Systemic and Intravitreal Delivery of Dendrimers to Activated Microglia/Macrophage in Ischemia/Reperfusion Mouse Retina

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PURPOSE. Microglial activation and associated neuroinflammation play a key role in the pathogenesis of many diseases of the retina, including viral infection, diabetes, and retinal degeneration. Strategies to target activated microglia and macrophages and attenuate inflammation may be valuable in treating these diseases. We seek to develop dendrimer-based formulations that target retinal microglia and macrophages in a pathology-dependent manner, and deliver drugs, either intravenously or intravitreally.

METHODS. Retinal uptake of cyanine dye (Cy5)-conjugated dendrimer (D-Cy5) was assessed in normal and ischemia/reperfusion (I/R) mouse eyes. Microglia/macrophage uptake of the dendrimer was assessed with immunofluorescence using rabbit Iba-1 antibody with Cy3-tagged secondary antibody (microglia/macrophage). Uptake in retina and other organs was quantified using fluorescence spectroscopy.

RESULTS. Clearance of D-Cy5 from normal eyes was almost complete by 72 hours after intravitreal injection and 24 hours after intravenous delivery. In eyes with activated microglia after I/R injury, D-Cy5 was retained by activated microglia/macrophage (Iba1⁺ cells) up to 21 days after intravitreal and intravenous administration. In I/R eyes, the relative retention of intravitreal and intravenous D-Cy5 was comparable, if a 30-fold higher intravenous dose was used.

CONCLUSIONS. Intravitreal and systemic dendrimers target activated microglia and show qualitatively similar retinal biodistribution when administered by either route. Results provide proof-of-concept insights for developing dendrimer drug formulations as treatment options for retinal diseases associated with microglia or macrophage activation such as age-related macular degeneration, diabetic retinopathy, and retinal degenerations.

Keywords: choroid, dendrimers, ischemia, microglia, retina

Dendrimers are tree-like, nanostructured polymers that are emerging as good candidates for drug and gene delivery vehicles for a wide range of applications. Dendrimers possess a high density of surface functional groups that can modulate the local environment to enable unique tissue interactions, which can be advantageous when developing novel therapies.¹ Generation-4 hydroxyl polyamidoamine (PAMAM-G4-OH) dendrimers have shown high levels of cellular uptake in previous studies² and are noncytotoxic and nonimmunogenic.³ Balakrishnan et al.⁴ and Kannan et al.⁵ have shown the therapeutic utility of dendrimer-based delivery systems in producing motor function improvements in a rabbit model of cerebral palsy. Targeting neuroinflammation, even in the postnatal period for a prenatal injury, was effective in improving motor function. Other related studies have identified activated microglia as one of the targets of the dendrimers in brain and demonstrated that dendrimers can be effective in treating neuroinflammation.⁶

Targeting activated microglia may have significant implications in retinal diseases. Microglia are the resident macrophages of the brain and retina, and they play an important role in immune defense against pathogens in the central nervous

system.⁷ They become activated in diseases such as diabetes and retinal degeneration, where cells die and microglia phagocytose cellular debris.^{8,9} Activation of retinal microglia occurs in a mouse model of ischemia/reperfusion injury (I/R), as well as in glaucoma,¹⁰ diabetic retinopathy,¹¹ and branch vein occlusion.¹² Retinal vascular occlusion, whether by high intraocular pressure in the I/R model or by thrombus in branch vein occlusion (BVO), causes a decrease in blood flow within the eye, resulting in retinal ischemia. This causes death of neurons, initiating further activation of microglia.⁹

This study explores the unique retinal biodistribution of systemically and intravitreally administered fluorescently labeled generation-4 hydroxyl polyamidoamine (D-Cy5) dendrimers. The ability of intravenously and intravitreally delivered dendrimers to target activated microglia in retina was investigated in normal and ischemic retinas. Activation of microglia was induced by I/R injury to retina, which damages neurons and blood vessels. Presently there are no systemic treatments available for ischemic retinopathies or age-related macular degeneration (AMD), and systemic delivery would have advantages beyond current treatments, including broader

adaptation, reduced cost, and better patient compliance. The standard of care for exudative AMD is intravitreal injections of antibody against VEGF, as frequently as monthly, so intravenous (IV) administration would avoid injections directly in the eye. Furthermore, this study demonstrates long-term retention of the dendrimer in activated microglia, which may reduce the frequency of injection. Using activated microglia as a target for the attenuation of inflammation may be an effective way to treat a number of diseases of the eye, including AMD, where inflammation plays a large part in the degeneration of photoreceptors, destroying the delicate environment of the retina and choroid.^{13–15}

MATERIALS AND METHODS

Synthesis and Characterization of Cy5-Labeled Dendrimers

The synthesis procedure for D-Cy5 and its physiochemical characterization is detailed in the Supplementary Material and Supplementary Figure S1.

