Scleral Plug of Biodegradable Polymers Containing Tacrolimus (FK506) for Experimental Uveitis

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PURPOSE. To evaluate the efficacy of a biodegradable polymeric scleral plug containing the immunosuppressive agent, FK506, in a rabbit model for experimental uveitis.

METHODS. The scleral plugs were prepared by dissolving poly(ol-lactide-co-glycolide; PLGA) and FK506 (weight, 8.5 mg; length, 5 mm; 1% FK506). The release of FK506 was evaluated in vitro by spectrophotometry on days 1, 3, 7, 14, 21, and 35. In vivo, FK506 concentrations of the vitreous were measured by high performance liquid chromatography 2 and 4 weeks after intravitreal plug implantation in pigmented rabbits. Sixteen pigmented rabbits were immunized twice subcutaneously with 10 mg of Mycobacterium tuberculosis H37Ra antigen. Twelve days later, the right eyes of all rabbits were challenged with an intravitreal injection of 50 μg of antigen. After the first challenge, the 16 eyes of 16 pigmented rabbits were divided into two groups. Scleral plugs were implanted into the vitreous of the right eye of eight rabbits. Eight control rabbits received a sham device. The aqueous protein concentrations and cell counts were determined on postchallenge days 7, 14, and 28. To simulated chronic inflammation, the eyes were rechallenged with intravitreal antigen on day 14 and were observed for 1 month. Inflammation of the anterior chamber and the vitreous were graded clinically by two masked observers. Retinal function was evaluated by electroretinography (ERG) and histologic examination.

RESULTS. Clinical scores (anterior chamber cells, flare, and vitreous opacity) showed that treated eyes had significantly less inflammation than untreated eyes (P < 0.001). Quantitative analysis of inflammatory cells (P < 0.001) and protein concentrations (P < 0.0001) in the anterior chamber showed significant decreases in treated eyes. Histopathologic examination showedmarked inflammation and tissue disorganization in the untreated eyes. No retinal toxicity was detected, histopathologically and electroretinographically. After antigen rechallenge, inflammation in experimental eyes was still less than in control eyes.

CONCLUSIONS. Intravitreal sustained-release of FK506 from a biodegradable polymeric scleral plug was highly effective in suppressing the inflammation of experimental uveitis in a rabbit model for at least 6 weeks. This device may be useful in the management of patients with severe chronic uveitis. (Invest Ophtalmol Vis Sci. 2003;44:4845–4852) DOI:10.1167/iovs.02-1228

Tacrolimus (FK506) is an immunosuppressive agent that has been isolated from the fermentation broth of Streptomyces tsukubaensis.1,2 It has a mechanism of action similar to that of cyclosporin A, but is more potent.3 In vitro, FK506 inhibits the generation of cytotoxic T lymphocytes and the production of interleukin-2 and -3 and interferon-γ at levels approximately 100 times lower than that of cyclosporin A.4 In vivo, FK506 showed a strong immunosuppressive effect in a variety of animal models of transplantation and in the treatment of experimental autoimmune uveoretinitis.5,6

The use of FK506 is of special interest in ophthalmology, because it may be effective in the treatment of immune-mediated disease, such as corneal graft rejection,6 keratitis, scleritis, ocular pemphigoid, and uveitis. In a previous study,7 oral FK506 was used to treat patients with refractory uveitis including Behçet’s disease. The adverse effects8–10 of systemically administered FK506, however, include nephrotoxicity, hypertension, hyperesthesia, muscular weakness, insomnia, tremor, photophobia, gastrointestinal symptoms, and central nervous system alterations. Long-term therapy is necessary for chronic uveitis, and the incidence of systemic side effects may be increased when FK506 is administered systemically. Furthermore, systemically administered drugs penetrate poorly into the intraocular tissues because of the blood–ocular barrier. Therefore, topical application of drugs is limited by the lipophilic physical and chemical characteristics of the drug and vehicle and results in poor intravitreal penetration.

Therefore, intravitreal injection is becoming a more accepted method of delivering drugs directly to the vitreous and retina. Frequent intravitreal injections are essential to maintain drug concentrations within a therapeutic range in the posterior segment, and this approach may be associated with a risk of complications including cataract, retinal detachment, vitreous hemorrhage, and endophthalmitis. In addition, the half-life of a single intravitreally injected drug was demonstrated to be insufficiently long for an effective clinical response. Therefore, we have attempted to develop controlled drug delivery systems10 for vitreoretinal tissue that are able to deliver a drug for long enough time after a single intravitreal injection. To avoid the complications mentioned herein, we prepared a scleral plug of biodegradable polymer containing FK506 as an intravitreal sustained-release device and investigated whether such an implantable biodegradable drug delivery device is effective in the treatment of severe panuveitis in a rabbit model.

