Inflammatory Response after Scatter Laser Photocoagulation in Nonphotocoagulated Retina

Atsushi Nonaka,1 Junichi Kiryu,1 Akitaka Tsujikawa,1 Kenji Yamashiro,1 Kazuaki Nisbijima,1 Hiroshi Kamizuru,1 Yoshiaki Ieki,1 Kazuaki Miyamoto,1 Hirokazu Nishiwa,1 Yoshibito Honda,1 and Yuichiro Ogura2

PURPOSE. Macular edema is one of the most serious adverse effects after retinal scatter laser photocoagulation. It has been suggested that the changes in the distribution of retinal blood flow or the inflammatory reaction after photocoagulation may be involved in the pathogenesis of macular edema, but little information is available about its exact mechanism. This study was designed to evaluate quantitatively leukocyte–endothelial cell interactions and vascular permeability in the nonphotocoagulated portions of the retina after partial scatter laser photocoagulation.

METHODS. Argon laser photocoagulation was performed in one half of the retina in male pigmented rats (n = 90). In the other half of the retina, leukocyte dynamics after photocoagulation were evaluated in vivo with acridine orange digital fluorography. Retinal vessel permeability was quantified by using Evans blue dye.

RESULTS. Scatter laser photocoagulation caused significant inflammatory leukocyte–endothelial interactions not only in the photocoagulated but also in the untreated half of the retina. In the nonphotocoagulated half of the retina, the number of leukocytes rolling along the major retinal veins increased after photocoagulation and peaked at 12 hours (14.3 ± 4.5 cells/min per vessel). Leukocyte accumulation in the untreated half of the retina increased after photocoagulation, with a peak of 47.5 ± 13.0 cells/mm² 24 hours after photocoagulation. Retinal vascular permeability in the untreated half of the retina gradually increased after photocoagulation.

CONCLUSIONS. Scatter laser photocoagulation increased leukocyte rolling and subsequent accumulation in both the photocoagulated and the untreated portions of the retina. The accumulated leukocytes may be involved in the augmented vascular permeability in the untreated retina, resulting in retinal edema after photocoagulation. (Invest Ophthalmol Vis Sci. 2002;43:1204–1209)

Scatter laser photocoagulation is widely used for the treatment of various retinal diseases, such as diabetic retinopathy, retinal artery or vein occlusion, and retinopathy of prematurity. Despite the treatment’s significant suppressive effect against neovascularization, macular edema is recognized as a potential side effect of scatter photocoagulation, resulting in transient or persistent visual disturbance.1 According to a recent report by Tsujikawa et al.,2 60% of eyes treated with scatter photocoagulation showed an increase in foveal thickness, detectable by a scanning retinal thickness analyzer, after photocoagulation. In general, scatter laser photocoagulation is used outside the retinal vascular arcade, but the retina at the posterior pole often increases in thickness after laser photocoagulation. Although it has been suggested that autoregulatory changes in distribution of retinal blood flow are involved in the pathogenesis of postlaser macular edema, the exact mechanism remains unclear.

A pathogenic mechanism for the development of macular edema has been suggested to be the postlaser inflammatory reaction in the retina.1 Recently, the inflammatory factors, in addition to direct effects on junctional permeability, were shown to cause inflammatory barrier disruption by leukocyte-mediated mechanisms.3 The inflammatory factors produced in the coagulated peripheral region may induce the upregulation of endothelial adhesion molecules in the nonphotocoagulated posterior pole retina as well as the coagulated peripheral retina, resulting in leukocyte recruitment to the macula. Subsequently, accumulated leukocytes in the posterior pole may contribute to the breakdown of the retinal endothelial cell barrier, resulting in macular edema after scatter laser photocoagulation.

