

Time-Dependent Changes of Cell Proliferation After Laser Photocoagulation in Mouse Chorioretinal Tissue

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Submitted: November 20, 2014
Accepted: March 22, 2015

Citation: Lee SH, Kim HD, Park YJ, Ohn YH, Park TK. Time-dependent changes of cell proliferation after laser photocoagulation in mouse chorioretinal tissue. *Invest Ophthalmol Vis Sci*. 2015;56:2696–2708. DOI:10.1167/iov.14-16112

PURPOSE. This study investigated the time course of cell proliferation after laser photocoagulation and analyzed the cell types of proliferating cells.

METHODS. C57BL/6J mice received unilateral laser photocoagulation. Intraperitoneal bromodeoxyuridine (BrdU) injection was performed, and mice were divided into two groups according to the injection paradigm: group 1 with continuous injection and group 2 with periodic injection. Each group was again divided into four subgroups according to injection period: 0 to 3 days ($n = 11$), 0 to 7 days ($n = 14$), 0 to 14 days ($n = 6$), and 0 to 28 days ($n = 6$) after laser photocoagulation for group 1; and 0 to 3 days ($n = 11$), 4 to 7 days ($n = 6$), 8 to 14 days ($n = 6$), and 15 to 28 days ($n = 6$) after laser photocoagulation for group 2. The eyes were examined with immunohistochemistry using anti-BrdU antibody and other various antibodies for identification of proliferating cells. Manual cell counting and flow cytometry were performed for quantification.

RESULTS. In group 1, the number of BrdU⁺ cells showed marked increase during the first 3 days of laser lesioning, reaching its maximum after 7 days ($P < 0.05$). Group 2 also demonstrated peak proliferation during the first 3 days, but a significantly reduced number of BrdU⁺ cells were detected during 4 to 7 days, 8 to 14 days, and 15 to 28 days of laser treatment ($P < 0.05$). BrdU⁺ cells colocalized with CD11b, F4/80, iba1, RPE65, CD31, and glial fibrillary acidic protein (GFAP) labeling, and CD11b⁺, F4/80⁺, and iba1⁺ cells constituted the main fraction of BrdU⁺ cells.

CONCLUSIONS. Laser photocoagulation induced cell proliferation mostly during the first 3 days, and many proliferating cells were identified as inflammatory cells, RPE cells, endothelial cells, and Müller cells.

Keywords: cell proliferation, chorioretinal tissue, laser photocoagulation

Laser photocoagulation is a well-established treatment for many retinal diseases. In proliferative diabetic retinopathy, panretinal photocoagulation (PRP) has been the mainstay for the treatment of retinal neovascularization since the publication of the Diabetic Retinopathy Study in 1976, whereas focal and grid laser treatments are one main optional treatment in cases of diabetic macular edema and branch vein occlusion.^{1–3} It is believed that purposeful destruction of a significant fraction of the photoreceptors, as well as the retinal pigment epithelial layers, underlies the therapeutic benefit of laser photocoagulation.^{4–6} And yet, laser photocoagulation can sometimes induce several complications such as epiretinal membrane,⁷ proliferative fibrotic scars,⁸ and macular edema.^{9–11} The exact cellular mechanisms leading either to the beneficial effect or to the complications of laser photocoagulation still need to be elucidated.

An increasing body of research has been conducted on the histologic analysis of lesions resulting from various laser photocoagulation regimes, and several studies have described specific cellular responses to laser photocoagulation.^{6,12–16} Recently, since the primary target of the laser therapy is the RPE layer,¹⁷ many studies have focused on examining the alteration of the RPE layer occurring after laser photocoagulation such as

proliferation and migration of RPE cells.^{18–21} Because of the inherent characteristics of the lasers used for photocoagulation, however, it is nearly impossible to confine the generated thermal energy to the RPE layer. This necessarily means that surrounding cells, notably overlying photoreceptors and underlying endothelial cells in the choroid, suffer collateral damage at clinically therapeutic energy settings.^{5,22–25} Thus, the thermal damage of the laser may induce various cellular processes not only in the RPE layer, but also in the neural retina as well as the choroid.

In light of the above, we investigated the time course characteristic of cell proliferation in the chorioretinal tissue after laser treatment by examining transverse sectional images. We further identified these proliferating cells using various primary antibodies at different time points, and examined the temporal changes of proliferative activity of each cell type.

MATERIALS AND METHODS

Animals

C57BL/6J mice (OrientBio, Inc., Sungnam, Korea) at 8 weeks of age were used. The animals were housed in a temperature- and humidity-controlled room with a 12-hour light/12-hour dark cycle

and were provided with food and water ad libitum. This study was approved by the Animal Care Committee of Soonchunhyang University Bucheon Hospital (Permit Number: SCH-animal-2009015), and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, with all efforts made to minimize suffering.

Laser Photocoagulation

Animals were anesthetized by intraperitoneal injection of a mixture of zolazepam/tiletamine (Zoletil, 40 mg/kg; Virbac, Carros, France) and xylazine (Rompun, 5 mg/kg; Bayer Healthcare, Leverkusen, Germany), followed by pupil dilation with 0.5% tropicamide and 2.5% phenylephrine (Mydrin-P; Santen, Emeryville, CA, USA). Only the right eyes of mice were used for laser treatment. Laser photocoagulation (200- μ m spot size, 0.04-second duration, and 100-mW laser power) was performed with the slit lamp delivery system of a PASCAL diode laser (OptiMedica, Inc., Santa Clara, CA, USA), which provides irradiation of frequency doubled neodymium:yttrium-aluminum-garnet (Nd:YAG) laser diode with 532-nm wavelength. To neutralize corneal and lenticular dioptric power, a handheld flat-glass coverslip was used as a contact lens with application of 0.5% methylcellulose (Gentel; Novartis, East Hanover, NJ, USA) in front of mice eyes. Ten laser spots were distributed in a concentric pattern around the optic nerve head (Fig. 1A). If a lesion produced a gaseous bubble, indicating Bruch's membrane rupture or hemorrhage, the animals were discarded from the experiment. Ten mice did not receive laser treatment to serve as a control.

Fundus Fluorescence Angiography (FFA) and Infrared Reflectance (IR) Imaging

The lesioned retinas were examined by FFA and IR images using a confocal scanning laser ophthalmoscope (HRA2; Heidelberg Engineering, Heidelberg, Germany) immediately after laser photocoagulation and at day 7 of laser photocoagulation. Specifically, animals were anesthetized and pupils were dilated. Fluorescein and IR images were captured at 3 to 5 minutes after intraperitoneal (IP) injection of 0.1 mL 2% fluorescein sodium (Fluorescite; Akorn, Lake Forest, IL, USA) and used to identify any vascular leakage on laser lesion, which would indicate the formation of choroidal neovascularization.

Intraperitoneal Injection of Bromodeoxyuridine (BrdU)

Intraperitoneal bromodeoxyuridine (BrdU; 100 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA) was performed to detect cell proliferation induced by laser treatment. Mice were given IP injection of BrdU in PBS twice a day, and animals were divided into two experimental groups according to BrdU injection paradigms. The mice from group 1 received continuous IP BrdU injection from the time of laser photocoagulation to the day they were killed, and the animals from group 2 received periodic IP BrdU injection during certain time points after laser treatment. Each group was again divided into four subgroups based on BrdU injection period (1a–1d for group 1, and 2a–2d for group 2). The injection period for subgroups from group 1 were as follows: group 1a, 0 to 3 days ($n = 11$); group 1b, 0 to 7 days ($n = 14$); group 1c, 0 to 14 days ($n = 6$); and group 1d, 0 to 28 days ($n = 6$) of IP BrdU injection after laser treatment (Fig. 1B); and the injection period for subgroups from group 2 were as follows: group 2a, 0 to 3 days ($n = 11$); group 2b, 4 to 7 days ($n = 6$); group 2c, 8 to 14 days ($n = 6$); and group 2d, 15 to 28 days ($n = 6$) of IP BrdU injection after laser lesioning (Fig. 1C). The contralateral unlasered eyes with IP BrdU injection given for

3 days (laser⁻/BrdU⁺, $n = 18$) served as controls, and the additional groups of mice not receiving BrdU injection after the laser treatment (laser⁺/BrdU⁻, $n = 5$) and mice receiving neither BrdU injection nor laser treatment (laser⁻/BrdU⁻, $n = 5$) were also added as controls. Six animals in each subgroup were used for transverse section preparation, and five animals in group 1a, group 2a, and laser⁻/BrdU⁺ control group were used for whole-mount preparation. For flow cytometric analysis, eight animals from group 1b and laser⁻/BrdU⁺ control group were used.

