Subconjunctival Nano- and Microparticles Sustain Retinal Delivery of Budesonide, a Corticosteroid Capable of Inhibiting VEGF Expression

Uday B. Kompella,1,2 Nagesh Bandi,1 and Surya P. Ayalasomayajula1

PURPOSE. The purpose of this study was to determine whether budesonide inhibits expression of vascular endothelial growth factor (VEGF) in a retinal pigment epithelial cell line (ARPE-19) and to determine whether subconjunctivally administered budesonide nano- and microparticles sustain retinal drug levels.

METHODS. The effect of budesonide (100 pM to 10 μM) on VEGF secretion, expression of VEGF mRNA, and cytotoxicity were determined in ARPE-19 cells by ELISA, RT-PCR, and a cell-viability assay, respectively. To determine the involvement of glucocorticoid receptor in the observed effects of budesonide, secretion and mRNA expression studies were performed in the presence of a glucocorticoid receptor antagonist (RU486). α-Polylactide (PLA) nano- and microparticles containing budesonide were prepared by a solvent evaporation technique, and the particles were characterized for size, morphology, encapsulation efficiency, and in vitro release. Budesonide-PLA nano- and microparticles were administered subconjunctivally to one eye of Sprague-Dawley rats and drug levels in the retina, vitreous, lens, and cornea of both eyes were determined at the end of 1, 7, and 14 days.

RESULTS. At concentrations devoid of cytotoxicity, budesonide inhibited VEGF secretion as well as mRNA expression in ARPE-19 cells in a dose-dependent manner. RU486 treatment prevented budesonide-mediated inhibition of VEGF secretion and VEGF mRNA expression. Budesonide-PLA nano- (345 nm) and microparticles (3.6 μm), with an encapsulation efficiency of 65% and 99%, respectively, sustained budesonide release in vitro. After subconjunctival administration, both budesonide-PLA nano- and microparticles produced sustained budesonide levels in the retina and other ocular tissues.

CONCLUSIONS. Budesonide is capable of inhibiting VEGF expression through glucocorticoid receptor activity. Subconjunctivally administered budesonide-PLA nano- and microparticles sustain retinal drug delivery. (Invest Ophthalmol Vis Sci. 2003; 44:1192–1201) DOI:10.1167/iovs.02-0791

Corticosteroids, such as budesonide, are the drugs of choice for the treatment of inflammatory disorders, including asthma, inflammatory bowel disease (IBD), and arthritis. Budesonide (molecular weight [MW], 430.5; log P = 3.2), a potent nonhalogenated corticosteroid that is currently in clinical use for treatment of asthma, allergic rhinitis, and IBD, has a relative glucocorticoid receptor affinity of 935 compared with 100 for dexamethasone and a 200-fold higher glucocorticoid receptor affinity and a 1000-fold higher topical anti-inflammatory potency than cortisol.1 Through its ability to inhibit the expression of several proinflammatory genes, such as interleukin (IL)-6, IL-8, and tumor necrosis factor-α, budesonide inhibits inflammatory symptoms such as edema and vascular hyperpermeability at nanomolar concentrations.2,3 In airway and alveolar epithelial cells, we have shown that budesonide inhibits the expression of vascular endothelial growth factor (VEGF),4 a potent endothelial cell mitogen capable of inducing inflammatory symptoms such as vascular hyperpermeability, edema, and angiogenesis. Indeed, VEGF is implicated in the pathogenesis of inflammatory disorders such as asthma, IBD, and arthritis.5–7 In the eye, VEGF has been implicated in the progression of several vascular disorders, such as diabetic retinopathy, cystoid macular edema, choroidal neovascularization, and macular degeneration, which affect the posterior segment of the eye and resemble inflammatory disorders.8–13 As a first step to determine the applicability of budesonide to these disorders, one objective of this study was to determine the effect of budesonide on VEGF expression in a cultured human retinal pigment epithelial cell line (ARPE-19). Retinal pigment epithelial (RPE) cells form the outer blood–retinal barrier and express VEGF constitutively.14 We have demonstrated the ability of ARPE-19 cells to secrete VEGF.15 Drug delivery to the posterior segment of the eye including choroid, retina, and vitreous is a challenging task, because of the formidable obstacle posed by the blood–retinal barrier and the extraocular epithelia. Only a minute fraction of the drug, administered orally or by other extravascular routes (e.g., subcutaneous and intramuscular injections), reaches the target retina, requiring large and often toxic doses for therapeutic effectiveness.16 This challenge is further aggravated because the drug levels must be sustained for prolonged periods at the target site. Furthermore, because budesonide undergoes extensive hepatic first-pass metabolism to the extent of 85%, use of this drug necessitates frequent administration and/or high doses by the oral route.17 One possible approach to improve retinal drug delivery is to facilitate localized delivery to the posterior segment by using injectable, sustained-release, biodegradable particulate systems.

