Visualization and Quantitative Analysis of Leukocyte Dynamics in Retinal Microcirculation of Rats

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Purpose. Recent studies have demonstrated that leukocytes play an important role in microcirculatory flow disturbances. A few methods are available to investigate leukocyte dynamics in retinal microcirculation. The authors explored leukocyte dynamics in the retina of rats with acridine orange digital fluorography.

Methods. Acridine orange digital fluorography produces high-resolution images from a scanning laser ophthalmoscope with the use of a fluorescent nuclear dye of acridine orange, which has been used for staining nucleic acids of cells in histochemical and cytochemical studies. The images were recorded on S-VHS tapes and were investigated with a personal computer-based image analysis system.

Results. Fluorescent leukocytes in the retinal microcirculation were visualized. Highly magnified images could be obtained because of the high dioptric power of the rat eye compared with the primate eye. It was possible to observe leukocyte deformation in narrow capillaries and nuclei of vascular endothelium. At arteriolar bifurcations, leukocytes moved preferentially into the branch with the higher flow rate. "Preferential channels" were identified in which predominantly leukocytes were delivered; the channels were characterized by high flow velocity and a straight, short capillary route. The average leukocyte velocities of the arteries, veins, and capillaries are 29.5 ± 7.3, 17.4 ± 5.3, and 1.4 ± 0.4 mm/second, respectively.

Conclusions. This study demonstrates that microcirculatory dynamics of leukocytes can be visualized and analyzed quantitatively in rats in vivo with acridine orange fluorography. This method may be a promising tool to reveal how leukocytes contribute to retinal flow disturbances under various pathologic conditions. Invest Ophthalmol Vis Sci. 1996;37:1341-1347.

Increasingly, experimental data support the concept that leukocytes are involved in tissue injury associated with blood flow disturbances.1-6 Because the diameter of a leukocyte exceeds that of the average capillary,7 leukocytes are subject to considerable deformation during passage through the capillary network. Their stiff viscoelastic properties8 and their natural tendency to adhere to vascular endothelium and subsequently to release oxygen-derived free radicals or proteolytic enzymes also cause problems.9-11 Furthermore, activated leukocytes increase blood flow resistance.12 Under normal physiological conditions, high perfusion pressure prevents leukocytes from plugging capillaries and causing vascular damage. Under pathologic conditions, such as diabetic retinopathy7 and vascular occlusive disease,13 the hemodynamic alterations attributed to leukocytes include an increase in postcapillary resistance caused by leukocyte adhesion in venules and a restriction of flow caused by capillary plugging. Therefore, an analysis of leukocyte rheology in the retinal microvascular network is of considerable clinical interest.

The hemodynamics of retinal microcirculation have been studied by various techniques. Fluorescein angiography has been useful to detect capillary leakage or complete blockage; however, it cannot reveal microcirculatory dynamics of circulating blood cells. Although laser Doppler velocimetry14 can be used to evaluate the speed of red blood cell flow in capillaries, it is not suitable for analyzing the movement of individ-
Animal Preparation

Acridine Orange Digital Fluorography was induced by intramuscular injection of a mixture with eye drops of 0.5% tropicamide and 2.5% phenylephrine hydrochloride. A contact lens was used to retain corneal clarity throughout the experiment. Heart rates and rectal temperatures were monitored. For the duration of the experiments, heart rates were maintained at more than 250 beats per minute, and body temperatures were kept between 37°C and 39°C.

Acridine Orange Digital Fluorography

We used metachromatic fluorochrome of acridine orange, a widely used probe to determine quantity and conformation of nucleic acids in biochemical and cytchemical studies. The dye emits a green fluorescence when it binds with double-stranded nucleic acids (DNAs). The spectral properties of acridine orange–DNA complexes are similar to those of sodium fluorescein, with an excitation maximum at 502 nm and an emission maximum at 522 nm. When acridine orange solution is injected intravenously, leukocytes are stained selectively among circulating blood cells. Nuclei of vascular endothelial cells also are stained.22

Acridine orange (0.1% solution in saline; WAKO, Osaka, Japan) was injected continuously through a tail vein at 1 to 2 ml/minute. The maximum dose was 10 mg/kg. Retinal images were generated by a scanning laser ophthalmoscope (Rodenstock Instrument, Munich, Germany). The argon blue laser was used for the illumination source, with a regular emission filter for fluorescein angiography. We studied the peripapillary retinal vessels with a 20° or 40° field. The obtained images were stored on S-VHS videotape (30 frames/second). Using replayed videotape images, leukocyte dynamics in the retinal vessels were studied.