Tissue Preparation

At the end of each injection period, mice were deeply anesthetized by IP injection of 4:1 mixture of zolazepam/tiletamine (80 mg/kg) and xylazine (10 mg/kg), and were intracardially perfused with 0.1 M phosphate buffer (PB) containing 1000 U/mL heparin, followed by 4% paraformaldehyde (PFA) in 0.1 M PB. Eyeballs were enucleated, and eye tissues were trimmed with the removal of the anterior segment and the lens by cutting through the limbal cornea. To determine the orientation of eyecups, nictitating membrane was left attached to the nasal side of the limbus. Eyecups were fixed with 4% PFA in 0.1 M PB at pH 7.4 for 2 hours. The eyecups were then transferred to 30% sucrose in PBS, incubated overnight, and then embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA). The serial sagittal sections (in 12 to 6 o'clock plane) with thickness of 10 μ m were taken from the embedded eyecups, and then mounted on adhesive microscope slides (Histobond; Marienfeld-Superior, Lauda-Königshofen, Germany). By visually scanning the all serial sections, the transverse sections with the furthest disruption at the RPE-photoreceptor junction were designated as the center of the laser lesion (Fig. 1A). For retinal whole-mount immunohistochemistry, 10 lasered eyes from group 1a and group 2a (five from each group) were dissected into posterior eyecups with the neural retina removed. The eyes were then fixed in 4% PFA and prepared as flattened whole-mounts with four equidistant cuts.

BrdU Assay

Using central transverse sections from the each laser spot, tissue DNA denaturation was done with 1 N of hydrochloric acid for 20 minutes at 37°C, and then neutralized with 0.1 M borate buffer twice, for 5 minutes each time. Antigen retrieval using 0.1% trypsin was performed for 3 minutes at 37°C, and at the end of each step, slides were washed with 0.1 M PB. The tissues were first blocked with 5% goat serum in PBS for 15 minutes at 37°C, followed by 2 hours application of primary antibodies against BrdU. The primary antibodies used in the study were a mouse anti-BrdU antibody and a rat anti-BrdU antibody at 1:200 dilution (Table). Subsequently, the samples were washed with 0.1 M PB three times, for 5 minutes each, and incubated with secondary antibodies, AlexaFluor 568-goat anti-mouse immunoglobulin G (IgG; Molecular probes, Grand Island, NY, USA) for 1 hour at room temperature. Using 0.1 M PB, samples were again rinsed three times, for 5 minutes each. The results were examined and photographed using an Axioplan microscope with $\times 200$ magnification (Carl Zeiss AG, Oberkochen, Germany) and a 1500-ms exposure time. The images were digitalized with a monochromatic charge-coupled device (CCD) camera (Axio-CamMRm; Carl Zeiss AG), and each central transverse section was captured using image-capture software (Axiovision; Carl Zeiss AG). Nomarski images were also taken for each transverse section to identify the layers of the neural retina, the RPE, the choroid, and the sclera. For the control mice without laser treatment, the images were taken from the regions corresponding to approximately the same distance from the optic nerve head at which the laser photocoagulation was performed.

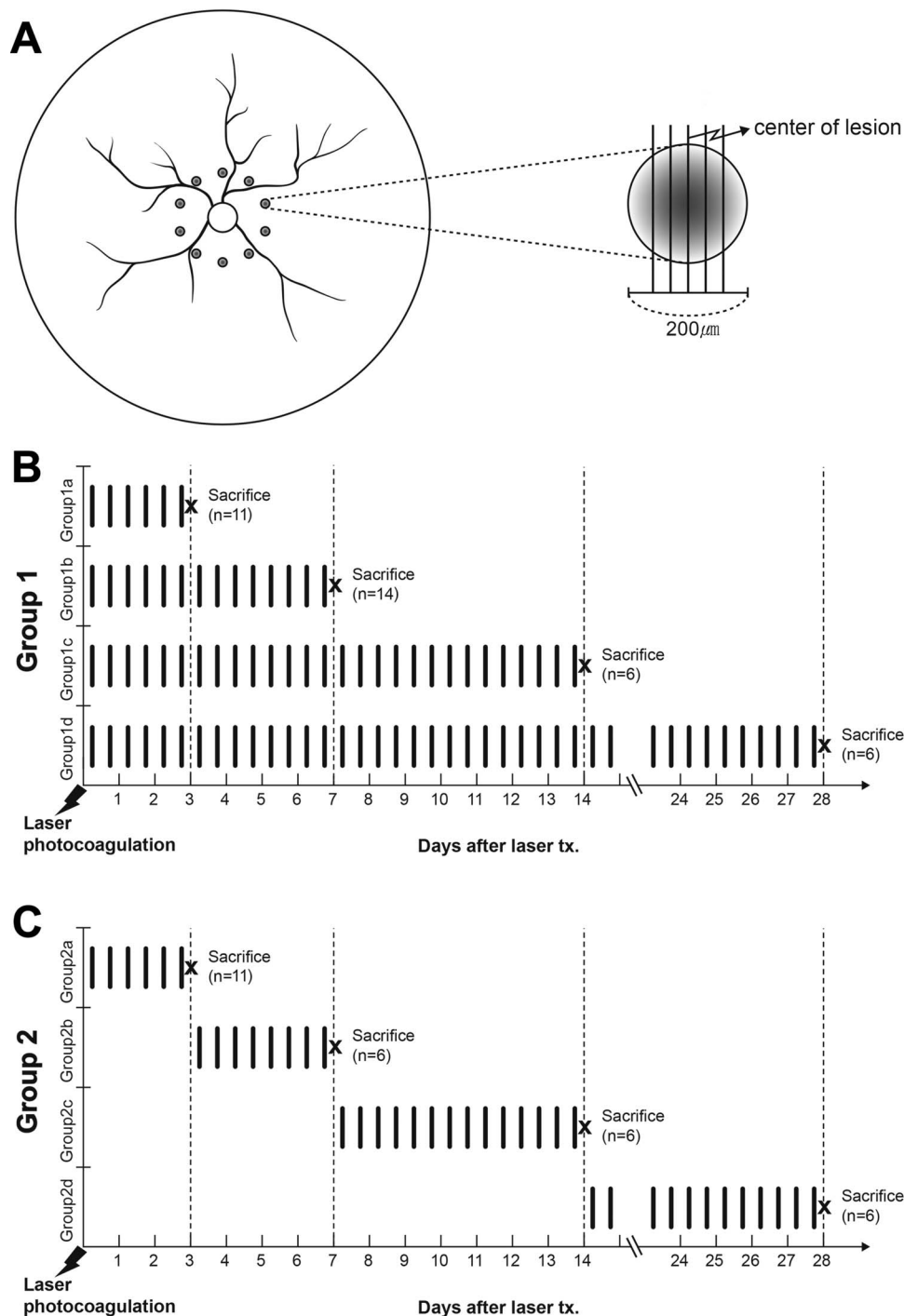


FIGURE 1. The schematics of the distribution of laser burns and the experimental settings according to the BrdU injection paradigm. The drawings demonstrate the concentric distribution of laser burns around the optic nerve head in mouse fundus and the central transverse section selected for BrdU⁺ cell counting (A). Regarding the experimental settings, group 1 was subjected to continuous IP BrdU injection from the time of laser photocoagulation to the day they were killed, which was performed at 3 days, 7 days, 14 days, and 28 days of laser lesioning (B). Group 2 received periodic IP BrdU injections at the following intervals: 0 to 3 days, 4 to 7 days, 8 to 14 days, and 15 to 28 days of laser photocoagulation, and mice were killed at the end of each injection interval (C). Laser photocoagulation was performed at day 0.

Immunohistochemistry

Immunohistochemistry with transverse sections of the retina was performed and examined with confocal microscopy (LSM510 meta; Carl Zeiss AG). For double immunostaining for BrdU with other various cell markers (Table), slides were incubated with an anti-BrdU antibody and antibodies for each cell marker at 37°C for 2 hours after DNA denaturation.

