In Vivo Imaging and Quantitative Evaluation of the Rat Retinal Nerve Fiber Layer Using Scanning Laser Ophthalmoscopy

Ichiro Kawaguchi, Tomomi Higashide, Sbinji Obkubo, Hisashi Takeda, and Kazubisa Sugiyama

PURPOSE. To determine whether scanning laser ophthalmoscopy (SLO) is useful for in vivo imaging and quantitative evaluation of rat retinal nerve fiber layer (RNFL) using an optic nerve crush model.

METHODS. The optic nerve of the right eye was crushed intraorbitally with a clip. The left eye served as the untreated control. Fundus images of both eyes were recorded by SLO using an argon blue laser before and 1, 2, and 4 weeks after optic nerve crush. The focused plane was sequentially moved by changing the refractive values in the SLO setting. The range of refractive values (ΔF) in which the RNFL reflex was clearly observed was determined. The RNFL thickness in retinal sections was measured and compared to the ΔF value from SLO images taken before histologic preparation.

RESULTS. Striations of RNFL radiating from the optic disc were clearly visible by SLO. No obvious changes in the RNFL reflex were observed 1 week after optic nerve crush. However, striations of RNFL became uniformly darker and thinner 2 weeks after the crush and were barely visible 4 weeks after the crush. The ΔF value was unchanged 1 week after the crush, but then decreased significantly and progressively after the second week. ΔF was unchanged in the control eyes during the experimental period. The ΔF value correlated significantly with the histologically determined RNFL thickness.

CONCLUSIONS. SLO is a useful and valuable tool for in vivo imaging and quantitative evaluation of rat RNFL. (Invest Ophtalmol Vis Sci. 2006;47:2911–2916) DOI:10.1167/iovs.05-1169

Retinal nerve fiber layer (RNFL) defects are one of the most critical factors to assess in the evaluation of the degree and distribution of retinal ganglion cell loss in patients with glaucoma. In routine fundus examinations, red-free or green light rather than white light is suitable for visualization of RNFL, because of the optical properties of RNFL.1 A scanning laser ophthalmoscope (SLO; Rodenstock Instruments, Munich, Germany) provides argon blue laser illumination and confocal apertures which are ideal for RNFL observation as the wave-length of the laser is optimal for RNFL visualization, and a small confocal aperture allows high-contrast imaging by reducing the scattered light arising from defocused tissues.2 Accordingly, highly reproducible RNFL evaluation can be performed by SLO.2 However, there are no detailed reports on RNFL in rat eyes visualized with an SLO, although in one study an SLO was used for imaging the rat fundus and assessing retinal circulation.3

Ocular hypertension, ischemia–reperfusion, and optic nerve crush rodent models have been used to elucidate the pathophysiology of glaucoma and other optic neuropathies.4 In these models, loss of retinal ganglion cells has usually been evaluated histologically by counting the number of cell bodies in the retina or their axons in the optic nerve.5–8 For intraretinal axons of retinal ganglion cells, qualitative assessment of the effect of axotomy has been performed by staining the axons in the flatmount retina.9,10 However, there have been no reports of in vivo evaluation of RNFL changes in rodent models of optic nerve injury.

In this study, we determined whether the SLO is useful for in vivo imaging and quantitative evaluation of the rat RNFL using the optic nerve crush model.

METHODS

Animals

Male Brown Norway rats, 12 weeks of age and weighing 200 to 250 g, were used in this study. The rats had free access to food and water and were maintained in cages in an environmentally controlled room with a 12-hour light–dark cycle. All experiments were conducted on rats anesthetized by an intraperitoneal injection (65 mg/kg) of sodium pentobarbital (Somnopentil; Schering-Plough Animal Health, Omaha, NE). All animals were treated in accordance with the ARVO statement for the Use of Animal in Ophthalmic and Vision Research.

Optic Nerve Crush Model

The conjunctiva of the right eye was incised in the superotemporal quadrant to expose the optic nerve by careful blunt dissection under an operating microscope. The optic nerve was crushed 2 mm behind the globe for 30 seconds with a 60-g vascular clip (Micro Vascular Clip; Roboz Surgical Instrument Co., Gaithersburg, MD). Special care was taken not to damage the blood supply to the eye traveling along the inferior side of the optic nerve.10 Immediate recovery of retinal blood supply after removal of the clip was observed by indirect ophthalmoscopy in each eye. The left eye served as the untreated control.

