In Vivo Evaluation of Laser-Induced Choroidal Neovascularization Using Spectral-Domain Optical Coherence Tomography

Andrea Giani,1,2 Aristomenis Thanos,1,2 Mi In Rob,1,2 Edward Connolly,1
George Trichonas,1 Ivana Kim,1 Evangelos Gragoudas,1 Demetrios Vavvas,1
and Joan W. Miller1

PURPOSE. To describe the in vivo evolution of laser-induced choroidal neovascularization (CNV) in mice using spectral domain optical coherence tomography (SD-OCT).

METHODS. Laser photocoagulation was applied to the mouse fundus using a 532-nm diode laser (100, 150, and 200 mW; 100-μm diameter, 0.1-second duration). SD-OCT examination was performed immediately after laser application and at days 3, 5, 7, 14, 21, and 28 after laser. Fluorescein angiography (FA) was performed at day 5, 7, 14, and 28. Acquired SD-OCT images were analyzed to describe morphologic features, measure CNV size and retinal thickness, and assess the frequency of lesions resulting in fluid accumulation. Finally, SD-OCT images were compared to fluorescein angiograms and histologic sections with immunostaining at similar time points.

RESULTS. SD-OCT allowed visualization of the initial laser damage and the subsequent stages of the injury response. CNV formation reached its maximum size at day 5. By day 7, significant size reduction was observed (P < 0.001), continuing through days 14 and 28. Exudation signs, such as fluid accumulation and increase in retinal thickness, followed the same time course, with a peak at day 5 and a decrease by day 7. Delivery of higher laser energy levels to the RPE/choriocapillary complex resulted in a significant percentage of lesions demonstrating excessive chorioretinal damage without CNV formation.

CONCLUSIONS. SD-OCT is a fast and reliable tool for the in vivo evaluation of laser-induced CNV, allowing quantification of lesion size and exudation parameters. Moreover, it provides morphologic information that correlates with histologic findings. (Invest Ophthalmol Vis Sci. 2011;52:3880–3887) DOI:10.1167/iovs.10-6266

From the 1Angiogenesis and Laser Laboratories, Retina Service, Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts.

2These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Supported by Massachusetts Lions Research Fund Inc; Massachusetts Eye and Ear Infirmary Neovascular Research Fund (JWM); and Research to Prevent Blindness.

Submitted for publication July 23, 2010; revised November 4 and 29, 2010; accepted December 10, 2010.

Disclosure: A. Giani, None; A. Thanos, None; M.I. Rob, None; E. Connolly, None; G. Trichonas, None; I. Kim, None; E. Gragoudas, None; D. Vavvas, None; J.W. Miller, Alcon Laboratory (C), P

Corresponding author: Joan W. Miller, Massachusetts Eye and Ear Infirmary, Harvard Medical School, 243 Charles Street, Boston, MA 02114; jwmiller@meei.harvard.edu.

Choroidal neovascularization (CNV) is the hallmark of neovascular age-related macular degeneration (AMD) and is responsible for most vision loss seen in this disorder. CNV is characterized by the abnormal growth of new vessels originating from the choroidal vasculature and their subsequent growth under the retinal pigment epithelium, subretinal space, or a combination of both. As the population ages, AMD will be a more common cause of vision loss than will diabetic eye disease and glaucoma combined.1–4 Strategies for preventing vision loss in AMD have improved dramatically over the past decade, especially with the advent of anti-VEGF agents. Despite this progress, a significant proportion of patients with AMD will still experience significant vision impairment. Therefore, it becomes clear that there is still a need to better understand CNV pathogenesis and to further identify novel therapeutic targets.

