Appendix E1

Materials and Methods

General

All chemicals were purchased from Sigma-Aldrich (St Louis, Mo) unless otherwise stated. No-carrier-added fluorine 18 (^{18}F) fluoride was produced by the ^{18}O (p, n)^{18}F nuclear reaction on a PETtrace cyclotron (GE Medical Systems, Milwaukee, Wis). ^{18}F-P3BZA was prepared on a TRACERLab FX FN module (GE Medical Systems). Pig eyeballs were purchased from Animal Technologies (Tyler, Tex) for isolation of pRPE cells. The amelanotic hRPE cell line ARPE-19 was used as a control and obtained from American Type Culture Collection (Manassas, Va). All of the analyses of sequential radioactivity for ^{18}F-P3BZA quantifications in vivo and in vitro were decay corrected.

Radiosynthesis of ^{18}F-P3BZA

Melanin-targeted PET probe ^{18}F-P3BZA was prepared according to previously published procedures (Z.C. and L.B. have a patent pending for ^{18}F-P3BZA) (21,22). Briefly, 5-bromo-*N*-[2-(diethylamino)ethyl]-picolinamide (5 mg) was reacted with K^{18}F-$K_{2.2.2}$ complex in 200 μL of anhydrous dimethyl sulfoxide at 110°C for 10 minutes with stirring. The radiolabeled product was isolated with a semi-preparative high-performance liquid chromatograph, and the fractions containing the product were collected and combined. The radiochemical yield was calculated by dividing the amount of the obtained product by the injected activity into the high-performance liquid chromatograph. The final product ^{18}F-P3BZA was reconstituted in phosphate-buffered saline (PBS) (0.1 mol/L, pH = 7.4). To determine the retention time, radiochemical purity, and specific activity, the final product was injected into an analytical high-performance liquid chromatograph with a flow rate of 1 mL/min by using a Vydac (Chrom Tech, Apple Valley, Minn) protein and peptide column (5 μm, 250 × 4.6 mm). The final radiochemical purity was determined by injecting the product onto a radio- high-performance liquid chromatograph column. The radioactive fraction was measured in a dose calibrator for specific activity calculation.

The ultraviolet absorbance was monitored at 218 nm, and the identification of the small molecules was confirmed on the basis of the ultraviolet spectrum acquired with use of a photodiode array detector. Before use in in vitro and in vivo animal experiments, the final radioactive product was passed through a 0.22-μm filter into a sterile vial.

pRPE Cell Isolation and Culturing

Melanotic pRPE and amelanotic ARPE-19 cells were used as positive and negative controls, respectively, for the evaluation of ^{18}F-P3BZA in vitro and in vivo. pRPE cells were isolated in our laboratory according to a previously published procedure (25). Briefly, fresh eyeballs were cleaned by removing all surrounding tissues. The eyeballs were sterilized by incubating in betadine solution (Purdue Products, Stamford, Conn) for 15 minutes and then washed with sterilized PBS. The anterior part of the eye and the neural retina, approximately 3.5 mm posterior to the limbus, was removed. Then, 0.25% trypsin was added to the posterior eyecup and it was

incubated at 37°C for 1 hour. The enzyme solution was pipetted up and down a few times to loose pRPE cells from choroid and sclera, and the pRPE cells were collected into a centrifuge tube. Culture medium (Dulbecco's modified Eagle high-glucose medium with low glucose [DMEM low glucose], 10% fetal bovine serum, antibiotic/antimycotic; Gibco Life Sciences, Grand Island, NY) was added to the cells to stop enzyme reaction. To obtain pure pRPE population, the cell suspension was placed on the top of 40% Percoll (Amersham Biosciences, Piscataway, NJ) cushion (in PBS) and centrifuged for 10 minutes at 300 g. The hRPE cells settled to the bottom of the centrifuge tube. The purified pRPE cells were resuspended into the culture medium and plated in culture dishes. Cell cultures were incubated at 37°C with 5% CO_2, and the medium was changed two to three times a week. Both pRPE and hRPE cells were cultured by using the same culture medium and incubator and grown to 100% confluence before attachment to gelatin microcarriers and implantation.

In Vitro Assays

Cell uptake of ^{18}F-P3BZA

pRPE (generation 3, 5×10^5) or control ARPE-19 cells were plated in a 12-well plate pretreated with or without L-tyrosine (2 mmol/L) for 24 hours. They were incubated with 74 kBq (2 μCi) per well of ^{18}F-P3BZA at 37°C for 15, 30, 60, and 120 minutes. The cells were washed three times with chilled PBS and lysed with 500 μL of 0.1 mol/L NaOH. The cell lysates were collected, and the radioactivity of the lysates was measured with a gamma counter (Perkin-Elmer, Waltham, Mass). The cell uptake was expressed as the percentage of total applied radioactivity. All experiments were performed with triplicate samples. Similarly, the uptake of ^{18}F-P3BZA at 30 minutes of incubation was determined in pRPE cells at different passage numbers (pRPE-P3, pRPE-P5, and pRPE-P8) and in control ARPE-19 cells, with or without pretreatment with L-tyrosine.

