Sustained Delivery of Retinoic Acid From Microspheres of Biodegradable Polymer in PVR

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Purpose. The aims were to obtain a controlled intravitreous release of retinoic acid (RA) by injecting drug loaded microspheres of biodegradable polymers and to study the potential use of this RA delivery system in a rabbit model of proliferative vitreoretinopathy (PVR).

Methods. The release of RA in vitro from 15 mg of 50-50 poly(DL-lactide-co-glycolide) (PLGA) in 1 ml of water at room temperature was measured with a spectrophotometer. In a rabbit model of PVR, 11 eyes were injected with 5 mg of microspheres containing 22 ng of RA/mg of PLGA, and seven control eyes were injected with microspheres of the same polymer that did not contain RA. In a third group, six rabbits were injected with 5 mg (n = 3) and 10 mg (n = 3) of microspheres containing RA.

Results. The initial concentration of RA was 20.8 μg/mg of PLGA. The release curve showed a fairly constant daily release of 7 μg/d for about 30 days. At 40 days, the release rate decreased to about 6 μg/d. After 40 days, 82.8% of the RA was released. Four of 11 treated rabbits (36%) and 7/7 (100%) controls showed tractional retinal detachment (TRD) (P < 0.01) after 2 months. Histopathologically, a mild, localized, foreign body reaction was observed.

Conclusions. The authors obtained a sustained release of RA from PLGA microspheres in vitro for 40 days. A single injection of RA-loaded microspheres in suspension in BSS was effective in reducing the incidence of TRD after 2 months in a rabbit model of PVR. Invest Ophthalmol Vis Sci 1993;34:2743–2751.

Proliferative vitreoretinopathy (PVR) causes a majority of failures of vitreoretinal surgery despite the use of silicone oil (SiO) as an extended tamponade.1-3 Therefore, the pharmacologic approach for the prophylaxis of PVR is gaining in importance. Antineoplastic agents, vitamin A, and drugs injected intravitreously for the treatment of PVR, endophthalmitis, and other vitreoretinal diseases are under investigation.1-4-6 Because these substances have relatively short retention times,4-6,10 and some have small therapeutic indices, repeated intravitreous injections are needed to maintain effective drug levels that can result in an increased incidence of complications.

The use of polymers in the form of microspheres that can dissolve into nontoxic metabolites is a promising sustained drug delivery system for intraocular use. Poly(lactic acid) (PLA) was the first polymer used in bioerodable implants in 1970.11 To date, the biodegradable polymers of lactic acid, glycolic acid, and their copolymers have a prominent place in studies on the prolonged delivery of pharmacologic agents in general. For the eye, the release from PLA and poly(lactic-co-glycolic)acid microspheres of 5-FU, a drug commonly used as an antiproliferative agent, has been studied in vivo and in vitro.12 Studies on the controlled release of adriamycin from PLA microspheres in fil-
tering glaucoma surgery and in an animal model of PVR have been published recently.\textsuperscript{13,14}

Retinoic acid (RA), a lipophilic derivative of vitamin A and a metabolite of the photoreceptor pathway, has likewise been found to have antiproliferative effects on the cells of the retinal pigment epithelium (RPE).\textsuperscript{15,16} In a rabbit model of PVR, when delivered as a single intravitreal injection dissolved in silicone oil (SiO), RA has been shown to be effective in decreasing the incidence of tracional retinal detachment (TRD).\textsuperscript{17} However, SiO is not always needed in vitreous surgery for retinal detachment with PVR. Scleral buckling procedures can be effective in treating a retinal detachment with PVR grades A to C.\textsuperscript{1,2} A system able to deliver antiproliferative substances such as RA into the vitreous cavity could be useful in the management of the proliferative changes when SiO is not needed.

In this paper, we report the kinetics of RA release from PLGA microspheres in vitro and the antiproliferative effect of intravitreal release of RA from a single injection of polymer microspheres in an animal model of PVR.