The development of the laser-induced CNV model has had a significant impact in our understanding of the molecular events involved in CNV development and has become the most widely used model in neovascular AMD research.5 The model itself has been validated in the preclinical testing of all current treatment modalities used today for AMD patients.6,7 Currently, the formation of experimental CNV and its response to investigational treatments is examined primarily in vivo.8–12 Spectral-domain optical coherence tomography (SD-OCT) is a technology that allows a very detailed, noninvasive, in vivo evaluation of tissues. It has been recently used in rodents to measure retinal thickness and to evaluate models of retinal degeneration,9 retinal tumors,10 and retinal detachment.11 In humans, it has become an essential tool in the clinical management of several pathologies, including neovascular AMD.12–15 To this aim, we sought to examine the value of SD-OCT as a tool for the assessment of laser-induced CNV in rodents. In this study we describe the in vivo evolution of laser-induced CNV in mice by means of a portable SD-OCT instrument.

METHODS

Animals

All animal experiments followed the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were...
approved by the Animal Care Committee of Massachusetts Eye and Ear Infirmary. Wild-type (WT) C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). A total of 70 male mice between 6 and 8 weeks of age were used for this study. For all procedures, anesthesia was achieved by intraperitoneal injection of 50 mg/kg ketamine hydrochloride (Phoenix Pharmaceutical, Inc., St. Joseph, MO) and 10 mg/kg xylazine (Phoenix Pharmaceutical, Inc.), and pupils were dilated with topical 0.5% tropicamide (Alcon, Humacao, Puerto Rico).

**Laser-Induced Model of CNV**

Laser photocoagulation was performed using a 532-nm laser (Oculight GLx Laser System, IRIDEX Corporation, Mountain View, CA) attached to a slit lamp, and a coverslip was used to align the cornea to allow view to the posterior pole of the eye. Four lesions were induced using the indicated laser settings. The lesions were located at the 3, 6, 9, and 12 o’clock meridians centered on the optic nerve and located two or three disc diameters from the optic nerve. Laser-induced disruption of Bruch’s membrane was identified by the appearance of a bubble at the site of photocoagulation. Laser spots that did not result in the formation of a bubble were excluded from the studies. Keeping the same spot size (100 μm) and duration (0.1 second), animals were equally divided into three laser power setting groups: 100, 150, and 200 mW.

To test interoperator variability, laser photocoagulation was executed in separate sessions by two investigators, and results were compared.

**Fluorescein Angiography**

Fluorescein angiography (FA) was performed using a commercial camera and imaging system (TRC 50 VT camera and IMAGEnet 1.55 system; Topcon, Paramus, NJ) at 5 days and at 1 and 2 weeks after laser photocoagulation. Photographs were captured with a 20-diopter lens in contact with the fundus camera lens after intraperitoneal injection of 0.1 mL of 2% fluorescein sodium (Akorn, Decatur, IL). Two masked retina specialists not involved in laser photocoagulation or angiography evaluated the fluorescein angiograms at a single sitting. Lesions of 0.1 mL of 2% fluorescein sodium (Akorn, Decatur, IL). Two masked retina specialists not involved in laser photocoagulation or angiography evaluated the fluorescein angiograms at a single sitting. Lesions were graded using a previously established scheme as follows: 0 (not leaky), faint hyperfluorescence or mottled fluorescence without leakage; 1 (questionable leakage), hyperfluorescent lesion without progressive increase in size or intensity; 2A (leaky), hyperfluorescence increasing in intensity but not in size; 2B (clinically significant leakage), hyperfluorescence increasing in intensity and in size.

**Optical Coherence Tomography**

OCT was performed using an SD-OCT system (Bioptigen Inc., Durham, NC) immediately after laser application and at days 3, 5, 7, 14, 21, and 28. Mice were positioned on a custom cassette that allowed three-dimensional free rotation to align the mouse eye for imaging. Hydration with normal saline was used to preserve corneal clarity. Volume analysis centered on the optic nerve head was performed, using 100 horizontal, radar, and consecutive B-scan lines, each one composed by 1200 A-scans. The volume size was 1.6 × 1.6 mm. The software was able to generate the en face fundus image using the reflectance information obtained from the OCT sections (volume intensity projection), so that the point-to-point correlation between OCT and fundus position was possible and accurate.

