Alterations of Retinal Microcirculation in Response to Scatter Photocoagulation

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PURPOSE. To perform acridine orange digital fluorography on rats after scatter photocoagulation to investigate alterations of retinal microcirculation at the capillary level, the authors used leukocyte dynamics as a parameter.

METHODS. Twenty-five pigmented rats (Long-Evans) were studied. Argon laser photocoagulation, extending 6 disc diameters from the optic disc, was delivered to one half of the retina, and the other half was untreated. The total number of burns was 200 ± 10. Leukocyte hemodynamics in retinal microcirculation were evaluated 4, 7, 14, and 28 days after photocoagulation by acridine orange digital fluorography. The fundus image was obtained by using an argon laser in a scanning laser ophthalmoscope and was recorded on magnetic tapes at a video rate. The images were analyzed by a personal computer-based image analysis system.

RESULTS. Leukocyte velocities in the retinal capillaries were significantly decreased immediately after photocoagulation. In the laser-treated area, mean capillary leukocyte velocities were 0.73, 0.92, 1.0, and 1.3 mm/second on days 4, 7, 14, and 28, respectively (velocity in normal control animals 1.4 mm/second). In addition, leukocyte hemodynamics were compromised in the untreated retina: Mean capillary leukocyte velocities were 0.88, 1.1, 1.2, and 1.3 mm/second on days 4, 7, 14, and 28, respectively. Twenty-eight days after photocoagulation, the velocities recovered to normal values in the treated and the untreated areas of the retina.

CONCLUSIONS. Retinal capillary hemodynamics were impaired after scatter photocoagulation, and the hemodynamics in the untreated retina were also affected. Photocoagulation to the retina may influence capillary hemodynamics by diffusible chemical substances and by direct tissue injury.

Laser photocoagulation is widely used to treat various retinal diseases. Alteration of retinal blood flow induced by panretinal photocoagulation (PRP) has been investigated by various methods, such as laser Doppler velocimetry and color Doppler imaging. The hemodynamics in the microcirculation have been investigated by evaluating mean circulation time by a dye dilution technique. Arend et al. used the scanning laser technique in combination with digital image analysis to study change in the blood flow velocities in the retinal perimacular capillaries. Their results showed significant reduction in blood flow velocities in patients with diabetes. The blue-field entoptic phenomenon allows evaluation of average leukocyte velocities in the perifoveal capillaries in humans. However, few data are available regarding retinal microcirculation after scatter laser photocoagulation, particularly in the acute phase.

Various side effects occur after PRP, including macular edema, serous detachment, macular capillary nonperfusion, and arteriolar obstruction. Although pathogenic mechanisms responsible for the breakdown of the blood-retinal barrier are thought to be inflammation and altered blood flow, the cause of the side effects of PRP is not fully understood.

We have developed a technique to visualize leukocytes in the retinal microcirculation in vivo. In acridine orange fluorography, it is possible to trace the movement of individual leukocytes and to evaluate leukocyte dynamics in the retinal microcirculation objectively and quantitatively. We delivered hemifundus photocoagulation to the rat retina and assessed alterations of the retinal microcirculation at the capillary level by evaluating leukocyte dynamics.

MATERIALS AND METHODS

Animal Preparation

The experiments were performed on 25 male pigmented rats (Long-Evans, 200–250 g each) in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A mixture (1:1) of 4 mg/kg xylazine hydrochloride and 10 mg/kg ketamine hydrochloride was used for intramuscular anesthesia. Each rat was stabilized by a mouth bar. The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine eye drops. The cornea was anesthetized with drops of 0.4% oxybuprocaine hydrochloride. A contact lens that had a radius of curvature of 5 mm was placed on the cornea to
prevent it from drying and to maintain transparency throughout the experiments. Heart rates were maintained at more than 250 beats per minute, and the body temperature was kept between 37°C and 39°C throughout the experiments.

Laser Photocoagulation

Argon laser photocoagulation was delivered through a slit lamp biomicroscope equipped with a 90-diopter (D) fundus lens. Scatter burns were placed in half the retina. The other half was untreated (Fig. 1). The laser treatment was applied using the following settings: spot size, 100 μm; duration, 0.05 second; and power, 20 mW to 40 mW. The spots were white centers surrounded by gray and were comparable with those of therapeutic PRP. In Figure 2 a fundus image of the rat 4 days after photocoagulation is shown. The spots were placed 1 or 1.5 spot diameters apart, and the treated area was extended 6 disc diameters peripherally from the optic disc. The total number of burns was 200 ± 10. Only one eye was treated in each animal.