MATERIALS AND METHODS

Animal Preparation

Seven pigmented Long-Evans rats, each weighing 200 to 250 g, were used. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anesthesia was induced by intramuscular injection of a mixture (1:1) of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). Pupils were dilated with eye drops of 0.5% tropicamide and 2.5% phenylephrine hydrochloride. A contact lens was used to retain corneal clarity throughout the experiment. Heart rates and rectal temperatures were monitored. For the duration of the experiments, heart rates were maintained at more than 250 beats per minute, and body temperatures were kept between 37°C and 39°C.

Data Analysis

Video recordings were analyzed by an image analysis system, which has been described in detail elsewhere,22 consisting of a personal computer (Apple Computer, Cupertino, CA) equipped with a video digitizer (Radius, San Jose, CA). The latter digitizes the video image in real time (30 frames per second) to 640 horizontal and 480 vertical pixels with an intensity resolution of 256 steps (8 bit).

Leukocyte positions were determined by interactively marking relevant points on the digitized image using imaging software (Adobe Photoshop; Adobe Systems, Mountain View, CA). To determine leukocyte velocities, the digitized images were processed by subtracting each frame from the previous one, so that only negative differences were displayed. This procedure resulted in a suppression of all stationary fluorescent structures, such as endothelial and retinal cells. Leukocyte velocities were determined in a frame-to-frame analysis of the cell position in the processed image. The center position of the leukocyte of the generated image was marked manually by a pixel on a computer monitor, and, with the use of image software (NIH Image 1.44, National Institutes of Health public domain software), the distance of the marked positions was measured in pixels as a straight line. A calibration factor was determined to convert the obtained data to the real value. In brief, three eyes of the rats were enucleated after the experiment, and the size of each optic disc was measured. The ratio between the actual size and the apparent value of the optic disc on a computer monitor was calculated. Leukocyte flow velocity was calibrated with this ratio.

At arteriolar bifurcations, leukocyte distribution was analyzed from the processed recordings by observing the distribution of 50 consecutive leukocytes from the parent vessel to each daughter vessel. The mean
Leukocyte Dynamics in Retinal Microcirculation

FIGURE 1. Numerous fluorescent particles are observed in the retinal vessels. Fluorescent leukocytes are highly visible in the artery, vein, and capillaries. Fluorescence of the vessels is minimal, and choroidal fluorescence is negligible.

leukocyte velocity in each daughter vessel was calculated by averaging velocities of 10 consecutive leukocytes. In arteriolar trees, leukocyte distribution was analyzed by counting all leukocytes passing the arteriolar trees until the number reached 200. Small arteriolar trees (those with three branches or fewer), were excluded. An arteriolar tree was divided into segments by its bifurcations. In each segment, leukocyte velocity was measured by averaging velocities of 20 consecutive leukocytes. Vessel diameters were measured from images digitized at time points when the vessels were well stained with acridine orange. A density profile was plotted across the vessel near the bifurcation (NIH Image 1.44). Each vessel diameter, in pixels, was calculated as the distance between the half-height points determined separately on each side of the density profile of the vessel image. The obtained data were converted into real values using the ratio mentioned above.

RESULTS

Acridine Orange Digital Fluorography

Fluorescent leukocytes in the retinal microcirculation were visualized (Fig. 1). Each leukocyte could be traced from the arterioles, through the capillaries, to the venules (Fig. 2). Velocity changes of a leukocyte in the microcirculation were analyzed frame by frame (Fig. 3). Additionally, individual leukocytes were recognized in the arteries and the veins in spite of their faster flow rates. All visible leukocytes, however, could not be followed consecutively in arteries and veins. Retinal vascular structures were observed simultaneously because the staining of the endothelial nucleus made the vascular walls fluorescent, whereas dye-containing plasma was less fluorescent. Background fluorescence from the choroid was negligible.