Detection of the signal was achieved by incubation with the appropriate combination of AlexaFluor 488 and AlexaFluor 568 conjugated secondary antibodies, and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.1 μg/mL; D9542; Sigma-Aldrich Corp.) stain was used to label cell nuclei with 3 minutes of incubation time. To perform double-label immunohistochemistry on whole-mount preparations, the samples

TABLE. Antibodies Used for Immunohistochemistry in This Study

Target	Host	Manufacturer	Target Cell	Catalog No.	Dilution
BrdU	Mouse	Sigma-Aldrich Corp., St. Louis, MO, USA	Proliferating cells	B8434	1:200
BrdU	Rat	Serotec, Oxford, UK	Proliferating cells	OBT0030G	1:200
CD11b	Rat	Serotec, Oxford, UK	Monocyte	MCA711G	1:2000
F4/80	Rat	Serotec, Oxford, UK	Macrophage	MCA497GA	1:2000
Iba1	Rabbit	Wako Chemicals, Osaka, Japan	Microglia	019-19741	1:1000
RPE65	Mouse	Novus Biologicals, Littleton, CO, USA	RPE	NB100-355	1:1000
CD31	Rat	BD Transduction Laboratories, Lexington, KY, USA	Endothelial cell	550274	1:200
GFAP	Rabbit	Millipore, Billerica, MA, USA	Müller cell	AB5804	1:1000

were treated as described above except that DAPI-staining procedure was omitted, and they were subsequently mounted and examined under LMS510 confocal fluorescence microscope. The images were captured using image-capture software (LSM Image Browser; Carl Zeiss AG) with $\times 200$ and $\times 400$ magnification for transverse sections, and $\times 200$ magnification for whole-mounts.

Cell Counts

To quantify the cell proliferation, the number of BrdU⁺ cells from the central transverse sections were assessed as the BrdU labeling from the central transverse section represents the proliferative activity of the whole laser lesion. BrdU⁺ cells were manually counted by two independent experienced colleagues (JRO and JWH) who were unaware of the groups of the animals. Cell count was done separately for two different layers: the neural retina/RPE and the choroid/sclera. Five laser spots with typical morphology from each eye were selected for the quantitative image analysis. Additionally, using the captured confocal images, the numbers of proliferating cells for each cell type were also evaluated through manual cell counting, and the percentages of CD11b⁺/BrdU⁺, F4/80⁺/BrdU⁺, iba1⁺/BrdU⁺, RPE65⁺/BrdU⁺, and CD31⁺/BrdU⁺ cells with respect to the total number of BrdU⁺ cells were calculated. All values are given as mean $\pm$ SD.

Flow Cytometry

To confirm the results from the manual cell counting, expression of CD11b, F4/80, iba1, and RPE65 were assessed by flow cytometry. Eight eyes (two eyes for each antibody) from group 1b were enucleated, and the posterior eyecups were separated. After incubation in 0.25% trypsin for 1 hour at room temperature, a single cell suspension was isolated from the chorioretinal tissue using a Pasteur pipette. For flow cytometric analysis of the RPE cells, the RPE layer was isolated from the chorioretinal tissue and dissociated into single cells after incubation in 0.25% trypsin. CD11b, F4/80, iba1, and RPE65 conjugated with AlexaFluor 488 were used to label inflammatory cells and RPE cells, and BrdU conjugated with AlexaFluor 647 was used to label proliferating cells. Digested tissue was collected and washed twice using 0.1% borate buffer. Eight laser⁻/BrdU⁺ eyes were used as controls. Data were acquired using an EPICS XL flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed with CellQuest software (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Using 48 IP BrdU-injected animals, one-way ANOVA with Tukey's post hoc analysis was conducted to compare the number of BrdU⁺ cells between the four subgroups in each group and the control group. The numbers of BrdU⁺ cells were compared separately for the neural retina/RPE and the

choroid/sclera. Moreover, the percentages of each proliferating cell type with respect to the total number of BrdU⁺ cells were compared between the four subgroups in each group. Statistical analysis was conducted using SPSS software version 20.0 for Windows (SPSS, Inc., Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

RESULTS

Laser Lesions

Laser burns could be identified in vivo using FFA and IR imaging immediately after laser photocoagulation and after 7 days of laser photocoagulation (Figs. 2A, 2B). In FFA images, discrete hyperfluorescent laser lesions were located concentrically around the optic nerve head without any definite leakages, and IR images showed laser sites with central hyper-reflectance surrounded by peripheral hypo-reflectance (Fig. 2A). After 7 days of laser treatment, FFA image did not reveal any hyper-fluorescences in the previously visible laser spots, indicating no choroidal neovascularization formation, while IR image demonstrated granular hyper-reflectance at the laser sites (Fig. 2B).

Cell Proliferation After Laser Photocoagulation

The eyes from the control group (laser⁻/BrdU⁻, laser⁻/BrdU⁺, and laser⁺/BrdU⁻) did not reveal any cell proliferation (Figs. 3A-C, 3E-G). On the other hand, the central transverse sections of laser burns after 3 days of laser treatment (groups 1a and 2a) demonstrated a burst of BrdU labeling indicating marked increase in cell proliferation (Fig. 3D). When merged with Nomarski image, BrdU⁺ cells were observed in both the neural retina/RPE and the choroid/sclera with more numerous BrdU⁺ cells located in the choroid/sclera than in the neural retina/RPE layer (Fig. 3H).

To determine the time course of cell proliferation after laser photocoagulation, mice were divided into two groups and further divided into four subgroups each. Group 1 reflects the accumulated number of proliferating cells at different time points at which the animals were killed for each subgroup, whereas group 2 represents the cell population undergoing proliferation during different periods of BrdU injection for each subgroup. For example, BrdU⁺ cells observed in group 2b (IP BrdU-injected mice during 4–7 days after laser photocoagulation) correspond to the cells that became proliferative during 4 to 7 days of laser treatment. The transverse sections from group 1a showed a burst of BrdU labeling around the laser site, and the number of BrdU⁺ cells leveled off until 28 days after laser photocoagulation (Figs. 4A–D). Group 2a demonstrated an identical pattern of BrdU labeling to that shown in group 1a (Fig. 4I). However, the number of BrdU⁺ cells declined markedly in group 2b (Fig. 4J), and only scant BrdU labeling was observed in group 2c and group 2d (Figs. 4K, 4L). In all

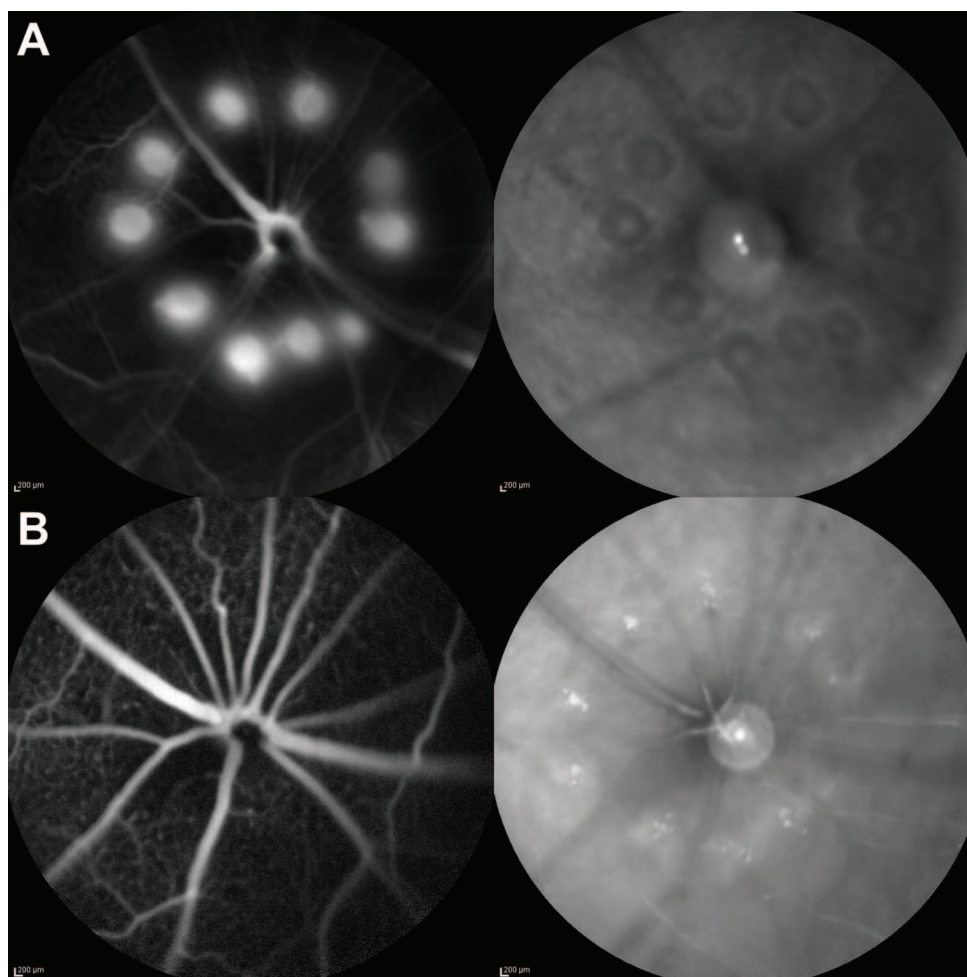


FIGURE 2. The FFA and IR image of a mouse fundus. Fundus fluorescence angiography and IR images taken immediately after laser treatment displayed 10 discrete hyperfluorescent laser spots without definite leakage (A). After 7 days of lesioning, FFA images did not show any leakage around the laser lesions, indicating no choroidal neovascularization formation, while IR images demonstrated mild hyper-reflectances at laser spots (B).

subgroups (1a-1d and 2a-2d), BrdU⁺ cells were examined mainly in the choroid/sclera, and only a minor portion of BrdU labeling was observed in the subretinal space and the RPE layer (Figs. 4E-H, M-P).