MATERIALS AND METHODS

Poly(ol-lactide-co-glycolide) with weight-average molecular weight of 65,000 and a 50:50 copolymer ratio of ol-lactic acid to glycolic acid...
The weight-average molecular weight was determined by gel permeation chromatography by the supplier. FR900506 (FK506) was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan).

**Preparation of Scleral Plugs Containing FK506**

The scleral implants were prepared by dissolving the polymer and FK506, which is a good solvent for the polymer and the drug. PLGA (50/50)-63,000 (247.5 mg) with FK506 (2.5 mg) was dissolved in 1,4-dioxane (20 mL). The resultant solution was sterilized by filtration (pore size, 0.22 μm; Durapore; Nihon Millipore Ltd., Tokyo, Japan) and lyophilized (FDU-830; Tokyo Rikakikai, Tokyo, Japan) in an autoclaved glass vial to obtain a homogeneous cake. In the isolator, the cake then was compressed into a scleral implant on a hot plate (Model HM-19; Koike Precision Instruments, Osaka, Japan) at a temperature ranging from 80°C to 100°C. It was confirmed that FK506 degradation did not occur during the preparation process of the implants. The implants had FK506 loading of 1 wt%. Using the same procedures, scleral plugs without FK506 (placebo plugs) were made. The scleral implant weighed 8.5 mg and was 5 mm in length and 1 mm in diameter. It was shaped similar to the metallic scleral plug using during vitrectomy (Fig. 1).

**Evaluation of In Vitro Release of FK506 from the Plug**

The implants were placed in 2 mL of phosphate-buffered solution (pH 7.4) in a closed vial and were immersed in a shaking water bath at 37°C. On days 1, 3, 7, 14, 21, and 35, approximately 2 mL of the released medium was removed and replaced with the same quantity of fresh medium. The amount of FK506 released into the medium was measured by high-performance liquid chromatography (HPLC). All chromatograms were obtained using commercially available components. The analytical column used was a 4.6-mm (inner diameter) × 25-cm prepackaged column (model ODS-A303; YMC, Kyoto, Japan). A pump (model LC-10AS; Shimadzu, Kyoto, Japan) was used at a constant flow rate of 1.0 mL/min with a pulse dampener and degasser (model KT-16; Showa Denko, Tokyo, Japan). The eluent consisted of 30 mM PBS and 5 mM heptane sulfonate sodium salt in 2% acetonitrile-water (1:49; pH 2.0). The column oven (model CTO-10A; Shimadzu) was equipped and set at 45°C. A spectrophotometric detector (model SPD-6A; Shimadzu) was used at a wavelength of 254 nm.

**Implantation of the Scleral Plug and Evaluation of In Vivo Release of FK506**

We used pigmented rabbits weighing 1.5 to 2.0 kg each in the study. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbits were anesthetized with an intramuscular injection of pentobarbital sodium (20 mg/kg) before treatment. The pupils were dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride eye drops. The ocular surface was then anesthetized with a topical instillation of 0.4% oxybuprocaine hydrochloride.

After the sclera was exposed, a 1-mm sclerotomy was performed with a V-lance 2 mm from the limbus. The FK506-loaded scleral plug was inserted at the sclerotomy site. The conjunctival wound was sutured with 8-0 dixon. One drop of topical 0.5% gentamicin solution was instilled into the eye after surgery for the prevention of infection.

At weeks 2 and 4 after intravitreal implantation, FK506 concentrations in the vitreous were determined. Five rabbits were used at each time point. The intravitreal FK506 concentration was determined.
opacity was also graded on a 0 to 4 scale based on the examination of a 0.5-mm2 beam during slit lamp examination. The vitreous opacity was also graded on a 0 to 4 scale based on the cell number and opacity observed reported grading system.\textsuperscript{13,14} Anterior chamber cells and fragments of the severity of the inflammation were evaluated. We determined the anterior chamber cells, flare, and vitreous opacity to be quantifiable measurements of the severity of the inflammation according to a previously reported grading system.\textsuperscript{13,14} Anterior chamber cells and flare were graded on a 0 to 4 scale based on the cell number and opacity observed in a 0.5 × 2-mm beam during slit lamp examination. The vitreous opacity was also graded on a 0 to 4 scale based on the examination of the posterior pole with an indirect ophthalmoscope and a 20-D aspherical lens.