In a 20° field, more detailed observation was possible, although the thickness of the retina made it difficult to focus the image (Fig. 4). The spindle-shaped fluorescent nuclei of vascular endothelium were recognized (Fig 4). Some leukocytes had been deformed (shapes ranged from spherical to cylindrical) during the passage through the narrow and flexed capillaries. The deformation could be shown only in video play.

Leukocyte Distribution in Arteriolar Trees and Capillaries

Twenty arteriolar trees in five rats (four arteriolar trees in each rat), were selected and leukocyte distribution was analyzed. The diameter of the analyzed arterioles ranged from 9.6 to 19.2 μm. Figure 5 shows a scatterplot of the relationship between leukocyte distribution and velocity in a pair of daughter vessels divided from a parent arteriole. Fractional leukocyte number and fractional leukocyte velocity between daughter vessels showed significant correlation ($r^2 = 0.836; P < 0.0001$). Leukocytes flowed preferentially into the branch with the higher velocity at arteriolar bifurcations. Figure 6 shows an arteriolar tree with each arteriolar segment. The segments were determined by arteriolar bifurcations. Table 1 shows the distribution of 200 consecutive leukocytes that passed through the arteriolar tree observed in Figure 6. The leukocyte velocity in each arteriolar segment also is shown. According to Table 1, 149 (74.5%) of 200 leukocytes passed predominantly through one of the arteriolar routes (through route B to route E). The data suggested that “preferential channels” of leukocytes existed in the arteriolar tree. Preferential channels were identified in 6 of 16 arteriolar trees analyzed. They were likely to be straight, with high velocity and a short capillary portion. In the capillary network, leukocytes

FIGURE 2. Composite picture of consecutive digital images with a time interval of 1/30 second. Other fluorescent leukocytes were erased with the aid of computer graphic software. Long arrows indicate the direction of flow. Short arrows show the leukocyte flowing in capillaries. Open arrow indicates the leukocyte flowing in the marginal stream in the vein.
were nonhomogeneously distributed. In some capillaries, no leukocytes were recognized during the 3-minute recording period, whereas many leukocytes passed through other capillaries.

**Leukocyte Velocities**

Average leukocyte velocities were evaluated in an artery, a vein, and 40 to 50 capillaries in each rat eye (five rats) (Table 2). One hundred leukocytes were studied to determine whether each leukocyte could be followed between consecutive frames in an artery or a vein. In an artery, 92 (92%) leukocytes could be followed consecutively, whereas 95 (95%) leukocytes could be pursued consecutively in a vein. In the arteries, leukocytes moved so quickly that not all the fluorescent particles could be analyzed. The velocity of the arteries and veins ranged widely.

**DISCUSSION**

The current study demonstrated that acridine orange digital fluorography is useful to investigate leukocyte rheology in retinal microcirculation in vivo. Leukocytes were well visualized in the rats, and a detailed analysis was possible. The advantages of acridine orange digital fluorography are exclusive fluorescent leukocytes among circulating blood cells, low fluorescent particles could be analyzed. The velocity of the arterioles and veins ranged widely.

**FIGURE 3.** Leukocyte velocity changes were calculated by analyzing their movement between consecutive fluorescent dots shown in Figure 2.

**FIGURE 4.** Digital image in a 20° field. The fluorescent spindle-shaped nuclei of the endothelium or pericytes are visible along the vessels.

**FIGURE 5.** Scatterplot of fractional leukocyte number related to fractional leukocyte velocity between daughter vessels. Twenty arteriolar bifurcations in five rat eyes were analyzed. The diameter of the arterioles analyzed ranged from 9.6 to 19.2 μm. P = parent vessel, D1 and D2 = daughter vessels; n1 and n2 = number of leukocytes passed through D1 and D2; v1 and v2 = velocity of leukocyte in D1 and D2. The regression line formula, $y = -2.16 + 3.71X (r^2 = 0.836)$, demonstrates a significant correlation ($P < 0.0001$).