To quantify cell proliferation in the neural retina/RPE as a function of time after laser lesioning, BrdU⁺ cells in the neural retina/RPE were counted from the central transverse sections of the lesion. The values for the number of BrdU⁺ cells in the neural retina/RPE for each subgroup from group 1 (represented as mean $\pm$ SD) were as follows: group 1a, 11.70 ± 2.54 ; group 1b, 20.60 ± 4.36 ; group 1c, 20.48 ± 4.47 ; and group 1d, 20.86 ± 5.69 ; and the mean values for the number of BrdU⁺ cells in the neural retina/RPE for each subgroup of group 2 were as follows: group 2a, 11.68 ± 2.44 ; group 2b, 6.40 ± 1.75 ; group 2c, 2.22 ± 1.16 ; and group 2d, 1.76 ± 0.86 . For group 1, the increase in the number of BrdU⁺ cells in the neural retina/RPE was most prominent during the first 3 days of laser treatment (group 1a), and the cell proliferative activity significantly increased until 7 days of laser photocoagulation ($P < 0.05$). There was no statistically significant difference in the number of BrdU⁺ cells after 7 days ($P > 0.05$), reaching its maximum at day 7 and maintaining the elevated number of BrdU⁺ cells until day 28 (Fig. 5A). A similar result was found for group 2, where the peak proliferative activity was noticed in group 2a. After the peak proliferative

period, however, a significantly fewer number of BrdU⁺ cells were detected during 4 to 7 days (group 2b) and 8 to 14 days of laser treatment (group 2c) ($P < 0.05$), and the decline in the proliferative activity persisted until the period of 15 to 28 days after laser treatment without statistical significance ($P > 0.05$) (Fig. 5B).

The number of BrdU⁺ cells in the choroid/sclera was also calculated to evaluate the proliferative activity underneath the RPE layer. The mean values for the number of BrdU⁺ cells after laser treatment for each subgroup of group 1 were as follows: group 1a, 18.76 ± 2.99 ; group 1b, 25.50 ± 4.47 ; group 1c, 26.63 ± 4.18 ; and group 1d, 27.13 ± 4.83 , and the mean values for the number of labeled cells for each subgroup of group 2 were as follows: group 2a, 18.76 ± 2.99 ; group 2b, 14.33 ± 2.04 ; group 2c, 4.49 ± 1.12 ; and group 2d, 3.79 ± 1.13 . The summarized data of BrdU labeling in the choroid/sclera showed a similar temporal pattern of cell proliferation with that observed in the neural retina/RPE, although a small difference was observed. In group 1, the number of BrdU⁺ cells in the choroid/sclera showed a gradual increase until 28 days of laser treatment (Fig. 5A). Groups 2b and 2c showed markedly reduced number of BrdU⁺ cells when compared with group 2a ($P < 0.05$), which was similar to the temporal pattern of cell proliferation observed in the neural retina/RPE (Fig. 5B).

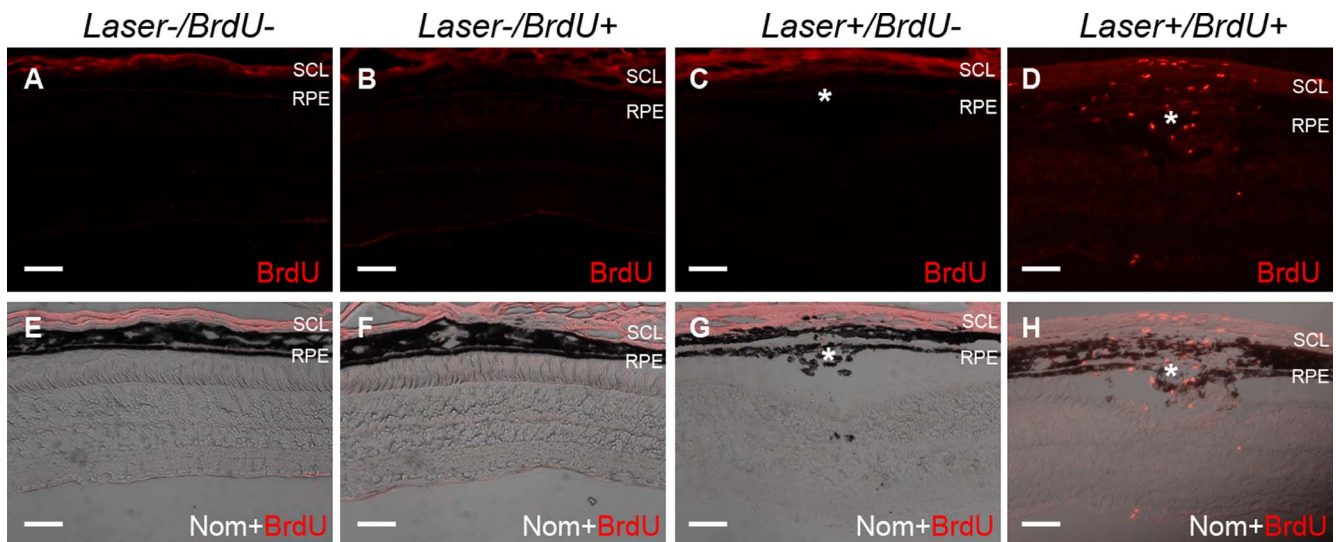


FIGURE 3. Transverse sectional images of BrdU labeling from control groups (laser⁻/BrdU⁻, laser⁻/BrdU⁺, and laser⁺/BrdU⁻) and group 1a. The transverse sections from control groups (laser⁻/BrdU⁻, laser⁻/BrdU⁺, and laser⁺/BrdU⁻) were devoid of BrdU labeling (A–C, E–G). Laser treatment induced robust cell proliferation in the mice chorioretinal tissue (D, H). SCL, sclera; Nom, Nomarski. Scale bars: 50 μ m. *Laser burn sites.

Identification of the Proliferating Cell Types

The BrdU⁺ cells, which are considered as cells with proliferative activity, were probed with various antibodies to identify the proliferating cell types. Mice from group 1a were used. To detect the change in proliferation of inflammatory cells, we performed immunolabeling for CD11b, F4/80, and iba1, which are well-characterized markers for microglia and macrophage. In the unlasered eyes, microglia and macrophage were restricted to the inner retina without any proliferative activity (Figs. 6A, 6C, 6E). After 3 days of laser photocoagulation, CD11b⁺ or F4/80⁺ cells were detected mainly in the subretinal space and in the choroid, and many BrdU⁺ cells in the neural retina/RPE and the choroid/sclera colocalized with CD11b and F4/80 expression, indicating that inflammatory cells undergo proliferative phase after laser photocoagulation (Figs. 6B, 6D). Immunolabeling for iba1, a specific marker for microglia, revealed many ramified, polarized, or amoeboid-shaped microglia in the subretinal space and in the choroid after laser photocoagulation. Double immunostaining with BrdU indicated that a considerable proportion of microglia found in the subretinal space and in the choroid had proliferative activity (Fig. 6F). Colocalization of BrdU expression with RPE65 and CD31 expression demonstrated that laser photocoagulation induced proliferation of RPE cells and endothelial cells in the choroid (Figs. 6H, 6J). Proliferating RPE cells were seen in the RPE layer as well as in the subretinal space and in the outer nuclear layer (ONL), indicating migration of RPE cells. The endothelial cells with proliferative activity were mainly seen in the choroid. Unlasered control eyes did not display any proliferative activities in the RPE layer, nor in the endothelial cells of the retina and the choroid (Figs. 6G, 6I).

To investigate the proliferative activity in Müller cells after laser photocoagulation, double immunolabeling of GFAP and BrdU was performed. This revealed a few BrdU⁺ Müller glia in the inner nuclear layer (INL) and the ONL of the lasered region, which are indicative results of Müller cell proliferation and migration after laser treatment (Fig. 6L). In the absence of laser treatment, GFAP expression was not detectable in Müller cells and was restricted to the astrocytes (Fig. 6K).

Double immunostaining with whole-mounts from group 1a revealed clusters of proliferating subretinal CD11b⁺, F4/80⁺, and iba1⁺ cells (Figs. 7B1–3, 7D1–3, 7F1–3). Laser-treated regions also demonstrated RPE65⁺/BrdU⁺ cells with the loss of

RPE cell integrity in the center of the lesion and elongation of surrounding RPE cells (Figs. 7H1–3). However, labeling of CD31 by endothelial cells was weaker than those of other cell markers, some colocalizing with BrdU labeling (Figs. 7J1–3). The control eyes were devoid of any immunoreactivity, except RPE65 (Figs. 7A, 7C, 7E, 7G, 7I).

