Transscleral-RPE Permeability of PEDF and Ovalbumin Proteins: Implications for Subconjunctival Protein Delivery

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PURPOSE. To investigate the in vitro, ex vivo, and in vivo transscleral-retinal pigment epithelium (RPE) permeability of PEDF and structurally related proteins for the exploration of novel routes of protein delivery to the retina.

METHODS. Monkey RPE cells were cultured on permeable supports to separate apical and basal compartments. Porcine scleral tissue was placed in Ussing chambers to separate uveal and orbital compartments. Transepithelial resistance and voltage were measured by an electrical resistance system, and paracellular tracer flux was evaluated with trypan blue. Subconjunctival administration in rat eyes was by injections of soluble protein or by implantation of polyvinyl alcohol devices containing protein. Fluorescein-conjugated (Fl) PEDF and ovalbumin were determined by spectrofluorometry, laser scanning, immunoblotting, epifluorescence, and confocal microscopy. Permeability was assessed by fluoresceinated-protein flux.

RESULTS. Transepithelial resistance, impermeability to trypan blue, and confocal microscopy confirmed functional and structural tight junction formation of RPE cells cultured on permeable supports. Full-length Fl-PEDF and Fl-ovalbumin proteins diffused through RPE cell monolayers from either the apical or basal side. Fl-ovalbumin diffused through scleral tissues at constant rates. Subconjunctival Fl-PEDF or Fl-ovalbumin administration in vivo revealed movement of full-length protein into the choroid and retina as early as 1 hour.

CONCLUSIONS. The sclera and RPE were permeable in vitro, ex vivo, and in vivo to PEDF and ovalbumin proteins. These large proteins can traverse through the sclera-RPE to reach the retina. In addition, these data prompt the proposal that subconjunctival protein delivery may represent a feasible and minimally invasive route for PEDF administration in the clinic.

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sceral explants ex vivo, and rat eyes in vivo, to evaluate the diffusion and transcleral-RPE permeability of proteins from subconjunctival injections or implants.

**Materials and Methods**

**Proteins**

Recombinant human PEDF protein was purified from BHK cells, which overexpress the full-length human PEDF cDNA. Ovalbumin (Ova) was purchased from Worthington Laboratories (Lakewood, NJ). PEDF and Ova were chemically coupled with fluorescein 5EX succinimidyl ester (Molecular Probes, Eugene, OR), as previously described. For characterization of fluorescein-conjugated proteins, see Supplementary Fig. S1. http://www iovs org/cgi/content/full/46/12/4383/DC1 Fluorescent molecular weight protein markers were from Invitrogen (Carlsbad, CA).

**Spectrophotometry and Fluorometry**

Fluorescein and trypan blue were measured with a combined multi-label counter-photorimeter-fluorometer (Witlab Alexander 1420; Perkin-Elmer, Boston, MA) at a wavelength of 560 nm for photometry and at an excitation wavelength of 485 nm and an emission wavelength of 535 nm for fluorometry. Samples were loaded in triplicate into 96-well plates. Fluorescein was also measured on a spectrophotofluorometer (Quantamaster; Photon Technology International, Lawrenceville, NJ) with Felix software, version 1.21; Photon Technology International, Santa Clara, CA) at an excitation wavelength of 495 nm and a 520 nm emission wavelength.

**Monkey RPE Cell Cultures**

Nontransformed monkey RPE cells from primary cultures (a generous gift from Bruce Pfeiffer, Bauch, and Lomb, Rochester, NY) at passages 8 to 10 were seeded on permeable membranes of 12 mm diameter and 0.4 μm pore size tissue culture–treated polylysine membranes placed in 12-well plates (Transwells; Corning-Costar, Cambridge, MA). Cells (10⁴ cells/well) were cultured in DMEM/F12, 200 mM L-glutamine, 100 mM Na-pyruvate, 10 mM nonessential amino acids, 10,000 U/mL penicillin, 10,000 μg/mL streptomycin (Cellgro, Herndon, VA), and 5% fetal bovine serum (Invitrogen-Gibco, Grand Island, NY) at 37°C with 5% CO₂ in a humid atmosphere. Basal (lower) and apical (upper) compartments were loaded with 1.5 and 0.5 mL of medium, respectively, for hydrostatic equilibrium. Cells were monitored for confluence with an inverted microscope (IX70-S8F; Olympus, Tokyo, Japan).

**Transepithelial Resistance**

Transepithelial resistance (TER) was determined with an electrical resistance system (Millicell; Millipore, Billerica, MA) according to the manufacturer’s instructions. Media were changed every other day. Briefly, the permeable inserts (Transwell; Corning-Costar) and electrodes in fresh medium were equilibrated to room temperature for 30 minutes. TERs of the permeable membranes were measured in ohms per square centimeter and subtracted from nonspecific TERs obtained without cells and expressed as specific TER (ΔTER).