**FIGURE 6.** Arrows indicate the routes of the flow in an arteriolar tree. Leukocyte distribution and velocities at arteriolar bifurcations were analyzed. Leukocytes were distributed to the branch with the higher flow rate. In the arteriolar tree, "preferential channels" were identified (see Table 1.)
TABLE 1. Leukocyte Distribution and Flow Velocities at Arteriolar Bifurcations*  

<table>
<thead>
<tr>
<th>Route</th>
<th>Number (%) of Leukocytes</th>
<th>Velocity (mm/sec) (mean ± SD)</th>
<th>Vessel Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>25 (11.5)</td>
<td>6.5 ± 1.1</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>177 (88.5)</td>
<td>11.1 ± 1.4</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>14 (7)</td>
<td>3.9 ± 0.5</td>
<td>13</td>
</tr>
<tr>
<td>D</td>
<td>14 (7)</td>
<td>2.8 ± 0.9</td>
<td>10</td>
</tr>
<tr>
<td>E</td>
<td>149 (74.5)</td>
<td>6.0 ± 1.5</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td></td>
<td>23</td>
</tr>
</tbody>
</table>

* The number of leukocytes passing through each route in Figure 6 was counted until the total number of leukocytes flowing into the arteriole reached 200. Mean velocity of 20 consecutive leukocytes was calculated in each route.
† The sum number of leukocytes in C, D, and E.
‡ Stem of the arteriole.

There are two advantages of using a rat model: first, we were able to obtain highly magnified images compared to those obtained in monkeys. One pixel was approximately equivalent to 3.2 μm in a 40° field or 1.6 μm in a 20° field in the rat eye, whereas the monkey eye yielded 4 μm in a 20° field. The area focused by the laser beam may be smaller in rats than in monkeys because of the dioptric difference between species. Deformation of leukocytes was observed in the narrow capillaries. The fluorescent portion (mainly the nucleus) of the leukocytes could be recognized. However, these phenomena were demonstrated only when the tape was played back on video monitors. It was difficult to show the deformation of leukocytes in a still picture. The spindle-shaped nuclei of vascular endothelium or pericytes also were visible. Second, leukocytes were recognized as distinct dots in high flow vessels, such as arterioles and venules. It was possible to investigate frame-by-frame movements of leukocytes in retinal microcirculation. We did not synchronize the results by electrocardiography. The measured velocities were average over cardiac cycles. Leukocyte flow velocities could be measured reliably in the arterioles and venules, as well as in the capillaries. Leukocyte distribution in arteriolar trees could be analyzed. Leukocyte movements in arteries and veins also were visualized, though all leukocytes could not be followed consecutively. The velocity of the arteries and veins ranged widely, possibly as a result of the pulsatile nature of the flow or of cell location across vessels. Leukocytes flowing closer to the axis are bound to flow faster, whereas leukocytes flowing in the marginal stream tend to be slower.

Limitations of acridine orange fluorography are as follows:

1. Digital processing using the scanning laser ophthalmoscope is restricted spatially and temporally, as described elsewhere. In brief, the spatial resolution theoretically is approximately 10 μm as a laser beam is focused to a 10-μm spot at the retina in humans. Spatial resolution, however, will be higher in the rat eye, as mentioned. Recorded scanning laser ophthalmoscope images are restricted temporally by video rates (30 frames per second). The measurement of leukocyte velocity in high flow vessels, such as arteries and veins, is especially difficult. In arteries and veins, fluorescent particles sometimes disappeared when consecutive frames were analyzed. In an artery, 92% of the analyzed leukocytes could be followed consecutively, whereas 95% could be followed consecutively in a vein. The lack of sharpness could have been caused by motion artifact, specifically motion between two lines of the scanning raster or scattering of light when the leukocytes are located deep inside the vessels. In addition, in large vessels, because of the absorption of excitation light by hemoglobin, the penetration would be limited. Therefore, acridine orange-labeled leukocytes moving at the center or near the back wall of the vessels may not be visualized as well as those moving near the front wall (near the vitreous). The relationship between leukocyte flow velocity and mean blood flow cannot be established with the current technique because this technique could not provide the information on the flow of erythrocytes or plasma.