Localized drug delivery to the posterior segment of the eye can be achieved by administering drugs through the subconjunctival route,18,19 which has been in clinical use for administering anti-inflammatory agents and anesthetics intended for ocular therapy, primarily for the anterior segment. The potential of this route for retinal delivery and the underlying mechanisms of delivery have been determined recently.20–22 The vitreous concentrations of 14-C mannitol are significantly higher when the drug is administered by the subconjunctival route, as opposed to the intracameral route,22 and the drug levels in the contralateral eyes are much lower after subcon-
Budesonide, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium salt) was obtained from Promega Corp. (Madison, WI) and the primers for VEGF, GR-α, GR-β, 18S rRNA were purchased from Sigma Chemical Co. (St. Louis, MO). PLA of intrinsic viscosity 1.1 was determined by the relative formation of formazan after budesonide treatment. The medium was changed every 2 days, and all studies were conducted with day 8 confluent cells of passages 20 to 30.

### VEGF Secretion Studies
On day 8, serum-containing medium was removed from ARPE-19 cells plated in 96-well cluster plates, fresh medium without serum was added, and the monolayers were allowed to remain in quiescence for 12 hours. After the quiescence period, the monolayers were incubated with budesonide (0–10 μM) in the presence and absence of a glucocorticoid receptor antagonist (RU486: 0.1, 1, and 10 μM) for 12 hours. At the end of 12 hours, secreted VEGF in the supernatants was quantified by an ELISA method capable of detecting VEGF_{165} and VEGF_{121} isoforms (Research Diagnostics Inc., Flanders, NJ).

### RT-PCR Studies
Using RT-PCR (Promega), we determined the effect of budesonide on VEGF mRNA expression in the presence and absence of RU486 (1 μM).

### MTT Assay
The effect of budesonide treatment on cytotoxicity was assessed by colorimetric MTT assay, as described previously. The cell viability was determined by the relative formation of formazan after budesonide treatments when compared with the control treated with budesonide-free medium.

### Budesonide-PLA Particle Formulation
Polymeric-budesonide nano- and microparticles were formulated by a solvent-evaporation method. Briefly, budesonide and the polymer were dissolved in chloroform and then dried to remove the solvent. The resulting powder was then dispersed in a solvent system (methanol and ethanol) and further dried to obtain the final formulation. The resulting particles were characterized for size, morphology, and drug loading efficiency.

---

### Materials and Methods

#### Chemicals

The ARPE-19 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cell culture medium (Dulbecco’s modified Eagle’s medium Ham’s-F-12 [DMEM]-F12), fetal bovine serum (FBS), penicillin-streptomycin, and L-glutamine were purchased from Gibco-BRL (Grand Island, NY). The cells were cultured, either in cell culture flasks (T-75 and T-25 cm²) or in 96-well plates obtained from BD Bioscience Labware (Franklin Lakes, NJ). An RT-PCR kit was obtained from Promega Corp. (Madison, WI) and the primers for VEGF, glucocorticoid receptor, and 18S rRNA were custom synthesized (DNA Core Facility, University of Nebraska Medical Center, Omaha, NE).

#### Table 1. Primers Used for RT-PCR Analysis in ARPE-19 Cells

<table>
<thead>
<tr>
<th>Product</th>
<th>Primer Sequences</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF_{165} and VEGF_{121}</td>
<td>Forward-TGAGTCCGACATTCTCTGTGC</td>
<td>584 and 452</td>
</tr>
<tr>
<td></td>
<td>Reverse-TACCGGCCTGGCCTGACAT</td>
<td></td>
</tr>
<tr>
<td>GR-α</td>
<td>Forward-ACAGGTTCAAGGATTTCTTT</td>
<td>557</td>
</tr>
<tr>
<td></td>
<td>Reverse-ACGCTCTGTTGGCAGA</td>
<td></td>
</tr>
<tr>
<td>GR-β</td>
<td>Forward-ACAGGTTCAAGGATTTCTTT</td>
<td>294</td>
</tr>
<tr>
<td></td>
<td>Reverse-GCCCAAGATTTGGTGGATGA</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Forward-GGACCAAGGAGGCAAGATTGCC</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>Reverse-TCAATCTGGCTGGCTGACG</td>
<td></td>
</tr>
</tbody>
</table>

---

 dl/g was obtained from Birmingham Polymers, Inc. (Birmingham, AL). Hydroxy-propyl-β-cyclodextrin was a gift from Cerestar USA, Inc. (Hammond, IN). The HPLC grade methylene chloride and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA).