**Flux across Cultured RPE Monolayers**

The method used is based on the notion that flux of substances through RPE monolayers is directly proportional to RPE permeability of such substances. Fl-protein (5 μg/mL) or trypan blue (0.04%) were loaded in apical, basal or both media. Flux was measured across duplicate permeable membranes by collecting fluid samples from both sides at intervals indicated in the figures and the solute concentrations measured in triplicate by fluorometry for fluorescein-conjugated proteins and photometry for trypan blue. Fresh media were reloaded on both sides of the monolayer after each sampling. Prior calibration correlating fluorescence values with fluorescein-conjugated protein concentration standards was obtained by serial dilutions of standards in medium. Protein concentrations in the media were determined from fluorometric measurements minus background (measurement of media alone) and from the measurements of Fl-PEDF concentration standards. Protein concentrations were expressed as μg/mL per time interval of the collected samples. Apical-to-basal and basal-to-apical ratios were calculated from these values. Solute concentrations in each compartment were compared to the theoretical concentration at equilibrium (i.e., when concentrations of both apical and basal compartments become equal).

**Flux of Ova across the Sclera**

Fresh porcine eyes were obtained from an abattoir (Dorsey Meats, Woodsboro, MD) and soaked for 15 minutes in standard antibiotic solution containing 10,000 U/mL penicillin and 10,000 μg/mL streptomycin in balanced saline (BSS Plus; Alcon, Fort Worth, TX). Under sterile conditions, scleral segments free of RPE-choroid were dissected from the temporal equatorial side of the eye, which has the thinnest sclera and lacks perforating vessels, and was stored at −80°C in optimal cutting temperature (OCT) compound (Tissue-Tek 4583; Sakura Finetek Inc., Torrance, CA). Under sterile conditions, the thawed sclera was sealed with an O-ring between the orbital and uveal compartments of a Ussing chamber (EasyMount; Physiologic Instruments, San Diego, CA). Temperature was maintained at 37°C with a circulating water bath, and 5% CO₂ and 95% O₂ were bubbled through the solution, to circulate and to maintain the pH. Leaks in our system were tested by applying hydrostatic pressure from the orbital side with culture medium. The minimum diameter of a scleral segment for a leak-free system was determined to be 8.5 mm, by adding Fl-Ova in balanced saline to the orbital chamber with the balanced saline alone in the uveal chamber. After a short time (<30 minutes) the sclera was removed, fixed, and sectioned for epifluorescence microscopy, to evaluate fluorescence in the borders of the O-ring area indicative of leaks.

Donor (5 mL of 50 μg/mL Fl-Ova in balanced saline, 10,000 U/mL penicillin, 10,000 μg/mL streptomycin, and complete miniprotease inhibitors [1 tablet per 10 mL of solution; Roche, Mannheim, Germany]) and receiving (5 mL balanced saline solution with supplements) solutions were loaded into the orbital and uveal chambers, respectively. The fluids from both compartments were sampled at 0, 12, 24, 30, 36, and 48 hours after incubation at 37°C. After each sampling, fresh donor and receiving solutions were reloaded to maintain a nearly identical concentration difference across the tissue at the start of each time interval and to minimize the effects of evaporation. Fl-Ova concentrations in each chamber, Cd (donor/orbital) and Cr (receiving/uveal), were measured by spectrophotometry. From the known chamber volume (V), the total amount of ova (M) diffusing over the sampling time (t) was calculated and reported as a diffusion flux (R), in micrograms per hour, where M = Cd × V and R = M/t. The diffusion flux, R, can be related to the diffusion coefficient (D) by the equation: R = D × A × (Cd − Cr)/L, where A is the exposed area of the scleral tissue in the Ussing chamber and L is the tissue’s thickness, estimated to be 0.83 ± 0.2 mm from 10 scleral measurements with a Vernier caliper. For the present sample times, Cr was always less than 1% of Cd, and so Cr was omitted from the calculations.