**Clinical Observations**

On days 3, 7, 14, 17, 21, and 28 after the first challenge, two masked observers used slit lamp biomicroscopy and indirect ophthalmoscopy to evaluate the severity of inflammation. We determined the anterior chamber cells, flare, and vitreous opacity to be quantifiable measurements of the severity of the inflammation according to a previously reported grading system.\textsuperscript{13,14} Anterior chamber cells and flare were graded on a 0 to 4 scale based on the cell number and opacity observed in a 0.5 × 2-mm beam during slit lamp examination. The vitreous opacity was also graded on a 0 to 4 scale based on the examination of the posterior pole with an indirect ophthalmoscope and a 20-D aspherical lens.

**Aqueous Protein Measurement and Cell Count**

Three animals in each group were used at 1, 2, and 4 weeks after the first challenge for aqueous protein measurement and cell count. Animals were anesthetized, and 0.2 to 0.3 mL aqueous humor was aspirated from the right eye of each of the rabbits with a heparin-rinsed glass syringe connected to a 27-gauge needle. Aqueous cell count was measured by hemocytometry. One drop of aqueous was placed on a microscope slide and stained with Wright stain for differential cell count. The remaining aqueous was centrifuged at 250g for 5 minutes, and the supernatant was used for protein measurement. The protein content of the aqueous humor was determined using a Bio-Rad (Richmond, CA) assay kit with bovine serum albumin as a standard dilution reference curve, according to the manufacturer’s recommendation.

**Electroretinographic Study**

Retinal function of FK506-treated group was evaluated by photopic electroretinography (ERG; model ERG-50; Kowa, Tokyo, Japan) on day 28 after the first challenge.

**Histopathologic Study**

Eight weeks after the first challenge, selected eyes of treated and untreated rabbits were enucleated and processed for histopathologic
The eyes were immediately fixed in phosphate-buffered 2.5% glutaraldehyde and 5% formaldehyde in 0.15 M phosphate buffered solution (pH 7.2) at 20°C. The specimens were dehydrated in a series of ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. Serial sections of the eye were cut at a thickness of 4.0 μm and mounted on glass slides. After being dewaxed in xylene, sections were hydrated in a series of descending concentrations of ethanol. The hydrated sections were stained with Mayer’s hematoxylin solution at 20°C for 10 minutes, rinsed in tap water for 15 minutes, immersed in 0.5% eosin solution at 20°C for 10 minutes, dehydrated in a series of ascending concentrations of ethanol, cleared in xylene, and mounted in synthetic resin solution (Harleco; Kokusai Shiyaku, Kobe, Japan). All sections were examined in detail by light microscopy (Provis AX 70 with U-photograph; Olympus Co., Japan).

Statistical Comparison
A Mann-Whitney nonparametric test was used to compare the grading of anterior chamber cells, anterior chamber flare, and vitreous opacity between treated and untreated eyes. An unpaired analysis was used to compare the quantitative determination of aqueous cell number and cell protein at different times after the initial challenge.

RESULTS
In Vitro Release of FK506 from the Scleral Plugs and In Vivo Release in the Rabbit Eye
The cumulative release of FK506 from the implants is plotted in Fig. 2A. The release lasted more than 35 days. FK506 concentrations in the vitreous after plug implantation are shown in Figure 2B. The PLGA (50/50)-63,000 scleral plug maintained a nearly constant FK506 level (480–350 ng/g) in the vitreous for 4 weeks.

Clinical Observations
The results of the clinical grading of anterior chamber cells, flare, and vitreous opacity after the first challenge are shown in
effects of FK506 based on ERG

In the FK506-treated group, there was no evidence of toxic

Electroretinographic and Histopathologic Study

the aqueous protein concentration and cell count re

Quantitative Measurement of Aqueous Cells and Protein

The aqueous protein concentration and cell count reflected the clinical observation (Fig. 6). The difference between control and FK506-treated eyes was statistically significant at 1 and 2 weeks after the first challenge, and this difference was maintained at 4 weeks.