MATERIALS AND METHODS

Poly(D,L-lactic-co-glycolic)acid (PLGA) (Resomer RG502, Boehringer, Germany) has an inherent viscosity approximately 0.2 dl/l in 0.1% chloroform, at 25°C and a viscosity-average molecular weight of 3000 d. Polyvinyl alcohol (PVA, [Polysciences, Inc., Warrington, PA]) has an average molecular weight of 78,000 d and is 88% hydrolyzed. All-trans RA (all-trans vitamin A acid, 98%) was supplied by Eastman Kodak Company (Rochester, NY).

Microsphere Preparation

The microspheres were prepared by a modified solvent evaporation method\textsuperscript{18} using a single emulsion. In a disposable glass test tube of 10 ml capacity, a solution of 400 mg of PLGA in 2 ml of methylene chloride (Fisher Scientific Company, Fair Lawn, NJ) was prepared. After the solution was clear, 10 mg of RA was added. When the new solution was clear, 2 ml of 1% PVA saturated with methylene chloride were added. This mixture was emulsified on a Vortex agitator (Vortex Genie, Springfield, MA) for 10 seconds at velocity.\textsuperscript{10} Immediately the emulsion was incorporated into 100 ml of 0.1% PVA and stirred on a magnetic stirrer (Corning Stirrer/Hot Plate, Corning, NY) under an aspirating hood at room temperature for about 3 hours until the methylene chloride evaporated.

The microspheres were filtered through sieves with apertures of 212, 106, 53, and 38 μm (Newark Wire Cloth Co., Newark, NJ), washed three times with distilled water to remove the PVA, centrifuged (1000g for 10 minutes) (Sorvall superspeed RC2-B, DuPont, Wilmington, DE) and freeze dried (Freeze dryer 5, Labconco Corp., Kansas City, MO).

The dried microspheres were stored over anhydrous CaSO\textsubscript{4} (W.A. Hammond Drierite Company, Xenia, OH) at 3°C until required for use.

Microsphere Evaluation

To identify the residual solvent present into the microspheres, we put 100 mg of microspheres each in four hermetically sealed vials (14 ml) at velocity 10. We stored two vials at 55°C and two at room temperature. A headspace technique consisting of collecting 1 ml of the air inside the vial with a microsyringe (S.G.E. 1B-FV56 syringe, Supelco, Inc., Bellefonte, PA) was used. The air was analyzed in a gas chromatography system (Auto system GC, Perkin Elmer, Norwalk, CT) with the following temperature setting: oven 50°C, injector 80°C, detector 100°C. Four new bunches of 100 mg of microspheres made with the same polymer were stored in a vacuum for 2 days, and the air was examined as described.

The size and the shape of the microspheres were checked by optic (BH2 Olympus, Tokyo, Japan) and scanning electron (AMR 1000A, Amray Inc., Bedford, MA) microscopy.

RA-loaded microspheres were dissolved in methylene chloride, and the RA content of the solution determined with a spectrophotometer (DU-70 Spectrophotometer, Beckman, Fullerton, CA) at 366 nm. The absorption was compared with a standard curve.

In Vitro Experiments

Because RA is insoluble in water, in vitro release kinetics were carried out to study the amount of RA left in the microspheres.

Fifteen mg of RA-loaded microspheres were suspended in 1 ml of distilled water in each of 14 polypropylene tubes with cap (Pipet Tips, Kew Scientific, Inc., Columbus, OH) of 1.5 ml capacity. The resulting suspensions were continuously agitated at room temperature on a shaker (Lab-line Instruments, Inc. Melrose Park, IL).

At 1, 4, 7, 14, 21, 30, 40 days, two samples were centrifuged, the supernatant was removed, and the remaining microspheres were washed three times in distilled water (100 ml) and freeze dried. They were dissolved in 20 ml of methylene chloride, and the concentration of RA was determined spectrophotometrically at 366 nm.

In Vivo Experiments

All in vivo experiments were conducted using 24 pigmented rabbits of both sexes weighing 2.7 to 3.6 kg.
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They were treated in compliance with the ARVO resolution on the use of animals in research.