Animals and I/R Injury

All procedures involving animals conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and Johns Hopkins University IACUC research guidelines. Male, 25-g, BALB/c albino mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for all experiments. For surgeries, mice were anesthetized with intraperitoneal ketamine (50 mg/kg; Bioniche Pharma, Lake Forest, IL, USA) and xylazine (10 mg/kg; Phoenix Pharmaceuticals, St. Joseph, MO, USA). Ischemia/reperfusion injury was performed in the left eye as previously described.^{16,17} Briefly, the anterior chamber was cannulated with a 30-gauge needle (BD PrecisionGlide, Franklin Lakes, NJ, USA) attached to a line from a saline reservoir at a height calibrated to yield 90 mm Hg. The IOP was elevated to 90 mm Hg for 90 minutes; I/R injury and choroidal nonperfusion was evident by blanching of the posterior segment via fundus examination. After ischemia, the needle was immediately withdrawn, allowing for rapid reperfusion; IOP was normalized, and reflow of the retinal vasculature was documented. The right eye had no I/R injury and served as a control eye. Six animals were used per group.

Dendrimer Injection and Animal Euthanasia

Six days post I/R injury, mice were injected with dendrimer, (Dendritech, Mainland, MI, USA) either intravitreally or intravenously. For intravitreal injections, 2 μ L containing 20 μ g D-Cy5 was injected into the vitreous chamber, using a glass needle aided with a compression injector (Harvard Apparatus, Holliston, MA, USA). For IV injections, 600 μ g D-Cy5 dissolved in 100 μ L sterile phosphate-buffered saline (PBS; Corning, Manassas, VA, USA) was injected via a 30-g needle into the femoral vein. Animals injected with free Cy5 or PBS served as positive or negative controls for this study. At appropriate time points (24 and 72 hours and 21 days) post dendrimer injections, 24 hours was used for dendrimer uptake and loading, 72 hours for evaluating retention and clearing, and 21 days to determine long-term retention. The retina repaired itself after 21 days in the I/R model, so it seemed an appropriate time to stop. At these time points, the animals were anesthetized using ketamine/xylazine and euthanized using a lethal dose of sodium pentobarbital (Lundbeck,

Deerfield, IL, USA). Eyes were immediately enucleated and processed for immunohistochemistry analysis.

Immunohistochemistry and Confocal Microscopy

Eyes for each condition (I/R and non-I/R; $n = 6$ for each group) were enucleated, fixed in 2% paraformaldehyde (EMS, Hatfield, PA, USA) in PBS for 1 hour at room temperature. The anterior chamber of the eye was removed, and posterior eye cup was cryopreserved with a sucrose gradient previously described.¹⁸ Eyes were frozen in a 20% sucrose/PBS and optimum cutting temperature compound (OCT) in a 1:2 ratio, respectively, using dry ice in isopentane. Cryoblocks were stored at -80°C until 8- μ m sections were cut using a cryostat (Microm, Walldorf, Germany). Four sections from each cryoblock were used for image analysis. Sections were incubated in rabbit anti-ionized calcium binding adapter 1 molecule (Iba-1; Wako Chemicals, Richmond, VA, USA), which is a microglia/macrophage cell marker, and then a goat anti-rabbit-Cy3 secondary antibody (Life Technologies, Grand Island, NY, USA) was applied. Sections were analyzed using a confocal microscope (model 510 unit; Carl Zeiss, Inc., Thornwood, NY, USA). Excitation and emission wavelengths and laser settings were identical for all tissues. Z-stacks of sections were taken and collapsed to give an image through the depth of the whole section. One representative image of all sections at each time point is provided in this report.