We have developed a method of acridine orange digital fluorography that allows us to visualize leukocytes and to evaluate quantitatively their behavior in the retinal microcirculation in vivo.4–8 Using this method, Hiroshiba et al.9 previously demonstrated impaired leukocyte flow in the untreated portion of the retina after partial scatter laser photocoagulation. We hypothesized that accumulated leukocytes in the nonphotocoagulated posterior pole would play a role in the pathogenesis of macular edema after scatter laser photocoagulation. In the present study, after application of scatter laser photocoagulation in one half of the retina, we evaluated quantitatively the inflammatory leukocyte–endothelial cell interactions in the untreated half of the retina. In addition, we also investigated the vascular permeability after photocoagulation in the untreated half of the retina by means of a recently reported sensitive method in which Evans blue is used to quantitate blood-retinal barrier breakdown.10

METHODS

Animal Model

All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male pigmented Long-Evans rats, weighing 200 to 250 g (n = 90), were anesthetized with a mixture (1:1) of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. A contact lens was

From the 1Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan; and the 2Department of Ophthalmology, Nagoya City University Medical School, Nagoya, Japan.

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Corresponding author: Junichi Kiryu, Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan; kiryu@kuhp.kyoto-u.ac.jp.

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1204
place on the cornea to maintain transparency, and body temperature was kept at between 38 ± 0.5°C throughout the experiments.

Argon laser photocoagulation was delivered through a slit lamp biomicroscope equipped with a 90-D fundus lens. Scatter laser burns were placed in half of the retina and the other half was left untreated. The laser treatment was applied at the following settings: spot size, 100 μm; duration, 0.05 seconds; and power, 40 mW.9 The spots were placed 1 or 1.5 spot diameters peripherally from the optic disc. Only one eye was treated in each animal.

**Acridine Orange Digital Fluorography**

At 4, 12, 24, 48, and 168 hours after 400 spots of photocoagulation were applied, acridine orange digital fluorography was performed. Six eyes of six different rats were examined at each time point. To examine a dose response with total number of photocoagulation in leukocyte dynamics, acridine orange digital fluorography was performed at 24 hours after 100, 200, and 300 spots of photocoagulation. Six eyes of six different rats were examined for each amount of photocoagulation. Six rats without photocoagulation served as the control.

Acridine orange digital fluorography has been described in detail.5,6 Acridine orange (0.1% solution in saline; Wako Pure Chemicals, Osaka, Japan) was injected continuously through a catheter inserted into the tail vein for 1 minute at a rate of 1 mL/min. The fundus was observed with a scanning laser ophthalmoscope (Rodenstock Instruments, Munich, Germany) in the 405 nm field for 5 minutes. Because the dye emits a green fluorescence when it interacts with DNA, the argon blue laser was used for the illumination source. Immediately after intravenous injection of acridine orange, leukocytes were stained selectively among the circulating blood cells. Nuclei of vascular endothelial cells also were stained. At 30 minutes after injection, the fundus was observed again to evaluate leukocyte accumulation in the retinal microcirculation. The images obtained were stored on an S-VHS videotape at a rate of 30 frames/sec for further analysis.

After the laser ophthalmoscopic images were obtained, rats were killed with an overdose of anesthesia. Eyes were enucleated to determine a calibration factor to convert data measured on a computer monitor (in pixels) into real data (in micrometers).

**Image Analysis**

The video recordings were analyzed with a computer-assisted image analysis system that obtains continuous high-resolution images of fundus stained by acridine orange, as described in detail elsewhere.5,6 In brief, the system consists of a computer equipped with a video digitizer (Radius, San Jose, CA) that digitizes the video image in real time to 640 horizontal and 480 vertical pixels with an intensity resolution of 256 steps. Using this system, we evaluated the diameters of the major retinal vessels, the flux of rolling leukocytes along the major retinal veins, and the number of leukocytes that had accumulated in the retinal microcirculation. We evaluated these parameters separately in either the photocoagulated or nonphotocoagulated half of the retina.

Diameters of major retinal vessels were measured at 1 disc diameter from the center of the optic disc. Each vessel diameter was calculated as the distance between two endothelial cells stained by acridine orange on each side of the vessel. The averages of the individual arterial and venous diameters in either the photocoagulated or nonphotocoagulated half of the retina were used as the arterial and venous diameters in each rat.

