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Blockade of Sphingosine-1-phosphate Reduces Macrophage Influx and Retinal and Choroidal Neovascularization

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Abstract

Sphingosine-1-phosphate (S1P) is a bioactive lipid molecule that stimulates endothelial cell migration, proliferation, and survival *in vitro*, and tumor angiogenesis *in vivo*. In this study, we used a humanized monoclonal antibody (sonopizumab) that selectively binds S1P to investigate its role in retinal and choroidal neovascularization (NV). Intraocular injection of sonopizumab significantly reduced macrophage influx into ischemic retina and strongly suppressed retinal NV in mice with oxygen-induced ischemic retinopathy. In mice with laser-induced rupture sites in Bruch's membrane, intraocular injection of sonopizumab significantly reduced the area of choroidal NV and concomitantly reduced fluorescein leakage from the remaining choroidal NV. Four weeks after intraocular injection of up to 1.8 mg of the sonopizumab in nonhuman primates, electroretinograms and fluorescein angiograms were normal, and light microscopy of ocular sections showed no evidence of structural damage. These data show for the first time that S1P stimulates both choroidal and retinal NV and suggest that sonopizumab could be considered for evaluation in patients with choroidal or retinal NV.

Introduction

Several proteins have been demonstrated to promote angiogenesis in various vascular beds, but lipid molecules such as sphingosine-1-phosphate (S1P) may also contribute. S1P acts through a family of five G protein-coupled receptors, S1P receptors 1–5 (Lee et al., 1998), first identified on vascular endothelial cells as the products of genes upregulated during differentiation of endothelial cells (endothelial differentiation genes, EDG) (Hla and Maciag, 1990). Acting through S1P receptors, S1P stimulates migration and survival of cultured vascular endothelial cells, and formation of cell-cell adherence junctions (Lee et al., 1998; Paik et al., 2001). Mice deficient in S1P₁ receptor die between embryonic day 13.5 and 14.5 due to lack of pericytes recruitment around developing vessels resulting in lethal hemorrhages (Liu et al., 2000). In adult mice, injection of S1P₁ receptor multiplex siRNAs into tumor xenografts or injection of a monoclonal antibody directed against S1P suppressed angiogenesis and tumor growth (Chae et al., 2004; Visentin et al., 2006) suggesting a role for S1P in tumor

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angiogenesis. However, bioactive lipids such as S1P have not previously been demonstrated to directly promote ocular angiogenesis.

In ischemic retina, S1P₂ receptor, but not S1P₁ or S1P₃ receptors, is strongly upregulated and compared to wild type controls, mice deficient in S1P₂ receptor develop significantly less ischemia-induced retinal neovascularization (NV) (Skoura et al., 2007). This suggests the hypothesis that an antagonist of S1P would inhibit ischemia-induced retinal NV and possibly other types of ocular NV. In this study, we used a humanized and optimized monoclonal antibody that binds S1P (sonopizumab) to test that hypothesis.

Materials and Methods

Mice

Pathogen-free C57BL/6 mice (Charles River, Wilmington, MA) were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Care and Use Committee at the Johns Hopkins University Medical School.

A humanized monoclonal antibody that specifically binds SP1

The generation and characterization of a monoclonal antibody directed against SP1 has been previously described (Visentin et al., 2006). A humanized version of the antibody (LT1009, sonopizumab, Lpath Therapeutics, Inc., San Diego, CA) was utilized in this study.

Intravitreal injections

Intravitreal injections were done under a dissecting microscope with a Harvard Pump Microinjection System and pulled glass micropipettes as previously described (Mori et al., 2001).