Acridine Orange Digital Fluorography

We used a membrane-permeable fluorochrome of acridine orange, which emits a green fluorescence when it binds with double-stranded DNA. The fluorescent spectrum of the acridine orange-DNA complex is similar to that of fluorescein sodium—an excitation maximum of 502 nm and an emission maximum of 522 nm. Because of this property, the fluorescence of the acridine orange-DNA complex was obtained through the filter used for fluorescein sodium. The leukocytes were selectively stained in the circulating blood cells, and the vascular endothelium was also stained.

Acridine orange (Wako, Osaka, Japan) was dissolved with saline solution to a concentration of 0.1%. The rats were catheterized, and acridine orange solution was continuously injected at a rate of 1 ml/minute. The maximum dose did not exceed 1 ml for each rat.

Retinal images were generated by a scanning laser ophthalmoscope (Rodenstock Instrument, Munich, Germany). The argon blue laser was used for excitation, equipped with the filter normally used for fluorescein sodium. The rat fundus was observed in a 40° field. The images were recorded on S-VHS videotapes at a rate of 30 frames/second. Leukocyte hemodynamics in the retinal microcirculation were evaluated on days 4, 7, 14, and 28 after photocoagulation. In addition, 0.1 ml 10% fluorescein sodium was injected after acridine orange fluorography. Fluorescein angiography was performed to determine whether capillary obstruction had occurred.

Image Analysis

The recorded images (30 frames/second) were analyzed by a personal computer-based image analysis system, which has been described in detail elsewhere. The recorded images were digitized by an analog-digital converter board (Video Vision; Radius, San Jose, CA) and were entered into a personal computer (Macintosh; Apple Computer, Cupertino, CA).

Typical flow pattern of leukocytes in the retinal circulation is as follows: Leukocytes first appear at the optic disc and flow fast through the arterioles. When they move from retinal arterioles to capillaries, their speed suddenly decreases. Leukocytes travel through capillaries at a fairly constant velocity and flow faster again when they enter the venules. An example of velocity change when a leukocyte moves from precapillary through capillary to postcapillary vessels is shown in Figure 3. This flow pattern was observed in normal rats and in laser-treated rats in our experiments. The leukocytes passing through the capillaries were determined by their flow pattern, described earlier.

After marking the center of the leukocytes on each frame, subtraction images were generated from the consecutive images by using imaging software (Adobe Photoshop; Adobe, Mountain View, CA). All stationary fluorescence from vessel epithelium or background retinal cells was subtracted, and only the fluorescence from the moving leukocytes remained throughout the procedure. The distances that the same leukocyte moved between two consecutive frames were measured in pixels, by imaging software (NIH image 1.44 shareware software; National Institutes of Health, Bethesda, MD). The total number of leukocytes investigated in the capillaries ranged from 50 to 80 in the treated and the untreated retinas of each rat. The average of the individual leukocyte velocities was calculated as a mean...
leukocyte velocity, and the averages and standard deviations of the mean leukocyte velocities were calculated in each rat. The distances were converted into real velocity, as previously reported. In brief, the eyes were enucleated after acridine orange fluorographic study, and the disc size was calculated under a microscope. The ratio of the real size to the measured value on the image was determined to convert the distance from pixels to real values. One pixel was approximately equivalent to 3.2 μm in a 40° field. Analysis of variance was used to assess the significance of the results. Error probability less than 0.05 was considered to be statistically significant. Velocity histograms were presented to show the distribution of leukocyte velocities in the capillaries. The standard deviations were obtained from all leukocyte velocities.