Counts of colocalization of dendrimers and retinal microglia/macrophages were accessed using surface function in IMARIS software (BITPLANE, Zurich, Switzerland). Briefly, Z-stack images of retinal sections (ora serrata to ora serrata) were converted to a three-dimensional (3D) model based on absolute intensity of Cy5 (dendrimers) and Cy3 (Iba-1⁺ cells). Two different surfaces were created for microglia and for D-Cy5, and counts of microglia with colocalization were done when both signals overlapped in the 3D model.

Biodistribution Analysis of D-Cy5

Each mouse was injected through the femoral vein with 600 μ g of D-Cy5 in 100 μ L sterile PBS or with 2 μ L (2 μ g D-Cy5) intravitreally at each time point. Mice were euthanized at 24 and 72 hours and at 21 days post injection ($n = 8$ /group), and vital organs (heart, lungs, spleen, kidney, liver, and eyes) were harvested and weighed. Organs were snap-frozen on dry ice and stored at -80°C . Upon analysis, tissues were thawed and samples of ~ 100 to 150 mg tissue were homogenized with 1 mL MeOH in low-DNA-binding Eppendorf tubes using a stainless steel bead and tissue homogenizer (Tissuelyzer LT; Qiagen, Hilden, Germany). The pulpy suspension was sonicated for 30 minutes, and appropriate volumes containing 100 mg tissue were diluted with methanol (Fisher, Pittsburgh, PA, USA) to 1 mL so that the tissue-to-volume ratio was the same for all samples. Samples were centrifuged at 10,000 rpm for 10 minutes at 4°C , and supernatants were analyzed using fluorescence spectroscopy (FLS). The amount of D-Cy5 in each organ was calculated from emission values (minus background values from organs injected with PBS), using calibration graphs, and then values were back-calculated to obtain the percentage of injected dose per organ, using whole-organ wet weight.

The anterior segments of enucleated eyes were removed by a 360° circumferential cut near the ora serrata. The posterior eye cups (retina plus choroid plus sclera) were snap-frozen in liquid nitrogen until homogenized using disposable polypropylene pestles (Bel-Art, Wayne, NJ, USA). Homogenates were dispersed in 100 μ L 0.05 M methanolic TCA solution and sonicated for 20 minutes. The solution was evaporated under vacuum and reconstituted with 100 μ L PBS, frozen, and then lyophilized to

FIGURE 1. Sections from control non-I/R eyes 24 hours after intravitreal injection. (A–D) Twenty-four hours after injection of D-Cy5 there was no dendrimer retained in normal retina. (E–H) Free Cy5 was present throughout the inner retina and in the inner plexiform layer (IPL), but none was associated with Iba1⁺ cells. Arrowheads indicate the internal limiting membrane (ILM). (I–L) Injection of PBS resulted in no fluorescence at the Cy5 wavelength. Blue = DAPI nuclear marker; red = D-Cy5 and Cy5; green = Iba-1; NFL, nerve fiber layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: 40 μ m.

obtain white powder. Powder was reconstituted with 100 μ L methanol, sonicated for 20 minutes, then centrifuged for 10 minutes at 15,000 rpm; and the supernatant was subjected to FLS measurements in low-volume cuvettes (StarnaCells, Atascadero, CA, USA). The amount of dendrimer retained in posterior eye cups was determined using a calibration graph of D-Cy5 ranging from 0.1 ng to 20 ng in methanol.

Statistical Analysis

Data were analyzed for significant differences using Student's *t*-test to determine the significance between two groups. A *P* value of ≤ 0.05 was considered significant.

RESULTS

Ischemia/Reperfusion: Differences in Microglia/Macrophage Population, Morphology, and Retinal Structure Changes

Because only Iba-1 was used as the marker and it labels all monocytic cells, it was not possible to identify positive cells as

microglia or macrophage, so we refer to these cells as Iba-1-positive (Iba-1⁺) microglia/macrophages. Iba-1⁺ cells in normal retina were reduced in number compared to that in I/R retina. Morphologically heterogeneous populations of microglia/macrophages were predominately found in choroid and retinal inner nuclear layer (INL), and very few of them were observed in the outer plexiform layer (OPL, between INL and outer nuclear layer) (Figs. 1A–D, 1I–L). Retinas had a normal lamination after intravitreal injection (Fig. 1). Ischemia/reperfusion injury led to crenulation (folding) of retina, and the retinas appeared to be thinner than normal retinas, suggesting neuronal and ganglion cell death (Fig. 2). At 24 hours post IR, retinal microglia/macrophages were activated and increased in number and were distributed in all retinal layers: inner plexiform layer (IPL), INL, outer nuclear layer (ONL), and the subretinal space (Figs. 2A–D).