Tracer studies with radiolabeled sonopizumab

[H³]-labeled sonopizumab was produced at LPath Therapeutics, Inc by radiolabeling with tritium [propionyl-³H] with specific activity of 2.0 mCi/mg[H³] by Vitrox. Three µg of a 1:4 mixture of labeled to unlabeled sonopizumab was injected into the vitreal cavity of each eye of 8–10 week old female C57BL/6 mice. At 1, 7, and 14 days after injection, mice were euthanized and eyes were removed. Anterior segments were removed and lenses, retinas, and eyecups (retinal pigmented epithelium, choroid, and sclera) were weighed and briefly sonicated in lysis buffer (phosphate buffered saline (PBS) containing 20 mM EDTA and 1% Triton X-100) and briefly solubilized in NaOH. Samples were added to vials containing scintillation fluid and radioactivity was counted.

Dosage amount and frequency of sonopizumab administration in mice

Five to 6-week-old female C57BL/6 mice were randomized and treated in masked fashion. One group of mice received an intraocular injection of 1 µl of PBS or PBS containing 0.05, 0.5, 1.0 or 3.0 µg of sonopizumab and the following day Bruch's membrane was ruptured at 3 locations in each eye. A second group of mice received an intraocular injection of PBS or 0.5 µg of sonopizumab one day prior to and 6 days after laser-induced rupture of Bruch's membrane.

Mouse model of choroidal NV

Choroidal NV was induced by laser photocoagulation-induced rupture of Bruch's membrane as previously described (Tobe et al., 1998). Briefly, 5 to 6-week-old female C57BL/6 mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and pupils were

dilated. Laser photocoagulation (75µm spot size, 0.1 second duration, 120 mW) was performed in the 9, 12, and 3 o'clock positions of the posterior pole of each eye with the slit lamp delivery system of an OcuLight GL diode laser (Iridex, Mountain View, CA) and a handheld cover slip as a contact lens to view the retina. Production of a bubble at the time of laser, which indicates rupture of Bruch's membrane, is an important factor in obtaining choroidal NV; therefore, only burns in which a bubble was produced were included in the study. After 14 days, the mice were perfused with 1 ml of PBS containing 50 mg/ml of fluorescein-labeled dextran (2×10^6 average molecular weight; Sigma-Aldrich, St. Louis, MO) and choroidal flat mounts were examined by fluorescence microscopy. Images were captured with a Nikon Digital Still Camera DXM1200 (Nikon Instruments Inc, NY). Image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to measure the total area of choroidal NV at each rupture site with the investigator masked with respect to treatment group.

Sonepcizumab administration in mice with ischemic retinopathy

C57BL/6 mice were placed in 75% oxygen at P7 and at P12 were returned to room air and given an intraocular injection of 1 (1 of a 3 (g/l solution of sonepcizumab in one eye and PBS in the fellow eye. The quantification of retinal NV was performed as previously described (Shen et al., 2007). Briefly, P17 mice were given an intraocular injection of 1 (1 of rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1) antibody (Pharmingen, San Jose, CA) under a dissecting microscope with a Harvard Pump Microinjection System and pulled glass micropipettes. Mice were euthanized 12 hours after injection and eyes were fixed in PBS-buffered formalin for 5 hours at room temperature. Retinas were dissected, washed, and incubated with goat-anti rat polyclonal antibody conjugated with Alexa 488 1:500 (Invitrogen, Carlsbad, CA) at room temperature for 45 minutes and then flat mounted. An observer masked with respect to treatment group measured the area of neovascularization per retina by image analysis.

Effect of sonepcizumab on macrophage recruitment in mice with ischemic retinopathy

C57BL/6 mice were placed in 75% oxygen at P7 and at P12 they were returned to room air and received a 1 µl intraocular injection of 3 µg of sonepcizumab in one eye and PBS in the other eye. At P17, the mice given an intraocular injection of 1 µl of F4/80 labeled with phycoerythrin (EBioscience, San Diego, CA) and after 8 hours, mice were euthanized and eyes were removed and fixed in phosphate-buffered formalin at room temperature for 5 hours. Retinas were dissected and washed with PBS containing 0.25% Triton X-100 and whole mounted. Slides were viewed with a Nikon Fluorescence Microscope and the number of macrophages per retina was counted by image analysis.

