinolone (Kenakolot-A; Bristol Pharmaceuticals KK, Tokyo, Japan) was performed away from the filtration site to decrease postoperative inflammation and ofloxacin ointment (Santen, Pharmaceutical Co., Ltd., Osaka, Japan) was applied. The follow-up period was 4 and 12 weeks in Experiments 1 and 2, respectively.

In Vivo Examinations

After topical anesthesia of 0.4% oxybuprocaine hydrochloride (Santen Pharmaceutical Co., Ltd.) IOP was measured using a rebound tonometer (TonoVet; Tiolat Oy, Helsinki, Finland) at baseline and twice a week during the postoperative period in all experimental groups. An average of three tonometer readings, with a maximum 5% standard deviation (SD), were recorded per eye. The IOP of all the experimental rabbits was measured at approximately 6 PM throughout the experimental period.

Following the IOP measurements, the anterior segment of the eye was observed under general anesthesia described above using a surgical microscope and images were recorded with a digital video (GVD-1000; SONY Co., Tokyo, Japan) weekly for 4 weeks after surgery. The size of the avascular area was measured in the digital video images of the blebs using Image-Pro Plus software (Version 7.0; Media Cybernetics Inc., Bethesda, MD, USA) by a single examiner (TO).

Following the surgical microscopic observations, bleb morphology was examined by UBM (UD-6000, Tomey, Nagoya, Japan) weekly for 4 weeks after surgery in the same manner described in our previous reports.^{17,18} In Experiment 2, these examinations were performed once every 2 weeks from 4 to 12 weeks after surgery.

Histological Evaluation

As described in our previous reports,^{17,18} animals were killed 4 weeks (Experiment 1) and 12 weeks (Experiment 2) postoperatively after all in vivo examinations were completed by an overdose intravenous injection of sodium pentobarbital. Corneoscleral blocks of the operation site (10×10 mm²) with overlying conjunctiva were dissected from all eyes. All blocks were fixed in 20% paraformaldehyde and embedded in paraffin. Sections through the operation site stained with hematoxylin and eosin or with Masson's trichrome were observed by light microscopy. Using sections stained with Masson's trichrome, subconjunctival collagen density was graded according to Perkins et al.³⁰: 0, no change (no stain); 1, minimal change (light blue); 2, mild change (mild blue); 3, moderate change (fairly blue); and 4, severe change (densely blue). In addition, the density of the blood vessels in the subconjunctival tissue (tubular structures filled with red blood cells) was graded: grade 0, no blood vessels per image; grade 1, 1 to 5 blood vessels per image; grade 2, 6 to 20 vessels per image; and grade 3, more than 20 vessels per image.

Figure 1. IOP changes over time in each treatment group. Postoperative IOP was significantly lower than baseline in all groups ($P < 0.001$). Postoperative IOP course was significantly different between the four groups ($P < 0.001$). Squares, control; triangles, PTX 50 μg ; diamonds, PTX 5 μg ; circles, PTX 0.5 μg .

Quantification of PTX Release

The release rate of PTX from the HPF and from a 14- μm thick smooth film in vitro was quantified by high-performance liquid chromatography (HPLC). A 14- μm thick smooth film was not laminated and was uniformly loaded with PTX. A film (6 $\times$ 6 mm), loaded with 0.5 μg PTX, was put into a tube containing 2 mL of phosphate-buffered saline (PBS, 20 mM NaPB, 150 mM NaCl, 0.1% NaN₃, pH7.4) and incubated at 37°C. Once a day, the tube was mixed by inverting. The remaining PTX in the film was quantified from 0 to 4 weeks, except at 3 weeks. A total of 32 films were used ($n = 4$ for each week per group). The film was transferred to a new tube to remove PBS and it was dried. An appropriate volume (125–500 μL) of solvent used for HPLC (55% [vol/vol] acetonitrile, 45% [vol/vol] 2 mM phosphoric acid) was added to the tube and mixed well. The solution was centrifuged to remove the film, filtered through a 0.45- μm membrane filter (Ultrafree-MC; Merck KGaA, Darmstadt, Germany), and 100 μL of the solution was directly injected into the HPLC.

