Comparative Flow Velocity of Erythrocytes and Leukocytes in Feline Retinal Capillaries

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**Purpose.** To study the flow velocity of erythrocytes and leukocytes in the same retinal capillaries in cat eyes.

**Methods.** Blood cells from cats were stained with fluorescent label and reinjected into the animals. An electro-optical device combined with a highly sensitive CCD camera detected the fluorescent signal from the labeled blood cells. The signal was stored on a videotape (VHS-PAL, 25 frames per second) and later analyzed digitally. Twenty leukocytes were identified, each flowing through a separate retinal capillary pathway. The capillary pathways were categorized as simple and complex according to their morphologic complexity. Observations were made on 10 simple and 10 complex capillary pathways. The passages of one leukocyte and 20 erythrocytes in each capillary path were recorded and analyzed. The blood cell velocity ratio was calculated from the number of frames needed for each cell to complete a passage through the capillary pathway (higher velocity = fewer frames).

**Results.** The erythrocytes traveled faster than the leukocytes in both the short and long capillary pathways. The frames ratio (mean percent ± standard deviation) of erythrocyte–leukocyte passage in the same capillary pathway was 84.55% ± 2.0% in the short ones and 66.21% ± 7.1% in the complex ones. The main deceleration in leukocytes flow was noted in the looping parts of the complex capillary pathways.

**Conclusions.** The velocity of leukocytes is slower than that of erythrocytes in a given retinal capillary pathway. The structural complexity of a capillary pathway has a greater effect on leukocytic velocity than on erythrocytic velocity. Invest Ophthalmol Vis Sci. 1996; 37:1854–1859.

Blood flow disturbances, tissue ischemia, and consequent blindness can result from various pathologies. Greater insight into retinal hemodynamics might contribute to our understanding of these pathologies, but few methods are available for retinal blood flow measurements, and none bear the necessary precision for providing conclusive evidence. The blue-field entoptic phenomenon probably is related to the leukocyte flow in the retinal perifoveal region. Scanning laser ophthalmoscopy uses measurements of hyperfluorescent dots (leukocytes) and hypofluorescent segments (erythrocytes Rouleaux) that are visible in the perifoveal capillary net during fluorescein angiography. Because leukocytes and erythrocytes are different in structure and quantity, it is unclear whether they share retinal capillary flow parameters. Laser Doppler velocimetry is used widely for retinal blood flow measurements that usually are performed in large retinal vessels, such as a large vein near the optic disc. The blood flow correlation between the large retinal vessels and the retinal microcirculation is unknown; therefore, relying on the interpretation of these measurements for understanding the microcirculation hemodynamics is not adequate. Fluorescent blood cell angiography enables a clear differentiation to be made between labeled leukocytes, labeled erythrocytes, and plasma from the time of entry to a capillary to the time of exit. A capillary pathway begins where the blood cell flow turns into a single-cell row and ends where two cells or more flow simultaneously in the same plane. Two structurally different retinal capillary pathways were identified by us in a previous study. The current study compares the erythro-
Animal Preparation

Retinal Capillary Blood Cell Velocity was induced by 50 mg ketamine HCl given intramuscularly. A plastic tube (pediatric airway) was inserted into the animal’s mouth to fix its tongue and to secure its airway while its head was fixed to a bench. Anesthesia was maintained with intravenous ketamine HCl 0.4% (Localine; Fischer Pharmaceuticals, Israel), the eye was maintained open with a pediatric lid retractor. The pupil was dilated with tropicamide 0.5% (Mydramide; Fischer) drops. A 10-mm diameter contact lens with a 1-mm rubber rim was positioned on the center of the cornea. The rubber rim of the contact lens was fixed to the lid retractor by adjustable silk sutures. A small amount of methyl cellulose was instilled every few minutes between the contact lens and the cornea, and one drop of Localine was added every 15 minutes.

Blood Cell Labeling

PKH26-GL (Sigma, St. Louis, MO) is a molecular probe that, once incorporated into the cell membrane, becomes trapped because of its insolubility in an aqueous environment. It will neither leak into the surrounding medium nor transfer to other cells. It appears not to affect in vivo blood cell survival or cellular volume changes in response to hypotonic shock. The wavelength of peak excitation of the PKH26-GL is 551 nm, and the wavelength of peak emission is 567 nm. Ten milliliters of whole blood was collected in a syringe, and 0.5 ml of sodium citrate was used as anticoagulant. One milliliter of whole blood was diluted 1:10 with Ca- and Mg-free phosphate-buffered saline (PBS) and then washed twice in PBS (pellet + 10 ml PBS). A centrifugal force of 400g was used for 10 minutes to pellet the cells. The rest of the blood was centrifuged at 4000g to separate the cells from the plasma that was removed and mixed with 40 ml PBS. Staining solution was prepared just before use from 10 ml of diluent C (Sigma) with 80 μl stock solution of PKH26-GL. Cells were incubated with the staining solution for 2 minutes at room temperature, after which 10 ml PBS-plasma solution was added to stop the reaction. An additional 10 ml PBS-plasma was added to the mixture 1 minute later. Labeled cells were washed again twice with PBS-plasma solution and twice with PBS only. The pellet was resuspended in 4 ml PBS to be returned into the animal’s circulation.