RESULTS

Evaluation of Capillary Flow by Using Leukocyte Dynamics

When acridine orange was injected though the catheter in the tail vein, the leukocyte dynamics were well observed. Retinal vasculature was clearly visualized in the early phase. It was possible to trace individual leukocytes moving in the retinal circulation consecutively. In Figure 4, a sequence of eight successive angiographic images are shown, tracing an individual leukocyte moving from the arteriole to the capillaries in the photocoagulated retina. The interval between two frames was 1/30 of a second. Figure 5 is a computer-assisted composite made by overlaying the successive images in Figure 4. The laser burn had no influence on the visibility of leukocyte movement. There was no apparent difference in leukocyte dynamics inside and outside the laser burns. Leukocytes moving in the overlapping retinal vessels at the laser spots were also well observed. Fluorescein angiographic study showed no retinal capillary occlusion in the laser-treated or the untreated area on any experimental day. The movement of leukocytes in the retinal circulation was most visible within the first 60 seconds. The mean leukocyte velocity in the retinal capillaries of five normal rats was 1.4 ± 0.09 mm/second (mean ± SD).

Effect of Panretinal Photocoagulation on Leukocyte Velocity in the Retinal Capillaries

The leukocyte velocities in the retinal capillaries were significantly decreased immediately after photocoagulation and then gradually recovered to normal as time elapsed. The changes in mean leukocyte velocity in capillaries in the laser-treated area were 0.73 ± 0.11 mm/second (P < 0.001), 0.92 ± 0.07 mm/second (P < 0.001), 1 ± 0.04 mm/second (P < 0.001), and 1.3 ± 0.08 mm/second (P = 0.12), on days 4, 7, 14, and 28, respectively (Fig. 6). P was calculated by comparing these measurements with the mean velocity measured in normal rats. The velocity 28 days after photocoagulation was not significantly reduced from that in the normal control animals.

Leukocyte hemodynamics were compromised in the untreated retina. The change in mean leukocyte velocities in capillaries of the untreated retinal area were 0.88 ± 0.07 mm/second (P < 0.001), 1.1 ± 0.07 mm/second (P < 0.001), 1.2 ± 0.07 mm/second (P < 0.002), and 1.3 ± 0.07 mm/second (P = 0.2), on days 4, 7, 14, and 28, respectively (Fig. 7). P was calculated by comparing the velocities in the untreated retinas with those measured in normal rats. The difference between the velocity on day 28 and that in normal control animals was not significant. The leukocyte velocities were smaller in the treated retina than in the untreated retina on days 4 (P < 0.002), 7 (P < 0.005), and 14 (P = 0.003). The difference was not significant 28 days after photocoagulation (P = 0.75).

Changes in Velocity Histograms of Leukocytes

The velocity histograms of leukocytes in the retinal capillaries are shown in Figure 8. The most frequent leukocyte velocities were 1.4 to 1.6 mm/second in control eyes and 0.6 to 0.8 mm/second in treated eyes on day 4. The variations in the velocities of all leukocytes were calculated and are described in Figure 8. An F test was performed. The variation in the velocity on day 4 was smaller than that in normal animals (P = 0.01 in the treated retina and P = 0.03 in the untreated retina). There was no significant difference between variations in velocity recorded on day 28 and those recorded in normal animals (P = 0.35 in the treated retina and P = 0.30 in the untreated retina).

DISCUSSION

An experimental rat model was used to study acute alterations of retinal circulation after scatter photocoagulation. The advantages of using the rat were that evaluating the effect of photocoagulation on the leukocyte movements in the untreated retina was easy because the retinal circulation of the rat eye is symmetric and that evaluating the early effect of scatter photocoagulation was possible because the treatment was completed at one time for one eye, whereas it usually needs several times to complete panretinal photocoagulation on the human eye.

![Figure 3. A typical pattern of leukocyte velocity change in retinal microcirculation. Leukocytes travel through capillaries at a constant velocity.](image-url)
Our results show that leukocyte dynamics may be a good marker for evaluating microcirculation in capillaries. There is heterogeneity in erythrocyte movement in capillaries. Erythrocytes move faster than leukocytes when there are no leukocytes in the capillaries. Blood cells flow in single file in capillaries because there is no room for them to flow side by side. They cannot pass leading cells. Erythrocytes catch up with leukocytes and create a train formation when leukocytes are present in a vessel. The velocity of erythrocytes equals that of leukocytes in capillaries only when leukocytes obstruct their movements. Results of an in vivo study have shown that leukocyte velocity is markedly reduced when the capillary diameter is as narrow as 4 $\mu$m to 5 $\mu$m, although leukocytes could pass through capillaries of 4-$\mu$m diameter. Leukocytes have larger diameters than capillaries, even in the normal state; therefore, the ability to deform is important for them to pass through capillary networks. Even slight changes in the conditions of the microcirculation may have critical importance in leukocyte dynamics. We detected changes
in the microcirculation correlated with changes in leukocyte velocity.