HPLC was performed as described by Choung with some modifications.²⁰ The HPLC system used was a TOSOH system (HLC-803D; TOSOH Corp., Tokyo, Japan) with a spectrophotometer (UV-8 model II; TOSOH Corp.). A column MGII ($\Phi 4.6 \times 150$ mm, 5 μm ; Shiseido Co., Ltd., Tokyo, Japan) was used with a column oven (CTO-6A; Simadzu Corp., Kyoto, Japan) at 40°C. PTX was detected at 227 nm and the flow rate was 1 mL/min. Standards of PTX were used with concentrations of 50, 200, 500, and 1000 ng/mL for calibration.

Next, the release rate of PTX from a HPF in vivo was quantified by HPLC. An experimental filtration surgery using a HPF loaded with 0.5 μg of PTX was performed for four rabbit eyes. Surgery was performed with the same procedure as described above. A HPF, which was embedded on the sclera, was removed at 2 and 4 weeks after surgery ($n = 2$ for each week), rinsed with PBS and dried. PTX remaining in the film was also quantified by HPLC. The film had to be removed from the surgical site in this test. Therefore, the test cannot be performed simultaneously in other in vivo tests in which all eyes were subjected to histological preparation.

Statistical Analysis

Surgical failure was defined as either less than 20% IOP reduction at two consecutive measurements, or by the disappearance of the filtration space in UBM images. As described in our previous reports,^{17,18} Kaplan-Meier survival analysis with a log-rank test was performed with surgical failure as the end point in Experiment 1. IOP levels were compared between groups, or between different time points, by two-way repeated measures analysis of variance (ANOVA) in Experiment 1. In Experiment 2, IOP levels were compared between different time points by one-way repeated measures ANOVA. The release rate of PTX from films was compared between groups by two-way repeated measures ANOVA. The size of the avascular area in the bleb was compared between groups by one-way ANOVA. A post-hoc analysis of ANOVA was performed with the Bonferroni test. The scores of fibrosis and vascularity in the subconjunctival tissue determined from histological sections were compared at 4 weeks after surgery between four groups using the Kruskal-Wallis test with Steel-Dwass post-hoc analysis. Statistical analysis was performed using SPSS software (Version 13.0; SPSS Inc., Chicago, IL). For all analyses, P values less than 0.05 were considered statistically significant.

Results

No intraoperative complications occurred in any animals and, therefore, no animals were excluded from this study.

Experiment 1 (4-Week Follow-Up): Results of Filtration Surgery

Postoperative IOP was significantly lower than baseline in all groups (Fig. 1). In addition, postoperative IOP course was significantly different between the four groups ($P < 0.001$). Surgical failure occurred

Figure 2. Series of photos and UBM images of blebs in groups 1 and 2 taken weekly after surgery. (A) Group 1. (B) Group 2. *Upper images:* bleb photos; *lower images:* UBM images. The IOP is indicated in the upper left corner of each UBM image. In group 1, UBM images show a shallow filtration space at 4 weeks after surgery (white arrows in a UBM image of 1). Black arrows in the bleb photos at 4 week postoperatively indicate a large avascular bleb. The highly reflective lines in the bleb indicate the presence of the film (white arrows in a UBM image of 2).

in two of five eyes at 3 weeks after surgery in the control group. In these two failed eyes, there was less than 20% IOP reduction in two consecutive measurements and the filtration space disappeared in UBM images. In contrast, no eyes with PTX failed. Bleb survival was not significantly different between the four groups ($P = 0.096$).