Retinal Imaging System

The method used was described earlier. Briefly, a magnifying system combined with sets of filters to match the excitation and emission of fluorescein sodium and PKH26-GL were incorporated into it. A night-vision CCD camera detected the fluorescent signal from the labeled blood cells and transferred it to a video system for an on-line view and simultaneous recording (analog mode). By using a corneal contact lens, the instrument enables a noninvasive view of the circulating fluorescing cells. The attached stand permits proper alignment with the eye of the anesthetized animal. To avoid phototoxicity, the light intensity of an operating microscope was taken as the upper limit. Short exposures of 2 to 3 minutes were performed each time, and 30 minutes of darkness were provided for recovery between exposures to decrease further any possible light damage.

Data Analysis

A video digitizing and editing system (miro VIDEO DC1 tv; Miro Computer Products AG, Germany) was used with a personal computer (CPU-486DX4, 100 MHz; Intel) with a hard disc of 2.5 gigabytes for data storage. The video recording method used was VHSPAL, and the image acquisition rate was 25 frames per second. Fast-traveling blood cells were captured as lines of fluorescence instead of as dots because these cells had an advancing speed that was faster than the decay time of the light intensifier detector of the system. The recorded data were displayed on a computer monitor, and short video clips (up to 500 consecutive frames)—each one of them containing a complete passage of a leukocytes—were isolated (Mediastudio 1.0; Ulead Systems). The segment containing the leukocyte passage was isolated within these video clips, and each frame was transformed to a single image file (TGA format). The same procedure was followed for 10 segments of one complete erythrocyte passage in the same capillary pathway. The files were processed by an image processing software package (Photoshop 3.01; Adobe), which enabled enhancement and marking of the fluorescence marks of the individual cells in consecutive frames (Fig. 1). After superimposing consecutive frames by placing one on top of the other, the advancement of a tagged cell in the capillary path-
FIGURE 1. The movement of blood cells in a short capillary pathway. (left) Leukocyte flow through the capillary path as a combination of nine frames superimposed on each other. Note that the recorded signal was originally bright, and no computer image enhancement was necessary. (right) The flow of an erythrocyte through the same path. Only seven frames were counted (instead of nine for the leukocyte). Note the difference between the bright leukocyte and the relatively faint erythrocyte as originally observed.

way was plotted and stored as an independent file (Fig. 2). Because the capillary pathway is the same for the leukocyte and the erythrocytes and because image acquisition speed (25 frames per second) does not change, the number of frames represents the flow velocity. To compensate for possible velocity fluctuations, 20 measurements of the passage of an erythrocyte through the capillary pathway were taken: all 20 took place within 5 seconds from the onset of passage of the leukocyte in that capillary pathway. The average number of frames representing the erythrocytes' crossing of the pathway was calculated and compared to the number of frames recorded for the passage of a leukocyte. These measurements were repeated for 10 complex and 10 simple capillary pathways. The erythrocytes' passage time in these capillary pathways was calculated as a percentage of one leukocyte's passage time in the same capillary pathway.

RESULTS

The leukocytes were identified by their larger size and brighter fluorescence. Because only a few thousand leukocytes exist and become stained in 1 ml of blood (compared to $10^9$ erythrocytes), the passage of labeled leukocyte is far less apparent than that of labeled erythrocytes. Therefore, any given leukocytic passage in a fully visible capillary pathway (from terminal arteriole to collecting venule) was used as the leukocyte database ($n = 1$). Twenty of these leukocytic passages were chosen, 10 in the short, structurally simple capillary pathways (Fig. 1) and 10 in longer, structurally more complex capillary pathways (Fig. 2). The passage of a leukocyte in a capillary net is continuous and smooth. Deceleration can be observed in the parts of the pathway that loop, and acceleration can be seen in the relatively straight parts. Erythrocytes are smaller and less bright and could be detected in the field at all times. The passage of 20 labeled erythrocytes ($n = 20$) was documented in the capillary pathway that was marked for the leukocyte passage. The average number of frames for the passage of one erythrocyte passage in the capillary pathway was calculated for each of these capillary pathways. Fewer frames were counted for the passage of erythrocytes than for leukocytes (which means that the erythrocytes traveled faster) for all pathways. For erythrocytes to cross a given short capillary pathway, the average number of frames needed was $84.5\% \pm 2.0\%$ of the number of frames needed for leukocytes to cross the same pathway. For a complex pathway, this ratio was $66.2\% \pm 7.1\%$. Even the slowest erythrocytes were faster than any leukocyte in a given capillary pathway. Table 1 summarizes the results of the above measurements and the frames ratio calculations. The main location of the flow velocity deceleration for all blood cells was the looping parts of the capillary pathways. As could be expected, this phenomenon was observed mainly in the complex capillary paths in which the existence of looping partly defines the nature of these pathways. Although the image was detected on a two-dimensional video monitor, the deviation from the focal plane resulted in a fuzzy appearance of the moving fluorescing cell
FIGURE 2. The movement of blood cells in a complex capillary pathway. (top) Leukocyte flow, a combination of 18 frames. (bottom left) The flow of erythrocytes in the same capillary pathway. Cs = start of the capillary pathway; Ce = end of the capillary pathway. The erythrocyte crossed that path in only 12 frames. (bottom right) Computer enhancement of the faint signal of the labeled erythrocyte shown in Ery-O.

until its return to the focal plane level. Fine movements in the forward–backward direction enabled the determination of what was deeper and what was superficial to the focal plane.