To further investigate the proliferative activity of each cell type at different time points, we also performed double immunostaining with transverse sections from group 2b (4–7 days), group 2c (8–14 days), and group 2d (15–28 days). Colocalization of CD11b, F4/80, and iba1 expression with BrdU labeling confirmed that the proliferation of microglia and macrophages persisted until 15 to 28 days of laser photocoagulation, with markedly decreased proliferative activity (Figs. 8A–I). However, RPE65⁺/BrdU⁺, CD31⁺/BrdU⁺, GFAP⁺/BrdU⁺ cells were detected only during 0 to 3 days of laser treatment and were not visible during 4 to 7 days, 8 to 14 days, and 15 to 28 days of laser lesioning (Figs. 8J–O).

Quantitative Evaluation of Cell Types

To quantify the numbers of proliferating cells for each cell type, CD11b⁺/BrdU⁺, F4/80⁺/BrdU⁺, iba1⁺/BrdU⁺, RPE65⁺/BrdU⁺, and CD31⁺/BrdU⁺ cells were manually counted from confocal images, and the percentages of proliferating cells for each cell type with respect to the total BrdU⁺ cells were calculated. The percentages of each proliferating cell type from group 1 remained relatively constant throughout the whole experimental period (CD11b⁺/BrdU⁺: 54.75%–58.02%, F4/80⁺/BrdU⁺: 51.38%–56.87%, iba1⁺/BrdU⁺: 38.50%–43.00%, RPE65⁺/BrdU⁺: 34.99%–37.59%, and CD31⁺/BrdU⁺: 21.00%–23.00%; $P > 0.05$) (Supplementary Figs. S1A–E). For group 2, however, a slight decrease without statistical significance in the percentage of proliferative inflammatory cells was observed in group 2b, group 2c, and group 2d when compared with group 2a (CD11b⁺/BrdU⁺: 54.75% (group 2a) to 37.00% (group 2d), F4/80⁺/BrdU⁺: 51.37% (group 2a) to 35.00% (group 2d), and iba1⁺/BrdU⁺: 38.5% (group 2a) to 29.00% (group 2d); $P > 0.05$) (Supplementary Figs. S1F–H). Since markedly reduced numbers of BrdU⁺ cells were detected in groups 2b, 2c, and 2d when compared with group 2a, the percentages of proliferating cells for each cell type in groups 2b, 2c, and 2d do not indicate the exact proliferative activity. RPE65⁺/BrdU⁺ cells and

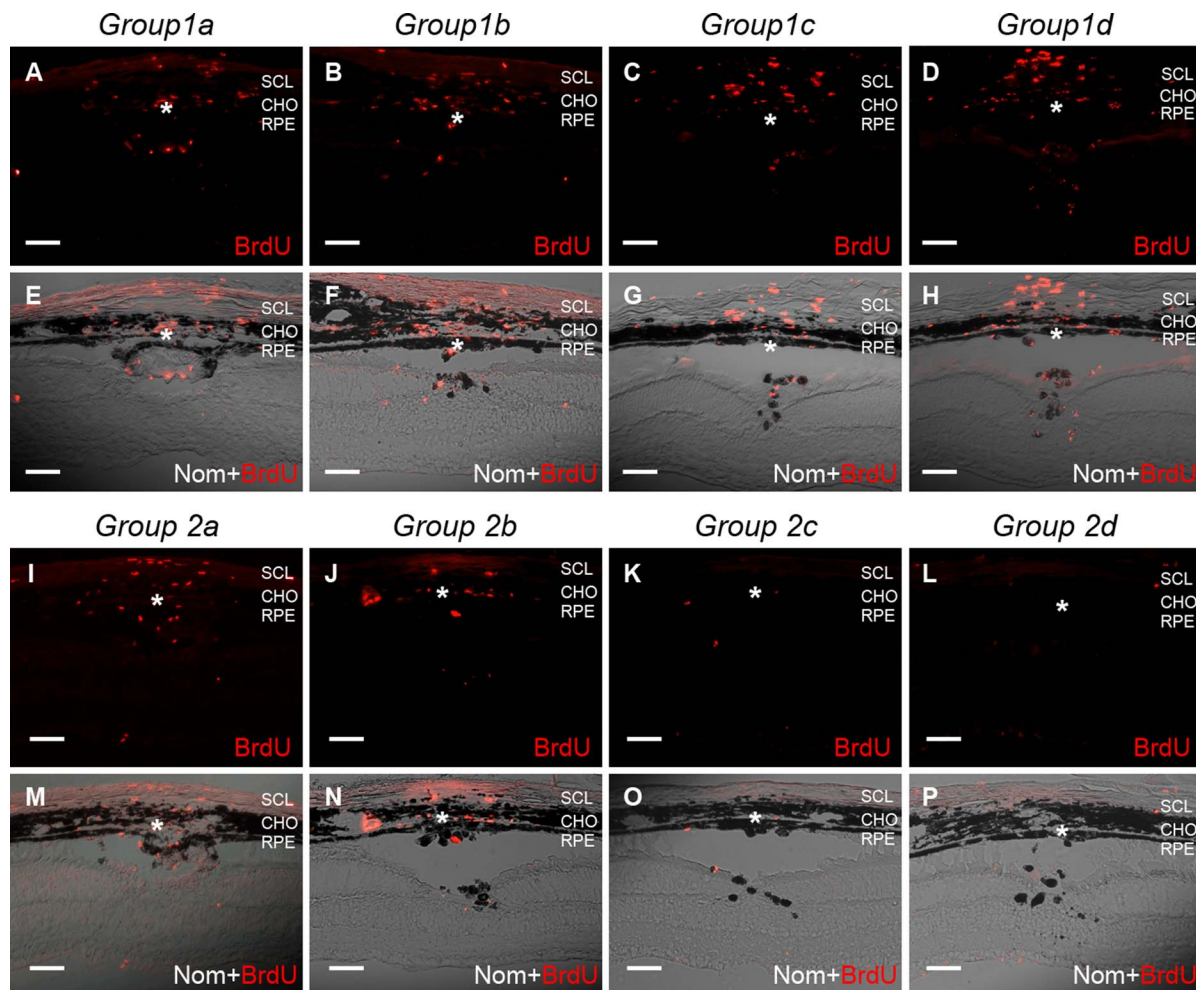


FIGURE 4. Representative images of transverse sections from four subgroups of group 1 and group 2. The four subgroups from group 1 received BrdU at each of the following intervals: group 1a, 0 to 3 days (A, E); group 1b, 0 to 7 days (B, F); group 1c, 0 to 14 days (C, G); and group 1d, 0 to 28 days after laser photocoagulation (D, H). BrdU⁺ cells were observed both in the neural retina/RPE and the choroid/sclera, with the choroid/sclera demonstrating more numerous BrdU⁺ cells than the neural retina/RPE layer. Robust BrdU expression was observed in group 1a (A, E), and the number of BrdU⁺ cells leveled off until 28 days of lesioning (B–D, F–H). The four subgroups of group 2 include the following: group 2a, 0 to 3 days (I, M); group 2b, 4 to 7 days (J, N); group 2c, 8 to 14 days (K, O); and group 2d, 15 to 28 days after laser lesioning (L, P). Merged images showed peak proliferation during the first 3 days (I, M) and markedly reduced BrdU labeling during 4 to 7 days of laser treatment (J, N). Scant BrdU labeling was observed during 8 to 14 days (K, O) and 15 to 28 days of laser lesioning (L, P). *Laser burn sites. Scale bars: 50 μ m.

CD31⁺/BrdU⁺ cells were only detected in group 2a (3.4% and 21%, respectively) and were not observed in groups 2b, 2c, and 2d (Supplementary Figs. S1I, S1J).

To confirm these results, flow cytometry was conducted. The eyes from group 1b were used for the analysis since maximal accumulated cell proliferation was observed after 7 days of laser treatment. The whole chorioretinal tissue was used for flow cytometric analysis of inflammatory cells, and the RPE layer was used for RPE cell analysis. Representative dot plots from flow cytometric analysis of the whole chorioretinal tissue and the RPE sheet after 7 days of laser treatment are shown in Supplementary Figure S2. CD11b⁺/BrdU⁺ cells and F4/80⁺/BrdU⁺ cells constituted approximately 60% of total BrdU⁺ cells (CD11b⁺: 63.2% and F4/80⁺: 61.6%, respectively), and iba1⁺/BrdU⁺ cells accounted for 43.0% of total BrdU⁺ cells. These results were similar to the results from manual cell counting. Since the RPE sheets were used for flow cytometric analysis of the RPE cells, most of BrdU⁺ cells were RPE65⁺/BrdU⁺ cells, constituting up to 96.6%. No double-positive cells were found from flow cytometry of control eyes.