#### Cell Culture

ARPE-19 cells were cultured in DMEM-F12 containing 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. The medium was changed every 2 days, and all studies were conducted with day 8 confluent cells of passages 20 to 30.

---

The peak subretinal fluid concentrations of dexamethasone in humans after oral (7.5 mg), peribulbar (5 mg), and subconjunctival (2.5 mg) administration were 12.3 ± 1.61, 82.2 ± 17.6, and 359 ± 82.2 ng/mL, respectively, indicating that use of the subconjunctival route delivers greater amounts of drug to the retina. In addition, subconjunctivally administered carboxiplatin effectively inhibits intraocular tumor growth in a dose-dependent fashion in transgenic murine retinoblastoma models. Besides the retinal delivery of low-molecular-weight molecules, an osmotic pump delivery system connected to the subconjunctival space has been shown to be useful in sustaining detectable levels of a large macromolecule in the tissues of the posterior segment of the eye.

A clinically applicable way to achieve sustained drug delivery is to use drug-encapsulating polymeric microparticles (1–1000 μm) or nanoparticles (1–1000 nm). By controlling drug diffusion and/or polymer degradation, these systems can prolong drug release. Artificial or natural polymers that are biocompatible and biodegradable are often used for the preparation of particulate systems. Such polymers include polylactide (PLA), poly (lactide-co-glycolide) (PLGA), acrylic polymers or copolymers, hyaluronic acid derivatives, and alginites. Among the available biodegradable polymers, the PLA and PLGAs are the most widely used. In vivo, the lactide-glycolide polymer chains are hydrolyzed to form natural metabolites (lactic and glycolic acids) that are eliminated from the body through the Kreb’s cycle. Depending on their composition and molecular weight, the poly (lactic acid) and its copolymers with glycolic acid provide degradation rates ranging from months to years.

Thus, there is compelling evidence that sustained drug delivery to the retina can be achieved by administering drugs through the subconjunctival route. Thus, as a second objective of this study, we tested the hypothesis that subconjunctival administration of budesonide-PLA nano- and microparticles can sustain budesonide delivery to the retina.

---

The ARPE-19 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cell culture medium (Dulbecco’s modified Eagle’s medium Ham’s-F-12 [DMEM]-F12), fetal bovine serum (FBS), penicillin-streptomycin, and L-glutamine were purchased from Gibco-BRL (Grand Island, NY). The cells were cultured, either in cell culture flasks (T-75 and T-25 cm²) or in 96-well plates obtained from BD Bioscience Labware (Franklin Lakes, NJ). An RT-PCR kit was obtained from Promega Corp. (Madison, WI) and the primers for VEGF, glucocorticoid receptor, and 18S rRNA were custom synthesized (DNA Core Facility, University of Nebraska Medical Center, Omaha, NE). Budesonide, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; triazolyl blue), and polyvinyl alcohol (PVA) were obtained from Sigma Chemical Co. (St. Louis, MO). PLA of intrinsic viscosity 1.1 was determined by the relative formation of formazan after budesonide treatment. The medium was changed every 2 days, and all studies were conducted with day 8 confluent cells of passages 20 to 30.
were dissolved in 1 mL dichloromethane, and this solution was added to 10 mL of an aqueous PVA (2% wt/vol) solution. The resultant mixture was sonicated for 1.5 minutes at 20 W or 5 minutes at 50 W with a probe sonicator (Misonix Inc., Farmingdale, NY), to obtain an oil-and-water (O/W) emulsion for microparticle and nanoparticle formulations, respectively. The O/W emulsion was immediately added drop-wise to 125 mL of an aqueous PVA (2% wt/vol) solution. The contents were stirred overnight at room temperature to evaporate the methylene chloride, allowing the formation of a turbid particulate suspension. The nano- and microparticles were separated by ultracentrifugation (35,000g for 1 hour) and centrifugation (1000g for 30 minutes), respectively. The pellets were washed two times, resuspended in deionized water, and freeze dried to obtain lyophilized particles.