Effect of intraocular sonepcizumab on leakage from choroidal NV

C57BL/6 mice (n = 10) had rupture of Bruch's membrane by laser photocoagulation in 3 locations in each eye and then received an intraocular injection of 1 µl containing 3 µg of sonepcizumab in one eye and 1 µl of PBS in the fellow eye. Seven days after laser, the mice were given an intraperitoneal injection of 12 µl/g body weight of 1% fluorescein sodium (Alcon, Fort Worth, Texas) and after 5 minutes they were euthanized. Eyes were removed and fixed in phosphate-buffered formalin at room temperature for 5 hours. Retinas were dissected, washed, and incubated with rat anti-PECAM-1 antibody (1:100, BD Biosciences, San Jose, CA) for 2 hours at room temperature. After washing, retinas were stained with a donkey anti-rat polyclonal antibody conjugated with Cy-3 (Jackson ImmunoResearch Lab, West Grove, PA). Retinas were washed, flat mounted and examined by fluorescence microscopy. Using the red channel, the area of PECAM-1-stained choroidal NV was measured by image analysis and using the green channel the area of fluorescein was measured. The area of fluorescein leakage was calculated by subtracting the area of choroidal NV from the area of fluorescein.

Safety studies in primates

Sixteen male and 16 female Cynomolgus monkeys (*Macaca fascicularis*), age 2–7 years, were randomized to receive an intraocular injection of 50 μ l of vehicle (n=10) or vehicle containing 0.25 (n=6), 0.7 (n=6), or 1.8 mg (n=10) of sonepcizumab per eye on days 1, 15, and 29. The end of the dosing phase was 7 days after the last injection (day 36). Ocular exams, fluorescein angiograms, and electroretinograms (ERGs) were done prior to dosing, near the middle and at the end of the dosing phase, and during a 4 week recovery phase.

For fluorescein angiograms, animals were anesthetized with ketamine, intubated, and maintained on sevoflurane. Pupils were dilated and after an intravenous injection of fluorescein, fundus photographs were taken every few seconds for about a minute. Stereo photographs of the posterior pole were taken at approximately 1 to 2, and 5 minutes. Between approximately 2 and 5 minutes, nonstereoscopic photographs will be taken of two mid-peripheral fields (temporal and nasal) of each eye. ERGs were performed on each eye. Animals were dark adapted for two hours, anesthetized with ketamine and xylazine, and eyes were dilated. After scotopic ERGs, monkeys were light adapted for 10 minutes and then photopic ERGs were done followed by visual evoked potentials

Some monkeys were euthanized for necropsy at the end of the dosing phase and the remainder at the end of the recovery phase. Eyes were enucleated with a long segment of optic nerve and embedded in paraffin. Ocular and optic nerve sections were stained with hematoxylin and eosin and examined microscopically. .

Results

Entry of sonepcizumab into retina, retinal pigmented epithelium (RPE), and choroid after intravitreal injection

In order to assess entry and exit of sonepcizumab into the retina and choroid after intravitreal injection, radiolabeled sonepcizumab was injected into the vitreous cavity and after 1, 7, or 14 days, mice were euthanized and the lens, retina, and eyecup (RPE and choroid) were dissected and homogenized. The protein concentration of homogenates was determined and radioactivity was counted. The mean radioactivity per mg protein was significantly greater at 7 days after injection compared 1 or 14 days after injection (Figure 1), indicating that most of the sonepcizumab entered the tissues from the vitreous between 1 and 7 days, and most of the sonepcizumab exited the tissues between 7 and 14 days. The levels were similar in retina and RPE/choroid and somewhat less in lens.

Intravitreal injection of sonepcizumab suppresses growth of and leakage from choroidal NV

Mice were given an intraocular injection of PBS or 0.05, 0.5, 1, or 3 μ g of sonepcizumab and the following day Bruch's membrane was ruptured in 3 locations by laser photocoagulation. After two weeks, compared to eyes injected with PBS, the mean area of choroidal NV at Bruch's membrane rupture sites was significantly less in eyes injected with 0.5, 1 or 3 μ g of sonepcizumab, but not those injected with 0.05 μ g of sonepcizumab (Figure 2).