**In Vivo Quantification of Size and Exudation Parameters in Laser-Induced CNV**

CNV size was measured using both the volume and the cross-sectional area of the lesions. To evaluate the volume of CNV, each single section passing through the lesion was evaluated. ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) was used to delineate and measure the area of the CNV in each image, defined as the subretinal hyperreflective material above the RPE layer. Since the vertical interval between each section corresponded to one pixel, the total volume resulted from the sum of each single sectional area. To evaluate the cross-sectional size of each lesion in OCT images, the sections passing through the center of the CNV were chosen. The center of the lesion was defined as the midline passing through the area of RPE-Bruch’s membrane rupture. To consistently identify this point, we used the en face fundus reconstruction tool provided with the Bioptigen SD-OCT system. This software tool allows one to generate a fundus image using the average reflectivity from each single OCT A-scan. The axial interval in OCT images used to create this reconstruction is customizable. For our purpose we narrowed and positioned it to the level of RPE/choroid complex, so that the area of the RPE-Bruch’s membrane rupture was easily identifiable in the fundus image as a hyperreflective spot (Fig. 1A). For each time point, the same spot was used to evaluate the size of the CNV. The sectional area of the lesion was delineated and measured in the same manner used for the volume evaluation.

Exudation from lesions was assessed by both identifying the fluid accumulation and evaluating retinal thickness at days 3, 5, 7, 14, and 28. The presence of fluid was evaluated in all the sections passing through each lesion. For each time point the frequency of lesions showing fluid accumulation was assessed. The same images used to assess CNV size were used to evaluate retinal thickness (RT), defined as the distance between the inner limiting membrane and the outer limit of the RPE layer.

**Choroidal Flatmount Preparation and Ex Vivo CNV Volume Quantification**

Flatmount preparation and ex vivo CNV volume quantification were performed as follows. Mice were anesthetized at the indicated time points and perfused through the left ventricle with 10 mL PBS followed by 10 mL of 5 mg/mL FITC-dextran (MWt, 2 × 10⁶; Sigma, St. Louis, MO) immediately after laser application and at days 3, 5, 7, 14, 21, and 28. The distance between the inner limiting membrane and the outer limit of the RPE layer was measured using a previously established scheme as follows: 0 (not identifiable), faint hyperfluorescence or mottled fluorescence without leakage; 1 (questionable leakage), hyperfluorescent lesion without progressive increase in size or intensity; 2A (leaky), hyperfluorescence increasing in intensity but not in size; 2B (clinically significant leakage), hyperfluorescence increasing in intensity and in size.

**Figure 1.** Evaluation of CNV evolution using SD-OCT. Identification of the center of the lesion seen as a hyporeflective area on fundus reconstruction (A). OCT image showing the formation of the subretinal bubble soon after laser application as an indicator of Bruch’s membrane rupture (B). By day 1, retinal hyperreflectivity acquired a distinctive butterfly-like shape (C). Evolution of the choroidal fibrovascular tissue, followed over time with serial OCT sessions (D–H).
MO). Eyes were enucleated and fixed with 4% paraformaldehyde for 30 minutes at 4°C. Before enucleation, a suture was placed at the 12 o’clock meridian to keep the orientation of the eye and to facilitate comparison of the individual CNV with OCT measurements. The anterior segment and retina were removed from the eyecup, and four relaxing radial incisions were made. The remaining RPE-choroid-sclera complex was flatmounted with mounting medium (Vectorshield; Vector Laboratories, Burlingame, CA) and coverslipped. Flatmounts were examined with a scanning laser confocal microscope (TCS SP2; Leica, Heidelberg, Germany). Vessels were visualized by exciting with blue argon laser wavelength (488 nm) and capturing the emission between 515 and 545 nm. Horizontal optical sections (1-μm step) were obtained from the surface of the RPE-choroid-sclera complex. The deepest focal plane in which the surrounding choroidal vascular network connecting to the lesion could be identified was judged to be the floor of the lesion. Any vessel in the laser-treated area and superficial to this reference plane was judged as CNV. The area of CNV-related fluorescence was measured using ImageJ software. The summation of the whole fluorescent area in each horizontal section was used as an index for the volume of CNV.