Melanin quantification analysis

In vitro measurement of melanin content was performed for pRPE and ARPE-19 cells in parallel with the ^{18}F-P3BZA cell uptake assay according to previously reported methods, with slight modifications (28). The cells were harvested after pretreatment with or without 2 mmol/L L-tyrosine for 24 hours and washed with PBS; then, they were incubated overnight in 500 μL of 1 nmol/L NaOH at room temperature. The solution was pipetted repeatedly to homogenize the extracts. The concentration of total protein was determined by using a protein assay kit (micro BCA; Pierce Biotechnology, Rockford, Ill), and the concentration of samples was adjusted to 0.4 μg/μL. Extracts (100 μL) were added into 96-well plates in triplicate. The relative melanin content of samples was determined by measuring their absorbance at 405 nm by using a plate reader (Safire; Tecan, Research Triangle Park, NC). Results are expressed as absorbance of 405 nm per mg protein (A 405 nm per milligram protein).

Cell Implantation

Attachment of cells to gelatin microcarriers

RPE cells (pRPE and control ARPE-19 cells) were passively adsorbed or attached to gelatin microcarriers (Cytodex 1, 131–220 μm, dextran beads, Sigma) according to typical methods (11). Briefly, 24 hours before the attachment study, dry gelatin microcarriers were hydrated in a 1.5-mL microcentrifuge tube with calcium- and magnesium-free PBS (Invitrogen, Grand Island,

NY) for a minimum of 1.5 hours and then autoclaved (121°C, 15 psi, 30 minutes) for sterilization. Sterilized gelatin microcarriers were then re-suspended and washed twice with fresh PBS and stored in complete pRPE medium. After being harvested by means of trypsinization and mechanical agitation, RPE cells (≥90% cell viability) was mixed with gelatin microcarriers in complete RPE medium to obtain the suspension with a final concentration of 1×10^6 cells per 10 mg of gelatin microcarriers in a 1.5-mL micro-centrifuge tube, and the resulting mixture was placed for 15–18 hours in a 37°C incubator. After incubation, the RPE-gelatin microcarrier suspensions were gently washed with PBS to remove any unattached RPE cells. A small volume (10 μL) of sample was taken and treated with a Dispase solution to break down gelatin microcarriers, and the samples were assessed for cell viability and concentration by using the trypan blue exclusion method. Only RPE–gelatin microcarrier suspensions passing the minimum criteria (≥90% cell viability and ≥2000 cells/μL RPE–gelatin microcarrier suspension) were selected for implantation. Gelatin microcarrier suspensions were treated in a similar manner as controls.

Surgical procedure

Twenty female Wistar rats weighing 176–200 g were used for the surgical procedure. All animal work was conducted in accordance with the Administrative Panel on Laboratory Animal Care at Stanford University. The implantation procedure was performed following the surgical procedures described in a previous report (29). Animals were anesthetized by using isofluorane and were implanted at left striatum with RPE–gelatin microcarriers by using the following (flat-skull) coordinates: anteroposterior, −0.4; mediolateral, ±3.5; dorsoventral, −5.0. The control gelatin microcarriers alone were implanted on the contralateral side of the striatum by using the same procedure. Before the implantation procedure, solutions contained RPE cells (melanotic pRPE or amelanotic ARPE-19 cells, approximately 12 000–15 000 cells in 10 μL) were drawn into a sterile 50-μL syringe (Hamilton, Reno, Nev).

Dynamic PET

To assess the in vivo kinetics of ^{18}F-P3BZA in normal rat brain, dynamic PET imaging (10 × 1 minute, 10 × 2 minute, 2 × 5 minute, 3 × 10 minute; total of 25 frames) was initiated immediately after administration of the probe and terminated 70 minutes later in normal rats (n = 4).

CT (632 sections at 206 μm) was performed immediately after PET for both photon attenuation correction and image co-registration with PET image data for anatomic information. PET images were reconstructed with a two-dimensional ordered-subsets expectation maximization algorithm (OSEM 2D). Regions of interest were drawn in bilateral striatum by using the Inveon Research Workspace software. The average radioactivity concentration in the regions of interest was obtained from the mean pixel values within the region of interest volume. These data were converted to counts per milliliter per minute by using a predetermined conversion factor. The results were then divided by the ID to obtain an image region of interest–derived percentage ID per gram of tissue, and the time-activity curve was then calculated and obtained.

Static PET/CT

To investigate the efficacy of ^{18}F-P3BZA for imaging of implanted pRPE cells in vivo, static PET (10 minute) was performed in normal rats with pRPE–gelatin microcarriers (n = 4) or ARPE-19–gelatin microcarriers (n = 4) implanted in left striatum, parallel with gelatin microcarriers only as controls in the right striatum. PET/CT scans were obtained 1 hour after tail vein injection of ^{18}F-P3BZA. Rats were sacrificed immediately after PET/CT, and rat brain sections were prepared for autoradiography and hematoxylin-eosin staining.