In another experiment, mice had rupture of Bruch's membrane at 3 locations in each eye and then 3 μ g of sonepcizumab was injected in one eye and vehicle was injected in the fellow eye. Seven days later the mice were euthanized 5 minutes after an intraperitoneal injection of 12 (l/g body weight of 1 % fluorescein sodium and eyes were rapidly removed and fixed. Choroidal flat mounts were stained for PECAM-1 allowing image analysis measurement by a masked investigator of the area of PECAM-1-stained choroidal NV as well as the area of staining from albumin-bound fluorescein that had leaked out of the choroidal neovascularization. The area

of fluorescein leakage was calculated by subtracting the area of PECAM-1 staining from the area of fluorescein staining. Sonepcizumab-injected eyes appeared to have smaller areas of fluorescein staining (Figure 3A) compared to PBS-injected eyes (Figure 3B) and smaller areas of PECAM-1-labeled choroidal NV (Figure 3C versus 3D). Merged images (Figures 3E and F) showed double-labeled CNV surrounded by fluorescein that had leaked into surrounding tissue. Image analysis confirmed that compared to PBS-injected eyes, those injected with sonepcizumab had significantly smaller areas of PECAM-1-stained CNV (Figure 3G) and significantly less fluorescein leakage from the choroidal NV (Figure 3H).

Intravitreal injection of sonepcizumab suppresses ischemia-induced retinal NV and reduces influx of macrophages

After 5 days in 75% oxygen, P12 mice were returned to room air and given an intravitreal injection of 3 μ g sonepcizumab in one eye and PBS in the fellow eye. At P17, the mean area of retinal NV on the surface of the retina was significantly less in eyes injected with sonepcizumab compared to PBS-injected fellow eyes (Figure 4). The mean number of macrophages per retina was also significantly less in eyes injected with 3 μ g sonepcizumab compared to fellow eyes injected with PBS (Figure 5).

Intraocular injection of sonepcizumab does not cause identifiable retinal toxicity in primates

Monkeys received an intraocular injection of 50 μ l of vehicle (n=10) or vehicle containing 0.25 (n=6), 0.7 (n=6), or 1.8 mg (n=10) of sonepcizumab per eye on days 1, 15, and 29. Slit lamp exams showed no intraocular inflammation at any time after injections and fundoscopic examinations showed no identifiable retinal abnormalities. Fluorescein angiograms 4 weeks after initiations of injections showed no leakage or window defects in any of the monkeys (not shown). Compared to pre-injection baseline tests, there was no statistically significant difference in scotopic ERG a- or b-waves, photopic b-waves, or visual evoked potentials. Light microscopy of ocular sections showed normal-appearing retinas (not shown).

Discussion

Retinal NV occurs in diseases such as diabetic retinopathy in which there is ischemia from closure of retinal capillaries. Diabetic retinopathy is the most common cause of severe vision loss in working age individuals in developed countries (Klein and Klein, 1995). Ischemia results in elevated levels of several proteins that contribute to retinal NV including vascular endothelial growth factor (VEGF), platelet-derived growth factor-B (PDGF-B), and stromal-derived factor-1 (SDF-1) (Aiello et al., 1995; Kelly et al., 2003; Lima e Silva et al., 2007; Ozaki et al., 2000). Another protein that is increased in ischemic retina is one of the receptors for the lipid signaling molecule S1P, S1P₂ receptor, and mice that are deficient in S1P₂ receptor develop less ischemia-induced retinal NV than wild type controls (Skoura et al., 2007). In this study, we have confirmed that S1P contributes to ischemia-induced retinal NV, because it was suppressed by intraocular injection of a monoclonal antibody that neutralizes S1P. Intraocular injection of the S1P neutralizing antibody also significantly reduced macrophage influx into ischemic retina and since macrophages play an important stimulatory role in ischemia-induced retinal NV (Shen et al., 2007) this is one mechanism of action of the antibody.