In the FK506-treated eyes, the biodegradable scleral plug containing the FK506 device suppressed the mean aqueous protein concentration at all time points compared with the control eyes. Similarly, in the treated eyes, the mean aqueous cell count was significantly lower at all time points than in the control eyes.

Electroretinographic and Histopathologic Study

In the FK506-treated group, there was no evidence of toxic effects of FK506 based on ERG findings, and the b-wave amplitude remained normal throughout the study. Histopathologic analysis did not show any evidence of drug-released toxic effect in the implanted scleral plug group. Mild foreign body reactions were observed around the degrading plug in both groups. The reaction appeared to the same degree in both

DISCUSSION

In this study, a biodegradable scleral plug containing FK506 was effective in suppressing uveitis in a rabbit model. Implanted scleral plugs containing FK506 significantly reduced the uveitis ophthalmoscopically and histopathologically and the aqueous protein and number of cells in the anterior chamber were reduced. The device prevented experimental chronic uveitis for a prolonged time after the first challenge.

This scleral plug delivers drug into the vitreous as the polymer degrades. The degradation of the matrix depends on
its molecular weight and polymer composition. The release rate increased with the decrease of the molecular weight and lactide content of PLGA. Because we found the remaining polymer composed of scleral plug at the implantation site after completion of drug release (data not shown), in this study we chose poly(lactic-glycolic acid) in which the ratio of lactic acid to glycolic acid was 50:50, and the molecular mass was 63,000 Da as a relatively rapidly degradable polymer. In this study, the in vitro release studies showed stable, long-term sustained release of drug.

In vivo experiments showed that intravitreal FK506 remained at a constant level for at least 1 month after intravitreal device implantation. The concentrations required to control immune-mediated diseases of the eye have not been established; however, the recommended minimum FK506 serum level in patients undergoing organ transplantation is 2 to 10 ng/mL. This drug concentration could be considered the minimum effective concentration to control the immune response in immune-mediated eye diseases, but the therapeutic range of the intravitreous concentration necessary for treatment of uveitis is unknown. In our study, the scleral plug was effective in delivering significant drug concentration to the vitreous (480–350 ng/g) for 4 weeks. These FK506 concentrations in the vitreous were effective for treatment of experimental severe uveitis. In addition, retinal damage due to FK506 was not detected electroretinographically or histopathologically.

FK506 is an immunosuppressive agent that has been shown to have immunosuppressive activities similar to those of cyclosporine. It suppresses in vitro immune systems at approximately 100 times lower concentrations than cyclosporin A. Many experimental and clinical studies of FK506 have been reported. Systemic toxicity of FK506 was reported to be dose dependent. It included diarrhea and left ventricular bleeding in investigations in the dog and atrophy of the thymus, renal calcification, and tubular degeneration in the rat. Clinically, the previous reports also indicate that systemic administration of FK506 induces a variety of side effects, such as renal impairment (28.3%), neurologic symptoms (18.9%), gastrointestinal symptoms (20.8%), and hyperglycemia (13.2%). Therefore, it is difficult to use the systemic administration of

![Figure 6. Aqueous cell count (A) and protein concentration (B) at 1, 2, and 4 weeks after the first challenge. Data are the mean ± SEM or results in eight animals. (A) *P = 0.0003, **P = 0.0017, unpaired t-test; (B) *P = 0.0001, unpaired t-test.](image-url)
FK506, because the range of the trough level of the drug that produces maximum effectiveness and minimum side effects is considered to be between 15 and 25 ng/mL. Considering these data, we therefore investigated topical administration with the intravitreal sustained release of FK506 for uveitis.

Several intravitreal sustained-release devices in the treatment of experimental uveitis have been reported. Dose-dependent inflammation was found with concentrations of tuberculin antigen varying from 20 to 50 μg. Posterior segment inflammation was observed with each dose, but significant anterior segment inflammation was observed only with 50 μg. To maximize the ability to detect a significant treatment effect on both anterior and posterior segment inflammation, the 50-μg dose was chosen for subsequent experiments. Our results showed that the clinical grades of experimental uveitis with and without treatment with the FK506-loaded scleral plug were similar to those in previous reports. The result of our experiment clearly shows that the biodegradable scleral plug containing FK506 provided effective, prolonged treatment of uveitis in a rabbit model.

This device may be useful in the treatment of patients with severe chronic uveitis who are intolerant to currently available therapies.

References