Rolling leukocytes were defined as leukocytes that moved at a velocity slower than that of free-flowing leukocytes. The number of rolling leukocytes was calculated from the number of cells crossing a fixed area of the vessel per minute at a distance of 1 disc diameter from the optic disc center. The flux of rolling leukocytes was defined as the total number of rolling leukocytes along all major veins in either the photocoagulated or nonphotocoagulated half of the retina.

The number of leukocytes that accumulated in the retinal microcirculation was evaluated at 30 minutes after acridine orange injection, as described previously.5 Briefly, an observation area around the optic disc was determined by drawing a polygon surrounded by the adjacent major retinal vessels. The area was measured in pixels on a computer monitor, and the density of trapped leukocytes was calculated by dividing the number of trapped leukocytes, which were recognized as fluorescent dots, by the area of the observation region. The densities of leukocytes were calculated in four peripapillary observation areas in the nonphotocoagulated half of the fundus. The average density of individual areas was used as the number of leukocytes accumulated in the retinal microcirculation for each rat.

**Measurement of Retinal Vessel Permeability Using Evans Blue**

Retinal blood vessel leakage in rats was quantitated at 12, 24, 72, 168, and 336 hours after 400 spots of photocoagulation (n = 6 at each time point) using Evans blue dye (Sigma, St. Louis, MO), as previously described in detail.10 Albumin leakage into the retinal tissue was estimated by measuring extravasated Evans blue dye, which noncovalently binds to plasma albumin in the blood stream.11 The dye was dissolved in normal saline (30 mg/mL), sonicated for 5 minutes, and filtered through a 5-μm filter. Ninety minutes after injection of Evans blue (30 mg/kg) through a tail vein catheter, the chest cavity was opened and the left heart ventricle cannulated. Each rat was then perfused with citrate-buffered 1% paraformaldehyde (37°C) for 2 minutes to remove the dye, maintaining the physiological pressure of 120 mm Hg. The eyes were immediately enucleated and the nonphotocoagulated portions of the retina were carefully dissected with the aid of an operating microscope. Each retina was incubated in 0.3 mL formamide for 18 hours at 70°C to extract extravasated Evans blue. The extract was ultracentrifuged at a speed of 70,000 rpm for 45 minutes at a temperature of 4°C. The absorbance of the supernatant was then measured with a spectrophotometer at 620 nm, the absorption maximum for Evans blue in formamide. The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide and normalized to the wet retinal weight.

**Statistical Analysis**

Data are expressed as the mean ± SEM. The data were analyzed using analysis of variance, with posthoc comparisons tested with the Fisher protected least-significant difference test. Spearman’s rank correlation was used to relate the total number of photocoagulation applications to postlaser alterations, such as leukocyte behavior and vessel dilation. P < 0.05 was considered significant in all statistical analyses.

**RESULTS**

**Diameters of Major Retinal Vessels**

After laser photocoagulation, significant vasodilation occurred in arteries and veins in the treated half of the retina (Fig. 1A). Figure 1B shows the time course of major retinal vessel diameters in the nonphotocoagulated retina at various time points after scatter laser photocoagulation. Photocoagulation caused similar vasodilation in arteries and veins in the untreated half of the retina. In the arteries, vasodilation occurred after photocoagulation and reached a peak at 24 to 48 hours (104%–107%; P < 0.01, compared with the control value). In the veins, vasodilation was more marked than in the arteries, with venous vasodilation reaching a peak 24 to 48 hours after photocoagulation (134%–155%; P < 0.01, compared with the control value).

**Leukocyte Rolling**

Immediately after acridine orange had been infused intravenously, leukocytes were stained selectively among circulating blood cells. Laser photocoagulation induced active leukocyte-endothelial interactions along the major retinal veins in the
coagulated half of the retina (Fig. 2A). Among the many free-flowing leukocytes, some were observed to be rolling slowly along the major retinal veins, making intermittent adhesive contact with vascular endothelial cells. No rolling leukocytes were observed along any of the major retinal arteries throughout the experiments.