All the rabbits were anesthetized with 0.5 ml of chlorpromazine HCl (25 mg/ml) and 1 ml of ketamine HCl (100 mg/ml) per kg intramuscularly. Proparacaine HCl 0.5% eye drops were instilled as a topical anesthetic before surgery. Mydriasis was obtained with 10% phenylephrine HCl and 1% cyclopentolate eye drops. Gas compression of the vitreous body with 0.4 ml of perfluoropropane gas (C₃F₈) (PCR, Gainesville, FL) was performed as previously described.⁵⁵,⁵⁶ After 4 days, the rabbits were reanesthetized and the gas was exchanged with a suspension of microspheres in balanced salt solution (BSS). A tuberculin syringe with a 27-gauge needle was used to inject the suspension 4 mm posterior to the limbus at the 11 o'clock position, and a 30-gauge needle was inserted at the 2 o'clock position to allow the gas to escape.

In group 1, 11 rabbits (11 eyes) received a single intravitreous injection of 5 mg of microspheres containing RA (22 μg/mg of PLGA). In 8 eyes, the microspheres were suspended in 1 ml BSS and in 3 eyes, in 1 ml of 0.08 mg/ml gentamicin solution in BSS.

In group 2, 7 control rabbits (7 eyes) received a single intravitreous injection of 5 mg of microspheres not containing RA. In 5 eyes, the microspheres were suspended in 1 ml BSS, and in 2 eyes the microspheres were suspended in 1 ml of 0.08 mg/ml gentamicin solution in BSS.

In group 2 (control group), 6 rabbits (6 eyes) were injected with microspheres containing RA (20 μg/mg of PLGA). Three eyes received 5 mg and 3 eyes received 10 mg to study how the microspheres interacted with the intraocular environment in a clinical fashion and whether they could give any histopathologic adverse reaction.

At the end of this procedure, group 1 and group 2 received 150 000 homologous fibroblasts in 0.1 ml of culture medium injected through a 30-gauge needle to produce periretinal proliferation.⁵¹ The cells were derived from subconjunctival tissue and cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 5000 units of penicillin-streptomycin, and 14.6 mg of glutamine. Leftover material was replenished in fresh media and checked for viability and contamination. Trypan Blue Exclusion Test⁶² was performed to determine cell viability, which was always found to be greater than 95%.

After every surgical procedure, Bacitracin zinc (400 U)-Neomycin sulfate (5 mg)-Polymixin B sulfate (10 000 U) (Fougera, Melville, NY) and Dexamethasone Na phosphate (0.05%) (MSD, West Point, PA) ophthalmic ointments were applied topically.

In groups 1 and 2, the rabbits were examined at the slit lamp and with the indirect ophthalmoscope before surgery, 1, 4, 7 days after surgery, and then every week for 2 months. In group 2, the rabbits were examined before surgery, 1, 4, and 7 days after surgery, and then were sacrificed 2 (n = 2), 3 (n = 2), and 8 (n = 2) weeks after surgery with an intravenous overdose of sodium pentobarbital. The proliferative changes were classified in five stages according to Fassenberg.²¹

Absence of TRD: grades 1 and 2 were considered successful; grades 3, 4, and 5, or the presence of TRD, were considered failures.

Chi-square analysis with Yates correction was used to determine statistical significance. If expected frequencies were < 1, the Chi-square value with correction for continuity was used. A value < 0.05 was considered statistically significant.

Histologic Evaluation

All eyes were enucleated and immediately fixed in formalin solution 10% (Sigma diagnostics, St. Louis, MO). Selected eyes were embedded in paraffin, stained with hematoxylin-eosin, and examined by light microscopy.

Some eyes were macroscopically dissected, and segments of the posterior ocular wall were embedded in resin, stained, and examined with the light microscope.