Electron Microscopy of Budesonide-PLA Particles

Nanoparticles. Transmission electron microscope (TEM) was used to analyze the morphology of budesonide-PLA nanoparticles. Initially, carbon-coated grids were floated on a droplet of the nanoparticle suspension on a flexible plastic film (Parafilm; Pechiney Plastic Packaging, Neenah, WI), to permit the adsorption of the nanoparticles onto the grid. After the grid was blotted with a filter paper and air dried for 5 minutes, it was transferred onto a drop of the negative stain. After this, the grid was blotted with a filter paper and air dried for 5 minutes. Ammonium molybdate was used as a negative stain in these experiments. Finally, the samples were examined with an electron microscope set at 10 kV (SEM51S; Phillips, Eindhoven, The Netherlands).

Microparticles. The morphology of the budesonide-PLA micro-particles was analyzed by scanning electron microscope (SEM). Briefly, a small quantity of microparticles were layered on the SEM stubs and coated with gold palladium under an argon atmosphere using a gold-sputter module in a high-vacuum evaporator. Samples were then observed for their surface morphology with a scanning electron microscope set at 10 kV (SEM51S; Phillips).

Particle Size and Drug-Loading Measurement of Budesonide-PLA Particles

Size. The particle size of budesonide-PLA nano- and microparticles was determined with a particle-size analyzer (Brookhaven Instruments Corp., Holtsville, NY). Briefly, a 0.5-mg sample of polymeric-budesonide was suspended in 5 mL of distilled water, and the diluted suspension was then subjected to particle size measurement.

Drug Loading. The loading efficiency of budesonide in the polymeric particles was determined by extracting and quantifying the encapsulated budesonide. Briefly, budesonide-polymeric particles (2 mg) were placed in a glass tube, and 2 mL methylene chloride was added and mixed thoroughly at room temperature for 16 hours. The resultant solution was evaporated to dryness under nitrogen, and the dried residue was reconstituted with 1000 μL acetonitrile-water mixture (70:30). This reconstituted solution was vortexed for 1 minute and centrifuged at 12,000g for 5 minutes, and 100 μL of the supernatant was injected onto the HPLC column.

In Vitro Drug Release

The in vitro release of budesonide from the PLA particles was performed at 37°C with dialysis membrane bags (molecular weight cutoff: 10,000; Spectrum Laboratory, Gardenia, CA) as described previously.33 Briefly, a 0.5-mL suspension of the budesonide-PLA particles containing 250 μg budesonide was taken into the dialysis bag, and the unit was allowed to float in 50 mL of release medium (phosphate-buffered saline; PBS, pH 7.4) containing 0.025% sodium azide as a preservative). At discrete time intervals, 1 mL of the release medium was removed and replaced with fresh release medium. The released budesonide was analyzed by HPLC assay.

Subconjunctival Administration

Sprague-Dawley rats weighing 180 to 200 g were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). After this, 50 or 75 μL of either solution (containing 10% wt/wt of hydroxypropyl-β-cyclodextrin as a solubilizing agent) or particulate suspension of budesonide (50 μg for nanoparticles and 75 μg for microparticles), with PBS (pH 7.4) used as the vehicle, was injected into the subconjunctival space of one eye (ipsilateral) by 27-gauge needle. The other eye (contralateral) served as the control. At the end of 1, 3, 7, and 14 days the animals were killed, the eyes were enucleated, frozen immediately, and stored at −80°C. The frozen eyes were dissected, and the ocular tissues including retina, vitreous, lens, and cornea were isolated and the drug levels estimated with a HPLC method described in the next section. The animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Budesonide HPLC Assay

The isolated ocular tissues were homogenized in 200 μL of PBS buffer (Tissue Tearor; Fisher Scientific). To the homogenate, 2.5 μL of a 40-μg/mL solution of celecoxib was added as an internal standard and mixed thoroughly. Methylene chloride (2 mL) was added and mixed thoroughly at room temperature for 5 minutes. The resultant solution was evaporated to dryness under nitrogen, and the dried residue was reconstituted with 150 μL acetonitrile-water (70:30) mixture. This reconstituted solution was vortexed for 1 minute and centrifuged at 12,000g for 5 minutes, and 100 μL of the supernatant was injected on an HPLC system (Waters, Milford, MA) equipped with a pump (TM616; Waters), a controller (600S; Waters), an autoinjector (717plus; Waters), and a PDA detector (996; Waters). The peak areas were integrated on computer (Millennium software, ver. 2.15.01; Millennium Software, Torrance, CA). The drugs were separated with a 25-cm long C-18 column (Discovery; Supelco, Emeryville, CA) with a particle diameter of 5 μm and a pore size of 10 nm. The mobile phase for the assay consisted of acetonitrile and aqueous buffer mixture (70:30 vol/vol). The buffer was 0.1% acetic acid in water at pH 3. The aqueous samples obtained from drug loading, and in vitro release studies were analyzed under the same HPLC conditions. The limit of detection of the HPLC assay was 1 ng, and the budesonide recovery from the tissues with the extraction procedure was more than 95%.

