Tracking Retinal Microgliosis in Models of Retinal Ganglion Cell Damage

Shu Liu,1,5 Zhi-wai Li,1,2,5 Robert N. Weinreb,3 Guibua Xu,1 James D. Lindsey,3 Cong Ye,1 Wing-bo Yung,4 Chi-Pui Pang,1 Dennis Shun Chi Lam,1 and Christopher Kai-shun Leung1

PURPOSE. To investigate the longitudinal profiles of microgliosis after optic nerve injury induced by optic nerve crush and acute elevation of intraocular pressure (IOP).

METHODS. A confocal scanning laser ophthalmoscope was used to image the retinal microglia of the CX3CR1GFP/+ transgenic mice in vivo at baseline, 3 days and then weekly for 4 weeks after optic nerve crush (n = 3), and after elevating the IOP to 110 mm Hg for 30 (n = 3) or 60 (n = 3) minutes.

RESULTS. After optic nerve crush, the density of microglia increased by 2.43 ± 0.19-fold at week 1 and then gradually declined with 2.04 ± 0.24-, 1.69 ± 0.25-, and 1.29 ± 0.11-fold increases at week 2, 3, and 4, respectively. Microgliosis followed a similar pattern after acute IOP elevation and the increase in microglia was associated with the duration of IOP elevation. There were 1.35 ± 0.17- and 2.03 ± 0.08-fold increases in microglia at week 1, and 1.15 ± 0.11- and 1.11 ± 0.10-fold increases at week 4, after 30 and 60 minutes of acute IOP elevation, respectively. The morphology of microglia changed from ramified to ameboid form in 1 week, and then returned to ramified form in the subsequent weeks. There was a significant negative association between the number of surviving retinal ganglion cells (RGCs) and the extent of microgliosis during the follow-up period (R² = 0.72, P = 0.004).

CONCLUSIONS. Longitudinal in vivo imaging of the retinal microglia can provide an effective approach to study microgliosis and its association with RGC degeneration. (Invest Ophthalmol Vis Sci. 2012;53:6254–6262) DOI:10.1167/iovs.12-9450

Microglial cells were originally described as a group of neuroglia and migratory leukocytes stained with silver carbonate.1 In the retina, microglia play a major role in mediating phagocytosis during the development and degeneration of retinal ganglion cells (RGCs).2 Resting microglia show ramified morphology with long branching processes and a small cell body with lamellar distribution in the inner retina.3 Upon activation in tissue injury, microglia transform into an ameboid shape capable of phagocytosis and secretion of cytotoxic factors and proinflammatory molecules.2–4 Notably, published data on the longitudinal profile of microgliosis is sparse. The introduction of the CX3CR1 knockout transgenic mice has facilitated in vivo visualization of retinal microglia.5 CX3CR1 is a chemokine receptor expressed by the microglia. By replacing one copy of the fractalkine receptor gene with a copy of the green fluorescent protein (GFP), it is possible to image the fluorescent microglial in vivo. Applying laser photocoagulation on the retina, Eter et al.6 have shown that microglial cells migrate to and accumulate in laser spots with a confocal scanning laser ophthalmoscope (CSLO). With time-lapse confocal microscopy on retinal explants, Lee et al.7 have demonstrated that microglia increase their motility and transform from symmetric to polarized form toward the laser spot after focal laser injury. While these studies provide valuable information on the migration pattern of retinal microglia after focal laser injury to the retina, the longitudinal profile of microgliosis after optic nerve injury has not been investigated. In this study, we imaged the CX3CR1GFP/+ transgenic mice with a CSLO in vivo and followed the changes of retinal microglia after optic nerve crush and acute elevation of intraocular pressure (IOP) with an objective of investigating the longitudinal profiles of microgliosis following RGC damage.

METHODS

Animals

Homozygous CX3CR1GFP/GFP transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Three- to 6-month-old CX3CR1GFP/+ transgenic mice were generated by breeding to a wild-type mice with a C57BL/6 background. The environment was kept at 21°C with a 12-hour light and 12-hour dark cycle. All mice were fed ad libitum. Animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by Animal Experimentation Ethics Committee of the Chinese University of Hong Kong (No. 10/047/MIS).