Bleb Appearance of Representative Cases

One week after surgery, a diffuse bleb was observed in all groups (Figs. 2A, 2B, 3A, 3B). In groups 2 and 3 of PTX-treated eyes, the majority of the bleb area became avascular and transparent (Figs. 2A, 3A). The bleb persisted until 4 weeks after surgery. In contrast, there were no avascular areas

detected during the postoperative period in group 4 (Fig. 3B). UBM images showed a subconjunctival filtration space in all PTX-treated eyes throughout the study period. In control eyes (group 1), there were no avascular areas detected during the postoperative period (Fig. 2A). UBM images showed a shallow or no filtration space at 4 weeks after surgery.

Histology of Surgical Sites at 4 Weeks Postoperatively

In PTX-treated eyes, the conjunctival epithelium in a large avascular area of the bleb (Figs. 4D, 5A) was markedly attenuated in all eyes in groups 2 and 3 (Figs. 4E, 5B). In contrast, the conjunctival epithelium in the bleb with no avascular area (Fig. 5D) was almost normal in all eyes in group 4 by histology (Fig.

Figure 3. Series of photos and UBM images of blebs in groups 3 and 4 taken weekly after surgery. (A) Group 3. (B) Group 4. *Upper images:* bleb photos; *lower images:* UBM images. The IOP is indicated in the upper left corner of each UBM image. Black arrows in the photos at 4 week postoperatively indicate a large avascular bleb. The highly reflective lines along the inner border of the bleb wall indicate the presence of the film (white arrows in a UBM image of 2).

5E). However, the subconjunctival connective tissue was loose and hypocellular in the blebs of all PTX-treated eyes (Figs. 4F, 5C, 5F). In addition, the sclerotomy site was associated with less fibrosis and cellular infiltration (Figs. 4F, 5C, 5F). In control eyes, the sclerotomy site was closed by moderate fibrosis, which was accompanied by moderate subepithelial cellular infiltration (Figs. 4B, 4C). No signs of toxicity to the surrounding ocular tissues were observed in PTX-treated eyes.

Comparison of Morphological Parameters Between Groups

Surgical Microscopy

The mean avascular area was significantly larger in groups 2 and 3 than groups 1 and 4 at 4 weeks after

surgery (Table 1). All control eyes and eyes treated with 0.5 μ g PTX developed no avascular area in the bleb postoperatively.

Histology at 4 Weeks After Surgery

The scores of subconjunctival fibrosis were 3.2 ± 0.5 , 0.6 ± 0.6 , 1.0 ± 0.7 , and 1.2 ± 0.4 in groups 1, 2, 3, and 4, respectively. The scores of subconjunctival fibrosis were significantly different between control and all PTX-treated eyes (Table 2). However, the scores of subconjunctival fibrosis were not significantly different between PTX treatment groups. The scores of vascularity in the subconjunctival tissue were 3.0 ± 0.0 , 0.6 ± 0.6 , 1.0 ± 0.0 , and 2.4 ± 0.6 in groups 1, 2, 3, and 4, respectively. Vascularity in the subconjunctival tissue was significantly larger in groups 1 and 4 than in groups 2 and 3 (Table 3).

Figure 4. Bleb morphology in groups 1 and 2 four weeks after surgery. (A–C) From an eye in group 1. (D–F) From an eye in group 2. (A) A bleb showing no avascular area over the filtration site. (D) A bleb photo showing an avascular area over the filtration site. (B, E) Histological sections stained with hematoxylin and eosin. Arrows indicate markedly attenuated conjunctival epithelium. (C, F) histological sections stained with Masson's trichrome. Mild collagen deposits and cellular infiltration are sparse in the bleb of group 1. Open arrows indicate the scleral flap (E, F). The area of scleral resection is shown by a pair of arrows (F). Scale bars: 500 μ m.