Migration of RPE Cells

The fate of the migrated proliferating RPE cells observed after 3 days of laser treatment was investigated by examining the transverse sections from the later time points. A few migrated RPE cells survived until 28 days of laser treatment from the clusters of migrated proliferating RPE cells seen in the subretinal space and in the ONL during the early period of laser photocoagulation (Fig. 9). However, the transverse sections from group 2 with periodic BrdU injection did not demonstrate any migrated proliferating RPE cells after 3 days of laser photocoagulation, indicating that the proliferating RPE cells migrated into the subretinal space and the ONL during the 0 to 3 days of laser photocoagulation.

DISCUSSION

Recently, a number of published studies have described specific cellular responses to laser treatment,^{6,13–15,26} shedding more light on the effect of laser photocoagulation on the retina at the molecular basis. Among these studies, however, only a

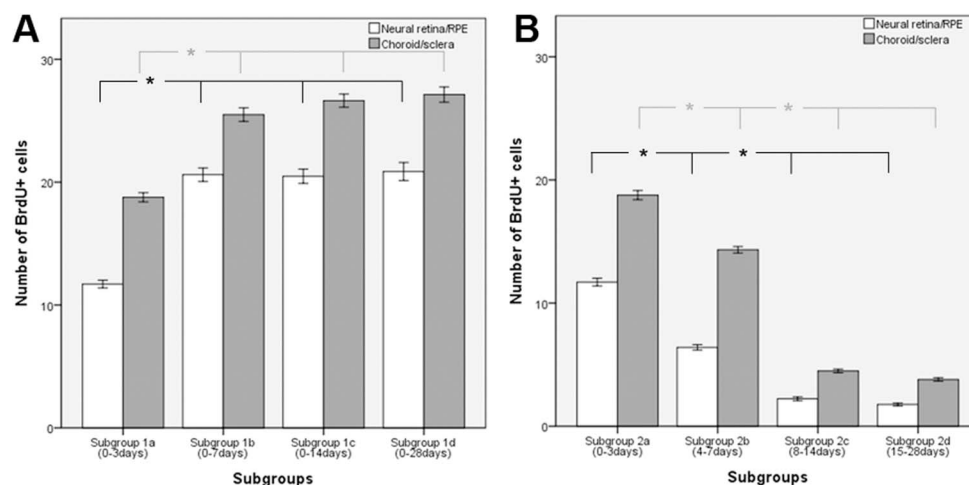


FIGURE 5. Summarized data of proliferating cells in the neural retina/RPE and the choroid/sclera. BrdU⁺ cells were counted separately for the neural retina/RPE and the choroid/sclera. In group 1, the number of BrdU⁺ cells in both the neural retina/RPE and the choroid/sclera showed marked increase during the first 3 days (group 1a). After day 7, the number of BrdU⁺ cells in the neural retina/RPE reached its maximum with significant increase from group 1a ($P < 0.05$), while the number of BrdU⁺ cells in the choroid/sclera layer showed a gradual increase until day 28 without statistical significance ($P > 0.05$) (A). However, group 2, which represents the proliferative activity during different intervals after certain periods of laser treatment, showed the peak increase in the number of BrdU-labeled cells in both the neural retina/RPE and the choroid/sclera during the 0 to 3 days interval (group 2a), and statistically significant decline in the number was observed until 14 days of laser treatment ($P < 0.05$) (B). Data are expressed as the mean $\pm$ SEM. Significant differences between the subgroups ($*P < 0.05$) are indicated: *black* and *gray lines* and *symbols* are used for the *white* and *gray diagrams*, respectively. Tested by one-way ANOVA test with Tukey's post hoc analysis.

limited number of reports have investigated the temporal changes of cell proliferation occurring after laser photocoagulation using whole-mounts¹⁸ or RPE cell lines.¹⁹ Using transverse sections, the present study provides a systematic assessment of cell proliferation changes over time in laser photocoagulation lesions of mice. Our results suggest that the photothermal and photodisruptive mechanism of RPE damage by laser treatment induces cell proliferation not only in the RPE layer itself, but also in the surrounding neural retina and in the underlying choroid and sclera. This indicates that there may be complex interactions between the multiple layers of retina and the choroid during the healing process after laser photocoagulation.

Laser-tissue interactions are affected by the wavelength, energy delivered, spot size, and duration of application.²⁷ Before conducting the present study, a preliminary assessment was performed to define the appropriate laser parameter that effectively destroys the photoreceptor cell layer without inducing Bruch's membrane rupture. In many cases, laser power higher than 100 mW or pulse duration longer than 0.04 seconds induced Bruch's membrane rupture, and the parameters used in this experimental setup (100 mW of power and 0.04 seconds duration) were found to be optimal to obtain reproducible spots with complete disruption of the photoreceptor cell layer without Bruch's membrane rupture as shown in the figures. Our recent studies have also demonstrated an adequate photocoagulation effect and successful results using the mouse and the rabbit retina with similar laser parameters.^{28,29}

After laser photocoagulation, many proliferating cells in the subretinal space and around the RPE layer were positive for CD11b, F4/80, and iba1, the major cell markers for microglia and macrophage. It was previously well documented that laser photocoagulation, along with the direct damage of the RPE and the photoreceptor layer by the thermal energy, can induce inflammatory processes such as infiltration of macrophages,^{15,30} morphologic change/migration of microglia into the lesion,^{31,32} and up-regulation of various proinflammatory cytokines and immunologic markers.^{15,26,33–37} Although differ-

ent experimental regimes were used for these previous studies, common inflammatory responses upon laser treatment appear to be evident when compared with our findings. Moreover, our study is the first coordinated investigation to demonstrate the proliferative activity in inflammatory cells induced by laser photocoagulation, which was found to constitute up to approximately 60% of the total BrdU⁺ cells.

The major novel finding demonstrated in this study is the temporal pattern of cell proliferation after laser photocoagulation. Selective reports have demonstrated that proinflammatory cytokines are upregulated during 3 days after laser treatment,^{26,37} which is entirely consistent with the peak proliferation period of inflammatory cells shown in this study. However, BrdU⁺ microglia and macrophages were also detected in group 2b, group 2c, and group 2d, constituting up to 30% to 40% of total BrdU⁺ cells. These results are indicative of inflammatory cell proliferation lasting more than 14 days after laser photocoagulation. Laser photocoagulation also caused proliferation of endothelial cells in the choroid during the first 3 days of laser lesioning. The role of these activated endothelial cells is unclear, but since endothelial cells in the choroid are known to engage in the early process of local inflammation,^{38–40} these activated endothelial cells may have engaged in recruiting inflammatory cells from the blood flow to the site of injury. Many BrdU⁺ inflammatory cells in the choroid and the sclera shown in this study may support this idea. In whole-mount preparation, however, CD31 expression was weakly detected only around the laser sites. This is probably owing to the hypopigment change of RPE cells during the healing process after laser photocoagulation, making underneath antibody detection possible.¹⁸

Laser photocoagulation also induced up-regulation of the intermediate filament GFAP, and a small portion of BrdU⁺ cells expressed GFAP. Induction of GFAP by Müller cells occurs pursuant to any pathologic situation and is considered the best characterized response of the retina to laser photocoagulation.^{6,13,14,41} Several previous studies have reported proliferation and migration of Müller cells during the early time points after laser photocoagulation, and the results from