In Vivo CSLO Imaging

The HRA2 (Heidelberg Engineering, GmbH, Dossenheim, Germany) (illuminating wavelength = 488 nm) was used to image the retina of the CX3CR1GFP/+ transgenic mice. A 55° wide-field lens was mounted to the camera to increase the field of view of the fundus. The scan rate of the HRA2 was 16 frames per second. Eye-tracking (a retinal recognition technology enabling the same retinal location “locked on”) was activated during imaging. Fifteen images at the same retinal location were captured, averaged automatically by the built-in software to
augment the signal-to-noise ratio, and simultaneously displayed on a computer screen. Each image frame represents approximately 40% of the total retinal area at an optical resolution of 5 μm to 10 μm. Pupils were dilated with topical mydriatic agents (tropicamide and phenylephrine, 0.5% each) to approximately 2 mm in diameter. The animal was restrained manually by a technician without using any general anesthetic agent. A 10-second break was given for every 15 seconds of imaging to allow eye blinking to keep the corneal surface moist.

**Acute IOP Elevation**

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg; Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (9 mg/kg; TranquilVed; Vedco, Inc., St. Joseph, MO). The anterior chamber was cannulated with a fine glass pipette connected by polyethylene tubing to a reservoir containing phosphate-buffered saline (PBS). The IOP was raised to 110 mm Hg by elevating the reservoir for 30 or 60 minutes. At the end of the IOP elevation, the glass pipette was removed. Each mouse had one eye with IOP elevation and the fellow eye (control eye) was cannulated without elevating the reservoir. Topical antibiotic and steroid ointment (TobraDex; Alcon Laboratories, Inc., Fort Worth, TX) was applied to the conjunctival sac.

**Optic Nerve Crush**

After anesthetizing the mice by intraperitoneal injection of ketamine and xylazine as described, limbal conjunctival peritomy was performed in the inferior region by gently peeling back to allow access to the posterior region of the globe. The optic nerve was then exposed through a small window made between the surrounding muscle bundles and fatty tissue by gentle blunt dissection. Care was taken not to damage muscles or the vortex veins. At a site approximately 1 mm posterior to the globe, the optic nerve was clamped with a pair of Dumont No. 5 self-closing tweezers (Ted Pella Inc., Redding, CA) for 2 to 3 seconds. Each mouse had one eye with optic nerve crush, and a sham procedure (exposing but not crushing the optic nerve) was performed on the fellow eye (control eye). Antibiotic ointment was applied to the surgical site. In the postoperative period, mice exhibited normal eating and drinking behavior.

**Immunohistochemistry and Confocal Laser Scanning Microscopy on Retinal Whole Mount**

Mice were anesthetized as described and exsanguinated by perfusion with oxygenated mammalian Ringer’s solution containing lidocaine hydrochloride (0.1 mg/mL, Xylocaine; Astra USA, Inc., Westborough, MA) and heparin sodium (500 units/mL, Heparin; Elkins-Sinn, Inc., Cherry Hill, NJ). Transcardial perfusion was then continued with fixative (approximately 20 mL 4.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4). Retinas were dissected after identifying the inferior area of the retina by the inferior position of the ophthalmic artery. For immunohistochemical staining of retinal microglia, the retinas were incubated in a blocking solution containing 10% fetal bovine serum (FBS), and 0.5% Triton X-100 in PBS (pH 7.4). The primary antibody anti–β-tubulin (TUJ1) was diluted 1:400 in 5% FBS, 0.4% Triton X-100. Retinas were incubated in primary antibody overnight in 4°C. Secondary antibody conjugated with Rhodamine red (1:400; Life Technologies, Grand Island, NY) was applied for 2 hours. The retinas were mounted in GB-mount (GBI Life Science, Inc.). The microglia and the RGCs were imaged at ×40 magnification by a confocal laser scanning microscope (Nikon C1 Confocal System, Tokyo, Japan).