Experiment 2 (12-Week Follow-Up)

Postoperative IOP was significantly lower than baseline ($P < 0.001$; Fig. 7). Surgical failure occurred in three of eight eyes at 12 weeks after surgery. Although a diffuse bleb was observed by UBM examination until 10 weeks after surgery, the bleb became flatter after a further 2 weeks (Fig. 7). There were no avascular areas detected during the postoperative period. By histology, the conjunctival epithelium was almost normal in all eyes. In addition, the sclerotomy site was associated with mild fibrosis and cellular infiltration. The scores of subconjunctival fibrosis and vascularity were 2.4 ± 0.7 and 2.7 ± 0.5 , respectively.

Quantification of PTX Released From Films

PTX in HPF and in a 14- μ m thick smooth film was quantified at 1, 2, and 4 weeks after they were incubated in PBS at 37°C. PTX was released from the films with an initial burst-release for 1 week followed by a slow-release phase until 4 weeks (Fig. 6). Almost all PTX was released by 4 weeks. Release of PTX from HPF was slower than that from a 14- μ m thick

smooth film ($P < 0.001$). For in vivo examinations, the residual ratio of PTX in a HPF, which was embedded on the sclera, was 0.32 ± 0.02 at 2 weeks and 0.10 ± 0.04 at 4 weeks after surgery, respectively ($n = 2$ for each week).

Discussion

Bleb-related endophthalmitis due to avascular bleb formation after trabeculectomy with MMC is the most serious complication of this surgery. As one of the solutions for this serious complication, our previous study showed that the concomitant use of HPF and MMC suppressed avascular bleb formation without compromising filtration.¹⁷ However, this procedure could not prevent avascular bleb formation completely. Therefore, we focused on PTX. Because the extended release of PTX from coated coronary stents led to decreased rates of restenosis of coronary arteries in cardiovascular intervention,²³ PTX was shown to provide MMC-like antifibrotic effects during conjunctival wound healing,²⁷ and was effective in promoting the success of experimental filtration surgery with a drug delivery system.^{19,26}

Figure 5. Bleb morphology in groups 3 and 4 four weeks after surgery. (A–C) From an eye in group 3. (D–F) From an eye in group 4. (A) A bleb photo showing an avascular area over the filtration site. (D) A bleb showing no avascular area over the filtration site. (B, E) Histological sections stained with hematoxylin and eosin. Arrows indicate markedly attenuated conjunctival epithelium. (C, F) Histological sections stained with Masson's trichrome. Open arrows indicate the scleral flap (B, C, E, F). The area of scleral resection is shown by a pair of arrows (C, F). Scale bars: 500 μm .

Despite the concomitant use of HPF, avascular blebs were reproduced in this study when using 50 and 5 μg PTX as well as in other rabbit models of filtration surgery with MMC.^{30–33} Jampel et al.²⁶ reported that bleb infection occurred in 50% of rabbit eyes even when 10 μg PTX powder was placed on the sclera approximately 5-mm posterior to the limbus. This indicates that the use of 5 μg PTX in this study might be an overdose. Charles et al.³⁴ reported that sectoral corneal edema and conjunctivitis were notably

decreased in eyes treated with a low dose of MMC (0.02 mg) in a polyanhydride carrier than with a higher dose (0.06 mg) in a rabbit model of full-thickness filtration surgery. Clinically significant ocular toxicity was not noted with a lower dosage of MMC. This result might indicate that MMC overdosing is problematic with the use of delivery methods. In addition, Heldman et al.³⁵ reported that the frequency of histological changes, including medial wall cell necrosis, increased with increasing

Table 1. Size of Avascular Area in the Bleb 4 Weeks After Surgery (mm^2)

Group		
1	0	**
2	21.9 ± 14.2	* **
3	19.9 ± 8.8	
4	0	

Data are shown as mean $\pm$ SD.

* $P = 0.01$, ** $P = 0.005$, by one-way ANOVA with a Bonferroni post hoc test.