RESULTS

Microsphere Characterization

In vitro. After 3 hours at 55°C and 2 days at room temperature, traces of the solvent were found in microspheres not stored in a vacuum. No traces of the solvent were found either at 55°C and at room temperature if the microspheres were stored in a vacuum for at least 2 days. Thereafter, the microspheres were stored in the presence of anhydrous CaSO₄ and in a vacuum to eliminate any trace of residual solvent in the polymer.

After filtration of the microsphere suspension through sieves, approximately 50% of the microspheres ranged in size from 54 to 105 μm (average about 60 μm). Figure 1 illustrates the distribution of sizes in one sample. The microspheres were uniformly round with smooth surfaces and some small pores and fracture lines on the surface. After 21 days in water, some of the microspheres disintegrated and some remained intact with porous surfaces.

In vivo. Two months after inoculation, the microspheres were still visible with the indirect ophthalmoscope in 16/22 (73%) rabbits.

In histology preparations of control eyes in which fibroblasts were not injected (Fig. 2), the microspheres appeared by light microscopy at 2 and 3 weeks as single particles located in proximity to the retina. The microspheres were uneven; some were broken in parts...
FIGURE 1. A representative sample of PLGA microspheres observed with a scanning electron microscope. At time 0, the microspheres were uniformly round and had smooth surfaces and average diameters of approximately 60 µm. Some pores and fracture lines can be noted on the surface of the microspheres.

and some were eroded and had irregular surfaces. Two weeks after the injection, there were few red blood cells or inflammatory cells, and a thin eosinophilic capsule was noted around clusters of microspheres. At 2 months, few microspheres were found, and a mild, inflammatory reaction was still visible.

In Vitro Release of RA From PLGA Microspheres.

Figure 3 shows the release profile of RA from PLGA microspheres. Each point represents the amount of RA remaining in the microspheres. At day 0, the microspheres contained 20.8 µg of RA/mg of PLGA. There was a constant daily release of RA without any significant burst effect (Fig. 4). After 40 days, 82.8% of the total RA was released from the microspheres.

Biologic Activity of the RA/PLGA Microspheres

Two rabbits were excluded from the study because of vitreous hemorrhage noted immediately after surgery. After the intravitreous injection, we observed some microspheres left on the vial walls and in the syringe used to inject them. By weighing the vial and the syringe before and after the suspension was made and aspirated, we calculated that the real amount of microspheres injected into the eye was 2.5 ± 0.2 mg.

After injection, the microspheres were uniformly distributed in the vitreous cavity, partly obscuring a clear view of the fundus. After 4 days they appeared as a fine powder with some small clusters that settled on the inferior retina from 4 to 8 o'clock.

In one eye, a small amount of microspheres

FIGURE 2. Three weeks after injection of 5 mg of RA-loaded PLGA microspheres. The three sections obtained from the same eye show an inflammatory reaction surrounding the microspheres and their fragments (A) (hematoxylin and eosin, X200). Some microspheres in contact with the retina were surrounded by glial cell proliferation (B) (hematoxylin and eosin, X200). A reaction that resembled an epiretinal membrane was noted in some sections (C) (hematoxylin and eosin, X400).
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DISCUSSION

The retinoids, a group of compounds related to vitamin A, exhibit inhibitory effects on cellular proliferation and differentiation. They modulate gene expression possibly by interacting with intracellular nuclear retinoid-binding proteins.23-25 Recently, retinoids became important as chemopreventive agents of tumorigenesis. They have been used in dermatology,26 hematology,27 cancer research and therapy,26,28 and embryonal development.29,30

Retinoids can inhibit the proliferation15 and the migration16,31 of RPE cells in vitro. Among them, retinoic acid has been shown to be the most effective.15 Retinal pigment epithelium cells, glial cells, and macrophages seem to play an important role in the proliferation of the fibrovascular membranes associated with retinal detachment.1-32 Campochiaro et al35 reported a modulatory effect of RA on RPE cell morphology and a density-dependent growth arrest. The normal level of retinoids in RPE cells is moreover significantly reduced by retinal detachment.36

RA is required by all vertebrates for growth, life reproduction, vision, maintenance of differentiated epithelia, and mucus secretions.37 Because it is practically insoluble in water (0.2 μM at room temperature and pH 7.3),38 RA dissolved in SiO has been used previously in this laboratory in an animal model of PVR,17 and a statistically significant decrease in the incidence of TRD was observed in the treated eyes compared to controls.