There are possible causes for decreased leukocyte velocities in capillaries: When the diameter of capillaries is reduced, the resistance to leukocytes passing through increases. Inflammation that occurs after laser photocoagulation causes increased vascular permeability and induces tissue edema, and high hydrostatic pressure then narrows the capillaries. Fluorescein leakage, which indicates hyperpermeability of vessels, is sometimes observed in fluorescein angiographic studies immediately after PRP. The leakage usually disappears within a month. The leakage of serum albumin was observed in immunohistochemical analysis in the outer retina and retinal pigment epithelium at 1, 3, and 7 days, and it disappeared within 14 days after photocoagulation. Hyperpermeability may play a major role in the reduction of leukocyte velocity because it also recovers on day 28. Capillary contraction or swelling of capillary endothelial cells induced by inflammation after laser delivery may occur, increasing resistance. This increased resistance in capillaries is mechanically induced.

Increased leukocyte-endothelium adhesion is another possible cause of decreased velocity. Free radical activity and platelet activating factor have been reported to increase after PRP. Both activate leukocytes and vascular endothelium and promote the receptor-mediated interaction. These substances are also thought to produce direct and indirect damage to vascular endothelium, leading to breakdown of the blood-retinal barrier and to leakage. Increased vascular permeability will cause serous detachment and retinal edema.

When leukocytes stiffen, it is more difficult for them to pass through the capillary network. Various inflammatory substances activate leukocytes and increase their stiffness. Those with high viscosity and low deformability have much difficulty in traversing capillaries, because they must become elongated to pass through these vessels. Increased viscosity related to inflammation induced by photocoagulation may be another cause of decreased leukocyte velocity in capillaries.

Breakdown of the blood-retinal barrier after PRP has been investigated by using magnetic resonance imaging or vitreous fluorophotometry. Various serum components are released into the vitreous, including transforming growth factor-β. In our study, leukocyte velocities in the capillaries at the untreated area were also affected. Inflammatory substances diffusing in the vitreous after the breakdown of the blood-retinal barrier may be one of the causes. As the velocity histograms in the present study show, a general decrease in leukocyte velocity resulted in decreased mean velocity in the treated and untreated retinas. This finding supports the concept that photocoagulation has a generalized effect on leukocyte behavior and is suggestive of the existence of diffusible substances. Histograms showed a reduced standard deviation in velocities in the photocoagulated retina on day 4, perhaps because of an increase in viscosity linked to inflammation after photocoagulation.

Diversion of retinal blood flow evaluated from the dynamics of red blood cells has been reported. The blood flow after scatter photocoagulation in the untreated retina was increased in the main retinal vessels, whereas other investigators reported unchanged flow in the untreated retina. Our results
show the acute decrease and gradual recovery of leukocyte velocities in treated and untreated retinas. The discrepancy may be caused by the difference in dynamics of erythrocytes in the main vessels and leukocytes in the capillaries or a difference in the time measured. It is possible that increased vascular resistance induced by capillary leukocytes may in turn cause decreased retinal blood flow in the acute phase.

Leukocyte and blood vessel characteristics in such pathologic states as diabetes are different from those in normal states. For example, leukocytes are less deformable in patients...
with diabetes and in experimental animal models, and they tend to plug capillaries. Intercellular adhesion molecule-1 is upregulated in the retina of patients with diabetes. Therefore, adhesive force between leukocyte and vascular endothelium is higher than it is in the normal state. Viscosity of whole blood and plasma and fibrinogen levels were significantly increased in diabetic cats. In addition, the autoregulatory capacity may be impaired in patients with diabetes, especially in patients who have progressive diabetic retinopathy, however, this notion is controversial. Thus the observed effects may be different and more severe in pathologic states.

The results in the present study demonstrated that retinal capillary hemodynamics were significantly impaired after scatter photocoagulation and that the hemodynamics in the untreated retina were also affected. They suggest that photocoagulation to the retina may influence capillary hemodynamics by diffusible chemical substances and by direct tissue injury.

References