Table 2. The Scores of Subconjunctival Fibrosis 4 Weeks After Surgery

Group		
1	3.2 ± 0.5	*** ** *
2	0.6 ± 0.6	
3	1.0 ± 0.7	
4	1.2 ± 0.4	

Data are shown as mean $\pm$ SD.

* $P = 0.031$, ** $P = 0.032$, *** $P = 0.027$, by a Kruskal-Wallis test with a Steel-Dwass post-hoc test.

drug dose in a porcine model of coronary intervention using a PTX eluting stent. Therefore, overdosing of drugs appears to be problematic in other fields such as drug delivery using stents. However, we succeeded in preventing avascular bleb formation by reducing the dose of PTX to 0.5 μg . This result indicates that the use of a HPF with 0.5 μg PTX might be able to reduce the risk of postoperative infection in filtration surgery. In histology, 0.5 μg PTX suppressed subconjunctival fibrosis significantly compared with the control group in this study. In other reports, PTX and MMC were both shown to inhibit fibroblast migration and proliferation in the subconjunctival space in rabbit eyes²⁷ and inhibit human Tenon's fibroblast migration and proliferation in an in vitro study.²⁸ These features are consistent with this study. On the other hand, there was avascular bleb formation in groups with less fibrosis such as the 50 and 5 μg PTX rabbits. This result indicates that 50 and 5 μg PTX might be excessive to prevent conjunctival damage. In addition, no infection was seen in any of the groups in this work. Our previous study showed that a HPF, which was placed subconjunctivally over the filtration site, reduced avascularity and conjunctival damage in the bleb in rabbit filtration surgery with MMC.²¹ Therefore, we considered that a HPF, which protects from conjunctival damage compared with the Jampel study²⁶ where 50% infection rates with 10 μg PTX were observed, might prevent postoperative infection. Given that a HPF with 0.5 μg PTX provided sustained IOP reduction and prevented avascular bleb formation without compromising filtration, the use of this drug delivery material might represent a better method than MMC application in the traditional manner regarding reduction in the risk of postoperative infection.

In Experiment 2, some blebs became flatter starting at 10 weeks after surgery. There are some reports about bleb survival in MMC-treated eyes in a rabbit model of filtration surgery. Bergstrom et al.³⁶ reported that survival rate was only 25% (2/8 eyes) at 3 months follow-up. Jampel et al.²⁶ reported that survival rate was 83% (5/6 eyes) at 7 weeks follow-up. Other reports indicated survival rate ranged from 0% to 50% at various follow-up periods (from 4–6 weeks).^{16,37–39} From these results, it appears to be difficult to achieve greater than 90% bleb survival (human 1-year goal) at 3 months in a rabbit model of filtration surgery with MMC, because the wound healing reaction is more aggressive in rabbits than in humans. Accordingly, the results of experiment 2

indicate that a HPF with 0.5 μg PTX provided sustained IOP reduction and favorable bleb survival even after a long follow-up period. However, a more prolonged release of PTX, or concomitant use of topical steroid, may be necessary for longer-term bleb survival in a rabbit model.

In this study, IOP changes were significantly different between each of the groups. The scores of subconjunctival fibrosis were 3.2 ± 0.5 , 0.6 ± 0.6 , 1.0 ± 0.7 , and 1.2 ± 0.4 in groups 1, 2, 3, and 4, respectively. In addition, the mean avascular area in the bleb was significantly larger in groups 2 and 3 than in groups 1 and 4 at 4 weeks after surgery. No avascular areas in the bleb were observed postoperatively in all control eyes and eyes treated with 0.5 μg PTX. The differences of fibrosis and avascular formation between each group might produce a significant difference in IOP reduction.

An ideal drug delivery system consists of a biodegradable material and should aid the local release of drug without local or systemic toxicity. In this study, the HPF worked well as a drug delivery device and kept sustained local release of PTX. PTX was released gradually for about 4 weeks from a HPF. Jampel et al.⁴⁰ reported that wound healing reactions occur most actively during the first 2 weeks postoperatively. According to the other reports, inflammatory cell infiltration was resolved within 2 weeks following glaucoma filtration surgery.^{14,41} Therefore, the release profile of PTX in our HPF carrier may be appropriate to suppress the major proliferative stage of wound healing.