In vitro. The release rate of drugs from polymer microspheres is dependent upon the molecular weight of the polymer,39 the environmental conditions,40 the solubility of the drug present in the matrix,41 the drug loading42-43 and the size40-42 of the microspheres.

In group 2, the histopathologic evaluation after 3 weeks revealed signs of a mild, localized foreign body reaction. Focal macrophage infiltration and multinucleated giant cells surrounding microsphere fragments were found in the preretinal vitreous. Increased numbers of cells morphologically similar to glial cells characterized the inner retinal layers underlying the foci of inflammation. All the remaining ocular structures were normal. At 2 months, the histopathologic findings were not significantly different from those at 3 months.

FIGURE 3. In vitro release profile of retinoic acid from 15 mg PLGA microspheres. Each point represents the amount of RA remaining in the microspheres. Solid and open circles represent two different sets of experiments. The line represents the average of the two measurements.

FIGURE 4. In vitro release rate of RA. Approximately 7 μg of RA was released per day from PLGA microspheres for about 30 days. At 40 days, the release rate has decreased to about 6 μg/d. Solid and open circles represent two different sets of experiments. The line represents the average of the two measurements.
delivery system. Gesser and Sanderson\(^41\) showed that about 70% and 40% after 1 day and 80% and 45% after day. In the same experiment, the release of 5-FU from microspheres for drugs with a solubility range from 4000 \(\mu\)g/ml to 1600 days. The release patterns from polymer microspheres reported by others for hydrophilic drugs are characterized by initial bursts. The release of 5-FU from 30:70 PLGA of MW 3300 d was almost 90% after 1 day. In the same experiment, the release of 5-FU from microspheres of PLA of MW 3400 d and 4700 d was about 70% and 40% after 1 day and 80% and 45% after 2 days, respectively.\(^12\)

The hydrophobic drugs lomustine and aclacinomycin showed nearly constant release rates from PLA microspheres for 26 and 30 days, respectively.\(^12\)\(^-\)\(^44\) Our results show a nearly constant release of RA from 50:50 PLGA microspheres for 40 days. It has been shown that drug solubility affects the duration of the delivery system. Gesser and Sanderson\(^41\) showed that for drugs with a solubility range from 4000 \(\mu\)g/ml to 0.02 \(\mu\)g/ml, the lifetime of the system was from 30 to 1600 days.

The near-linear release of drugs has been correlated with a corresponding linear decrease of molecular weight of the polymer and to the fast progression in pore formation.\(^45\) A surface erosion process with minimal diffusion release of the drug would be much more desirable because the total area of the microspheres decreases uniformly. Diffusion of the drug from the deeper layers of the microsphere to the surface might explain in part the faster release from the lower molecular weight polymers. Drug release from bioerodible polymers can occur by diffusion and by erosion of the matrix. For hydrophobic drugs, the most important mechanism of release seems to be the degradation of the polymer matrix. It has been hypothesized that the microspheres could have small channels or pores in the matrix formed upon evaporation of the solvent. Water molecules would be allowed to penetrate into these channels or pores and hydrate the polymer, resulting in matrix degradation.\(^49\)

**In vivo.** The problem of sterilization of injectable polymer microspheres has not yet been resolved. Grandfils et al\(^46\) used ethylene oxide to sterilize microspheres for arterial embolization. Other polymeric products such as sutures can be sterilized by high-energy radiation (x-rays, gamma rays, neutrons, electrons) or high-intensity ultrasonic vibrational energy or can be disinfected with alcohol.\(^43\) When applied to a polymer microspheres drug delivery system, each of these methods can affect the polymer or the drug. Moreover, the gas can, in part, remain entrapped in the polymer matrix. A previous report of intraocular injection of microspheres\(^12\) does not mention any method of sterilization.