In Vivo Imaging of RNFL over Time Using SLO

Before and 1, 2, and 4 weeks after optic nerve crush, the retinal images of both eyes were recorded by SLO (SLO 101; Rodenstock Instruments) with argon blue laser illumination (wavelength: 488 nm, output: 190–210 μW) in 21 rats. The eyes were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceuticals, Osaka, Japan). To preserve corneal clarity throughout the experiment, a custom-made contact lens with a radius of curvature

From the Department of Ophthalmology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan.

Supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

Submitted for publication September 1, 2005; revised February 3 and March 5, 2006; accepted May 1, 2006.

Disclosure: I. Kawaguchi, None; T. Higashide, None; S. Obkubo, None; H. Takeda, None; K. Sugiyama, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Ichiro Kawaguchi, Department of Ophthalmology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa, Ishikawa 9208641 Japan; ichiro-k@mub.biglobe.ne.jp.
of 2.75 mm and a diameter of 5.0 mm (Kyoto Contact Lens, Kyoto, Japan) was placed on the cornea after topical anesthesia with 0.4% oxybuprocaine hydrochloride eye drops (Santen Pharmaceuticals). A rat was placed on a heating pad (Deltaphase Isothermal Pad; Braintree Scientific, Inc., Braintree, MA) and its head was gently held manually to keep the eye in position for illuminating the fundus evenly with the argon blue laser.

Fundus images with a field angle of 40° were recorded by digital video (GV-D1000; Sony Co., Tokyo, Japan). First, the widest confocal aperture (C3) was used for qualitative observation of RNFL. Then, for the quantitative evaluation of RNFL thickness, the narrowest confocal aperture (C3) was used to maximize the image contrast of the focused plane and the axial resolution. The focused plane was sequentially moved through the retina, sclera to vitread, by changing the refractive values (maximum range, −20 D to +20 D) in the SLO setting (i.e., altering the setting of the ametropic corrector). The fundus images were recorded with a nominal 1-D step and the range of refractive values (ΔF) for RNFL thickness was determined by a masked observer.

As the focus measurements in this study were subjective, representative SLO images of RNFL with different focused planes, as shown in Figure 1, were used as the reference images for determining ΔF. For all 1-D steps of SLO images of each rat fundus, we looked carefully at the retinal location, approximately 1 disc diameter apart from the edges of the optic disc and determined the most scleral focal plane where the radial striations of RNFL become just visible. We also determined the most vitread focal plane with the striations of RNFL reflex just before being covered by the dark area around the optic disc which enlarges with larger refractive values. For evaluation of the reproducibility and reliability of these subjective focus measurements, we calculated intra- and interobserver agreement in the measurements using the SLO image series of 24 rat fundus (six image series each at baseline and 1, 2, and 4 weeks after optic nerve crush). One group of rats was used for each time point. For baseline data, the optic nerve was not crushed in the remaining group of rats, and the right eye was processed similarly. Immediately after the recording of the fundus by SLO, the eyes were enucleated after administration of an anesthetic overdose of intraperitoneal pentobarbital sodium. The anterior segment was removed and a small marking cut was placed on the edge of the posterior eye cup to identify the superior retinal portion. The eye cup was fixed in 4% paraformaldehyde-0.5% glutaraldehyde and 0.1 M phosphate-buffered saline for 2 hours at room temperature, and was embedded in mounting compound (Tissue-Tek OCT; Sakura Finetech- nical, Tokyo, Japan) followed by freezing with dry ice. Serial frozen sections (16 μm thick) were collected along the vertical meridian of the globe. After they were stained with hematoxylin-eosin, the retinal sections used for RNFL thickness measurements and calculated the mean number of cells in each eye.