Retinal Biodistribution of D-Cy5 After Intravitreal Administration

Intravitreal D-Cy5 showed differential biodistribution between normal and I/R retinas. In normal retinas, at 24 hours post

FIGURE 2. Sections from ischemia/reperfusion eyes 24 hours after intravitreal injection. (A–D) D-Cy5 (red) is present in Iba-1⁺ microglia/macrophages (arrows). Arrow with asterisk indicates retinal Iba-1⁺ cell with dendrimer shown in *inset*. (E–H) Higher magnification of D-Cy5 (red) in Iba-1⁺ microglia. Arrow with asterisk indicates subretinal Iba-1⁺ cell with dendrimer shown in *inset*. (I–L) Cy5 or free dye is seen throughout the inner retina and not associated with Iba-1⁺ microglia (arrowheads). Blue = DAPI; green = Iba-1; red = D-Cy5 and Cy5. Scale bars: 40 μ m (A–D, I–L); 20 μ m (E–H).

injection of D-Cy5, there was minimal fluorescence in retina and choroid (Figs. 1A–D), suggesting that dendrimers were quickly cleared. In contrast, free Cy5 remained in inner retina 24 hours after injection, but it was not colocalized with Iba-1 (Figs. 1E–H). In I/R-injured retinas at 24 hours, significant fluorescence from D-Cy5 was present (Figs. 2A–H). D-Cy5 colocalized with Iba-1⁺ microglia/macrophages in the subretinal space, the ONL and the INL and in the vicinity of the internal limiting membrane (ILM) of retina (Figs. 2A–H). At 72 hours post intravitreal injection, D-Cy5 was cleared from other cells and from vitreous in I/R eyes (Figs. 3A–H) and was found only within Iba-1⁺ cells near the ILM, in inner retina, and in subretinal space, as well as in retinal pigment epithelium (RPE) (Figs. 3A–H, arrows). However, in free-Cy5-injected animals, Cy5 was seen in inner retina and appeared to be concentrated in blood vessels near the ILM in both I/R and normal eyes (Figs. 2I–L, arrowheads) but was completely cleared by 72 hours (data not shown). Free Cy5 did not exhibit any cellular uptake at any time after this point. D-Cy5 was specifically retained in Iba-1⁺ cells in the photoreceptor layer and the IPL and near the ILM at 21 days post injection (Fig. 4).

Retinal Biodistribution of D-Cy5 After IV Administration

D-Cy5, free Cy5, or PBS was injected IV 6 days after I/R injury in one eye, and the fellow eye served as an uninjured control. In I/R eyes at 24 hours post IV, D-Cy5 had entered the retina from the circulation and was found within Iba-1⁺ cells throughout retina and in the subretinal space. However, in both the normal and the I/R eyes 24 hours post administration, free Cy5 appeared to be present predominantly in the choriocapillaris and choroid (Figs. 5E–H, arrowheads) but was cleared thereafter. However, D-Cy5 was present in choroidal macrophages in non-I/R eyes (Supplementary Fig. S2), suggesting that dendrimers had traversed the normal choriocapillaris fenestrations. Interestingly, we did not find any fluorescence signal from D-Cy5 in non-I/R retina, indicating that D-Cy5 was rapidly cleared, without uptake. Seventy-two hours post IV D-Cy5 injection, D-Cy5 was selectively localized and retained in microglia/macrophages in I/R retina as well as in the subretinal space (Figs. 6A–H, arrows). Even though Iba-1⁺ cells were in all