Choroidal NV occurs in diseases in which there are abnormalities in Bruch's membrane and/or the retinal pigmented epithelium, the most common of which is age-related macular degeneration (AMD) (Klein et al., 1992). Choroidal NV due to AMD is the most common cause of severe loss of vision in patients over the age of 60 in developed countries. Hypoxia or ischemia have not been demonstrated in diseases in which choroidal NV occurs, but macrophages have been shown to contribute (Killingsworth et al., 1990; Tsutsumi et al., 2003). In this study, we found that intraocular injection the S1P neutralizing antibody

suppresses growth of choroidal NV. It also suppressed leakage of fluid from the choroidal NV which results in collection of fluid in the macula, the major cause of acute loss of vision in patients with choroidal NV.

Blockade of S1P is likely to suppress retinal and choroidal NV by multiple mechanisms, because in addition to its macrophage recruiting effects, S1P has direct stimulatory effects on endothelial cells. S1P promotes survival, migration, and tube formation of cultured vascular endothelial cells (Kwon et al., 2001; Lee et al., 1998; Lee et al., 1999). There are also interactions between S1P signaling and that mediated by peptide growth factors, including VEGF. Transactivation of VEGF receptors mediates some of the angiogenic activity of S1P (Endo et al., 2002; Tanimoto et al., 2002). Conversely, VEGF sensitizes endothelium to the effects of S1P by upregulating S1P₁ receptors (Igarashi et al., 2003) and causes translocation and activation of sphingosine kinase resulting in elevation of S1P levels (Pitson et al., 2005). Thus, there may be advantages to combined blockade of VEGF and S1P.

Intraocular injections of ranibizumab, an Fab that binds all isoforms of VEGF-A, causes substantial visual improvement in 34–40% of patients with choroidal neovascularization due to AMD (Brown et al., 2006; Rosenfeld et al., 2006). The percentage of patients who experience improvement may be increased by simultaneously targeting VEGF and other molecules that contribute to choroidal NV. Since S1P may have both independent and overlapping effects with VEGF, it is a particularly appealing target. Intraocular injection of sonepcizumab shows a good safety profile in nonhuman primates and thus it is reasonable to investigate the effect of S1P blockade in patients with diseases characterized by ischemia mediated retinal NV such as diabetes and in patients with neovascular AMD.

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Figure 1. Measurement of radioactivity in ocular tissues 1, 7 and 14 days after Intravitreal injection of [^3H]sonopizumab

Four week old C57BL/6 mice were given an intravitreal injection of [^3H] sonopizumab in each eye and euthanized 1, 7, or 14 days after the injection. Eyes were removed and lens, retina, and eyecup consisting of the retinal pigmented epithelium, choroid, and sclera were dissected and homogenized. The protein concentration was measured and radioactivity was counted in a scintillation counter. Each bar represents the mean ($\pm$ SEM) calculated from 20 eyes and values are represented as [^3H] CPM/mg tissue. Lens, retinas and eyecups dissected from the mice exhibited the highest level of total radioactivity per milligram of tissue at day 7 after intravitreal dosing of [^3H]sonopizumab. The level of radioactivity was significantly higher at day 7 than at day 1 and day 14. Levels in retina and eyecup were comparable.

Statistical comparisons were made by ANOVA with Dunnett's correction for multiple comparisons, ($P < 0.05$).

Figure 2. Intravitreal sonepcizumab suppresses choroidal neovascularization (NV)