As a possible solution to the problem of sterilization, we used a wide-spectrum antibiotic in BSS. A low prophylactic dose of gentamicin with an injection of 5-FU loaded liposomes has been used in an animal model of PVR.\(^48\) In our study, the three rabbits injected with RA-loaded microspheres suspended in a solution of a nontoxic dose of gentamicin\(^49\) in BSS showed no retinal detachment after 8 weeks and no signs of adverse retinal changes by histopathology. The influence, if any, of the antibiotic solution on the release of the drug and on the clearance of the microspheres was not investigated. The two controls injected with the gentamicin solution showed TRD after 1 week. Their clinical and histopathologic findings did not differ from the other animals.

The amount of injected microspheres corresponded to a total dose of RA of 55 \(\mu\)g delivered for a period of more than 40 days. Data calculated from the in vitro release study show that 5 \(\mu\)g of RA should have been released after 4 days in vivo. So, during the critical period of 2 to 5 days for the proliferative processes described by Fischer et al,\(^50\) an effective amount of RA\(^17\) would have been delivered into the vitreous cavity. Moreover, a constant theoretical amount of approximately 1.4 \(\mu\)g/d of RA was delivered for approximately 30 days.

A previous study with a hydrosoluble drug as 5-FU in PLGA microspheres has reported a clearance time from the vitreous cavity of 14 ± 2.4 days in vitrectomized eyes and 48 ± 5.2 days in nonvitrectomized eyes, respectively.\(^12\) The degradation of polymers can be expected to be faster for low molecular weight polymers and for copolymers. Among copolymers, the 50:50 PLGA has the shortest half-life.\(^51\) To obtain a fast clearance of the microspheres from the vitreous cavity, we selected a 50:50 PLGA copolymer of molecular weight 3000 d. Nonetheless, the microspheres were still visible in the vitreous cavity after 2 months in 73% of the rabbits. The difference in water solubility of 5-FU and RA would in part explain this observation. Inside the vitreous cavity, the release of RA and the
dissolution of the microspheres in contact with a biologic environment could be affected by the presence of enzymes, ions, and cells.

Although microspheres of biocompatible biodegradable polymers have been studied before as a sustained drug delivery system, this is the first report of their use for intravitreal delivery of RA. The results of our study indicate that a single intravitreal injection of microspheres can provide therapeutic levels of RA and does not require the presence of SiO as a reservoir, nor does it require surgical maneuvers to implant or remove the drug delivery system. The system hydrolyzes to inert moieties as they degrade to their natural metabolites, glycolic and lactic acids.

A foreign body reaction around the microspheres and their fragments, and a glial proliferation around the microspheres that were in contact with the retina, were observed at 3 weeks, and a mild, inflammatory reaction was still visible at 2 months. These histopathologic changes resemble the reaction produced by intravitreal SiO and perfluoroether. A foreign body reaction to polymer microspheres with macrophages and giant cells has been already described after intramuscular injection in the rat. Even if eyes do not show any clinical sign of inflammation, this is a cautionary note for the intravitreal use of these polymers until further studies evaluate the histopathologic reaction of the eye after microspheres have completely degraded.

In conclusion, the results of our study showed that when used as a system for the delivery of RA in an animal model of PVR, PLGA microspheres can significantly reduce the incidence of TRD by 64% at the end of 8 weeks. Even though the degradation products of PLGA microspheres have been reported to be inert, in our study with RA-loaded microspheres, a foreign body reaction was observed histopathologically at 3 weeks and at 2 months of follow-up. Clinically, no inflammatory signs were noted, but histopathologic findings suggest that further studies are required to evaluate the long-term reaction of the eye to polymer microspheres.

Key Words
drug delivery, poly(DL-lactide-co-glycolide), microspheres, retinoic acid, PVR

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