DISCUSSION

It is widely accepted that retinal perfusion disturbances cause tissue ischemia, with consequent vision-threatening pathologies.\textsuperscript{15,16} Because erythrocytes are the main carriers of $O_2$–$CO_2$ in the circulation, it follows that tissue perfusion should be influenced by erythrocytic flow disturbances in the microcirculation. Laser Doppler velocimetry is one method used for retinal blood flow measurements. Unfortunately, this method is limited to the detection of regional blood cell flow in the retina. Although the region involved is small, the resolution of the method at the capillary level is limited; moreover, it offers no spatial resolution at the level of the retinal and the optic disc microcirculation.\textsuperscript{10} Two other techniques used in conjunction with retinal capillary blood cell flow measurements are scanning laser ophthalmoscopy\textsuperscript{7,8,17} and entoptic blue field simulation.\textsuperscript{1–6,18} Both methods are limited to the macula and the perifoveal capillary net. The source of the signal in the entoptic blue field simulation is assumed to be the movement of the leukocyte in the center of the macula. Nonetheless, the results were used for retinal blood flow assessment in diffuse retinal microvasculopathies.\textsuperscript{2–5,18} The scanning laser ophthalmoscope uses the speed of hypofluorescent\textsuperscript{17} or hyperfluorescent\textsuperscript{7,8} segments within the capillary lumen as indicators of the blood flow. It is noteworthy that the speed of the hypofluorescent segments in the perifoveal capillaries was found to be different from that of the hyperfluorescent segments\textsuperscript{10,20} The origin and nature of these hypofluorescent and hyperfluorescent segments is unclear. The data obtained by these methods should, therefore, be
TABLE 1. Number of Frames for Blood Cell Passage in a Capillary Path and Calculations of Frames Ratios

<table>
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<tr>
<th>Capillary Path</th>
<th>Structure</th>
<th>Frame-Leu (n = 1)</th>
<th>Frame-Ery (n = 20)</th>
<th>Range-Ery</th>
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Values are the number of frames captured in a complete passage of the labeled cells through a capillary pathway. S = simple capillary pathway; C = complex capillary pathway; Frame-Leu = number of frames for complete passage of labeled leukocyte (n = 1) in a capillary pathway; Frame-Ery = average number of frames of the passage of 20 erythrocytes (n = 20) through the same capillary path; Range = fewest and greatest number of frames recorded for the complete crossing of a given capillary pathway; Ery/Leu = ratio (percentage) between the number of frames needed for erythrocytes and leukocytes to cross the same capillary pathway.

The fluorescent blood cell angiographic method enables an accurate identification of the movement of an individual blood cell in the retinal capillary net. The current study showed that, under the same systemic conditions, leukocytes have a slower flow velocity than erythrocytes in a given nonmacular, capillary pathway. Results also indicate that the structural complexity of the capillary pathway affects the retinal microcirculation hemodynamics. Complex capillary pathways, characterized by their being longer and coiled (loops), showed a greater decelerating effect on the leukocytic velocity than on the erythrocytic one. The differences in size between leukocytes and erythrocytes provide a simplistic explanation for this phenomenon: When they flow into a tube (capillary) that is narrow even for erythrocytes, the larger leukocytes face greater resistance, especially in the coiled parts of the tube. Although this speculation appears reasonable, it has yet to be proved, and, until then, it is no better or worse than any other logical explanation for this observation. Furthermore, little is known about the blood cell flow in the retinal (nonperifoveal) capillary net. The source of current knowledge on retinal blood flow is mainly from large retinal vessels. Blood flow in large vessels has been analyzed in terms of nonideal fluid flow in a tube. This model seems to be applicable as long as the size of the particles (blood cells) is negligible in comparison to vessel size. It is reasonable to assume that this is not the case for capillaries, the small terminal arterioles, or collecting venules where a single blood cell occupies a significant part of the vessel diameter. It becomes necessary, therefore, to obtain the data directly from the retinal microvasculature. Because cats have no macula and because the method is limited to animals, further studies in animals that do have maculae are needed to obtain more data on macular, retinal, and optic disc capillary blood cell flow and how each of these correlates to the other.

Key Words
blood flow, capillaries, fluorescence, retina

References
1. Sinclair SH, Azar-Cavanagh M, Soper KA, Tuma RF, Mayrovitz HN. Investigation of the source of the blue