FIGURE 3. Seventy-two hours after intravitreal injection. (A–D) D-Cy5 is still present in microglia (arrows) and RPE cells (left, linear fluorescence) in I/R eyes. Arrow with asterisk indicates Iba1⁺ cell in inner retina with dendrimer shown in inset. (E–H) Higher magnification of D-Cy5 in microglia/macrophage (arrows). Arrow with asterisk indicates subretinal Iba1⁺ cell with dendrimer shown in insets. (I–L) D-Cy5 was not present in non-I/R control eyes. Blue = DAPI; green = Iba-1; red = D-Cy5 and Cy5. Scale bars: 40 μ m (A–D, I–L); 20 μ m (E–H).

retinal layers, dendrimers were retained mostly in microglia/macrophages in the subretinal space (Figs. 6E–H). At 21 days post injection, D-Cy5 was retained in a few scattered microglia/macrophages in retina and choroid. At 21 days, the Iba-1⁺ cells with D-Cy5 had a ramified morphology but retained D-Cy5 (Figs. 7E–H).

Quantification of Iba-1⁺ Cells and D-Cy5

IMARIS software was used to count Iba-1⁺ cells in 8- μ m cryosections from ora serrata to ora serrata. Four sections from each group were counted. There were significantly more Iba-1⁺ cells in I/R eyes than in non-I/R eyes (Fig. 8A). The software counts included not just a single label but cells with two labels colocalizing. Figure 8B demonstrates the cells selected by the software that had both labels (Fig. 8, white arrows) after setting parameters that only cell somas would be counted and not delicate processes. We determined that a significant number of Iba-1⁺ cells had D-Cy5 at all time points with both modes of D-Cy5 delivery (Figs. 8C, 8D), whereas no cells were double-labeled in non-I/R retinas.

Quantitative Whole-Body Biodistribution of D-Cy5 After IV Administration

Quantitative vital organ biodistribution (liver, kidney, spleen, heart, lungs, and serum) of IV D-Cy5 after I/R injury was determined using fluorescence quantification as previously published.¹⁹ D-Cy5 was stable in human plasma at 37°C in vivo and also following methanol extraction protocol, which yielded a D-Cy5 recovery of 96% from tissue.²⁰ Upon IV injection, considerable D-Cy5 was rapidly cleared from circulation via kidney. Twenty-four hours post IV, most D-Cy5 was cleared from plasma but was retained in some vital organs (Supplementary Fig. S3). At 24 hours, FLS analysis demonstrated that only $\sim 0.2\%$ of the injected dose was still in blood. The injected D-Cy5 conjugates were cleared from the major organs by 24 hours, but some accumulated in the kidneys ($5.5 \pm 1.5\%$ at 72 hours) (Supplementary Figs. S3, S4). This is in good agreement with the previous results based on fluorescence measurements¹⁹ and radiolabeling.³ D-Cy5 uptake and retention in kidney was $29.9 \pm 2.5\%$, $11.2 \pm 2.2\%$ in liver, and $3.3 \pm 1.3\%$ in spleen (Supplementary Fig. S3). Heart and lungs had minimal accumulations of D-

FIGURE 4. Twenty-one days after intravitreal injection. (A–D) D-Cy5 remains in Iba1⁺ cells in I/R eyes (arrows). Some appear to be subretinal macrophages (arrows). Arrow with asterisk indicates subretinal Iba1⁺ cell with dendrimer shown in inset. (E–H) Retina of D-Cy5-injected eyes after I/R at higher magnification. Arrow with asterisk indicates Iba1⁺ cell in inner retina with dendrimer shown in inset. (I–L) D-Cy5-wavelength fluorescence is not present in non-I/R eyes receiving PBS (fluorescence control). Blue = DAPI; red = D-Cy5 and Cy5; green = Iba-1. Scale bars: 40 μ m (A–D, I–L); 20 μ m (E–H).

Cy5 (0.0049% and 0.01%, respectively) at 24 hours. Free Cy5 was rapidly cleared from plasma and had significantly lower accumulation ($0.8 \pm 2.9\%$) in kidneys at 24 hours and could not be detected in other organs. Free Cy5 was not detectable in any of the organs, indicating that either it was either cleared from the body or the amount was below detection.