**Episcleral Injections and Surgical Implantations**

All animal experiments were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Brown-Norway retired breeder rats were obtained from Charles River Laboratories (Raleigh, NC). Animals were anesthetized with a combination (1:1) of ketamine (40–80 mg/kg) and xylazine (10–12 mg/kg) by intraperitoneal administration. Additional anesthetic was administered as appropriate, to maintain a proper level of surgical anesthesia, which was determined by palpebral or hind leg withdrawal reflex. Proparacaine (0.5%) was administered topically as corneal anesthesia. For injections, 10 μL of Fi-PEDF or Fi-Ova, containing indicated protein amounts, were injected subconjunctivally (Hamilton syringe; Hamilton Co., Reno, NV), in the right postequatorial superior temporal quadrant.
of rat eyes. The left eyes were injected with 10 μL of PBS (control). Two or four animals were used per point. For implantations, subconjunctival-matrix-type implants were fabricated with medical grade 10% polyvinyl alcohol and 5% Fl-Ova (see Supplementary Fig. S2, http://www.iovs.arvojournals.org/cgi/content/full/46/12/4385/DC1). A 2.5 mm supertemporal limbal conjunctival incision was made with scissors, to expose the sclera, and a blunt dissection parallel to the scleral plane was made to insert a Fl-Ova implant or a sham implant in the postequatorial subconjunctival space. No suturing was required because of the small incision and the postequatorial position of the implant. Euthanasia was performed at indicated times after injection, and the eyes were enucleated.

**Protein Extraction and Fluoresceinated Protein Determination in Tissues**

Sclera, choroid-RPE and retinal tissues were dissected. The same tissues for each condition were pooled and immersed in 10 M urea, 10% SDS, 1.5 mM dithiothreitol (DTT), and 100 mM Tris-Cl (pH 8.0), at 400 μL per two eyes. Alternatively, tissue protein extraction reagent (T-PER, Pierce, Rockford, IL) was used at a concentration of 100 μL/mg total tissue. Scleras were homogenized (Polytron PT 3000; Brinkmann, Herzogenrath, Switzerland) two times for 20 seconds each at 12,000 rpm. RPE-choroid and retina tissue suspensions were sonicated (Misonix Inc., Farmingdale, NY) in ice-water bath with five bursts of 75%/1-second cycles and an output between 8 and 9 (corresponding to 24 and 27 microns of amplitude using a 2-inch-cup horn in the Misonix XL2015 model). A total of 20 μL of each sample was subjected to SDS-PAGE. In addition, the homogenized or sonicated protein extracts were centrifuged at 2800g at 4°C for 10 minutes. The Fl-Ova concentrations in the supernatants were determined by spectrophotometry. A prior calibration, correlating fluorescence intensities with Fl-Ova protein concentrations was obtained by serial additions of known Fl-Ova concentrations to tissue extracts from animals not exposed to fluoresceinated protein. Concentrations calculated from the spectrophotometric values and calibration curves were expressed in micrograms of Ova per milliliter of extract and then converted to micromolars of Ova per milligram of tissue, based on the measured weight of tissue in each extraction solution.

**Protein Concentration Assays, SDS-PAGE, and Western Blot Analysis**

Protein concentration was determined with a protein assay (Bio-Rad Laboratories, Hercules, CA). SDS-PAGE was performed with 10% to 20% polyacrylamide gradient gels in SDS-tricine running buffer (Novex, San Diego, CA). Western transfers were performed as described elsewhere. Immunoreactions were performed with primary anti-fluorescein rabbit IgG fraction (Oregon Green; Molecular Probes) and detected by enhanced chemiluminescence (ECL; Lumi-Light®; Roche). The fluorescent signal in the gels was visualized with a fluorescence scanner (Typhoon 9410 Variable Model Imager; GE Healthcare, Arlington Heights, IL).

**Confocal and Epifluorescent Evaluation**

Monkey RPE cells in selected wells were fixed, labeled with phalloidin Alexa Fluor 568 and 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; Molecular Probes), and visualized by laser scanning confocal microscopy (Leica SP2, Leica Microsystems, Bannockburn, IL), to evaluate cell morphology and monolayer formation.

Enucleated eyes were fixed in 4% paraformaldehyde (Powder 30525-89-4 EM Grade; Polysciences, GmbH, Heppenheim, Germany) in phosphate-buffered saline (PBS), for 3 hours, oriented in Optimal Cutting Temperature compound (OCT, Tissue-Tek 4583; Sakura Finetek, Inc.) and stored at −70°C for 24 hours. Horizontal sections of 12 μm thickness were cut with a cryostat (Leica CM3050S; Leica Microsystem Inc., Bannockburn, IL) for visualization by epifluorescence microscopy (ECLIPSE E800; Nikon Instrument Group, Melville, NY), working at an excitation bandpass of 390 to 490 nm and an emission of 515 to 555 nm. Specimens were also analyzed by confocal microscopy. Samples were scanned in sequential scan mode to reduce bleed-through artifacts. Files were imported into Photoshop and converted to PSD format for layout purposes.