**In Vivo Imaging and Counting of Retinal Microglia and RGCs**

In vivo images of retinal microglia were captured at baseline, day 3, and then weekly for 4 weeks after optic nerve crush or acute IOP elevation. Images were exported to a computer for microglial cell counting by using Photoshop (7.0; Adobe Systems Incorporated, San Jose, CA). In each retina, 4 different square regions with clear visualization of the microglia, each measuring 400 μm × 400 μm, were analyzed. Microglial cells in the same retinal locations in each eye were counted manually before and after the injury. At 4 weeks, the retinas were dissected and stained with anti–β-tubulin (TUJ1) (described above). The number of TUJ1-positive RGCs was counted from 20 to 30 fields, each measuring 250 μm × 250 μm, for each eye. RGC and microglial cell counting were performed by a blinded observer.

The area under the microgliosis time curve (see Figs. 2D, 3D, 4D) was measured to evaluate the degree of microgliosis during the follow-up period after the injury. The microgliosis time curve was constructed with y-axis representing the fold change of microglia compared to the baseline and x-axis representing the follow-up duration after the injury. The association between the area under the microgliosis time curve and the number of TUJ1-positive RGCs measured at 4 weeks after the injury was evaluated with linear regression analysis. P < 0.05 was considered to be statistically significant.

**Results**

A total of 18 eyes from nine mice were serially imaged at baseline, 3 days and then weekly for 4 weeks after optic nerve injury. In vivo CSLO imaging allows visualization of individual microglial cells, including their cellular processes (Fig. 1). In resting state, the microglia exhibited a ramified form with cellular processes extending from the cell bodies. The average
Microglia density at baseline was 178 ± 16 cells/mm² with a range between 152 cells/mm² and 191 cells/mm².

**Changes in Microglia after Optic Nerve Crush**

After optic nerve crush (n = 3), the density of microglia increased and the morphology of the microglia changed from ramified to ameboid form at week 1 as evident in the serial CSLO images (Figs. 2A, 2B). The density of microglia then gradually declined and returned to the ramified form at week 4 (Fig. 2C). There was an average of 2.43 ± 0.19-fold increase in retinal microglia at week 1 (Fig. 2D). The number of microglia gradually declined with 2.04 ± 0.24-, 1.69 ± 0.25-, and 1.29 ± 0.11-fold increases at week 2, 3, and 4, respectively. There were no significant changes in the density of microglia in the fellow eyes (see Fig. 5A).

**Changes in Microglia after Acute IOP Elevation**

The longitudinal profile of microgliosis followed a similar pattern after acute IOP elevation. The density of microglia increased in 1 week followed by a gradual decline in density in the following weeks after 30 (n = 3 mice) and 60 (n = 3 mice) minutes of acute IOP elevation. The change in cellular morphology, however, was less dramatic for eyes after 30 minutes of acute IOP elevation (Fig. 3B) than for those after optic nerve crush (Fig. 2B) and after 60 minutes of acute IOP elevation (Fig. 4B). The increase in microgliial cell density was related to the duration of IOP elevation (Figs. 3D, 4D). There were 1.35 ± 0.17- and 2.03 ± 0.08-fold increases in microglia at week 1, and 1.15 ± 0.11- and 1.11 ± 0.10-fold increases at week 4, after 30 and 60 minutes of acute IOP elevation, respectively. All mice showed no significant changes in the density of microglia in the fellow control eyes (Fig. 5B).

**Histologic Analysis**

All GFP-labeled cells were Iba1 positive at baseline, after optic nerve crush, and after acute IOP elevation (see Fig. 7). Similar to those observed in the in vivo image series, microglia changed from ramified form at baseline to ameboid form at week 1 after optic nerve crush and after acute IOP elevation (Figs. 6, 7) and then returned to ramified form again in the following weeks (Fig. 8). The average density of RGCs at week 4 was 447 ± 50 cells/mm² after optic nerve crush, and 2326 ± 440 and 1490 ± 397 cells/mm² after 30 and 60 minutes of acute IOP elevation, respectively. There was a significant
negative association ($R^2 = 0.720, P = 0.004$) between the number of TUJ1-positive RGCs measured at week 4 and the area under the microgliosis time curve during the 4-week follow-up period ($n = 9$, including eyes after acute IOP elevation and after optic nerve crush) (Fig. 9). Increased microgliosis was associated with reduced number of surviving RGCs.