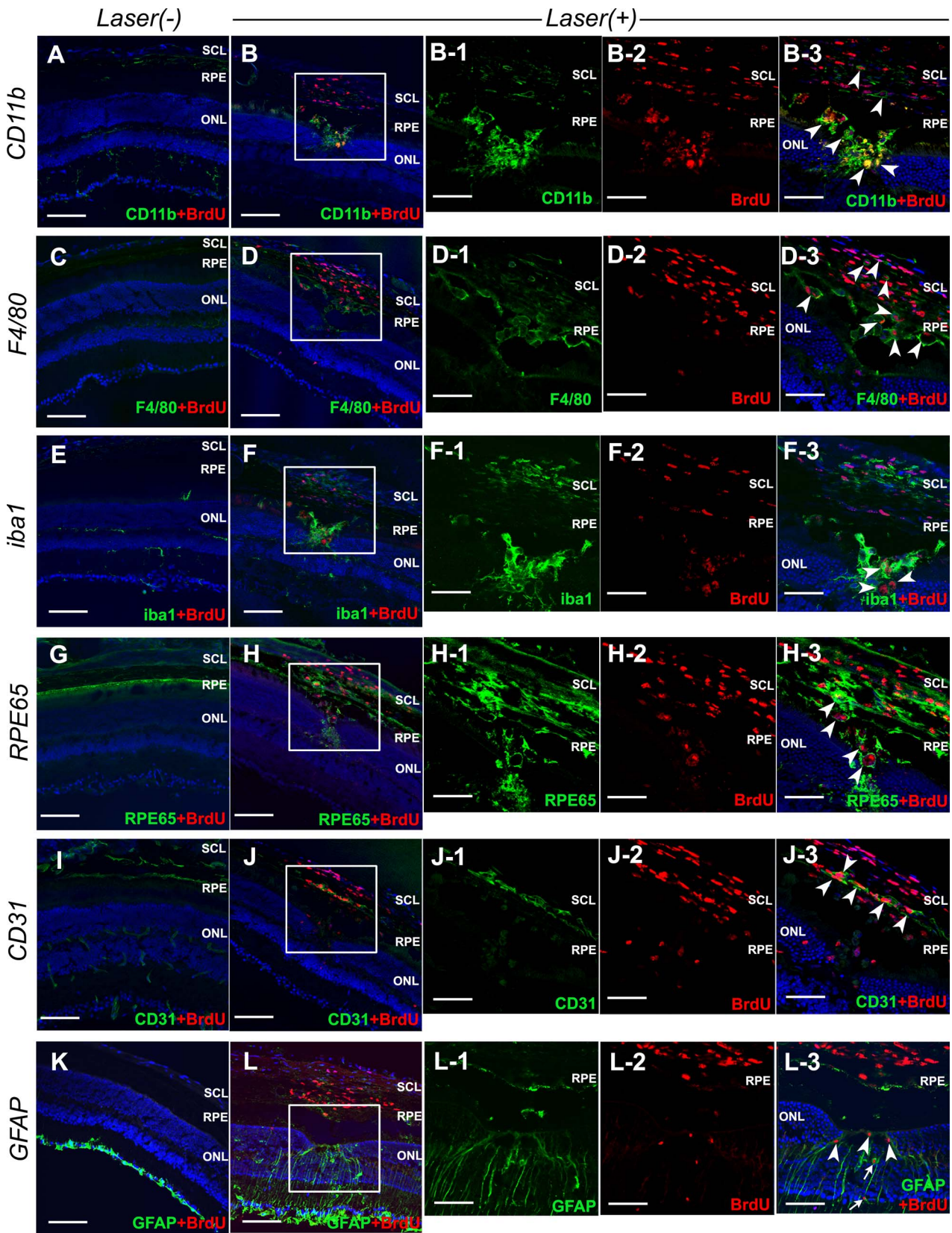


FIGURE 6. Representative confocal microscopic images from transverse sections at 3 days after laser photocoagulation immunostained with six different antibodies (CD11b, F4/80, iba1, RPE65, CD31, and GFAP; green) and BrdU (red). Transverse sections from unlasered retina did not reveal any BrdU expression (A, C, E, G, I, K). In laser-treated eyes, proliferative activities were detected in microglia and macrophage (CD11b, F4/80, and iba1) (boxes in [B, D, F]; arrowheads in original magnification $\times 2$ in [B3, D3, F3]), RPE cells (RPE65) (box in [H]; arrowheads in original magnification $\times 2$ in [H3]), and endothelial cells in the choroid (CD31) (box in [J]; arrowheads in original magnification $\times 2$ in [J3]). Moreover, a small number of proliferative Müller cells were found in the INL (box in [L]; arrows in original magnification $\times 2$ in [L3]), and a portion of these cells

migrated into the ONL at 3 days of laser photocoagulation (*arrowheads* in original magnification $\times 2$ in [B1]). DAPI was used for nuclei staining to visualize retinal layers (shown in *blue*). (A–L) *scale bars*: 100 μm ; and (B1–3, D1–3, F1–3, H1–3, J1–3, L1–3) *scale bars*: 50 μm .

this study correspond well to these earlier findings.^{13,14} Additionally, with the two different BrdU injection paradigms, this study also clearly demonstrated that the proliferative activity in Müller cells was only evident during the first 3 days of laser treatment.

Following laser photocoagulation, the proliferation of RPE cells was noted, and some of these cells migrated into the neural retina. In the present study, the temporal changes in RPE cell proliferation could be specifically analyzed by injecting BrdU during a certain period of time after laser

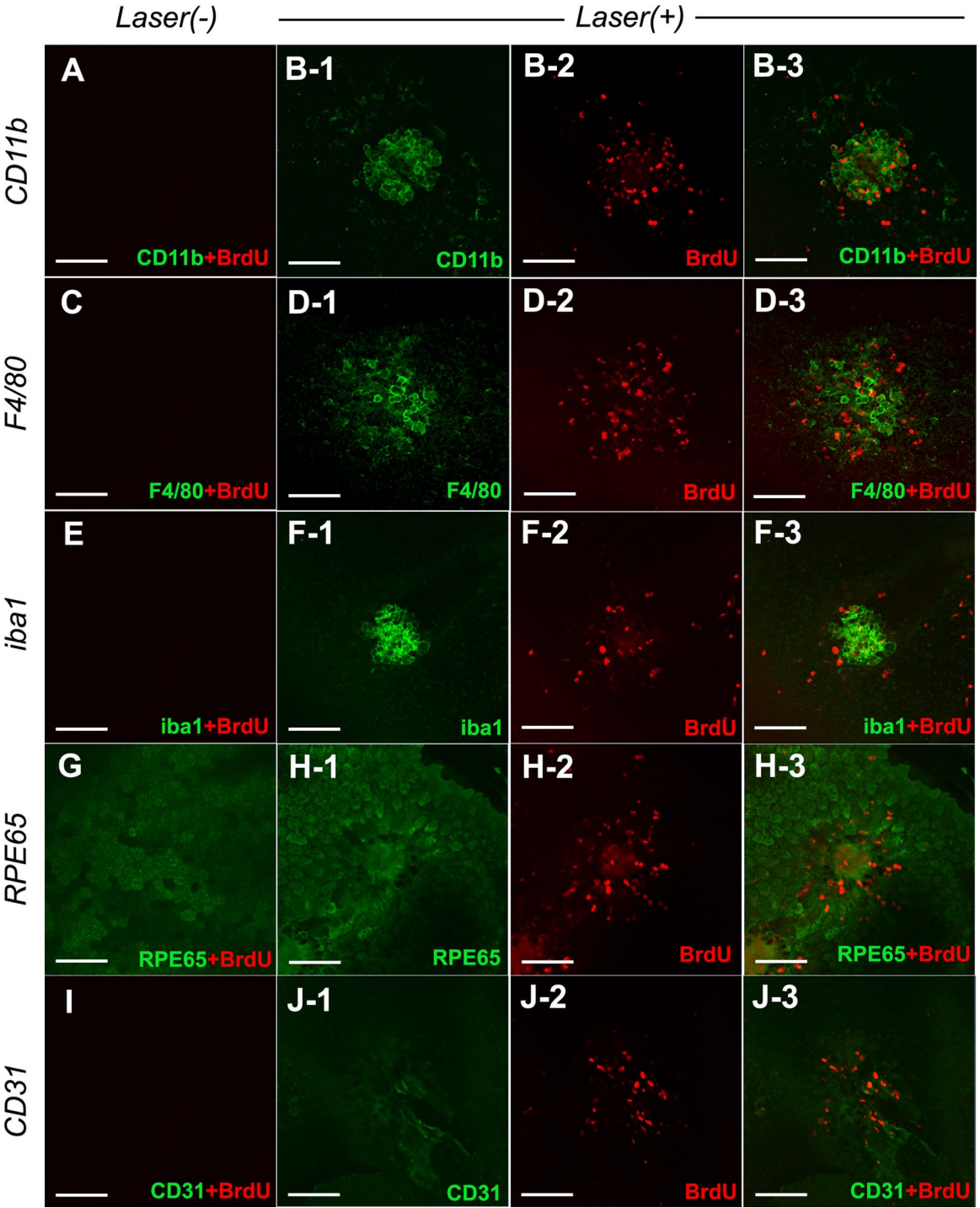


FIGURE 7. Identification of BrdU (*red*)-positive cells from whole-mount preparation at 3 days after laser photocoagulation using five different antibodies (CD11b, F4/80, iba1, RPE65, and CD31; *green*). Unlasered areas of retina did not show any BrdU expression (A, C, E, G, I). Lasered regions of retina demonstrated clusters of proliferative inflammatory cells (CD11b, F4/80, and iba1) (B1–3, D1–3, F1–3), and several proliferative RPE cells (RPE65) and endothelial cells (CD31) were also detected (H1–3, J1–3). *Scale bars*: 50 μm .