Longitudinal PET/CT

To evaluate the potential of ^{18}F-P3BZA for monitoring the survival and function of implanted RPE cells, longitudinal PET/CT scans were acquired 2, 9, and 16 days after implantation of gelatin microcarrier–bound pRPE in the left striatum and gelatin microcarriers alone as control in the right striatum in normal rats (n = 12). PET/CT started at 1 hour after tail vein injection of ^{18}F-P3BZA. Immediately after PET/CT at each time point (days 2, 9, and 16), four rats were sacrificed and the brain slices were prepared for immunohistochemistry and Fontana-Masson staining.

Postmortem Analysis

The rat brains were removed and embedded in optimum cutting temperature compound (Tissue-Tek; Sakura, Torrance, Calif). Subsequently, 12-μm-thick axial blocks were cut by using a cryostat microtome (HM 500M; Microm, Heidelberg, Germany). The sections were mounted on microscope slides (Fisherbrand Superfrost Plus, Fisher Scientific, Vernon Hills, Ill). The representative coronal sections through the striatum (including visible injection sites) were used for autoradiography immediately or preserved in −80°C for other postmortem analyses.

Ex Vivo Autoradiography

After static PET/CT, frozen brain sections were prepared immediately for autoradiography. The sections were air-dried for 10 minutes and then exposed to a radioactivity-sensitive storage phosphor screen (Perkin-Elmer) for 24 hours at 4°C. The imaging plates were analyzed by using a variable mode imager (Typhoon 9410; Amersham Biosciences, Salt Lake City, Utah), and the image data were visualized and processed with Image J software (image processing and analysis software in Java).

Hematoxylin-Eosin Staining

After static PET/CT, the brain sections adjacent to those for the autoradiography study were stained with hematoxylin-eosin by using a stain kit (H&E Stain Kit; BBC Chemical, Dallas, Tex) and following the instruction to identify the implantation sites and the anatomy of brain sections.

Fontana-Masson Staining

After longitudinal PET/CT, slices from the block containing a representative RPE cell profile on initial review were stained with Fontana-Masson stain (AMTS, Lodi, Calif). Fontana-Masson staining was conducted at each time point to verify the presence of melanin. The staining was performed according to the manufacturer's recommendations step by step, with slight modifications. Fontana silver solution was prepared by adding the concentrated ammonium hydroxide solution drop by drop to a mixture with one vial of Fontana silver solution and 27 mL of distilled water (10% silver nitrate solution), accompanied by continuous stirring. Frozen tissue

slides were fixed in pre-cooled acetone (–20°C) for 10 minutes, then the fixative were poured off and acetone was allowed to evaporate from the tissue sections for more than 20 minutes at room temperature. The tissue sections were quickly rinsed with running tap water followed by another thorough rinse with distilled water. The slides were placed in ammoniacal silver solution and incubated in a 58°–60°C water bath for 35 minutes. After incubation, the slides were gently rinsed with distilled water and placed in 0.1% gold chloride for 1 minute. The tissue sections were then rinsed twice with distilled water and placed in 5% sodium thiosulfate for 1–2 minutes. After being gently rinsed twice in running tap water for 2–3 minutes, the slides were placed in Nuclear Fast Red stain (Sigma, St Louis, Mo) for 5 minutes, followed by gentle rinses with running tap water and dehydration with three changes of fresh absolute alcohol. The slides were cleaned by means of three changes of fresh xylene or xylene substitute and covered with a coverslip by using a permanent mounting media.

Immunohistochemical Staining

To identify the implanted pRPE cells, tissue sections adjacent to those for Fontana-Masson staining underwent immunohistochemistry staining by using RPE65 (Chemicon, Temecula, Calif), a mouse monoclonal antibody that specifically reacts to a 65-kD protein on RPE cell membranes. The staining was performed according to the manufacturer's recommendations. After a 10-minute fix in ice-cold acetone (–20°C) and a 20-minute air dry at room temperature, the sections parallel to Fontana-Masson staining were quenched in 3% hydrogen peroxide in PBS for 15 minutes, and they were subsequently treated with a blocking solution containing 0.5% Tween-20 (Sigma) and 10% normal donkey serum. Sections were incubated overnight in a humidified chamber at 4°C with anti-RPE65 monoclonal antibody (1:10) diluted in blocking solution. On the next day, sections were washed three times with PBS containing 0.05% Tween-20 for 3 minutes each and treated with a donkey anti-mouse secondary antibody (1:300; Jackson Immunoresearch, West Grove, Pa) in the presence of 2% goat serum for 1 hour at room temperature. The slides were washed three times with distilled water and covered with a coverslip by using a permanent mounting media. The slices (except for those for the autoradiography study) were scanned with a slide scanner (Histalim-Hamamatsu's Nanozoomer; Histalim, Montpellier, France).