**DISCUSSION**

Using a CSLO to image the CX3CR1\(^{GFP/+}\) transgenic mice, we demonstrated the dynamic changes of retinal microglia after optic nerve crush and retinal ischemia induced by acute IOP elevation. The density of retinal microglia increased at week 1, which was followed by a gradual decline approaching to a level near the baseline at week 4. The extent of microgliosis during the follow-up period, which is represented by the area under the microgliosis time curve, is negatively associated with the number of surviving RGCs. To our knowledge, this is the first study to measure the longitudinal profile of microgliosis after optic nerve injury. This finding would be pertinent to address the role of microgliosis in relation to RGC degeneration.

Retinal microglia are resident macrophages that have been implicated in phagocytosis, antigen presentation, and secretion of various cytokines and growth factors.\(^{2,8-10}\) Secreting both positive- (e.g., brain derived neurotrophic factor [BDNF], glial cell line-derived neurotrophic factor [GDNF]) and negative-acting factors (e.g., TNF-$
\alpha$, IL-1$\beta$),\(^{11-13}\) microglia can exert both protective and deleterious effect on the RGCs. Activation of retinal microglia has been demonstrated in immunohistochemical studies following optic axotomy and IOP elevation. Zhang and Tso\(^{14}\) have shown that there is an increase in OX-42- and 5D4-positive microglia in the inner retinal layers and that the OX-42-positive microglia become ameboid and ovoid 1 week after optic axotomy. In another study by Zhang et al.,\(^{15}\) a significant increase of OX-42-, ED1-, and OX6-positive microglia is observed one day after retinal ischemia induced by elevating the IOP to 120 mm Hg for 60 minutes. Three to 14 days after the IOP elevation, scattered amoeboid OX-42-, 5D4-, and ED1-positive cells are found in the subretinal space. In a rat glaucoma model prepared by cauterizing the episcleral veins, Naskar et al.\(^{16}\) report that microglia take up the fluorescent dye released by dying RGCs as early as 3 days after IOP elevation and that Iba1-positive microglia are present in proximity to RGCs. These findings suggest an association between microgliosis and RGC degeneration. However, the exact relation between microgliosis and RGC degeneration remains obscure.

Elevating the IOP above the retinal perfusion pressure is a common approach to induce ischemia-reperfusion damage to RGCs. It has been shown that RGC loss can be detected after 60 to 90 minutes of acute IOP elevation to 110 mm Hg in rodents.\(^{17,18}\) In this study, there was a close association between microgliosis and RGC survival. The degree of microgliosis was related to the duration of acute IOP elevation and there was a significant association between surviving RGCs.
Figure 4. Serial in vivo imaging of retinal microglia before (A) and at 1 week (B) and 4 weeks (C) after 60 minutes of acute elevation of intraocular pressure. The microglia increased in number and changed from ramified to ameboid form in a week and then gradually returned to near-the-baseline level at week 4. The longitudinal profiles of microgliosis of the treated (solid) and the control fellow (dotted lines) eyes are shown in the microgliosis time curve (D). Scale bar = 200 μm.

Figure 5. Serial in vivo imaging of retinal microglia of the sham control eyes before (left panel), 1 week (middle panel) and 4 weeks (right panel) after optic nerve crush (A) and 60 minutes of acute elevation of intraocular pressure (B). Scale bar = 200 μm.
FIGURE 6. Confocal scanning laser microscopy images of retinal whole mounts dissected from a healthy CX3CR1-GFP+/− transgenic mouse. The GFP-positive cells (green) (left panel) were microglial cells and the TUJ1-positive cells (red) (middle panel) were retinal ganglion cells. The overlaid image is shown in the right panel. 4’,6-Diamidino-2-phenylindole (DAPI) staining is indicated in blue in the overlaid image. Scale bar = 100 μm.

