Progressive loss of retinal blood vessels in a live model of retinitis pigmentosa

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ABSTRACT ●

Objective: To assess retinal blood vessels in a live retinitis pigmentosa (RP) model with rd1 mutation and green fluorescent protein (GFP) expressed in vascular endothelium.

Methods: Homozygous (hm) Tie2-GFP mice with rd1 mutation and known retinal degeneration were crossed with wild-type CD1 mice to generate control heterozygous (ht) Tie2-GFP mice. The retinas of 16 live hm mice were evaluated at 2 weeks and 3, 5, and 8 months of age, and compared with age-matched control ht and CD1 mice by optical coherence tomography (OCT) and confocal scanning laser ophthalmoscopy (cSLO). Fluorescence intensity was measured and compared between strains at 3, 5, and 8 months. In vivo findings were validated by immunostaining with collagen IV and isolectin histopathology.

Results: All hm Tie2-GFP mice showed progressive outer retinal degeneration by OCT. Loss of small branches of blood vessels and then larger main vessels was seen by cSLO. Retinal tissue and vessels were preserved in control ht mice. At all ages, measurements of fluorescence intensity were reduced in hm compared with ht mice ($p < 0.001$). In all strains, intensity at 8 months was reduced compared with 3 months ($p < 0.001$) and 5 months ($p = 0.021$). Histopathological studies confirmed in vivo findings and revealed a pattern of blood vessel regression in the deep plexus, followed by intermediate and superficial retinal plexuses.

Conclusions: This is the first evidence of progressive loss of retinal blood vessels in a live mouse model of RP. These findings may be highly relevant to understanding retinal degeneration in RP to prevent blindness.

Retinitis pigmentosa (RP) is the most common cause of inherited retinal degeneration and a leading cause of blindness in several developed countries.[1] The degenerative process initially involves photoreceptor cells and then extends to outer retina.[2–4] The visual dysfunction starts with night blindness, resulting from rod photoreceptor degeneration, and peripheral vision loss followed by central vision loss, resulting from severe outer retinal degeneration.[4,5] Attenuation of retinal blood vessels is the characteristic finding in retinal fundi in RP patients.[6] Although recent retinal regenerative studies showed promising results using gene therapy and optogenetics,[7–11] there is no cure for this debilitating disease.[12] Thus, new models are needed to better understand the pathological processes and to test novel therapeutics.

Mutations in over 45 genes have been identified as causes of RP.[4] Currently, 2 inbred mouse strains, the FVB/N and C3H/HeOu, are commonly used models of RP.[13–15] They both carry the *Pde6b*^{rd1} (rd1) gene mutation encoding the B subunit of phosphodiesterase 6 (PDE6) cGMP and are phenotypically similar to human RP with autosomal-recessive mutation in *Pde6b*^{rd1}.[5,14–16] PDE6 plays a major role in the process of phototransduction in the rod photoreceptors, responsible for night and dim light vision.[14] The exact pathology underlying the progressive retinal degeneration remains poorly understood. Along with degeneration of neurons, the integrity of retinal blood vessels is essential to regulate the blood flow for optimal response to local metabolic needs. Furthermore, retinal vascular endothelial cells secrete factors such as platelet-derived growth factor B (PDGF-B) that preserve the retinal neurons.[16] To date, few studies have assessed retinal vessels in mouse models of RP.[17,18]

The homozygous (hm) Tie2-GFP mouse expresses green fluorescent protein (GFP) in vascular endothelium.[19] For this reason, it is widely used to evaluate blood vessels in the brain, heart, and tumours without any staining.[20–23] This mouse model was engineered by the introduction of GFP cDNA fused to a Tie2 promoter, resulting in GFP expression in vascular endothelial cells.[19] Tie2 receptor, together with its ligands, angiopoietins 1 and 2, plays an important role in endothelial cell survival, proliferation, and migration.[24–27] This model has been used in eye research to study endothelial cells in Schlemm's canal[28] and choroid.[29,30] Interestingly, the hm Tie2-GFP mouse carries the *Pde6b*^{rd1} mutation of RP and shows evidence of retinal degeneration.[31] There are very few studies of the retinal vessels in this strain—only 1 postmortem study at 2 months[31] and an in vivo study at

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https://doi.org/10.1016/j.jcjo.2017.10.014
ISSN 0008-4182/17

Canadian Ophthalmological Society | Société canadienne d'ophtalmologie
EYE PHYSICIANS AND SURGEONS OF CANADA | MÉDECINS ET CHIRURGIENS OPHTALMOLOGISTES DU CANADA

4 months without controls.[32] Here we perform the first systematic study to evaluate retinal vessels in this live mouse model of RP.

Materials and Methods

Animals

All animal experiments were conducted in accordance with the institutional Animal Care Committee policies and procedures and in compliance with the ARVO guidelines and the National Institutes of Health guide for the care and use of laboratory animals. Mice were housed in The Keenan Research Centre for Biomedical Science Animal Care facility, St. Michael's Hospital, on a 12-hour light–dark cycle and fed ad libitum.