Similarly, photocoagulation caused active leukocyte-endothelial interactions in the untreated half of the retina (Fig. 2B). A small number of leukocytes were observed rolling along the venous walls at 4 hours after photocoagulation. The flux of rolling leukocytes substantially increased and peaked at 12 hours after photocoagulation (14.3 ± 4.5 cells/min per vessel; \( P < 0.01 \), compared with the control value). No rolling leukocytes were observed 168 hours after photocoagulation.

**Leukocyte Accumulation**

At 30 minutes after acridine orange injection, we identified the accumulated leukocytes in the retinal microcirculation as distinct fluorescent dots with the highest contrast. Very few leukocytes were seen to have accumulated in the retinal microcirculation of the control rats. Scatter laser photocoagulation induced substantial leukocyte accumulation in the untreated half of the retina. Figure 3 shows the time course of the number of leukocytes accumulated in the retinal microcirculation after photocoagulation of the other half. Whereas few leukocytes were identified in the control rats, accumulation of leukocytes began to increase with time in the nonphotocoagulated half of the retina and peaked at 24 hours after photocoagulation (47.5 ± 13.0 cells/mm²; \( P < 0.01 \), compared with the control value).

**Examination of Dose Response to Total Number of Photocoagulation Spots**

Figure 4 indicates the dose response to the total number of photocoagulation spots in vasodilation of major retinal arteries and veins in the nonphotocoagulated half of the retina at 24 hours after photocoagulation. Major retinal vessels in the untreated half of the retina showed significant vasodilation in proportion to the total number of photocoagulation spots (relative prevalence 0.83 and 0.40, \( P < 0.01 \) and \( P < 0.05 \), respectively). Similarly, inflammatory leukocyte-endothelial interactions, such as rolling (Fig. 5) and accumulation (Fig. 6) after photocoagulation in the nonphotocoagulated half of the retina, showed a dose response to the total number of photocoagulation spots (relative prevalence 0.52 and 0.54, respectively, \( P < 0.01 \)).

**Retinal Vessel Permeability in Nonphotocoagulated Retina**

Figure 7 shows retinal vascular permeability in the nonphotocoagulated half of the retina at various time points after scatter photocoagulation. Retinal vascular permeability in the untreated half of the retina increased with time after photocoagulation. As early as 12 hours after photocoagulation, retinal vascular leakage increased to 142% in the untreated half of the retina (\( P = \text{NS} \) compared with the control value). Retinal vascular leakage increased to 360% at 168 hours after photocoagulation (\( P < 0.01 \), compared with the control value).
DISCUSSION

Blood-retinal barrier breakdown after scatter photocoagulation appears to contribute to various subsequent complications, primarily postlaser macular edema. In spite of its seriousness and frequency, however, little is known about the exact mechanisms of postlaser macular edema. Edema formation after scatter laser photocoagulation usually occurs inside the vascular arcade, especially in the macula, whereas photocoagulation is performed outside the arcade vessels. So far, although impaired blood-retinal barrier function after panretinal photocoagulation has been noted previously with fluorophotometry\textsuperscript{12-14} and magnetic resonance imaging,\textsuperscript{15,16} quantitative evaluation of the spatial distribution of barrier damage in the retina has been difficult. We therefore investigated the inflammatory response in the nonphotocoagulated half of the retina after half fundus photocoagulation.

Inflammation after scatter photocoagulation spots and the number of leukocytes accumulated in the retinal microcirculation at 24 hours after photocoagulation in the untreated portions of the retina (n = 6 in each group). Data are mean ± SEM. *P < 0.05 compared with control rats.