Confocal microscopy analysis of the kidney sections (Supplementary Fig. S4) revealed high D-Cy5 signal in the proximal tubules of the kidney cortex at 24 hours (Supplementary Fig. S4A), with this signal decreasing by 72 hours (Supplementary Fig. S4B), which is in good agreement with biodistribution data. High performance liquid chromatography (HPLC) of kidney extracts at 24 hours showed a small peak of free Cy5, but the major fraction of the peak was D-Cy5 (Supplementary Fig. S4D). Based on HPLC calibration, we estimate that 12% of the conjugated Cy5 was released by this time, suggesting that the conjugates are mostly intact in vivo. Hematoxylin and eosin staining of kidney sections from animals injected with D-Cy5 showed no neutrophil or monocyte infiltration,²¹ no

structural damage, or any signs of toxicity (Supplementary Figs. S4G–I).

Ocular Biodistribution of D-Cy5: Intravitreal Versus IV Administration

The IV dose of D-Cy5 was 30-fold higher than that of the intravitreal dose. Interestingly, the qualitative uptake and retention pattern in retina were similar after both modes of administration (Fig. 9). This demonstrates a relatively low uptake in the healthy control eye, followed by rapid clearance, and a much higher uptake in the fellow I/R eye and then sustained retention in the I/R eye. In fact, there were no significant difference in quantitative uptake/retention patterns between the two administration modes. Even though there was some choroidal presence after IV D-Cy5 in normal eyes, it appeared to be mostly cleared within 72 hours (Figs. 6I–L). In the I/R eye, after IV administration, ~40% of the D-Cy5 uptake observed after 24 hours was retained for up to 21 days. For intravitreal administration, ~16% of the D-Cy5 level from 24 hours was retained for up to 21 days.

FIGURE 5. Sections of retinas from I/R eyes 24 hours after IV injection. (A–H) D-Cy5 colocalizes with Iba-1 in labeled cells (green) in retina and also appears to be in RPE cells (bottom left). Arrow with asterisk indicates subretinal Iba1⁺ cell with dendrimer shown in inset. (E–H) Free Cy5 dye is still present in choroid after 24 hours. (I–L) There is no fluorescence in the Cy5 wavelength in eyes receiving PBS intravenously. Blue = DAPI; red = D-Cy5 and Cy5; green = Iba-1. Scale bars: 40 μ m (A–D, I–L); 20 μ m (E–H).

DISCUSSION

Ischemia/reperfusion injury has been used to model certain aspects of chronic glaucoma, diabetic retinopathy, and BVO. Ischemia/reperfusion injury causes occlusion of both retinal and choroidal blood vessels, resulting in reduced blood flow and tissue hypoxia.²² The above-mentioned conditions were reported to cause disruption of blood-retina barriers,^{9,16,22} activation of resident microglia/macrophages, infiltration of macrophages from blood, elevated production of cytokines (e.g., TNF- α , IFN- α , TGF- β , IL-1 β , and IL-6),²³ and death of retinal ganglion cells.²⁴ Activated microglia/macrophages have been associated with retinal diseases such as macular degeneration, diabetic retinopathy, glaucoma, and retinopathy of prematurity.^{9,24–26} The present study demonstrates the ability of PAMAM dendrimers to target the activated microglia/macrophages, a key cell type involved in retinal neuroinflammation after I/R injury.^{17,27} Retention by presumably activated microglia/macrophages was observed whether the dendrimer was delivered IV or intravitreally. Furthermore, Iba-1⁺ cells after I/R retained dendrimers, whereas other cell types did not take up the dendrimer. Dendrimers remained in Iba-1⁺ cells for 21 days, the longest time point evaluated in this study.

The D-Cy5 used in this study was well characterized in our previous study, allows for a high detection limit (0.1 ng), and is highly stable in vivo.^{19,28,29} ¹H-labeled proton nuclear magnetic resonance confirmed that one molecule of Cy5 was successfully conjugated to each dendrimer.^{28,29} High performance liquid chromatography analysis demonstrated the high purity of D-Cy5 conjugates and successful synthesis.^{28,29} The present study used near-infrared Cy5 to avoid tissue autofluorescence from retinal cells, especially activated microglia.