In this study, we intended to release PTX only toward the filtration space. Therefore, PTX was loaded only into a 7- μm thick smooth film, which was oriented toward the filtration space. Upon in vitro examination, the release rate of PTX from the stacked 14- μm thick HPF was slower than from a smooth 14- μm thick film, which was not laminated and was uniformly loaded with PTX. The difference of release rate of PTX from the 14- μm thick laminated HPF and smooth films might be attributed to a reduction of the release rate of PTX from the side of a 7- μm thick HPF without PTX. Accordingly, the 7- μm thick HPF without PTX may have blocked the release of PTX from the honeycomb side, which was facing the subconjunctival Tenon tissue in the filtration surgery.

The use of a drug-free HPF was also effective for reducing IOP and maintaining the bleb, as in our previous study.¹⁸ However, histological examination showed that 0.5 μg PTX suppressed subconjunctival

Table 3. The Scores of Vascularity in the Subconjunctival Tissue 4 Weeks After Surgery

Group		
1	3.0 ± 0.0	
2	0.6 ± 0.6	***
3	1.0 ± 0.0	**
4	2.4 ± 0.6	*

Data are shown as mean $\pm$ SD.

* $P = 0.024$, ** $P = 0.014$, *** $P = 0.036$, by a Kruskal-Wallis test with a Steel-Dwass post-hoc test.

fibrosis significantly compared with the control group in this study. There are reports about experimental filtration surgery with PLA or PLGA drug delivery implants, which were placed adjacent to the surgical site subconjunctivally.^{13,14,16} Rodríguez et al.¹⁶ reported that the use of drug-free PLGA implants was also associated with reduced IOP, corroborating the spacing effect of the implant. However, the difference of mean surgical survival between drug-free PLGA implants and the control was 1.2 days (approximately 13.1 vs. 11.9 days). Cui et al.¹³ and Yan et al.¹⁴

Figure 6. Release ratio of paclitaxel (PTX) from films. PTX in a HPF or a smooth film was quantified by HPLC at 1, 2, and 4 weeks ($n = 4$). Release of PTX from a HPF was slower than that from a 14- μ m thick smooth film ($P < 0.001$).

reported that there were no significant differences in IOP reduction and surgical survival between drug-free PLA or PLGA implants and control. From their results, these implants might not have a sufficient space-keeping effect. In this study, the persistence of blebs with the use of a drug-free HPF might be due to the effect as a wound adhesion barrier rather than a

Figure 7. IOP changes over time and bleb morphology in experiment 2. *Upper left:* IOP changes over time. *Lower left:* a bleb photo and an ultrasound biomicroscopy (UBM) image at 10 weeks after surgery. *Lower right:* a bleb photo and an UBM image at 12 weeks after surgery. *Upper right:* a histological section stained with Masson's trichrome. The area of scleral resection is shown by a pair of arrows. Scale bar: 500 μ m.

space-keeping effect because our 14- μ m thick film is far thinner than the materials used in other reports. Given that the use of a drug-free HPF reproduced the persistence of the bleb in our present study as well as in our previous study, the smooth surface of the film was considered to work as a mechanical adhesion barrier between the subconjunctival Tenon tissue and the sclera.

In conclusion, PTX released from a HPF promoted bleb survival and lowered IOP compared with the HPF alone. Blebs treated with higher doses of PTX (50 and 5 μ g) became avascular. However, the lowest dose of PTX (0.5 μ g) was effective for preventing bleb avascularity without compromising filtration in rabbits. Further investigations, including studies with a larger sample size and a longer follow-up period and design of a device with more prolonged PTX release are warranted before potential clinical application of the HPF in humans can proceed.

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