**RESULTS**

**Formation of Monolayers and Tight Junctions**

Monkey RPE cells were seeded on permeable membranes to form confluent cell monolayers with tight junctions and separated apical and basal sides. Because junctions can be evaluated structurally by immunolocalization of the junction-associated actin microfilaments, we evaluated the RPE cells by confocal microscopy after immunolabeling with phalloidin. Figure 1A shows that phalloidin outlined the actin cytoskeleton of the cells, forming a circumferential band at the level of the apical lateral complex of the RPE on the permeable supports. Transverse sections showed that the cells had fully developed actin cytoskeletons, with no overlapping of cell margins, an indication of a monolayer of confluent cells with tight junctions.

Junctions were evaluated functionally by measuring TER (i.e., resistance to ion flow) across the monolayer. TER measurements were performed every other day and immediately after changing the media, to avoid differences due to evaporation or chemical modifications of the media by cellular secretions. By 7 weeks, the specific TER reached 80 to 105 Ω/cm² (Fig. 1B) and was similar to that found previously in monkey RPE cells. These results demonstrate that our RPE cells formed tight junctions and were polarized.

Functional junction formation of RPE cells was also evaluated by flux of trypan blue, a paracellular tracer for testing impermeability of blood-brain and blood-retinal barriers. Trypan blue tracer flux was determined by movement of molecules through a concentration gradient that is inversely related to tight junction formation and directly related to permeability of monolayers. Trypan blue has low molecular weight (392 Da), is negatively charged, and can form complexes with serum albumin. The large trypan blue-albumin complexes, their negative charge, or both, prevent its passing through the monolayers. To test the impermeability of the RPE cells, a hydrostatic gradient was generated in the apical-to-basal direction by adding a volume of 0.04% trypan blue (1 mL) to the apical compartment, greater than necessary for hydrostatic equilibrium. In inserts without cells, the volume of the apical medium decreased significantly in 6 hours (Amaral J, personal observations, 2003). Moreover, trypan blue concentration increased in the basal side with time, approaching theoretical concentration at equilibrium by 12 hours (0.115 ± 0.02 mg/mL; Fig. 1C), indicative of an efficient diffusion toward the basal compartment through the permeable membrane. In contrast, on the membrane inserts, the apical volume did not decrease and trypan blue was not detected on the basal side (Fig 1D). Cultures impermeable to trypan blue had ΔTER of >60 Ω/cm² (Amaral J, personal observations, 2003). These results demonstrate that the RPE monolayers prevented bulk flow of the medium and the movement of trypan blue in the apical-to-basal direction through the permeable membrane, in agreement with tight junction formation in the RPE monolayers on permeable inserts.

**Flux of Fl-PEDF through RPE Monolayers**

RPE permeability of PEDF was tested on permeable inserts with monkey RPE monolayers that followed the criteria of ΔTER >80 Ω/cm² and with parallel cultures that were trypan blue impermeable. Solutions of 5 μg/mL Fl-PEDF were placed on one compartment and the Fl-PEDF concentrations determined in the opposite one after incubation at 37°C for 6- or 12-hour intervals. The theoretical equilibrium concentrations
of RPE cells in both directions. When 5 μg/mL FI-PEDF was loaded in both compartments of the inserts, without or with cells, the FI-PEDF concentrations on either compartment were not significantly different after 6 and 12 hours (Figs. 2G, 2H). The FI-PEDF concentrations after incubation were slightly lower than the original 5 μg/mL, probably due to nonspecific binding to the plastic walls or to the membrane support of the inserts or fluorescence quenching over time. Similar observations were obtained with Fl-Ova (Amaral J, unpublished results, 2003).

The integrity of the fluoresceinated proteins was evaluated by Western blot analysis of media from both compartments. After incubation with FI-PEDF for 12 hours, immunostaining of anti-fluorescein revealed protein with an expected apparent

for apical and basal loading were 1.25 and 3.75 μg/mL FI-PEDF, respectively. When loaded in the basal (Fig. 2A) or apical (Fig. 2D) compartments of inserts without cells, FI-PEDF concentrations reached equilibrium by 6 hours, indicating an efficient movement through permeable membranes. However, in inserts with cells, FI-PEDF concentrations in the compartment opposite the loading one increased with incubation time, so that after 6- and 12-hour incubations with basal loading, the apical FI-PEDF concentrations were 25% and 32% of the theoretical equilibrium values, respectively (Fig. 2B); and with apical loading, the basal FI-PEDF concentrations were 29% and 35.6% of the theoretical equilibrium values, respectively (Fig. 2E). These concentrations indicated flux through monolayers

as indicated by the schemes below each figure (Fig. 2A, B, C). The TERs were plotted versus incubation time. The specific TER (ΔTER) of the cells calculated by subtracting the control value (without cells) at 7 weeks ranged between 80 and 105 Ω/cm² (*P < 0.009, with versus without cells at 7 weeks). (C, D) Paracellular tracer flux was evaluated with and without mRPE monolayers. An excess of volume (1 mL) of trypan blue 0.04% was loaded in the apical compartment, to generate a hydrostatic gradient toward the basal compartment. The basal flux was calculated by photometry measurements after subtracting the background and is expressed in milligrams per milliliter. The theoretical equilibrium in both chambers was a concentration of 0.16 mg/mL trypan blue. Plots of trypan blue concentration on the basal side, opposite the loading side, of control inserts without cells (C) and with cells (D) over time are shown.