In contrast to the beneficial role of leukocytes with immunologic and antimicrobial activity, intense examinations have revealed that they are involved in the pathogenesis of various pathogenic conditions,\textsuperscript{21} including ischemia-reperfusion injury\textsuperscript{22} and diabetic retinopathy.\textsuperscript{23,24} Under such conditions, leukocytes accumulated in the microcirculation exhibit their cytotoxic properties by blocking blood flow\textsuperscript{25} and by releasing oxygen free radicals, proteolytic enzymes such as elastase or collagenase, and various inflammatory cytokines.\textsuperscript{21} Moreover,
crovascular permeability under leukocyte extravasation, may be responsible for increased micr

...tion in the untreated portions of the retina (n = 6 at each time point). Data are expressed as a percentage of control rat retina. Data are mean ± SEM. *P < 0.05 compared with control rats.

recent evidence suggests that leukocytes play a critical role in disrupting the microvascular barrier in various organs.3,26–34 These reports have suggested that, among several phases of leukocyte–endothelial interaction mediated by distinct adhesion molecules, leukocyte–endothelial contact, such as leukocyte adhesion on the vascular endothelium and subsequent leukocyte extravasation, may be responsible for increased microvascular permeability under inflammatory conditions.

Similar to findings in other organs, in the retina, blood-retinal barrier breakdown has been suggested to occur as a consequence of leukocyte infiltration under various pathologic conditions.23,33–35 A histologic study using a rat model of experimental autoimmune uveoretinitis demonstrated that infiltrated leukocytes cause blood-retinal breakdown in ocular inflammation.35 Another study suggested that interleukin-1β-induced blood-retinal barrier breakdown is significantly inhibited by leukocyte depletion.34 In addition, Miyamoto et al.23,35,36 have reported that prevention of increased leukocyte entrapment by inhibiting intercellular adhesion molecule-1 with its antibody reduces vascular leakage induced by diabetes or VEGF injection. Thus, the cytotoxic properties of leukocytes accumulated in the retinal microcirculation after photocoagulation may contribute to increased retinal vascular permeability in the nonphotocoagulated retina. As shown in Figures 2B and 3, the numbers of rolling and accumulated leukocytes began to increase substantially after scatter photocoagulation, with respective peaks at 12 and 24 hours. So far, little information is available about changes in the earlier phase that could cause impairment of barrier function. In our study, leukocyte accumulation in the retinal microcirculation occurred during a comparatively early phase after scatter photocoagulation.

To evaluate the spatial characteristics of postlaser vascular permeability, we used Evans blue in the present study. After half fundus photocoagulation treatment, vascular permeability due to the absence of macula was assessed in the whole portions of nonphotocoagulated retina in rat. Our results demonstrated a significant increase of vascular permeability in the nonphotocoagulated portions of the retina after half fundus photocoagulation. Contrary to our results, in an immunohistochemical study, Shiga et al.36,37 suggested that there was no detectable breakdown of the inner barrier after panretinal photocoagulation. In their study, however, only 200 spots of scatter photocoagulation were applied to monkey eyes. On the other hand, our study demonstrated that a significant dose response with total number of photocoagulation spots was present in the postlaser inflammation in the retinal microcirculation. This dose response may account for some of the conflicting results reported by Shiga et al.36 and us.

As shown in Figures 1A and 1B, retinal vessels showed remarkable vasodilation after photocoagulation. The vascular endothelium simultaneously produces a number of vasodilating factors (e.g., nitric oxide and prostaglandin-I2) and vasoconstricting factors (e.g., endothelin and thromboxane-A2), and their interactions mediate vascular tone.35 However, the inflammatory stimulus upregulates inducible-nitric oxide synthetase in leukocytes, which produce large amounts of nitric oxide, in contrast to the relatively small amounts of nitric oxide produced by constitutive nitric oxide synthetase in the vascular endothelium.38 Leukocytes accumulating after scatter photocoagulation and producing nitric oxide are likely to contribute to significant vasodilation in major retinal arteries and veins. In summary, scatter laser photocoagulation increased leukocyte rolling and subsequent accumulation in both the photocoagulated and nonphotocoagulated portions of the retina after half fundus scatter photocoagulation. The accumulated leukocytes may be involved in the augmented vascular permeability in the untreated retina, resulting in retinal edema after photocoagulation.

References