Correlation of ΔF and Histologically Determined RNFL Thickness

Sixteen rats were divided into four groups (four rats in each group). An optic nerve crush was performed in the right eye in three groups. Retinal sections of the right eye were prepared for RNFL thickness measurement at 1, 2, and 4 weeks after optic nerve crush. One group of rats was used for each time point. For baseline data, the optic nerve was not crushed in the remaining group of rats, and the right eye was processed similarly. Immediately after the recording of the fundus by SLO, the eyes were enucleated after administration of an anesthetic overdose of intraperitoneal pentobarbital sodium. The anterior segment was removed and a small marking cut was placed on the edge of the posterior eye cup to identify the superior retinal portion. The eye cup was fixed in 4% paraformaldehyde-0.5% glutaraldehyde and 0.1 M phosphate-buffered saline for 2 hours at room temperature, and was embedded in mounting compound (Tissue-Tek OCT; Sakura Finetech- nical, Tokyo, Japan) followed by freezing with dry ice. Serial frozen sections (16 μm thick) were collected along the vertical meridian of the globe. After they were stained with hematoxylin-eosin, the retinal sections were observed under an optical microscope (Eclipse TE300; Nikon Corp., Kanagawa, Japan) and were recorded as JPEG files with a digital cooled CCD camera (DS-5Mc-L1; Nikon) and a personal computer (Dimension 8300; Dell Inc., Round Rock, TX). For each eye, RNFL thickness of three consecutive sections derived from a location approximately 500 μm temporal from the center of the optic disc (~1 disc diameter from the edge of the optic disc) was measured using image-analysis software (Image-Pro Express 4.0; MediaCybernetics, Inc., Silver Spring, MD) and averaged. The RNFL thickness in the retinal sections was compared by the ΔF determined from SLO images.

We also counted the number of cells in the ganglion cell layer in the retinal sections used for RNFL thickness measurements and calculated the mean number of cells in each eye.

Statistical Analysis

The difference in ΔF was analyzed by the Wilcoxon signed-rank test for comparison between control and crushed eyes and by the Friedman test and post hoc tests for comparison of ΔF measured at different time points. The differences in RNFL thickness and the number of cells in the ganglion cell layer in retinal sections at different time points were
analyzed by one-way ANOVA and post hoc tests. Spearman’s rank-order correlation coefficient was calculated to determine the significance of the correlation between $H_9004 F$ and RNFL thickness measured in retinal sections. $P < 0.05$ was considered statistically significant. Data are expressed as the mean $\pm$ SD.

**RESULTS**

Striations of RNFL radiating from the optic disc were clearly visible in each eye by SLO under argon blue laser illumination when appropriately focused (Fig. 1). The fundus images were sharper, but darker through a C1 than through a C3 confocal aperture (data not shown). After the optic nerve crush, no obvious changes in RNFL reflex were observed 1 week later. However, 2 weeks after the crush, striations of RNFL became uniformly darker and thinner, suggesting diffuse loss of retinal ganglion cell axons. Disappearance of RNFL striations was more evident 4 weeks after the crush (Fig. 2). The RNFL changes after the optic nerve crush were consistent in all 21 rats. In contrast, RNFL appearance of control eyes was unchanged throughout the experimental period.

For the quantitative evaluation of RNFL thickness, the range of refractive values ($\Delta F$) in the SLO setting in which radial striations of RNFL was clearly observed near the optic disc was determined at each time point in both eyes of all 21 rats. The $\Delta F$ before and 1, 2, and 4 weeks after the optic nerve crush was $7.1 \pm 0.4, 7.2 \pm 0.7, 3.4 \pm 0.5$ and $1.2 \pm 0.4$ D, respectively (Fig. 5). The $\Delta F$ was unchanged 1 week after the crush, but then decreased significantly and progressively after the second week. No significant changes in $\Delta F$ were observed in the untreated control eyes during the experimental period.

To determine whether $\Delta F$ is a reliable indicator of actual RNFL thickness, the thickness was measured in frozen sections of the retina after the $\Delta F$ measurements by SLO (Fig. 4). The RNFL thickness at a location approximately 500 $\mu$m temporal from the center of the optic disc in retinal sections before and 1, 2, and 4 weeks after the optic nerve crush was $24.9 \pm 2.4$, $25.9 \pm 3.3$, $9.8 \pm 1.0$, and $3.9 \pm 0.2$ $\mu$m, respectively. The