FIGURE 1. Confocal imagery, TER, and paracellular tracer flux of monkey RPE cell monolayers. Monkey RPE were cultured on permeable membrane supports until confluent. (A) Cultures were evaluated by confocal microscopy. Sideview (Confocal xz scan) image of monkey RPE cell monolayer after 7 weeks of seeding on a polyester membrane. The actin cytoskeleton was stained with phalloidin (red) and the nuclei with DAPI (blue). Scale bar, 20 μm. (B) TER was measured immediately after the media were changed, every other day up to 7 weeks, and TERs were plotted versus incubation time. The specific TER (ΔTER) of the cells calculated by subtracting the control value (without cells) at 7 weeks ranged between 80 and 105 Ω/cm² (*P < 0.009, with versus without cells at 7 weeks). (C, D) Paracellular tracer flux was evaluated with and without mRPE monolayers. An excess of volume (1 mL) of trypan blue 0.04% was loaded in the apical compartment, to generate a hydrostatic gradient toward the basal compartment. The basal flux was calculated by photometry measurements after subtracting the background and is expressed in milligrams per milliliter. The theoretical equilibrium in both chambers was a concentration of 0.16 mg/mL trypan blue. Plots of trypan blue concentration on the basal side, opposite the loading side, of control inserts without cells (C) and with cells (D) over time are shown.

FIGURE 2. Flux of FI-PEDF across monkey RPE cell monolayers. FI-PEDF at 5 μg/mL was loaded in the basal, apical, or both compartments of permeable inserts. Every 6 or 12 hours, samples were removed, and fluorescein was measured with a plate reader fluorometer. The concentration of FI-PEDF calculated with fluorometry measurements after subtracting background is expressed in micrograms per milliliter. The averages of four measurements from each 6- and 12-hour interval were calculated from two experiments, one with duplicates, and the other in triplicates permeable inserts. The averages of these were calculated and plotted and are shown in (A), (D), and (G), for inserts without cells, and in (B), (E), and (H) for inserts with RPE cell monolayers. The y-axis correspond to FI-PEDF concentrations and the x-axis correspond to the time intervals. (E) Apical FI-PEDF concentration; (D) basal FI-PEDF concentration. *P < 0.0007; **P < 0.0003; ***P < 0.00001, each with respect to the time 0 hour in (B) and (E). Serum-free conditioned media at a 12-hour interval were collected from inserts with cells, and concentrated fivefold by ultrafiltration, and 20 μL of each were subjected to SDS-PAGE. Western blot analysis was performed with anti-fluorescein (diluted 1:200,000) by ECL. Blots in (C) are for basal, and in (F) for apical FI-PEDF loading, and in (I) for apical and basal FI-PEDF loading, as indicated by the schemes below each blot. Apical/basal and basal/apical ratios are summarized in Table 1.
molecular weight of 50,000 for Fl-PEDF in all the compartments under all conditions (Figs. 2C, 2F, 2I). Moreover, the apical–basal intensity ratios in Western analyses correlated with those from quantification by spectrophotometry (Table 1), confirming flow of full-length Fl-PEDF protein through RPE monolayers in both directions.

Confocal microscopy of selected inserts with RPE cells and Fl-PEDF loaded in the basal side showed distribution of fluorescein staining in the cytoplasm of confluent RPE cells, implying a Fl-PEDF flux across RPE cells (Fig. 3). Altogether, these results demonstrated the in vitro RPE permeability of Fl-PEDF from either direction of the monolayers.

**Ex Vivo Scleral Permeability of Fl-Ova**

Ova shares structural characteristics with PEDF protein (e.g., both fold like serpins without serine protease inhibitory activity). In addition, it was readily available in larger amounts (grams) and its conjugation yielded a higher ratio of fluorescein molecules per amount of protein (see Supplementary Fig. S1, http://www.iovs.arvojournals.org/cgi/content/full/46/12/4383/DC1), resulting in an easily detectable tracer. Sclera from porcine eyes is an excellent model for studying transcleral drug delivery because of its similarity to human sclera in thickness, composition, and permeability properties. The permeability of Fl-Ova across porcine sclera was tested with a Ussing diffusion device, to separate uveal and orbital compartments of the sclera. Fl-Ova at 50 μg/mL was added to the orbital medium and its diffusion rate was calculated from the concentration in the uveal medium over time. Fl-Ova diffused across the porcine sclera after reaching equilibrium at a constant rate with time (Fig. 4). The diffusion constant for porcine sclera was estimated to be $6.4 \times 10^{-7}$ cm²/s. These results demonstrate Fl-Ova movement from the orbital to the uveal side of porcine scleral explants and, consequently, the porcine scleral permeability of Fl-Ova.