Statistical Analysis

All data are expressed as the mean ± SD or the mean ± SE of the mean, and comparison of the mean values was performed using either Student’s t-test or ANOVA. Statistical significance was set at P < 0.05.

RESULTS

Effect of Budesonide on VEGF Secretion and VEGF mRNA Expression

VEGF Secretion. Compared with control VEGF secretion at the end of 12 hours in ARPE-19 cells (2297 ± 267 pg/mL), budesonide at 10⁻⁴, 10⁻³, 10⁻², 10⁻¹, 1, and 10 μM reduced VEGF secretion by 15%, 37%, 43%, 47%, 55%, and 52%, respectively (Fig. 1A). The effects observed at 10⁻³ to 10 μM were statistically significant.

VEGF mRNA Expression. mRNA expressions for VEGF and 18S RNA indicated bands at 584, 452, and 495 bp, corresponding to VEGF₁₆₅, VEGF₁₂₃, and 18S RNA, respectively. With budesonide treatment (10⁻⁴-10 μM), we observed a significant dose-dependent decrease in expression of VEGF₁₆₅ and VEGF₁₂₃ (Figs. 1B, 1C). The effects observed at 10⁻² to 10 μM were statistically significant.
Expression of Glucocorticoid Receptor mRNA in ARPE-19 Cells

RT-PCR analysis for GR mRNA expression in ARPE-19 cells indicated an intense band at 557 bp and a faint band at 294 bp, corresponding to GR-α and GR-β, respectively. (Fig. 2A).

Role of Glucocorticoid Receptor Activity in Budesonide-Mediated Effects

VEGF Secretion. Budesonide at 0.1 μM significantly reduced secretion of VEGF at the end of 12 hours to 67% in ARPE-19 cells. Cotreatment with 0.1, 1, and 10 μM RU486...
reversed secretion to 68%, 89%, and 98% of the control, respectively (Fig. 2B). The effects observed at 1- and 10-μM RU486 treatments were statistically significant.

**VEGF mRNA Expression.** Budesonide (0.1 μM) alone reduced VEGF mRNA levels, and cotreatment with 10 μM RU486 completely prevented this effect, suggesting the involvement of glucocorticoid receptor in budesonide-mediated effects (Fig. 3).

**Cytotoxicity**

Budesonide treatment up to 10 μM did not show any significant cytotoxicity at the end of 12 hours (Table 2).

**Particle Morphology, Particle Size, and Encapsulation Efficiencies of Budesonide-PLA Particles**

**Particle Morphology.** The morphology of the nano- and microparticles was assessed using TEM and SEM, respectively (Fig. 4). The TEM and SEM micrographs indicated mostly spherical particles with smooth surfaces.

**Particle Size.** The particle size of budesonide-PLA nano- and microparticles was measured with a laser light-scattering technique. The mean particle size of the nano- and microparticles was 345 ± 2 nm and 3.6 ± 0.01 μm, respectively (Table 3).

**Encapsulation Efficiency.** Budesonide encapsulation studies indicated that the encapsulation efficiency of budesonide in the PLA nano- and microparticle formulations was 65% and 99%, respectively (Table 3). The observed drug loading was 16.25% and 8.74% wt/wt budesonide in the nano- and microparticles, respectively.

**In Vitro Drug Release**

The release of budesonide from PLA nano- and microparticles into phosphate-buffered saline (PBS) was measured in vitro at 37°C (Fig. 5A). For the nanoparticles, after an initial burst release of 25% budesonide, the drug release was sustained over the 2-week study. The cumulative budesonide release at the end of 2 weeks was approximately 50% of the initial drug loading. The microparticles sustained release over the 6-week study period and the cumulative budesonide release at the end of 6 weeks was 23% of the initial drug loading. The microparticles were devoid of burst release. The release rates estimated between two consecutive time points in the cumulative drug release plots of nano- and microparticles are shown in Figure 5B.