Immunohistochemistry

At the indicated time points after CNV induction, eyes were enucleated and embedded in optimal temperature cutting medium (OCT; Tissue Tek; Sakura Finetek, Torrance, CA). During the enucleating and embedding procedure, the spatial orientation of the eye was preserved to identify CNV lesions of particular interest. Serial sections of 8-μm thickness were cut using a cryostat. Immunohistochemistry was performed as previously described. Antibodies were as follows: rabbit anti–CD31 (Dako, Carpinteria, CA); rabbit anti–mouse CD11b, rat anti–mouse Ly-6G (Gr-1), and rat anti–mouse CD31 (BD Biosciences, Franklin Lakes, NJ); rabbit anti–mouse glial fibrillary acidic protein (GFAP; Dako, Carpinteria, CA); and rabbit anti–mouse Ki-67 (ab15580; Abcam, Cambridge, MA). The Ki-67 index was calculated as the ratio of Ki-67+/nuclei divided by the total number of DAPI+/nuclei in the CNV complex. Endothelial cell proliferation index was calculated as the number of Ki-67+/CD31+ cells divided by the total number of DAPI+/CD31+ cells.

TdT-Mediated UTP Nick-End Labeling Assay

TUNEL staining was performed using an apoptosis detection kit (Apoptag Fluorescein In Situ Apoptosis Detection Kit; S7110; Chemicon International, Temecula, CA) according to the manufacturer’s instructions and was observed under a fluorescence microscope.

Statistical Analysis

The results are expressed as mean ± SE. The Mann-Whitney U test and Kruskal-Wallis test were used to assess differences among two and three groups, respectively, in CNV size, RT, and immunohistochemical cell proliferation studies. Wilcoxon signed rank test was used to assess differences among studied time points in CNV size and RT. Spearman correlation coefficient was used to analyze the relationship between RT and CNV size measured on SD-OCT and the severity of leakage on FA, and to assess the correlation between cross-sectional CNV size and CNV volume from SD-OCT and between the volume measurements using SD-OCT and choroidal flatmounts. Pearson’s χ² test was used to assess differences in unsuccessful CNV development and in lesions showing fluid frequency among laser power groups and between data obtained from two different laser operators and to assess the correlation between the severity of leakage on FA and the presence of subretinal fluid (SPSS 17.0; SPSS Inc., Chicago, IL). P < 0.05 was considered significant.

RESULTS

SD-OCT Imaging of Laser-Induced CNV Model and Correlation with Histology and Immunohistochemistry over Time

The immediate biomicroscopic evidence of laser injury is the appearance of a cavitation bubble, which indicates disruption of the Bruch’s membrane. OCT evaluation soon after laser application showed the bubble as a subretinal hyporeflective lesion that pushed the outer retinal layers upward (Fig. 1B). The bubble disappeared within several minutes. After resolution of the bubble, the retina showed a variable amount of swelling and an increase in reflectivity in the area corresponding to the laser spot and signs of subretinal fluid accumulation that appeared and regressed within the first 2 days. On day 1, we observed a butterfly-like hyperreflective reaction originating from the RPE and the outer photoreceptor layer and from the inner part of the photoreceptors and the outer plexiform layer (OPL; Figs. 1C, 2). This correlated with the histologic evaluation by hematoxylin and eosin (H&E) staining, which revealed disruption and necrosis of the outer retina, RPE, and underlygng choriocapillaris (Fig. 3, day 1). To further investigate the nature of the initial butterfly-shaped lesion, we performed immunohistochemistry for photoreceptors (anti-recoverin), TUNEL staining to assess apoptosis, and staining with anti–CD11b and anti–Gr1 antibodies to identify infiltrating macrophages and neutrophils, respectively. As seen in Figure 2, disruption and dislocation of the photoreceptors occurred 1 day after laser (Figs. 2A-C), and a substantial number of TUNEL-positive apoptotic cells were located primarily in the ONL (Figs. 3D-F). As noted by other investigators, neutrophils and macrophages infiltrated the site of injury (Figs. 2G–I). Thus, photoreceptor disruption and inflammatory cell infiltration likely contributed to the initial hyperreflective reaction seen in SD-OCT. To further assess regional retinal glial cell responses after laser injury, we performed immunostaining for GFAP (Fig. 3). On day 1, there was minimal activation of Müller cells, as seen by anti–GFAP staining. Additionally, few or no endothelial cells can be seen at the site of injury using anti–CD31 staining (Fig. 3, day 1).

