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. 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 showed marked inflammation and tissue disorganization in the untreated eyes. No retinal toxicity was detected, histopathologically and electoretinographically. 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 Ophthalmol 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.3 5

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 Behcet’s disease. The adverse effects8 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.

To avoid systemic side effects, topical application of an agent is preferred to the oral and intravenous routes. Unfortunately, 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 systems9 10 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 ε-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 was then 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 dexon. One drop of topical 0.3% 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
by the HPLC system. The rabbits were killed with an overdose of intravenous pentobarbital sodium, and the eyes were immediately enucleated. The eyes were stored at −85°C until processing. The retina and choroid separated from the vitreous while frozen and were homogenized after adding chilled acetonitrile (0.3 mL). The homogenate was centrifuged at 3000 rpm for 10 minutes (CF7D2; Hitachi, Ltd., Tokyo, Japan) and then the supernatant was dried under reduced pressure with a centrifugal concentrator (model VC-960; Taitec, Saitama, Japan). The residue was dissolved with 0.5 mL of the mobile phase eluent. Fifty milliliters of the solute was injected for analysis. Our methods provided FK506 sensitivity as low as 30 ng/mL.

Preimmunization with Antigen
A relatively severe, nonspecific experimental uveitis model was created according to modification of a previously published protocol.11,12 Sixteen pigmented rabbits, each weighing 1.5 to 2.0 kg, were injected subcutaneously with 10 mg of Mycobacterium tuberculosis H37RA antigen (Difco, Detroit, MI) suspended in 0.5 mL of mineral oil. One week later, a second injection of the same amount of subcutaneous antigen was given. In all animals, a firm, discrete nodule was identified at the injection site approximately 1 week after the second antigen injection, confirming the efficacy of immunization.

Uveitis Induction and Implantation of FK506-Containing Scleral Plugs
A microparticulate suspension of M. tuberculosis H37RA antigen was prepared by ultrasonication a suspension of the crude extract in sterile balanced salt solution.11,12 One day after scleral implantation, 50 μg of antigen suspended in 0.1 mL of balanced salt solution was injected into the vitreous cavity of the right eye of all rabbits under anesthesia (first challenge). To simulate chronic inflammation with exacerbations, eyes in the treated and untreated group were rechallenged with the same amount of intravitreal antigen on day 14 (second challenge). One drop of 1% atropine solution was then instilled into the eye to maintain postoperative cyclopia.

In the treated group, eight eyes of eight pigmented rabbits were used. Animals were anesthetized with an intramuscular injection of 0.3 mL ketamine hydrochloride (100 mg/mL) and 0.1 mL xylazine hydrochloride (100 mg/mL) per kilogram body weight. 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 dexon.

In the untreated group, eight eyes of another eight rabbits received surgical implantation of the placebo plug. One drop of topical 0.3% gentamicin solution was instilled into the eye after surgery for the prevention of infection.

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.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 measure by hemocytometer. 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
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 groups. In the control group eyes, marked inflammatory cells were seen on the surface of the retina and vitreous, corresponding to lesions of white exudates visible ophthalmoscopically. Destruction of retinal structures was seen in some areas (Fig. 7A). Histopathologic examination showed marked inflammation and tissue disorganization in the control eyes, whereas FK506-treated eyes had preserved architecture and greatly reduced inflammatory cells (Fig. 7B).
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 that does 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.
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 intravitreous 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