(A)

(B)

(C)

FIGURE 7. Confocal scanning laser microscopy images of retinal whole mounts dissected at week 1 after optic nerve crush (A) and after 30 minutes (B) and 60 minutes (C) of acute elevation of intraocular pressure. The colocalization (right panel) between GFP (green) (left panel) and Iba1 immunohistochemical staining (red) (middle panel) confirmed that the identity of GFP-positive cells were microglial cells. Scale bar = 100 μm.
measured at week 4 and the total number of microgliosis during the 4-week follow-up period (Fig. 9). Another important observation is that microgliosis persisted for a longer period after optic nerve crush than after acute IOP elevation. The area under the microgliosis time curve was generally larger for eyes after optic nerve crush than for those after acute IOP elevation. In a previous study using a blue-light CSLO to measure the number of RGCs in vivo, we have shown an exponential loss of Thy-1 cyan fluorescent protein (CFP)-positive RGCs 1 month after optic nerve crush.\textsuperscript{19} In contrast, no further loss of RGCs is observed after 1 week of acute IOP elevation for 90 minutes.\textsuperscript{18} It is unknown whether the microgliosis contributes to RGC neurodegeneration or whether microgliosis occurs in response to RGC death. It is plausible, however, that the persistence of microgliosis after optic nerve crush observed in this study would be related to the continued loss of RGCs in the initial weeks of the injury.

**Figure 8.** Confocal scanning laser microscopy images of retinal whole mounts dissected at week 4 after optic nerve crush (A) and after 30 minutes (B) and 60 minutes (C) of acute elevation of intraocular pressure. The GFP-positive cells (green) (left panel) were microglial cells and the TUJ1-positive cells (red) (middle panel) were retinal ganglion cells. The overlaid images with DAPI staining in blue are shown in the right panel. Scale bar = 100 μm.

**Figure 9.** A scatter plot showing the association between the area under the microgliosis time curve and the number of surviving retinal ganglion cells per mm\(^2\) after optic nerve injury induced by optic nerve crush and acute intraocular pressure elevation (n = 9).
To validate that the changes in microglia density and morphology were not consequential to eyeball manipulation, sham procedures (exposing without crushing the optic nerve and cannulating the anterior chamber without elevating the reservoir) were performed in the fellow eyes. Notably, there were no significant changes in microglial density or morphology in the fellow control eyes. This is different from the histologic study by Bodeutsch et al.\textsuperscript{20} showing that unilateral optic nerve crush leads to microglial proliferation in the injured eyes and also to a lesser extent, the fellow control eyes. Such discrepancy could be explained in part by the different degrees of optic nerve injury induced in the 2 studies. Bodeutsch et al.\textsuperscript{20} have performed blind optic nerve crush after making an incision in the conjunctiva, with the nerve being crushed for 10 to 12 seconds. By contrast, the duration of optic nerve crush in this study was shorter (2–5 seconds), and the optic nerve was identified and visualized for the crush, thereby minimizing collateral damage to the microvasculature. Damages incurred to the optic nerve in this study might not be sufficient to trigger microgliosis in the fellow control eyes. Being able to follow the same retinal areas longitudinally, in vivo imaging would be a more sensitive approach to study microgliosis compared with cross-sectional histologic analysis.

CX3CR1 signaling likely plays a major role in mediating microglia activation and RGC survival. CX3CR1 is expressed by the retinal microglia and functions as a chemotactic receptor for fractalkine, which has been implicated in the communication between neurons and microglia and the control of microglial neurotoxicity.\textsuperscript{21–25} In a recent study by Fuhrmann et al.,\textsuperscript{24} using two-photon in vivo imaging, knocking out the microglial chemokine receptor CX3CR1 has been shown to prevent neuron loss in a mice model of Alzheimer’s disease. Modifying the signaling pathway of CX3CR1 may thus be a potential therapeutic target to regulate the activation of microglia and enhance RGC survival.