On day 3, the butterfly-shaped lesion began to contract and became less reflective in its inner part. In the meantime, the OPL folded toward the ONL forming an arch pattern (Fig. 1D). At the same time, we observed increased reflectivity in the RPE and the outer photoreceptor region (Figs. 1D, 4A). This corresponded to the progression of the injury and the wound-healing response seen with H&E staining. Also evident with immunostaining was further activation of the Müller cells at the site of injury starting to extend their distal processes into the lesion, and the emergence of proliferating vascular endothelial cells confined mostly to the edges of the lesion (Fig. 3, day 3, Supplementary Fig. S1A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental). The Ki-67 and the endothelial cell proliferation index were found to be maximal at this time point and decreased thereafter (Kruskal Wallis test, P < 0.001; Supplementary Fig. S1B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental).

On day 5, the hyperreflective subretinal lesion was easily differentiated from the surrounding retina on SD-OCT (Fig. 1E) and subretinal hyporeflective areas, and intraretinal hyperreflective flecks appeared. This corresponded to a prominent subretinal fibrovascular complex seen on H&E staining (Fig. 3, day 5). SD-OCT images showed a hyperreflective layer starting to surround the lesion (Fig. 4B, black arrowhead). GFAP and CD31 immunostaining revealed Müller cells organizing their processes to form a horizontal boundary at the

Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933250/ on 06/24/2017
surface of the CNV membrane and the proliferation of endothelial cells within the neovascular complex, respectively (Fig. 3, day 5).

On day 7, SD-OCT revealed a decrease in size of the subretinal lesion and an increase in reflectivity (Fig. 1F). Hyporeflective spaces under the retina, consistent with subretinal fluid, started to dissipate from day 7 onward (Figs. 1F–H). Histologic evaluation revealed a smaller, more mature lesion surrounded by proliferating RPE cells that corresponded to the high reflectivity band seen on SD-OCT (Figs. 4C, 4D, black arrowheads). Furthermore, GFAP

\[ \text{GFAP} \]

and H11001 cells appeared to invade the fibrovascular complex (Fig. 3, day 7). Interestingly, the Ki-67 index of actively proliferating cells and the index of endothelial cell proliferation were minimal at this time point (Kruskal Wallis test, \( P < 0.001 \); Supplementary Fig. S1B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental).

The involution process of the CNV lesion and the reduction of subretinal fluid continued throughout day 14 and by day 28 were less visible by SD-OCT (Figs. 1G, 1H).

**Quantification of CNV Using SD-OCT**

**Size Evaluation.** Recently, CNV volume quantification has been used widely as an accurate tool to quantify the size of the neovascular complex. We were able to measure both the volume and the cross-sectional area of the CNV lesion with SD-OCT. For all the studied time points there was a strong correlation between CNV volume and cross-sectional area measured on SD-OCT (Spearman correlation, \( r = 0.87 \) and \( r = 0.93 \); \( P < 0.001 \); Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental). Considering these results we used the cross-sectional area as the reference SD-OCT indicator of the CNV size for this study.