**In Vivo Transscleral-RPE Permeability of Fl-PEDF and Fl-Ova**

To evaluate the transscleral-RPE permeability of PEDF and Ova, aliquots of fluoresceinated protein solutions were administered in the subconjunctival space in Brown Norway rat eyes, either by injections or from implants. Western blot analysis of protein extracts from scleral, choroidal-RPE, and retinal tissues prepared at given times after injection showed that the fluorescein fluorescence was detectable in the injected and orbital tissue. A circulating water bath maintained a constant temperature of 37°C, and CO₂ and O₂ were infused to the Ussing chamber. The orbital chamber was loaded with 5 mL of a 50-μg/mL Fl-Ova solution in balanced saline. The uveal chamber was loaded with 5 mL of saline alone. A circulating water bath maintained a constant temperature of 37°C, and CO₂ and O₂ were infused to circulate and to maintain physiologic pH levels. At given time points, the concentration of Fl-Ova was determined in the uveal chamber. Solutions were removed from both chambers after each time point and replenished with fresh ones. Plot of the cumulative amount of fluorescent protein, expressed in micrograms (∥axis), that diffused through a segment of sclera toward the uveal chamber versus the incubation time expressed in hours (∥axis).

**Table 1. Fl-PEDF Flux through Monkey RPE on Transwell Inserts**

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<th>Loading Compartment†</th>
<th>Time Interval (h)</th>
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* Ratios obtained from data in Figure 2.
† Fl-PEDF (5 μg/ml) applied in indicated compartments at time 0 hours.

**Figure 3.** Confocal image (x,y scan) of a representative insert of monkey RPE cell monolayer exposed to Fl-PEDF loaded in the basal compartment after 12 hours of incubation. Permeable inserts were fixed with 4% paraformaldehyde for 1 hour and incubated 3 hours with phalloidin, an actin marker (red) that outlines the cytoskeleton of cells. Nuclear content was stained with DAPI (blue). Fl-PEDF fluorescence (green) was detected at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Scale bar, 20 μm.

**Figure 4.** Ex vivo transscleral permeability of Fl-Ova. A segment of porcine sclera was secured between the orbital and uveal chambers of a Ussing chamber. The orbital chamber was loaded with 5 mL of a 50-μg/mL Fl-Ova solution in balanced saline. The uveal chamber was loaded with 5 mL of saline alone. A circulating water bath maintained a constant temperature of 37°C, and CO₂ and O₂ were infused to circulate and to maintain physiologic pH levels. At given time points, the concentration of Fl-Ova was determined in the uveal chamber. Solutions were removed from both chambers after each time point and replenished with fresh ones. Plot of the cumulative amount of fluorescent protein, expressed in micrograms (∥axis), that diffused through a segment of sclera toward the uveal chamber versus the incubation time expressed in hours (∥axis).
ceinated signal for Fl-PEDF and Fl-Ova migrated as 50- and 45-kDa proteins, respectively, consistent with permeability of full-length proteins (Fig. 5A), with both proteins being more intense in the RPE-choroid than in retina extracts. Coomassie blue staining of proteins in the gels showed that protein loading was equivalent among samples from the same tissues (Fig. 5B). The relative fluorescein-immunoreactivities from another set of animals injected with higher protein amounts than above were detected in the RPE-choroid and retinal extracts as early as 1 hour after injection but decreased with time and was not significant by 24 hours (Figs. 5C, 5D). The amount of Fl-Ova was also calculated from fluorescence measured spectrofluorometrically in each tissue extract and expressed as micrograms per milligram of tissue. Because the levels of fluorescence determined spectrofluorometrically at 1 and 3 hours paralleled those by laser scanning of proteins resolved by SDS-PAGE, it was assumed that most of the fluorescence corresponded to intact Fl-Ova protein. The levels of Fl-Ova decreased in the order sclera-RPE/choroid-retina at all time points after injection, suggesting a decreasing protein gradient from the sclera to the inner retina (Fig. 6). In addition, the fluoresceinated protein amounts decreased with time, as observed by Western blot analysis. At 6 hours, laser scanning and spectrofluorometric measurements were not directly proportional, suggesting protein clearance or degradation. These results demonstrate that Fl-PEDF and Fl-Ova proteins traversed in vivo from the subconjunctiva through the sclera, choroid-RPE, and retina. Thus, the sclera and RPE were permeable to these proteins. 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 (50 µg/implant) for subconjunctival