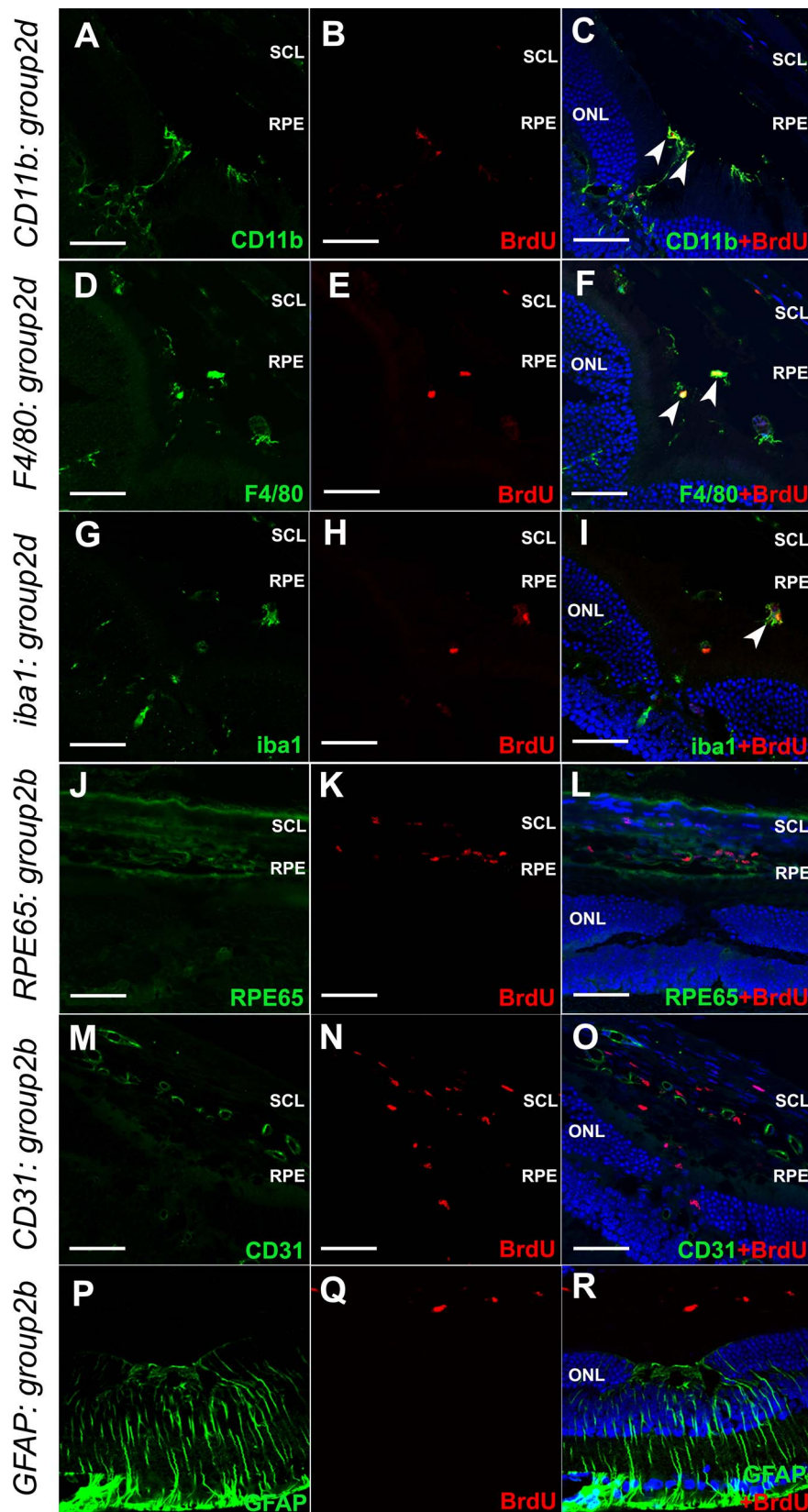


FIGURE 8. Representative images of mice from group 2b (intraperitoneal BrdU injection of 4–7 days after laser photocoagulation) and group 2d (BrdU injection of 15–28 days after laser lesioning) costained with six different antibodies (CD11b, F4/80, iba1, RPE65, CD31, and GFAP: *green*) and BrdU (*red*). Inflammatory cells (CD11b, F4/80, and iba1) underwent proliferation until 15 to 28 days of laser treatment (A–I), indicating persistent active neuroinflammation after 2 weeks of laser photocoagulation. However, RPE cells, endothelial cells, and Müller cells did not show any proliferative activity during 4 to 7 days after laser treatment (J–R). Scale bars: 50 μ m.

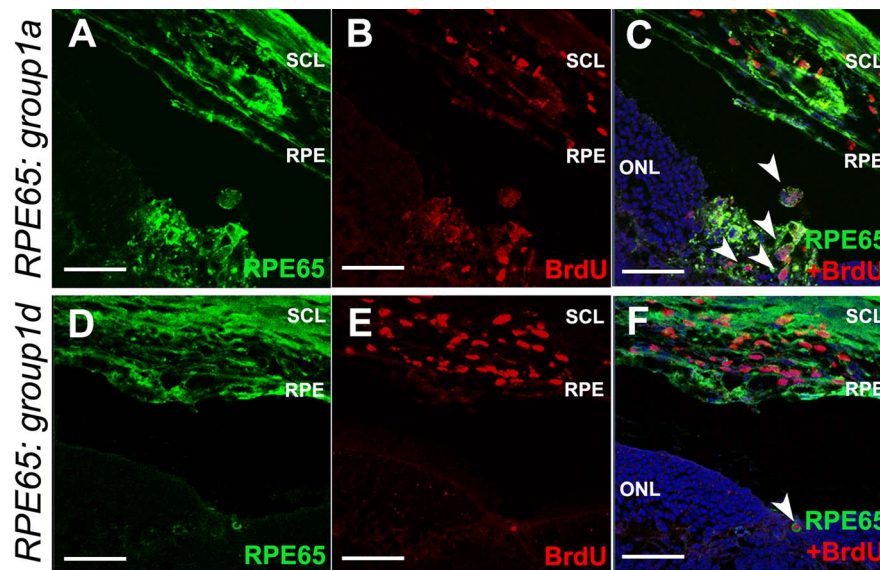


FIGURE 9. Immunolabeling for RPE cell marker RPE65 (green) and cell proliferation marker BrdU (red) at 3 days (group 1a), and 28 days (group 1d) after laser photocoagulation. Migrated proliferative RPE cells were observed in the subretinal space and the ONL at 3 days of laser photocoagulation ([C], arrowheads), and a few of these cells survived until 28 days after laser photocoagulation ([F], arrowheads). Scale bars: 50 μ m.

treatment, and the proliferative activity of the RPE cells was revealed to be reaching its maximum during the early time points after laser treatment. This finding implies that most of the proliferative activity of RPE cells takes place during the early period of retinal laser injury. Moreover, transverse sections revealed the migration of proliferative RPE cells into the subretinal space and the ONL as early as 3 days after the laser lesioning, and only a few of these migrated RPE65⁺/BrdU⁺ cells survived until 28 days of the treatment. It has been previously demonstrated that when the RPE layer suffers from a traumatic injury, RPE cells lose cell-to-cell contact, and these dislodged RPE cells undergo differentiation called epithelial-mesenchymal transition (EMT).⁴² EMT induces RPE cells to become dedifferentiated cells, and these dedifferentiated RPE cells may migrate and proliferate, thereby losing the characteristics of the RPE cells. These subsequent changes of the dislodged RPE cells after laser injury explain the decreased detection of RPE65⁺/BrdU⁺ cells during the late time points shown in this study.

In summary, this study demonstrated the temporal properties of cell proliferation in mouse chorioretinal tissue after laser photocoagulation. The results showed that most of the cell proliferation takes place during the early period of the laser treatment regardless of cell types, and that proliferation of inflammatory cells continues at least until 14 days after laser lesioning. This study may provide useful information on further evaluations of the biological processes occurring after laser photocoagulation and on investigations on the potential contribution of various cell types, especially RPE cells, to the benefits and side effects of laser photocoagulation.

Acknowledgments

The authors thank their colleagues in the Department of Ophthalmology, Soonchunhyang University, Bucheon Hospital (Jong Rok Oh and Jung Woo Han) for counting BrdU⁺ cells.

Supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the ministry of Education, Science, and Technology (Grant No. 2013R1A1A2009899), and by the Soonchunhyang University Research Fund.

Disclosure: S.H. Lee, None; H.D. Kim, None; Y.J. Park, None; Y.-H. Ohn, None; T.K. Park, None

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