**FIGURE 2.** Immunofluorescence staining of frozen sections 1 day after laser injury to identify components that contributed to the formation of the butterfly-like shaped hyperreflective reaction. Immunofluorescence using antibody against recoverin (A–C) showed significant photoreceptor disarray. TUNEL staining (D–F) revealed several TUNEL

\[ \text{TUNEL} \]

cells in the ONL and INL. Tissue necrosis and disruption triggered the infiltration of neutrophils (G–I) and CD11b

\[ \text{CD11b} \]

macrophages (J–L) to the site of injury. See Supplementary Figure S5A (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental) for nonimmune control staining. Scale bar, 100 \( \mu \)m (A–L).
Analysis of acquired images revealed the CNV size to be maximal on day 5 and decreased throughout until our last day of measurement, day 28. The biggest decline in size was observed between day 5 and day 7 (34.9%; Wilcoxon signed t-test; \( P < 0.001 \)). There was no statistically significant difference between days 7 and 14 (Wilcoxon signed t-test; \( P = 0.079 \)), whereas there was a statistically significant difference between days 7 and 28 (29.3% Wilcoxon signed t-test; \( P < 0.001 \); Fig. 5A).

In addition, there was significant correlation between the severity of leakage graded on FA with the size of CNV at days 5 and 14 (Spearman correlation, \( r = 0.761 \) [\( P < 0.001 \]) and \( r = 0.584 \) [\( P = 0.005 \)], respectively). On examining the effect of various laser powers (100, 150, and 200 mW) on CNV size, there was no significant difference in the regression pattern of the lesions, even though at each studied time point CNV size appeared to be directly dependent on the power intensity used to induce the lesion (Kruskal-Wallis test, all \( P > 0.05 \); Supplementary Fig. S2A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental).

Next, we compared CNV volume measurements obtained from SD-OCT with corresponding measurements from the choroidal flatmount preparation. At days 5 and 7, there was a fair correlation between CNV volumes measured using SD-OCT and choroidal flatmounts (Spearman correlation, \( r = 0.65 \) and \( r = 0.68 \) [\( P < 0.05 \); Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental]). However, at days 14 and 28, the two measures did not have a statistically significant correlation.

**Exudation and Retinal Thickness.** Signs of subretinal fluid accumulation that could be reliably distinguished and measured appeared starting from day 3. Measurement of the subretinal fluid revealed that the exudation reached a peak on day 5 and rapidly decreasing afterward, reaching a minimum on day 21 (Fig. 5B). The presence of subretinal fluid on SD-OCT did not correlate with the severity of leakage on FA (Pearson \( \chi^2 \) test, \( P = 0.225 \)).
There were no statistically significant differences between the three laser power groups at each time point, considering both subretinal fluid and RT (Kruskal-Wallis test, all \( P > 0.05 \); Supplementary Figs. S2B, S2C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental).

SD-OCT Evaluation of Excessive Chorioretinal Damage Produced by Laser Photocoagulation

Although the goal of laser injury was to rupture Bruch’s membrane, the optimal CNV development can be impaired when there is excessive damage to underlying choroidal or scleral tissue (Supplementary Figs. S3A–C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental). In these cases, there was discontinuity of choroidal tissue induced by the laser, resulting in dislocation of the retinal tissue to “plug” the defects as seen by SD-OCT and H&E staining (hereafter referred as chorioretinal excavations). Although these lesions were not CNV, they were difficult to differentiate using FA because chorioretinal excavations and “well-formed” CNV both show significant leakage (Supplementary Figs. S3D, S3E, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental). FA grading of the chorioretinal excavation lesions would result in a significantly higher percentage of grade 2B lesions both at day 7 (43.5% vs 78.6%, Pearson’s \( \chi^2 \) test; \( P = 0.028 \)) and at day 14 (21.7% vs 60.7%; \( P = 0.009 \)) compared with the well-formed CNV lesions (Supplementary Figs. S3F, S3G, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental). We also found that the laser power had an influence on the frequency of these chorioretinal excavations. In particular, when using 200 mW, the frequency of full-thickness chorioretinal damage was as high as 81.2%, but with 150 mW, this frequency decreased to 67.9%, and with 100 mW it dropped to 25% (Pearson’s \( \chi^2 \) test; \( P < 0.001 \); Supplementary Fig. S3H, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental). We also found that the laser power had an influence on the frequency of these chorioretinal excavations. In particular, when using 200 mW, the frequency of full-thickness chorioretinal damage was as high as 81.2%, but with 150 mW, this frequency decreased to 67.9%, and with 100 mW it dropped to 25% (Pearson’s \( \chi^2 \) test; \( P < 0.001 \); Supplementary Fig. S3H, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6266/-/DCSupplemental).