Five - six week old C57BL/6 mice were given an intravitreal injection in each eye consisting of 1 µl containing 0.05, 0.5, 1.0, or 3.0 µg of sonepcizumab or PBS 1 day before rupture of Bruch's membrane at 3 locations with laser photocoagulation. After two weeks, the mice were perfused with fluorescein-labeled dextran and choroidal flat mounts were examined by fluorescence microscopy. Compared to eyes injected with PBS (A), those injected with 0.05 µg of LT1009 (B) showed similar appearing choroidal NV at Bruch's membrane rupture sites, but eyes injected with 0.5 (C), 1.0 (D), or 3.0 (g) (E) seemed to show smaller areas of choroidal NV. The area of choroidal NV at each rupture site was measured by image analysis and the mean area of choroidal NV per eye was calculated to give a single experimental value. In (F), the bars show the mean ($\pm$ SD) for each group calculated from 20 experimental values and confirm that compared to eyes injected with PBS, the area of choroidal NV was significantly less in eyes injected with 0.5, 1.0, or 3.0 (µg of LT10029 (which were not significantly different from each other), but not those injected with 0.05 (µg of LT10029.

* $p = 0.01$ for difference from PBS by ANOVA with Dunnett's correction for multiple comparisons.

Figure 3. LT1009 reduces leakage from choroidal neovascularization (NV) at Bruch's membrane rupture sites

C57BL/6 mice (n = 10) had rupture of Bruch's membrane in 3 locations in each eye were given an intraocular injection of 3 μ g of sonepcizumab in one eye and PBS in the fellow eye. After one week, the mice were given an intraperitoneal injection of 12 μ l/g body weight of 1% fluorescein sodium and 5 minutes later, retinas were dissected, stained with anti-PECAM-1, and retinal flat mounts were examined by fluorescence microscopy. The area of fluorescein and PECAM-1 staining was measured by image analysis by a masked investigator and the area of leakage was calculated by subtracting the latter from the former. Eyes injected with sonepcizumab showed smaller areas of fluorescein staining (A) compared to eyes injected with

PBS (B). They also showed smaller areas of PECAM-1 staining (C versus D). Merged images from sonepcizumab-injected (E) and PBS-injected (F) eyes illustrate double labeling (yellow) in areas of choroidal NV and fluorescein that has leaked into surrounding tissue (F). Compared to eyes injected with PBS, those injected with sonepcizumab showed significantly smaller areas of PECAM-1 staining at Bruch's membrane rupture sites (G) and significantly less leakage (H). Statistical comparisons were made by unpaired t-test..

Figure 4. Intravitreal sonepcizumab suppresses ischemia-induced retinal neovascularization (NV)

C57BL/6 mice were placed in 75% oxygen at postnatal day (P) 7 and at P12 they were returned to room air and given an intravitreal injection of 3 μ g of sonepcizumab in one eye and PBS in the fellow eye. At P17, mice were given an intravitreal injection of rat anti-mouse PECAM-1 antibody and euthanized after 12 hours. Retinas were removed, washed, and incubated in 1:500 FITC-labeled goat anti-rat IgG and retinal flat mounts were examined by fluorescence microscopy. Retinas from mice treated with sonepcizumab (A) appeared to have less NV than retinas from mice treated with PBS (B). Image analysis by a masked investigator confirmed that compared to fellow eyes injected with PBS, eyes injected with sonepcizumab ($n=7$ for each) showed a significant reduction in mean ($\pm$ SD) area of NV per retina (C). Statistical comparison was made by unpaired t-test.

Figure 5. Intravitreal injection of sonopizumab reduces influx of macrophages into ischemic retina

C57BL/6 mice ($n = 8$) were placed in 75% oxygen at postnatal day (P) 7 and at P12 they were returned to room air and given an intraocular injection of 3 μ g sonopizumab in one eye and PBS in the fellow eye. At P17, mice were given an intravitreal injection of F4/80 antibody labeled with Alexa and after 8 hours retinal flat mounts were prepared and examined by fluorescence microscopy. Compared to fellow eyes treated with PBS which showed dense infiltration with macrophages (A), there were fewer macrophages in retinas from eyes injected with sonopizumab (B). This is most easily seen in the insets which are magnified views of the boxed areas. Image analysis confirmed that there was a significant reduction in the mean ($\pm$ SD) number of macrophages per retina in eyes treated with sonopizumab compared to those treated with PBS (C, $n = 8$ for each). Statistical comparisons were made by unpaired t-test.