![Figure 2. RNFL changes over time caused by optic nerve crush. SLO images at baseline (A, E) and 1 (B, F), 2 (C, G), and 4 (D, H) weeks after axonal injury. (A-D) Fundus images taken at the lower limit of refractive value for RNFL observation. (E-H) Fundus images taken at the upper limit of refractive value for RNFL observation. The refractive value of each SLO image is shown at top right.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932939/ on 08/21/2018)
thickness was unchanged 1 week after the crush, but then decreased significantly and progressively after the second week. There was a significant positive relationship ($r = 0.86$, $P < 0.001$) between $\Delta F$ and the histologically determined RNFL thickness (Fig. 5). In contrast, the cell counts in the ganglion cell layer expressed as a percentage of the baseline count (100% ± 6.7%) were 73.6% ± 6.6%, 41.4% ± 2.0%, and 22.8% ± 2.4% at 1, 2, and 4 weeks after optic nerve crush, respectively. This decrease in cell count from baseline was significant 1 week after the crush and progressed further thereafter ($P < 0.01$).

**DISCUSSION**

In contrast to the clinical significance of RNFL assessment for diagnosis and management of glaucoma and other optic neuropathies, there have been few reports dealing with the changes of the RNFL or intraretinal axons of retinal ganglion cells in rodent models with optic nerve injuries. An immunohistochemical study showed thick bundles of retinal ganglion cell axons converging toward the optic disc in wholemounts of normal rat retina.6 The striations radiating from the optic disc in rat retina observed by SLO imaging under argon blue laser illumination seemed quite similar to the intraretinal distribution of retinal ganglion cell axons.6 Given that the condition used for imaging the rat retina in this study was optimal for RNFL observation of human retina, the radial striations in SLO images were considered to be the reflex from the bundle of retinal ganglion cell axons.
The clinical usefulness of RNFL evaluation by SLO is limited because SLO evaluation of human RNFL remains qualitative. Other imaging modalities such as optical coherence tomography and nerve fiber layer polarimetry are more suitable for quantitative evaluation of RNFL thickness. However, there have been no reports of in vivo quantitative measurement of RNFL in rodent retina by these methods. The radial striations of RNFL in SLO images appeared to be much stronger in Brown Norway rats than in humans. We used pigmented rats for RNFL evaluation, as visualization of RNFL by argon laser is better in pigmented than in albino rats (data not shown). This better visualization by pigmentation is probably because most of the incident light is absorbed by pigments in the retinal pigment epithelium and the choroid and then reflected light from these tissues is greatly reduced.

We then attempted to evaluate rat RNFL quantitatively by SLO. The smallest setting of the confocal aperture in the SLO used in this study was 1 mm (C1), a setting considerably larger than those in tomographic instruments. Woon et al.11 measured the axial resolution of this SLO using a C1 confocal aperture in a human model eye and reported that the resolution was 300 μm in a 20° field of view. They used a model eye that consisted of a 16-mm lens and a micrometer mounted mirror. The pixel brightness of the SLO image of the mirror was then plotted against the axial displacement of the mirror. The full-width-at-half-maximum brightness was taken as the axial resolution. Even with this setting, the possibility of optical sectioning and in vivo three-dimensional reconstruction of the human fundus by SLO was reported.12 Furthermore, considerably improved axial resolution can be achieved with an SLO (Rodenstock). Fitzke et al.13 reported an optical section of 28 μm with a confocal aperture of 1 mm using a ×16 magnification system for the prototype SLO (Rodenstock).

A similar situation occurs when viewing the rat fundus by SLO. According to data of representative rat14 and human (Gullstrand’s schematic eye) eyes, the axial length of a rat eye is much shorter than that of humans (6.29 vs. 24.00 mm), and the total power of the rat eye is much greater than in humans (300.705 vs. 58.64 D). Therefore, lateral magnification of the rat fundus in SLO images should be approximately five times larger than that of the human fundus. We confirmed that SLO images of the rat fundus were approximately five times larger than images of the human fundus, with the size of the rat and human optic discs (~300 vs. 1500 μm in diameter) being similar in SLO images with the same size field of view. Given that axial magnification is determined by the square of the lateral magnification, the axial magnification of the SLO images of a rat fundus should therefore have been approximately 25 times larger than those of humans in a field of view of the same size. Therefore, when taking SLO images in a 40° field of view, as used in this study, the axial magnification of a rat fundus will be 6.25 times larger than human SLO images in a 20° field of view with the axial resolution of 300 μm.11 Accordingly, the axial resolution of the rat SLO images would have been approximately 50 μm in this study. Given that the thickness of human and rat retina are similar (typically 250 vs. 170 μm14), optical sectioning of a rat fundus by an SLO (Rodenstock) should be superior to that achieved in humans.