Interoperator Comparison

To assess intraoperator variability, another laser operator conducted the same series of experiments. There was no difference in size, exudation, RT, or chorioretinal excavation frequency between the two operators (Mann-Whitney \( U \) test; all \( P > 0.05 \)).

**DISCUSSION**

In this study, SD-OCT allowed a spatial and temporal in vivo analysis of the laser-induced CNV model in mice. CNV formation was monitored from its early induction, through the development of a leaking neovascular lesion, and its subsequent regression. To our knowledge, this is the first study to observe in vivo the sequence of events leading to the formation of the CNV after laser injury. We were able to identify three stages in this process that included an initial early reaction phase, a second phase of neovascular proliferation, and a third stage that involved the regression of the neovascular complex, in line with a study by Miller et al.\(^{16} \) using nonhuman primates. Nevertheless, the time course of CNV formation and regression may differ between species.

The subretinal bubble formation and hyperreflective reaction in the outer retina that are seen immediately after laser are likely to be consequences of mechanical and thermal damage.\(^{17} \) Changes in reflectivity that occur 1 day after laser injury, defined by the butterfly-like–shaped hyperreflective reaction on OCT, can be attributed to cellular necrosis and apoptosis that involve primarily the RPE/ONL, which further trigger the infiltration of inflammatory cells to the site of injury.\(^{16,17} \)
butterfly-like lesion appeared to be composed of symmetric hyperreflective reactions from the outer and inner aspects of the photoreceptor layer. This distinct shape after laser injury may arise from the pattern of thermal energy distribution to the surrounding cells, an assertion that is, however, difficult to prove experimentally. In the next 2 days, we observed a folding of the OPL toward the ONL, in an arch pattern similar to the one observed in human eyes that have undergone retinal photocoagulation.30 This folding of the OPL subsided over time and seemed to be directly correlated with the disappearance of the hyperreflective reaction in the inner part of photoreceptors and the reappearance of the ONL contour. At day 5, the outer part of the butterfly-like-shaped lesion showed an increase in reflectivity and shifted toward a well-delineated and organized subtinal material. The evolution of these OCT features may correspond to the initiation of CNV, where proliferation and accumulation of fibrovascular tissue fills the empty space generated by the laser-induced destruction of the RPE-choroidal complex.16

Another important novel observation of this study is that SD-OCT allowed the assessment of the CNV size and the exudation parameters of the neovascular complex, defined as the presence of subtinal fluid and the increase in total RT. The results from our correlation analysis supported the choice of referring to cross-sectional area values for the assessment of CNV size. This methodology was fast and reliable, and these values strongly correlated to volume measurements. We observed a significant decrease in CNV size between day 5 and day 7 after laser injury, which was further confirmed with choroidal flatmount preparation (Supplementary Fig. S4, http://www.iovs.orglookup/suppl/doi:10.1167/iov.s.10-62666/D/DCSupplemental). At these time points, there was a fair correlation between CNV volume obtained with SD-OCT and choroidal flatmounts. However, the correlation between the two methodologies was weaker at days 14 and 28 (Supplementary Table S1, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.10-62666/D/DCSupplemental). The latter can be partially attributed to a possible increased sensitivity of SD-OCT in detecting and measuring the CNV, especially when it has significantly regressed.