Hydroxyl PAMAM dendrimers, delivered either intravitreally or by IV injection, showed histopathology-dependent retinal distribution. Upon intravitreal injection, dendrimers were completely cleared from normal retina within 72 hours, whereas in I/R retinas, they specifically localized in activated microglia/macrophages (round or fusiform Iba-1⁺ cells) in INL and ONL and in the near vicinity of ILM. This can be attributed to the increased phagocytic activity of activated microglia cells and macrophages and the ability of the dendrimer to readily partition to the retina from the vitreous chamber. In normal retina, microglia are restricted to inner retinal layers, but under pathological conditions such as I/R injury, they exhibited dynamic behavior including migration to the injury site and engulfment of dead cells. In I/R injury, there are reports of a

FIGURE 6. Retina at 72 hours post IV D-Cy5 administration. (A–D) There are many Iba-1⁺ cells in I/R retinas, and a few in this field have D-Cy5 colocalized. (E–H) Colocalization (yellow) is shown at higher magnification. (I–L) No cells have D-Cy5 in non-I/R control eyes. Blue = DAPI; red = D-Cy5 and Cy5; green = Iba-1. Scale bars: 40 μ m (A–D, I–L); 20 μ m (E–H).

ganglionic and photoreceptor cell death, RPE atrophy, and accumulation of microglia in INL, ONL, and subretinal space.^{16,22,30} At 21 days post intravitreal injection in I/R eyes, dendrimers were still retained in microglia/macrophages, but the amount of D-Cy5 had decreased compared to that in 24 and 72 hours. This corresponded to a decline in number of round microglia/macrophages and a return to their quiescent, ramified morphology. Such changes in microglia morphology are in agreement with previous studies.^{17,27}

After IV administration, D-Cy5 was not found in normal retina, due either to the intact blood–retina barrier or to a lack of uptake by healthy retinal cells, but was found in normal choroid where it traversed the fenestrations in choriocapillaris (CC). Interestingly, most of the D-Cy5, whether injected intravitreally or IV, was cleared quickly from the major organs, yet I/R microglia/macrophages retained it for up to 21 days. All organs showed complete clearance of D-Cy5 by 72 hours, except for the kidney, which showed a small amount of retention after 72 hours. Furthermore, there was no indication of toxicity from the D-Cy5 at doses used in this study. Once taken up, the microglia/macrophages appear to retain the dendrimers over 21 days, even after they return to a ramified shape. The effectiveness of the IV D-Cy5 to target inflammation and the rapid clearance from major off-target organs make

dendrimer-based systemic delivery of therapeutics promising for retinal degeneration. Future dendrimer-therapeutic studies will focus on functional consequences of this therapy in this I/R model electroretinogram (ERG), vascular permeability, and quiescence of activated microglia.

Intravenous administration is safer than intravitreal administration, especially given the rapid clearance of dendrimers from the off target organs, but intravitreal administration is currently the standard of care for anti-VEGF therapies used in treating exudative age-related (wet) AMD and diabetic macular edema. D-Cy5 retention in microglia/macrophages at 21 days post femoral injection is also significant as this would decrease treatment frequency. The current anti-VEGF therapies require intravitreal injection monthly but have a half-life in the eye of only 4.9 days.³¹ Both the intravitreal and the IV D-Cy5 non-I/R controls at 24 hours post injection showed no fluorescence in retina, indicating that the dendrimer was already cleared from the retinas; these healthy controls had no activated microglia/macrophages. In I/R injury and in our cerebral palsy model, the blood–brain barrier is compromised as part of the pathology, but Cy5 alone administered intravenously or intravitreally was not localized to microglia. This difference in distribution between free and dendrimer-conjugated Cy5 suggests that the dendrimer retains its cargo and has a favorable distribution in

FIGURE 7. Retina and choroid 21 days after IV D-Cy5 injection. (A–D) Ischemia/reperfusion retina still has Cy5 colocalized with an Iba-1⁺ cell (arrow). (E–H) D-Cy5 colocalization with Iba-1 shown at higher magnification in a ramified Iba-1⁺ cell. (I–L) D-Cy5 administration to non-I/R control eyes. (A–H) Arrows mark colocalization of D-Cy5 (red) and Iba-1-labeled cells (green). Blue = DAPI; red = D-Cy5 and Cy5. Scale bars: 40 μ m (A–D, I–L); 20 μ m (E–H).

diseased and healthy retina (retention and clearance, respectively). Furthermore, free Cy5 was not present in microglia, indicating that it is the dendrimer and not the dye that was targeted to activated microglia.