**Tissue Levels of Budesonide after Budesonide-PLA Nanoparticle Administration**

Ocular tissue levels of budesonide were compared after a single subconjunctival injection of either budesonide solution

![Figure 2.](https://jo...
(containing 50 μg budesonide) or budesonide-PLA nanoparticles (containing 50 μg budesonide) at the end of days 1, 3, 7, and 14 (Fig. 6). At the end of day 1, there was no statistically significant difference between the tissue levels of solution and nanoparticle groups, except in the lens, wherein the nanoparticle group exhibited twofold higher drug levels. With administration of budesonide-PLA nanoparticles, the retinal levels of budesonide were two and nine times higher than the budesonide solution-treated groups at the end of 3 and 7 days, respectively (Fig. 6A). In the vitreous, there was no statistically significant difference between the two groups at the end of 3 days. However, the nanoparticle group had a 5.5 times higher vitreous budesonide concentration, compared with the budesonide solution-treated group at the end of day 7 (Fig. 6B). At the end of days 3 and 7, the corneal levels of budesonide with nanoparticle formulation were 4 and 27 times higher than that in the budesonide solution-treated group (Fig. 6C). In the lens, there was no difference between the two groups at the end of 3 and 7 days (Fig. 6D). On day 14, the drug was below the detection limits in both the solution and nanoparticle groups.

TABLE 2. Viability of ARPE-19 Cells after 12-Hour Budesonide Treatments as Determined by MTT assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>100 ± 6.71</td>
</tr>
<tr>
<td>Budesonide</td>
<td></td>
</tr>
<tr>
<td>10⁻⁴ μM</td>
<td>99 ± 5.6</td>
</tr>
<tr>
<td>10⁻³ μM</td>
<td>87 ± 8.2</td>
</tr>
<tr>
<td>10⁻² μM</td>
<td>93 ± 7.6</td>
</tr>
<tr>
<td>10⁻¹ μM</td>
<td>99 ± 4.7</td>
</tr>
<tr>
<td>1 μM</td>
<td>90 ± 8.7</td>
</tr>
<tr>
<td>10 μM</td>
<td>113 ± 4</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of results in eight experiments.

Tissue Levels of Budesonide after Budesonide-PLA Microparticle Administration

Ocular tissue levels of budesonide were compared after a single subconjunctival injection of either budesonide solution (containing 75 μg of budesonide) or budesonide-PLA microparticles (containing 75 μg of budesonide) at the end of days 1, 3, 7, and 14 (Fig. 6). On day 1, drug levels in the solution group were 3.5, 5.5, 3, and 2 times higher in the retina, vitreous, cornea, and lens, respectively, when compared with microparticle group (*P* < 0.05 for all tissues). On day 7, drug levels in the microparticle group were 37, 5, 4.2, and 3.4 times higher in the retina, vitreous, cornea, and lens, respectively, compared with the solution group (*P* < 0.05 for all tissues). Similarly, on day 14, drug levels in the microparticle group were higher compared with the solution group, wherein the drug levels were 3.5 ± 1.14, 0.8 ± 0.21, 1.9 ± 0.3, and 1.2 ± 0.25.

FIGURE 3. Budesonide inhibited expression of VEGF mRNA by ARPE-19 cells through its glucocorticoid receptor activity. (A) RU486 reversed budesonide-mediated inhibition of VEGF mRNA expression in ARPE-19 cells. Lane 1: untreated ARPE-19 cells; lane 2: ARPE-19 cells treated with budesonide (0.1 μM); and lane 3: ARPE-19 cells cotreated with budesonide (0.1 μM) and RU-486 (1 μM). (B) Densitometric analysis of VEGF mRNA band intensities normalized to 18S rRNA. Data are expressed as the mean ± SD of results in three experiments. *Significantly different from the control at *P* < 0.05.

FIGURE 4. Morphologic analysis of polymeric budesonide-PLA particles. (A) TEM micrograph of budesonide-PLA nanoparticles. Bar indicates 0.5 μm. (B) SEM micrograph of budesonide-PLA microparticles.
meric proteins, indicating that inhibition of VEGF expression is a viable strategy to inhibit retinal vascular changes. However, soluble receptor construct (VEGF TrapA), intravitreally administered VEGF-neutralizing antibody, and VEGF-receptor chimeric proteins, indicating that inhibition of VEGF expression is a viable strategy to inhibit retinal vascular changes. Therefore, retinal delivery of drugs capable of inhibiting VEGF expression is an attractive therapeutic option for alleviating retinal vascular changes. Indeed, retinal vascular changes were inhibited by intravenously administered VEGF-neutralizing soluble receptor construct (VEGF TrapA), intravitreally administered VEGF-neutralizing antibody, and VEGF-receptor chimeric proteins, indicating that inhibition of VEGF expression is a viable strategy to inhibit retinal vascular changes. However, antibodies and aptamers are macromolecules and have poor stability, limited permeability, and rapid clearance, prompting the need to identify and develop low-molecular weight drugs that can inhibit VEGF expression. To overcome these limitations with macromolecules, we are investigating low-molecular-weight drugs capable of inhibiting VEGF expression.