Figure 5. Western blot analysis of the RPE-choroid and retina of Brown Norway rats after subconjunctival injections of Fl-PEDF and Fl-Ova. At indicated postinjection time points, eyes were collected, and tissues (RPE-choroid and retina) were homogenized with denaturing solution and centrifuged and the supernatant subjected to SDS-PAGE for Western blot analysis against anti-fluorescein (diluted 1:200,000) by ECL. Noninjected eyes served as the control (Control). (A, B) A total of 10 µL containing either 14 µg of Fl-PEDF or 47 µg of Fl-Ova in PBS was injected per subconjunctiva. A volume of 20 µL of the supernatant (retina and choroid-RPE) was applied to a 10% to 20% gradient polyacrylamide gel. After transfer, gels were stained with Coomassie blue. Western blot analyses are shown in (A) and Coomassie Blue stained gel in (B). M, molecular weight markers; PpB, phosphorylase B; BSA, bovine serum albumin; Ova, ovalbumin; CI, carbonic anhydrase; TI, soybean trypsin inhibitor. (C, D) A total of 10 µL containing 95 µg Fl-Ova (C) or 57 µg Fl-PEDF (D) were injected per subconjunctiva. Western blot analysis of tissue homogenates were prepared, and the intensities of immunostained bands were measured with image-analysis software. Intensities relative to that of RPE-choroid at 1 hour after injection (100%) were calculated and plotted per each postinjection time for each tissue.

Figure 6. Spectrofluorometric measurements of Fl-Ova protein in sclera, RPE-choroid, and retina of rats after subconjunctival injections of Fl-Ova. A total of 10 µL containing 100 µg of Fl-Ova in PBS was injected per subconjunctiva. At indicated postinjection time points, two eyes were collected per point, and pools were made from the same tissues after dissection. Proteins from sclera, RPE-choroid and retina were extracted, and fluoresceinated proteins were analyzed by spectrofluorometry, to calculate Fl-Ova concentration. Each concentration point corresponds to calculations from the average of triplicate spectrofluorometric measurements. Data are the mean ± SD. SDS-PAGE and laser fluorescence scanning of Fl-Ova in sclera, RPE-choroid, and retina were also performed, and a fragment of the laser-scanned gel showing the only band detected is shown (top).
As expected for a matrix type implant, they released the fluoresceinated protein to PBS buffer at high rates during the initial hour or "initial burst" without a linear rate of diffusion (see Supplementary Material and Fig. S2, http://www.iovs.org/cgi/content/full/46/12/4383/DC1). Implants were placed in the temporal postequatorial subconjunctival space to prevent them from sliding out of the eye, thus avoiding the need for stitches and minimizing inflammatory responses. Fluorescein movement was followed by microscopy of the ocular sections. At 1 hour after implantation, fluorescein was found only in the temporal quadrant, with a gradient of fluorescence fading from the sclera to the choroid and retina, and from the area of the implant toward the optic nerve (Fig. 7A). By 24 hours after implantation, fluorescein was still detected homogeneously in both temporal and nasal retina–choroid with intense signal through the ciliary body (Fig. 7B). At higher magnification, a gradient of fluorescence was observed from the choroid to the inner retina at 8 hours (Fig. 7C). At 24 hours, the fluorescence signal was homogeneously distributed in the choriretinal tissues (Fig. 7E). The fluorescence signal was detected as a 45-kDa protein, indicating flux of full-length Fl-Ova by 24 hours after implantation (Fig. 8). These results imply that Fl-Ova implants in the subconjunctiva released full-length protein that traversed toward the retina.

**DISCUSSION**

We have demonstrated the permeability properties of the sclera and RPE to PEDF and Ova proteins. These properties
permit passage of these serpins from the subconjunctiva to the retina. This conclusion is based on the flow of PEDF through cultured monkey RPE cell monolayers (Figs. 2, 3), of ovalbumin across ex vivo porcine scleral explants (Fig. 4), and of Ova and PEDF in in vivo rat eyes (Figs. 5, 6, 7, 8). These results imply a potential use of PEDF proteins for local delivery to the choroid and retina as therapeutic agents. In addition, permeability of PEDF through the blood–retinal barrier plays an important role in the efficacy of systemic administration of PEDF protein to inhibit retina neovascularization.