As for the 1-D step of the ametropic corrector to evaluate RNFL thickness in this study, adding 1 D to the ametropic correction moves the plane of focus approximately 0.3 mm in the direction of the vitreous in the human eye, because adding 1 D to the total power of the human eye makes the posterior focal length approximately 0.3 mm shorter in Gullstrand’s schematic eye. In contrast, the posterior focal length in a rat eye14 is calculated to be approximately 10 μm shorter when adding 1 D to the total power of the rat eye. Thus, a 1-D step in the ametropic correction moves the focal plane approximately 10 μm, a step that is not too wide for measuring RNFL thickness (≥25 μm) with an axial resolution of approximately 50 μm.

In agreement with the theoretical consideration of the ability of the SLO for optical sectioning of a rat fundus, the focus measurement of rat RNFL (ΔF) using this technique was performed successfully with good intra- and interobserver reproducibility using a 1-mm confocal aperture.

In the next step, we compared the ΔF and the actual RNFL thickness in histologic sections to verify that ΔF is a reliable indicator of RNFL thickness. Although RNFL thickness may vary at different distances from the optic disc, we consistently measured the RNFL thickness at a location approximately 500 μm temporal from the center of the optic disc. At this location, the visibility of radial striations of RNFL in SLO images was suitable for determination of the ΔF value and also the RNFL was thick enough for measuring the changes in retinal sections.

Given a nominal 1-D step of the ametropic corrector corresponding to approximately 10-μm movement of the focal plane and the axial resolution of approximately 50 μm (full-width-at-half-maximum brightness) in SLO images of rat fundus, the ΔF of approximately 7 D for the 25-μm thickness of normal rat RNFL is reasonable. In contrast, ΔF was approximately 1 D for <5 μm of RNFL thickness in the retina 4 weeks after the optic nerve crush, although it should have been ≥5 D, theoretically. This discrepancy may be explained as follows. The half-maximum brightness of the normal rat RNFL reflex in SLO images may be similar to the threshold of seeing the reflex. However, for thinner RNFL, the visibility became lower, and then stricter focusing (i.e., smaller ΔF values) may have been needed to see the reflex.

The significant positive relationship between ΔF and the histologically determined RNFL thickness indicates that ΔF is a reliable indicator of actual RNFL thickness and, thus, quantitative analysis of rat RNFL can be performed by SLO.

The RNFL thickness was unchanged until the first week after the optic nerve crush in SLO images and retinal sections. Furthermore, we did not notice any qualitative changes of the RNFL reflex in either SLO images or intraretinal axon bundles in histologic sections between baseline and 1 week after the crush. As for the loss of retinal ganglion cell bodies after the optic nerve crush or axotomy, the time course and magnitude of cell death depends on the severity of the injury15,16 and the distance of the injury site from the ganglion cell bodies.17 When the optic nerve is damaged intraorbitally, ganglion cell death starts several days after injury,18 reaches 50% to 50% 1 week after,19–21 and progresses further thereafter. In this study, as an index of ganglion cell loss, we counted the number of cells in the ganglion cell layer in the retinal sections used for measuring RNFL thickness and found that the decrease in cell counts was significant 1 week after the crush. Thus, the result of this study suggests that loss of cell bodies may precede the loss of intraretinal axons of retinal ganglion cells after the optic nerve crush. The implication of dissociation between the time course of changes in intraretinal axons and cell bodies is currently unknown. Given that retinal ganglion cells of the adult rat die through apoptosis when axotomized,22 the clearance processes for apoptotic cell bodies may differ from those for intraretinal axons. Further studies are needed to examine this hypothesis.

In conclusion, SLO is a useful and valuable tool for in vivo imaging and quantification of rat RNFL. Evaluation of rat RNFL by SLO will be informative not only in optic nerve crush models but also in other models of optic neuropathy including glaucoma and for developing new therapies.
References


