Characterization of Fluorescein-Conjugated Proteins

Results

Before performing diffusion studies, PEDF and Ova were conjugated with fluorescein. Fluorescein-conjugated PEDF and Ova proteins migrated electrophoretically with the expected apparent $M_r$ of 50,000 and 45,000, respectively (Fig. S1A). Coomassie blue staining and immunostaining demonstrated that these samples contained highly pure proteins that immunoreacted with anti-fluorescein antibodies. The detection of fluorescein by fluorometry showed that fluorescence was linear and directly proportional to the amount of each protein in the range 0 - 20 µg/ml (Figs. S1B and S1C). From these curves the fluorescence units per concentration of protein were estimated and compared. Fl-Ova (350 F.U./µg/ml) had >10-fold fluorescence units per µg/ml than Fl-PEDF (22.5 F.U./µg/ml). These values reflected the number of exposed primary amines available for conjugation in each protein sample and implied that Fl-Ova would be a more sensitive tracer than Fl-PEDF.

Matrix Type Implants for Subconjunctival Delivery

Methods

**Intraocular implant fabrication.** A subconjunctival matrix type implant was manufactured using a solution of 10% polyvinyl alcohol (PVA) (w/v) formulated by placing 1.0 g of PVA (Celvol, Celanese Chemicals, Ltd., Dallas, TX) in 10 ml of molecular biology grade water (BIOfluids®, Biosource International, Camarillo, CA) in a 50 ml polypropylene conical tube (Falcon®, BD Biosciences, Franklin Lakes, NJ) and placed in a water bath at 100°C for 3 hours to dissolve all the PVA. The PVA solution was cooled to room temperature and 5 mg of Fl-Ova (~3 moles of fluorescein per mole of Ova; Molecular Probes, Eugene, OR) were added and stirred into the PVA mixture to produce a solution of 5% Fl-Ova(w/v) in 10% PVA (w/v). The mixture was poured onto a glass plate to produce a thin film as it dried at room temperature, from which disks of 1.5 mm diameter by about 0.7 mm thickness were made with a biopsy punch (Acu·Punch® 1.5 mm, Acuderm Inc, Ft. Lauderdale, FL) and contained approximately 40-50 µg of Fl-Ova/disk.

**In vitro protein release rate of implants.** In vitro release rates of Fl-Ova were determined by placing the PVA implants (n = 3) in 25 ml glass vials with 15 ml of phosphate buffered saline (PBS, pH 7.4) and stirring the saline with a magnetic bar at 150 rpm speed at room temperature. At indicated time points Fl-Ova concentrations in the solutions were determined by spectrofluorometry. After each assay, the solution was replaced with fresh PBS to simulate sink conditions for release. A calibration curve for Fl-Ova concentration was made using a spectrofluorometer. From the known solution volume in the sampling reservoir (V) 15 ml, and the sampled ova concentration (C) the amount of Fl-Ova (M) was calculated at each sample time (M = C x V) and the cumulative amount over total time was calculated by adding each successive sample amount.
Results

Because Ova was readily available in higher quantities than PEDF, and the fluorescein signal per molecule of Ova was also higher than for PEDF, we prepared matrix type implants with Fl-Ova to be placed in the subconjunctiva of rat eyes. Before implanting protein-releasing devices in animals, their release rates and kinetics were determined in vitro. Matrix implants containing about 50 µg Fl-Ova in PVA released the fluoresceinated protein to PBS buffer at high rates (23 µg Fl-Ova per hour) during the initial hour or “initial burst,” as expected for a matrix type implant (Fig. S2A). By 5 hours the release rates were one-tenth of the initial ones and then they declined slowly to last more than 60 hours, so that by 72 hours they were 0.06 µg Fl-Ova per hour. The total cumulative amount of Ova released by an implant is represented by the corresponding curve in figure S2B. It shows that about 40 µg of Ova was released during the first 5 hours. The implant prepared in this study may not be of much clinical value due to very short release times. Alternative delivery systems such as microparticle and nanoparticle systems are under study by other researchers showing sustained drug delivery to the retina for several days or weeks (Kompella UB, Bandi N, Ayalasomayajula SP. Subconjunctival nano- and microparticles sustain retinal delivery of budesonide, a corticosteroid capable of inhibiting VEGF expression. Invest Ophthalmol Vis Sci. 2003;44:1192-1201).