2. Scanning laser ophthalmoscopy produces only two-dimensional fundus images. Retinal vessel structures, however, have three dimensions; vascular passage also changes vertically to the retinal plane. In our method, this vertical vector of leukocyte velocity was not considered.

TABLE 2. Leukocyte Flow Velocities in Artery, Vein, and Capillary*  

<table>
<thead>
<tr>
<th>Number of Leukocytes</th>
<th>Leukocyte Velocity (mm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum</td>
</tr>
<tr>
<td>Artery</td>
<td>80</td>
</tr>
<tr>
<td>Vein</td>
<td>160</td>
</tr>
<tr>
<td>Capillary</td>
<td>240</td>
</tr>
</tbody>
</table>

* Leukocyte flow velocities were measured in an artery (15 to 20 leukocytes), a vein (30 to 35 leukocytes), and 40 to 50 capillaries in each rat eye (5 rats).
3. In the rat eye, the aberration of light prevents light from focusing on the peripheral retina, because the lens encompasses such a large volume compared to the total eye. Only peripapillary retinal circulation could be observed.

4. Acridine orange is suggested to be a mutagenic and carcinogenic agent. The phototoxicity also has been noted after exposure of acridine orange-stained cells to light. We do not think this method will be applicable to humans. However, acridine orange has long been used to study leukocyte rheology in various organs.

No undesirable effect on microcirculation of the dye has been reported.

It has become evident that leukocytes are involved in blood flow disturbances associated with various vascular diseases or organ transplantation. Leukocyte entrapment in microvessels during low perfusion pressure or ischemia has been demonstrated experimentally in various organs, such as skeletal muscle, brain, lung, kidney, liver, and heart. With regard to the retina, a few recent studies have suggested that leukocytes may be involved in flow disturbances associated with experimental retinal artery occlusion and diabetic retinopathy. However, these methods were histologic approaches. Acridine orange digital fluorography allows quantitative analysis of leukocyte dynamics in retinal microcirculation in vivo.

The distribution of leukocytes at microvascular bifurcations has been studied in some tissues that are accessible to intravital microscopy. All these studies show a preferential distribution of leukocytes at arteriolar bifurcations into the branch with the higher flow rate. This feature results in a nonhomogeneous leukocyte distribution in a capillary network. Our results showed that no leukocytes were recognized in some capillaries during a 3-minute recording period, whereas many leukocytes passed through other capillaries. The results are in good agreement with observations by Ley et al, demonstrating that no leukocytes were observed during the 5-minute recording period in rat mesentery capillaries. Our results show a significant relationship between fractional leukocyte number and fractional leukocyte velocity (r² = 0.836; P < 0.0001). Preferential channels were characterized by high flow rate, straight line, and short capillary portion. Preferential channels are low-resistance pathways. Leukocyte-shunting phenomenon will work as a protective mechanism because it is effective in preventing leukocytes from entering small capillaries, where they are likely to become plugged. Leukocytes tend to flow in the central stream in fast vessels such as arterioles, and a few leukocytes are distributed into branches. If this protective mechanism is broken under low perfusion pressure, leukocytes tend to flow along the vessel walls, and more leukocytes flow into the branches. Consequently, an increased number of leukocytes enter the capillary network, which, in turn, leads to the entrapment of leukocytes in capillaries and to subsequent vascular damage.

The results of this study demonstrate that microcirculatory leukocyte dynamics can be analyzed quantitatively in rats in vivo with acridine orange fluorography. Highly magnified images of the rat retina allow detailed information on retinal microcirculation.

Key Words
acridine orange digital fluorography, blood flow, leukocyte rheology, preferential channel, scanning laser ophthalmoscope

References
Leukocyte Dynamics in Retinal Microcirculation