The I/R model used herein represents some aspects of BVO. It also represents a retinal injury in which there is hypoxia, then tissue injury and death, followed by activation of microglia and inflammation. This model was used in a study by Zheng et al.¹⁷ to create a short-term model of some events in diabetic retinopathy, namely, creation of acellular capillaries, death of neurons, increased vascular permeability, and significant activation of microglia.

In chronic diseases like diabetic retinopathy, microglia are activated,⁹ and undoubtedly, they contribute to the neuroinflammation and death that occurs in diabetic retina. Repeated intravitreal injections to treat a chronic disease like diabetic retinopathy can further damage the vision. Marano et al.^{32,33} limited the number of intraocular injections by using an anti-VEGF oligonucleotide conjugated to a dendrimer, which permitted expression for 6 months. With IV injections of dendrimer, repeated injections would be possible without concern for secondary damage to the eye. In addition, retinal blood vessel leakage in diabetic retinopathy would allow

dendrimer entry and retention in the retina. Therefore, drugs bound to dendrimers could be released slowly over a prolonged period of time, decreasing the frequency of injections. For these reasons, Kannan et al.³⁴ suggested the use of dendrimers for delivery of therapies in many ocular diseases including AMD, where the outer retinal barrier is lost and activated microglia/macrophages are thought to contribute to the pathology. The therapeutic efficacy of systemically administered dendrimer-drug conjugates to activated microglia/macrophages in an animal model of cerebral palsy has been previously demonstrated.³⁵

In conclusion, the current study documents the retinal biodistribution of dendrimers upon intravitreal and systemic administration in mice, where one eye was healthy and the other eye had I/R injury. The qualitative biodistribution was similar after intravitreal and systemic administration of the dendrimer after I/R injury. There was a differential retinal biodistribution of dendrimers between the healthy eye and the injured fellow eye, with D-Cy5 localizing selectively in activated Iba-1⁺ microglia/macrophages in the injured eye only. Once localized in Iba-1⁺ cells, the dendrimers appeared to be retained for an appreciable time, up to 21 days. Twenty-one days was well beyond (3 $\times$) the half-life of popular injected

FIGURE 8. Quantification of Iba-1+ cells in retina. IMARIS software was trained to count Iba-1+ cells in sections of retina from ora serrata to ora serrata. (A) There was significant increase in the number of Iba-1+ cells in I/R retinas ($P < 0.01$). (B) Software was trained to select only soma of cells, not processes, in both the Iba-1-labeled cells (yellow arrows) and the Iba-1 and D-Cy5 cells in this 3D surface volume. The total number of microglia/macrophages (green) and those with D-Cy5 are shown at all three time points after intravitreal (C) and IV (D) administration to I/R eyes. These values were significantly greater than those in non-I/R retinas, where no cells in retina had D-Cy5 labeling.

FIGURE 9. Quantification of levels of D-Cy5 in posterior eye cups by fluorescence spectroscopy, after extraction of D-Cy5 from tissue. (A) Levels of dendrimers in non-I/R eyes show significant differences from those in I/R eyes after a single intravitreal injection of 20 µg D-Cy5. (B) D-Cy5 levels after a single IV injection of 600 µg; (C) comparison of dendrimer levels in I/R eyes in both the intravitreal and IV routes (at 30× higher dose) are comparable ($n = 8$; Student's t -test). For quantification, posterior eye cups were homogenized and lyophilized, and dendrimers were extracted into a small volume of methanol. Fluorescence was measured using previously established protocols, with appropriate D-Cy5 calibration and controls. D-Cy5 was near detection limit (NDL) in healthy eyes (3 and 21 days). * $P < 0.01$, I/R compared to non-I/R.

intraocular drugs like bevacizumab (Avastin; Genentech, San Francisco, CA, USA). The ability of the dendrimer to selectively target activated microglia/macrophages, even after systemic administration, may offer significant opportunities for targeted, sustained, systemic therapies for retinal degeneration like AMD and diabetic retinopathy, where the only therapies currently available require monthly intravitreal injections. Attenuation of the microglia and macrophage activation in AMD and diabetes would decrease the degeneration of photoreceptors and pathologic events.^{26,36,37} The relatively rapid clearance of the dendrimers from the healthy eye and off-target organs suggests that dendrimers may reduce side effects and short bioavailability associated with current ocular drugs.

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