The sclera separates uveal from orbital compartments, and is composed mainly of a matrix of collagen fibers and proteoglycans. Even though it is avascular and mostly devoid of cells, it is permeable to certain substances (e.g., albumin, insulin, and high-molecular-weight dextrans). There are no reports of diffusion coefficients for ovalbumin or porcine sclera. We have estimated the permeability coefficient (P) of porcine sclera (i.e., diffusion coefficient, $D = 0.64 \times 10^{-6}$ cm$^2$/s, divided by the thickness of tissue, 0.081 cm) for 40-kDa ovalbumin (45 kDa) to be $P = 7.7 \times 10^{-7}$ cm/s. This value compares with permeability values across human skin for dextrans with similar molecular masses reported by Kim et al. for 40-kDa dextran ($P = 7 \times 10^{-7}$ cm/s). However, it is 10 times lower than those reported by Olsen et al. for 40-kDa dextran, $P = 50 \times 10^{-7}$ cm/s. 70-kDa dextran, $P = 19 \times 10^{-7}$ cm/s. In any event, it appears that macromolecules such as Ova would be at least as permeable across human as it is across porcine sclera. Although the PEDF molecular radius has not yet been reported, it is estimated to be no larger than that of ovalbumin. Being that the molecular radius is a better predictor of permeability than is molecular mass, PEDF would be at least as permeable as ovalbumin across sclera. Their diffusion may be hindered somewhat as they pass through the complex matrix of the sclera (e.g., by binding of PEDF to collagens or glycosaminoglycans).

Another barrier to transport of macromolecules to the retina is the RPE-choroid. Recently, Pitkänen et al. compared the in vitro permeability coefficients for hydrophilic compounds across explants of human and rabbit sclera with those for bovine RPE-choroid. They reported that the RPE-choroid is approximately 10 to 100 times less permeable than the sclera to molecules with molecular radii ranging 1 to 6 nm. These data imply that the sclera would not be a rate-limiting barrier for transscleral delivery of drugs, including macromolecules, to the retina. The RPE forms the outer blood–retinal barrier by regulating transport between the fenestrated capillaries of the choroid and the neural retina. Highly specialized junctional complexes between RPE cells regulate transport through paracellular spaces and are critical features of this barrier. Together with the adherens junction and associated actin filaments, tight junctions form junctional complexes that completely encircle each cell near the apex of lateral membranes. Thus, junction permeability is regulated indirectly through cytoskeleton components. In the present study, structural, and functional evaluations revealed intact cytoskeleton with fully formed tight junctions of monkey RPE monolayers. Confluent monkey RPE monolayers generated significant ΔTER, similar to those reported previously in monkey, human, and rat RPE cells. To date, there is no information on protein permeability across the RPE, per se. The current reports are on permeability across choroid-RPE tissues of diffusible dextrans and small molecules. Pitkänen et al. concluded that bovine choroid-RPE has selective permeability for nonprotein lipophilic and hydrophilic macromolecules that is inversely related to their molecular radii. To our knowledge, our studies are the first to provide information on the permeability of PEDF protein across RPE cell monolayers.

Our in vivo data of subconjunctival delivery of protein has clinical implications. The in vivo transport of full-length PEDF and Ova proteins through the sclera toward the choroid-RPE and retina supports the in vitro and ex vivo permeability findings and is in agreement with data reported by Gehlbach et al. with 3 μg PEDF injected subconjunctivally in mice. Although retina permeability of PEDF and Ova can be extrapolated from our in vivo observations, actual permeabilities and the mechanism of protein transport across the inner retina remain unknown. The observed decreasing protein gradient from the sclera to the inner retina may be affected by several factors that include permeability properties, intraocular pressure, and clearance. The initial lag in scleral protein flux observed across the sclera ex vivo is in agreement with the intense protein signal in vivo and suggests protein saturation before constant diffusion can be achieved in the sclera. Differences in permeability between sclera and RPE result in gradient formation in an inward direction. Protein movement toward choroidal or retinal blood vessels and/or toward the vitreous and from there through the uveoscleral channels also contributes to gradient formation. Another observation is the half-life of the injected PEDF and Ova proteins. Protein detection in RPE-choroid and the retina ceased by 24 hours after injection, similar to a previous report with intravitreal injections of biotinylated PEDF in mice.

To guarantee protein release at a constant rate for a longer period, research on protein delivery devices is needed. We have shown here a first attempt with a matrix-type implant that somehow provided greater delivery of Ova than did a single injection. Even though this type of implant does not have a linear release rate (e.g., initial burst of diffusion), the feasibility of delivering constant protein levels to the choroid-RPE and retina by subconjunctival or episcleral routes is shown in this study.

In summary, our results lead us to conclude that (1) the sclera and choroid-RPE are permeable to PEDF and ovalbumin proteins and (2) these proteins can traverse the subconjunctiva to reach the retina. These conclusions suggest that a subconjunctival route of delivery is possible with this important protein and offers a feasible and minimally invasive route for administration in the clinic.

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References