CNV size and exudation parameters reached a maximum at approximately day 5 after laser injury. It is interesting that in this model we did not observe any intraretinal cystic edema, which is commonly seen in humans.12 Further comparison between SD-OCT findings and FA revealed that RT and CNV size correlated well with the severity of FA leakage (grade 2B) at days 5 and 14 after laser injury. This indicated that RT and CNV size, anatomic markers of cellular infiltration, edema, and fibrosis, correlated with the functional activity of CNV on FA.

Both fluid accumulation and retinal swelling started to resolve from day 7. These events also mark the start of regression of the CNV, in which an important role appears to play the proliferation and migration of RPE cells overlying the CNV. OCT showed evidence of splitting of the RPE layer at the edges of the lesions from day 5 and is unclear if this previously described phenomenon16,21,22 explains both the resolution of subtinal fluid and the contraction of the CNV lesion. The RPE barrier may be responsible for containing and removing the previously accumulated fluid, blocking further passage of the CNV to the subtinal space and inhibiting additional proliferation of new vessels at the same time.23 This may also be, to a certain extent, responsible for the absence of correlation between subtinal fluid accumulation and severity of FA leakage.

The initiation of the regression phase of the CNV was further confirmed by the observation that a significant decrease in CNV size occurred between days 5 and 7 and continued through day 28. Supportive of these data is the finding that the Ki-67 and endothelial cell proliferation indexes were found to be decreased at day 5 and 7.

Laser injury to the retina is known to induce dramatic changes in Müller cell GFAP expression.23 In contrast to previous studies,24 the observation that GFAP expression by Müller cells increased significantly within 5 days after CNV induction is novel and also warrants discussion. It is unknown whether the activation of Müller cells contributes to the formation of the neovascular complex or results from the retinal injury and the associated inflammatory response. However, the fact that VEGF is reported to be secreted by Müller cells25 and notable activation of Müller glia by day 5 coincides with the appearance of endothelial cells within the CNV complex, favors the hypothesis that activated Müller glia contributes, at least in part, to the formation of the CNV, thereby promoting retinocchoroidal healing. On the other hand, our data show that activated Müller cells only reach the margin of the neovascular complex by day 5 and then invade it by day 7. It is tempting to speculate that activated Müller cells may also be involved in the initiation of the regression phase of the CNV. GFAP-positive cells have been identified in excised human neovascular membranes.26–29 Similar to what we observed at day 5 in mice, Lopez et al.29 described the presence of VEGF-positive and GFAP-positive cells in the adherent retinal plaque attached to the inner surface of excised human CNV lesions. Further evidence is required to clarify the role of Müller glia in the evolution of CNV.

Finally, OCT analysis showed that differences in laser power settings did not affect the results regarding the size of the lesion and the amount of exudation. However, the risk of excessive retinal and choroidal damage increased proportionally to the laser power setting used, resulting in impairment of CNV development. In the 200-mW group, the frequency of well-formed CNV lesions was <20%. Notably, the frequency of fluorescein leakage evidence was higher than the well-formed ones when the disrupted lesions were analyzed. This leakage could have originated from choroidal fibrovascular tissue at the edges of the lesion but could also have represented dye leakage from the normal choroidal vessels in the area surrounding the lesion. Moreover, the amount of subtinal fluid accumulation in these excessive lesions was significantly lower than in the well-formed CNV. Considering these findings, attention should be paid to the amount of energy delivered to the RPE/choroidal complex. Our results cannot lead to a definition of a universal energy threshold because too many variables, such as laser wavelength, spot size, and duration, were involved in the laser settings and was not feasible for inclusion in this study.

In conclusion, our study provides evidence that OCT is a valuable tool for the in vivo evaluation of the laser-induced CNV model in mice. It provides the ability to evaluate not only the size but also the exudation of the neovascular complex, an important parameter that could not be assessed with other available techniques. OCT revealed morphologic features that are common to human pathology, whose thorough study will aid in better understanding of CNV pathogenesis.

References