CSLO imaging of the CX3CR1\textsuperscript{GFP/+} transgenic mice is an efficient approach to visualize the dynamic changes of retinal microglia. Although CX3CR1 can also be expressed by hematopoietic cells, NK cells, Th1 lymphocytes, and CD14\textsuperscript{+} monocytes,\textsuperscript{25} the colocalization between CX3CR1 and Iba1 in both resting and activated states confirmed that the identity of the fluorescent cells imaged in vivo were microglia. Using a confocal scanning laser microscope (CSLM) to image retinal explants of the CX3CR1\textsuperscript{GFP/+} mice, Lee et al.\textsuperscript{7} have demonstrated extension and retraction movements of the cellular process of microglia. Eter et al.\textsuperscript{6} have imaged the CX3CR1\textsuperscript{GFP/+} mice with a CSLO after focal argon laser photocogulation is applied to the retina. They have shown accumulation of microglia after 1 to 4 days of injury, and the number of accumulated microglial cells is proportional to the size and intensity of laser spots. Paques et al.\textsuperscript{55} have shown locomotion of individual microglia in vivo in the CX3CR1\textsuperscript{GFP/GFP} mice with a CSLO after focal laser damage to the retina. It is less certain, however, whether microglia deficient in fractalkine receptor respond differently upon activation. Liang et al.\textsuperscript{26} have found no differences in retinal microglial density, distribution, and cellular morphology between CX3CR1\textsuperscript{GFP/+} and CX3CR1\textsuperscript{GFP/GFP} transgenic mice, although microglial migration velocity is reduced in the CX3CR1\textsuperscript{GFP/GFP} strain in response to focal laser injury.

The key advantage of using a CSLO over a CSLM is the efficiency to perform in vivo imaging, permitting long-term repeated monitoring of microglial changes. Although the sample size was relatively small (n = 5 for each study group), the ability to follow-up the same retinal areas longitudinally had substantively reduced measurement variability secondary to interom and intereye differences in the number of microglia. Post hoc analysis revealed that the current sample size has a power of at least 78.2% to detect 15%-fold change in microgliosis (with a standard deviation of 0.15 and correlation coefficient between baseline and follow-up measurements of 0.9). This benefit, however, is at the expense of a lower image resolution than that for CSLM imaging on retinal explants. The in vivo resolution may not always be sufficient to differentiate ramified from ameboid form and the morphology of individual cellular processes was not as clear as that observed in the retinal whole mounts. It was impossible to determine in the in vivo images whether peripheral macrophages, or proliferation of resident microglia, contributed to the increase in cell density after the injury. Both resident microglia and blood-derived macrophages may have a role in influencing RGC survival after optic nerve injury.\textsuperscript{27,28} Although this study showed a negative association between microgliosis and the number of surviving RGCs, RGC damage could be a result of direct optic nerve injury at the early time points, independent of microgliosis. Inhibiting microgliosis would be useful to confirm the association between RGC degeneration and microgliosis. We are cross-breeding Thy-1-CFP and CX3CR1\textsuperscript{GFP/+} transgenic mice to generate a new strain that can facilitate in vivo imaging of both microglia and RGCs. The association between RGC degeneration and microgliosis can be better characterized by tracking the changes in density of RGC and microglia before and after optic nerve injury in the same retina.

In summary, in vivo CSLO imaging of the CX3CR1\textsuperscript{GFP/+} transgenic mice facilitated the investigation of the longitudinal dynamics of retinal microgliosis. Microgliosis was characterized by an increase in cell density with concomitant morphologic change from ramified to ameboid form during the first week of optic nerve injury. The microglia density then gradually declined to near-the-baseline level and the morphology returned to ramified form in approximately 4 weeks. Increased microgliosis was associated with RGC neurodegeneration. Investigating the dynamic profiles of microglia could provide mechanistic insights into RGC neuroprotection.

References

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