We crossed albino hm Tie2-GFP mice (Jackson Laboratory, Bar Harbor, Me) with wild-type albino CD1 mice (Charles River Laboratories, Montreal, Que.) to generate first-generation albino heterozygous (ht) Tie2-GFP mice. We included in our study hm Tie2-GFP, ht Tie2-GFP, and CD1 mice at 2 weeks and 3, 5, and 8 months of age.

In Vivo Infrared Reflectance and Blue-Laser Autofluorescence cSLO Imaging

For each of the 4 age groups (2 weeks and 3, 5, and 8 months), we imaged both eyes of hm Tie2-GFP mice (n = 4; 2 males, 2 females), in addition to ht Tie2-GFP mice (n = 4; 2 males, 2 females) and CD1 mice (n = 4; 2 males, 2 females), using a combined retinal confocal scanning laser ophthalmoscopy (cSLO) and optical coherence tomography system (Spectralis HRA + OCT, Heidelberg Engineering, Heidelberg, Germany).

Under general anaesthesia (2% isoflurane in O_2), pupils were dilated using tropicamide drops (Mydriacyl 1%; Alcon Canada Inc, Mississauga, Ont.) and both eyes of all mice were imaged using the Spectralis system mounted with a special 25-diopter lens (Heidelberg Engineering). Corneas were kept hydrated by topical application of artificial tears (GenTeal drops; Alcon Canada Inc) every 2 minutes. Mice were kept warm throughout the imaging procedure with a heating pad set to 38°C.

Three-dimensional OCT and retinal thickness maps (30°× 20° area) of live 2-week-old mouse retinas were generated using 25 single OCT scans with 61 μm steps and an average of 30 frames per scan. For the 3-, 5-, and 8-month-old mice, 3D OCT and retinal thickness maps (30°× 25° area) of live mice were generated using 31 single OCT scans with 69 μm steps and an average of 30 frames per scan.

With cSLO, blue laser autofluorescence (BAF) (λ = 486 nm) scans were used to assess the GFP-expressing retinal vasculature endothelium. BAF images were acquired with an average of 30 frames per scan. BAF-cSLO imaging of GFP signal in both ht and hm Tie2-GFP mice at 2 weeks of age was of poor quality due to the persistent hyaloid vessels and their small eyes.[33]

IR-cSLO scans (λ = 815 nm; 30 frames per scan) were used to assess the retinal vascular walls. This modality was useful especially in 2-week-old mice due to its longer wavelength and better penetration, which allowed imaging in spite of the persistence of hyaloid vessels.

Tissue Processing and Staining/ Immunohistochemistry

Mice at 2 weeks and 3 and 8 months of age were sacrificed under general anaesthesia (2% isoflurane in O_2) by perfusion with ice-cold phosphate buffered saline (PBS; pH = 7.4) and fixed by overnight immersion in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, Pa.) in PBS. Both eyes of each mouse were harvested and cryoprotected by immersion in sucrose (BioShop Canada Inc, Burlington, Ont.) (10% sucrose at 4°C for 24 hours and then in 20% sucrose at 4°C twice each for 24 hours); tissue was flash-frozen using 2-methylbutane (Thermo Fisher Scientific, Burlington, Ont.) and cooled by dry ice; globe specimens were embedded in OCT compound (Tissue-Tek, Sakura, Torrance, Calif.). The eyes were sectioned with a cryostat microtome (Leica CM1900, Cryostat; Leica Biosystems, Concord, Ont.) at 20 μm thickness and mounted on TruBond adhesive microscope slides (TruBond 380; Matsunami Glass Ind., Ltd, Osaka, Japan).

Selected sections were stained overnight at 4°C with isolectin GS-IB_4 Alexa Fluor 647 (1:50 dilution in 0.1% Triton X-100; Invitrogen, Carlsbad, Calif.) to visualize blood vessel endothelium and counterstained with DAPI nuclear stain (1:5000 dilution; Invitrogen).

Immunofluorescence staining was performed using the following primary antibodies: rabbit polyclonal anti–collagen IV antibody (1:200 dilution; ab6586; Abcam Inc, Toronto, Ont.) and rabbit polyclonal anti-GFAP antibody (1:500 dilution; Invitrogen) to detect basement membrane and astrocytes, respectively. Sections were incubated in 2% goat serum in PBS and 0.1% Triton X-100 for 40 minutes and then in the primary antibody overnight at 4°C, followed by secondary goat anti-rabbit IgG conjugated to Alexa Fluor 647 (1:1000 dilution; ab150079; Abcam Inc) with DAPI for 1 hour at room temperature. For negative controls, incubation with primary antibody was omitted.

Sections were then rinsed and coverslipped using aqueous mounting medium (DAKO; Agilent Technologies, Santa Clara, Calif.) and cover glass (No. 1.5; Fisher Scientific, Carlsbad, Calif.). The sections were imaged using a Zeiss LSM700 confocal microscope (Carl Zeiss Canada Ltd, Toronto, Ont.). Maximum intensity projection images were obtained from 16 z-stack slices using Fiji software.[34] Superficial, intermediate, and deep plexuses of retinal vessels were carefully assessed in each retina.

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