**Table 3. Size and Encapsulation Efficiency of Budesonide-PLA Nano- and Microparticles**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Size (nm)</th>
<th>Theoretical Drug Loading (% wt/wt)</th>
<th>Observed Drug Loading (% wt/wt)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoparticles</td>
<td>$345 \pm 2$</td>
<td>25</td>
<td>$16.25 \pm 1.2$</td>
<td>$65 \pm 5$</td>
</tr>
<tr>
<td>Microparticles</td>
<td>$3600 \pm 100$</td>
<td>8.81</td>
<td>$8.74 \pm 0.34$</td>
<td>$99.3 \pm 3.9$</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of results.

\(n = 3\), \(\dagger n = 5\), \(\ddagger n = 5\).

**Discussion**

Elevated retinal expression of VEGF, a potent 42-kDa endothelium-specific angiogenic mitogen is thought to play a role in the development of several vascular disorders of the eye, including diabetic retinopathy, cystoid macular edema, choroidal neovascularization, and age-related macular degeneration. Therefore, retinal delivery of drugs capable of inhibiting VEGF expression is an attractive therapeutic option for alleviating retinal vascular changes. Indeed, retinal vascular changes were inhibited by intravenously administered VEGF-neutralizing soluble receptor construct (VEGF TrapA), intravitreally administered VEGF-neutralizing antibody, and VEGF-receptor chimeric proteins, indicating that inhibition of VEGF expression is a viable strategy to inhibit retinal vascular changes. However, antibodies and aptamers are macromolecules and have poor stability, limited permeability, and rapid clearance, prompting the need to identify and develop low-molecular weight drugs that can inhibit VEGF expression. To overcome these limitations with macromolecules, we are investigating low-molecular-weight drugs capable of inhibiting VEGF expression.

In this study, budesonide inhibited VEGF protein secretion and VEGF mRNA expression in ARPE-19 cells (Fig. 1), an effect likely to facilitate its application as a new agent capable of inhibiting retinal VEGF expression. We observed an intense band corresponding to GR-1 mRNA in ARPE-19 cells (Fig. 2A). Budesonide inhibited VEGF secretion by its glucocorticoid receptor activity, as indicated by the prevention of budesonide-mediated inhibition of VEGF secretion upon cotreatment with RU486, a glucocorticoid receptor antagonist (Figs. 2, 3). We obtained similar evidence in airway and alveolar epithelial cells.

Retinal delivery of budesonide is attractive because of its favorable pharmacokinetic and pharmacologic properties. The rank of the local-to-systemic potency ratio of various corticosteroids is: budesonide > beclomethasone dipropionate > triamcinolone acetonide > flunisolide acetate. Also, the plasma elimination half-life of budesonide is short (2.3 hours), providing lower plasma accumulation of budesonide at steady state concentration and resulting in reduced systemic side effects such as adrenal suppression. In the eye, posterior subcapsular cataract has been reported in 9% of the patients with asthma treated with corticosteroids. These incidents often occur in patients previously exposed to oral corticosteroid therapy. However, in the case of budesonide, after a mean dose of 0.75 mg/d for a mean duration of 5 years in 95 patients, no posterior subcapsular cataracts were observed. Although, some corticosteroids including dexamethasone have been shown to cause ocular hypertension after either systemic, topical, or inhalation administration, no association was found between prolonged use of budesonide (nasal or inhalation modes of delivery) and ocular hypertension or open-angle glaucoma. Thus, the very high local-to-systemic activity ratio, low plasma half-life, and absence of reported ocular toxicity of budesonide in conjunction with its clinical availability provide impetus to develop budesonide for treating retinal disorders associated with elevation of VEGF.

Subconjunctival administration of budesonide solution resulted in detectable drug levels in the retina and vitreous (Fig. 6), suggesting the usefulness of this route to deliver drugs to the posterior segment of the eye. We have seen detectable drug levels only in the ipsilateral eye, and no drug was detected in the contralateral eye, suggesting that the contribution of systemic absorption and recirculation is minimal. Similarly, Lee and Robinson observed lower 14-C mannitol levels in the contralateral eye than in ipsilateral eye after subconjunctival administration. Thus, budesonide entered the intraocular tissues primarily through a nonsystemic pathway, possibly by
Budesonide was administered in the eyes of rats, either in the form of a solution (50 or 75 μg to one eye; small and large open circles, respectively), nanoparticles (50 μg to one eye; small filled circle), or microparticles (75 μg to one eye; large filled circle), and drug levels were estimated in (A) retina, (B) vitreous, (C) cornea, and (D) lens. Data are expressed as the mean ± SD of results in four experiments. Data are shown for the ipsilateral eye. Drug levels were below detection limits in the contralateral eye. Also, budesonide levels were below detection limits on day 14 in the solution and nanoparticles groups.

Because exposure to the drug in inflammatory and/or neo-vascular disorders is often needed for more than 3 days, development of sustained drug delivery systems for subconjunctival administration are desirable. To this end, there is growing interest in developing various sustained-release formulations, including gels, microparticles, nanoparticles, and liposomes. Simpson et al. recently demonstrated that a subconjunctivally injected fibrin sealant formulation can sustain retinal drug delivery. In the current study, we used injectable, sustained-release, biodegradable particulate systems for sustaining budesonide delivery. Although intravitreally administered microparticle systems sustained vitreous drug levels, the suspended particles in the vitreous increase polymeric burden in the eye and are likely to interfere with vision. Therefore, we administered budesonide particles by the subconjunctival route, which is less invasive to the eye than the intravitreal, subretinal, and subtenon modes of drug administration.

In this study, budesonide was encapsulated in PLA. Because of its biodegradability, biocompatibility, and formulation flexibility, PLA is used in the fabrication of several FDA-approved sustained release injectable products including doxycycline hyclate (Atridox; Atrix Pharmaceuticals, Ft. Collins, CO), leuprolide acetate (Lupron Depot; TAP Pharmaceuticals, Deerfield, IL), and goserelin acetate (Zoladex; Zeneca Pharmaceuticals, Wilmington, DE). The results of our studies indicated that the encapsulation efficiency of budesonide particles formulated using a solvent-evaporation technique was higher for the microparticles (99%) than the nanoparticles (65%; Table 3). The observed values for the budesonide microparticles and nanoparticles were comparable to those reported for sustained-release estradiol microparticles (99%) and sustained-release taxol nanoparticles (50%). The observed encapsulations are consistent with the previous reports that the encapsulation efficiency of particulate systems increases with their diameter. This can be explained by the fact that an increase in the particle diameter results in decrease in the surface area per unit volume, thereby reducing the possibility of drug loss by diffusion from the surface toward the continuous medium during the particle formation. In vitro release profiles indicated different profiles for nano- and microparticles (Fig. 5). The cumulative budesonide release from the nanoparticle formulations was higher, with an initial burst of approximately 25%, but the microparticles exhibited no discernible burst effect and maintained a steady release rate. The drug release rate declined more rapidly with the nanoparticles. Similarly, Berkland et al. reported lower burst release with larger particles. One possible reason for this difference is the decrease in surface drug due to a decrease in the surface area with increasing particle size.

The intraocular tissue concentrations of budesonide exhibited different trends after the administration of budesonide solution, budesonide nanoparticles, and budesonide microparticles (Fig. 6). On day 1, budesonide levels in the retina, vitreous, cornea, and lens achieved in the solution- and nanoparticle-treated groups were significantly higher than in the microparticle-treated group. The high burst release of budesonide from nanoparticles and/or diffusion of nanoparticles across the sclera is a likely reason for the equivalent tissue levels compared with the solution. On day 7, the retinal, vitreous, and corneal budesonide concentrations were significantly higher in the nanoparticles group than in the solution group, suggesting the ability of nanoparticles to sustain tissue drug levels. In the microparticle group, on days 7 and 14, the retinal, vitreous, corneal, and lens levels of budesonide were significantly higher than in the nanoparticle and solution groups (Fig. 6). This can be explained on the basis of a decline
in drug release rate from the nanoparticles below that of microparticles on these days.

In summary, budesonide, an FDA-approved corticosteroid for inflammatory disorders, is capable of reducing VEGF expression in retinal pigment epithelial cells through its glucocorticoid receptor activity. This indicates the potential value of budesonide for treating vascular disorders of the retina, wherein VEGF expression is elevated. Sustained-release nanoparticles for sustained drug delivery to the retina were successfully prepared with PLA, a biodegradable polymer. These particulate systems were found to be useful in sustaining retinal drug delivery.

References


