Erythrocyte and leukocyte dynamics in the retinal capillaries of diabetic mice

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Abstract

The purpose of this study was to analyse the dynamics of red blood cells (RBC) and white blood cells (WBC) in the retinal capillaries of C57BL/KS db/db mice, a genetic model of type 2 diabetes, and control mice, at different ages. Modified epifluorescence microscopy was used to analyse the capillary velocity of FITC-labeled RBC and rhodamine-labeled WBC in the retina. C57BL/KS db/db diabetic mice were compared to heterozygous non-diabetic mice at ages 8 and 18 weeks (\(n = 6\) in each group).

At 8 weeks, when hyperglycemia begins in db/db mice, no significant difference was found between average RBC and WBC velocity of the 2 groups. At 18 weeks, RBC velocity was significantly higher in diabetic mice compared to controls (1·21 \(\pm\) 0·29 versus 1·08 \(\pm\) 0·28 mm sec\(^{-1}\); \(p = 0·0003\)). No significant difference was found between WBC velocities (0·87 \(\pm\) 0·3 versus 0·85 \(\pm\) 0·3 mm sec\(^{-1}\)) even when normalized by RBC velocity values. Temporal and spatial coefficients of variation were significantly higher for WBC than RBC velocities (\(p < 0·0001\)) but were not significantly different in diabetic and control mice.

Direct measurement of RBC velocity with this new method showed that it was higher in the retinal capillaries of diabetic than control mice after 10–12 weeks of hyperglycemia, but not at the onset of hyperglycemia. This suggests that enhanced RBC velocity is not an immediate effect of hyperglycemia but a consequence of persistent hyperglycemia. The above results are in line with the hypothesis that microvascular flow increases in diabetes, as one of the first microvascular alterations. In contrast, WBC velocity was not different in diabetic and control mice.

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1. Introduction

The pathophysiology of diabetic retinopathy is still poorly understood. Recent studies have increasingly focused on dynamic analysis of the retinal circulation of blood cells in diabetes. Alterations in microcirculatory parameters seem to occur in the retina before the appearance of any clinical retinopathy and contribute to its appearance in diabetic animals (Higashi et al., 1998) and patients (Arai et al., 1998). Certain microcirculatory alterations have been found in the retina of diabetic rats, such as increased adherence of leukocytes to the retinal capillary endothelium (Miyamoto et al., 1998, 1999; Kinukawa et al., 1999; Nonaka et al., 2000), prolonged arteriovenous transit time (Bursell et al., 1992; Miyamoto et al., 1996) and reduced retinal blood flow (Clermont et al., 1994; Miyamoto et al., 1996). Reduced blood flow was also reported in diabetic dogs (Small et al., 1987). In diabetic patients, reduced perifoveal capillary flow velocity (Arend et al., 1994), prolonged retinal mean circulation time (Blair et al., 1982) and reduced blood flow (Sinclair et al., 1982; Patel et al., 1992; Bursell et al., 1996) were found in the retinal circulation. In contrast, increased blood flow has been reported in diabetic rats (Cringle et al., 1993) and in patients (Feke et al., 1985).

In vivo blood flow or velocity is usually measured by indirect techniques. The blue-field entoptic phenomenon,
probably related to the leukocyte flow, can only be used to study the human perifoveal region (Fallon et al., 1987; Sinclair et al., 1989; Lotfi and Grunwald, 1991). Hypofluorescent or hypofluorescent dots are visible in the perifoveal capillary net during fluorescein angiography with a scanning laser ophthalmoscope (Tanaka et al., 1991; Ohnishi et al., 1994), but their real nature is still controversial. Laser doppler velocimetry, used for retinal blood flow measurement, can be applied to large retinal vessels but not to capillaries (Green et al., 1983; Feke et al., 1989). The dye dilution technique and its variations are used to measure the retinal circulation time (Khoobehi et al., 1990) but only give an indirect measure of the retinal microcirculation, and are difficult to carry out. In recent years, several studies of the retinal microcirculation based on dye-labeled blood cells have been published (Nishiwaki et al., 1995, 1996; Fillacier et al., 1995; Kimura et al., 1995; Ben-Nun, 1996; Le Gargasson et al., 1997; Yang et al., 1997; Hossain et al., 1998; Khoobehi and Peyman, 1999; Paques et al., 2000). The advantages of labeled cell-based studies are to allow direct measurement in the capillaries and direct study of RBC perfusion, a crucial parameter for retina oxygenation, and to allow the study of WBC circulation parameters, which may differ from those of RBC and also depend on WBC–endothelium interactions. However, to our knowledge, none of the methods used by the authors allows simultaneous study of red blood cells (RBC) and white blood cells (WBC) in a simple and robust way in mice.

Most of these studies dealt with the variations in one parameter only: leukocyte dynamics, blood velocities or the morphology of retinal capillaries. However, interpretation of variations in one of these parameters without relating it to the others could lead to error, since they might interact with each other. For example, increased leukocyte adhesion can increase circulatory resistance and reduce blood flow, and conversely, reduced blood flow can increase leukocyte adhesion (Bienvenu and Granger, 1993).

In addition, most studies focused on type 1 diabetes models or patients. However, since the mechanism of microvascular complications may differ in the 2 types of diabetes, the results for experimental models of type 1 diabetes cannot be directly extrapolated to type 2 diabetes (Jaap and Tooke, 1995; Tooke, 1996).

In view of these considerations, we sought to analyse concomitantly the RBC and WBC circulations and retinal capillary angiogram in diabetic and control mice after the onset of hyperglycemia and 10 weeks later, using an original application of intravital microscopy. This in vivo study was carried out to address the following questions: is there any change, particularly in blood cell velocity, which is associated with experimental type 2 diabetes in the retinal microcirculation of mice, before any anatomic modification occurs? And if so, are such changes directly linked to the onset of hyperglycemia?

2. Materials and methods

2.1. Animals and their preparation

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male C57BL/Ks db/db mice (a model of spontaneous type 2 diabetes), and db/+ mice (controls) were purchased from CERJ (Le Genest St Isle, France). Each group was housed separately. All animals were fed with standard chow and had free access to food and water. Ten diabetic and 10 control mice were studied at age 8 weeks, which marks the onset of hyperglycemia in the C57BL/Ks db/db mouse (Kodama et al., 1994), and at 18 weeks. However, interpretable images were only obtained for 6 animals in each group at each time point.

Anesthesia was induced by intraperitoneal injection of a mixture of ketamine (100 mg kg\(^{-1}\)) and xylazine (25 mg kg\(^{-1}\)). Body temperature was kept at 37°C throughout the experiment by a homeothermic blanket control unit (Harvard Apparatus, Les Ulis, France). Pupils were dilated with topical tropicamide. Intravenous injections were performed in the corpus cavernosum.

Before each examination, animals were weighed, and their glycemia was measured on a capillary blood specimen obtained by a superficial cutaneous incision in the tail. No catheterization was done because we considered that catheter placement in the carotid artery might alter ocular blood flow. However, blood pressure was measured one week after the second examination by carotid catheterization of the same animals, using the same anesthetic protocol.

2.2. Plasma and blood cell labeling

RBC were labeled by a modified version of the Sarelius and Duling method (Sarelius and Duling, 1982). Briefly, arterial blood was taken from a donor animal using a heparinized syringe and centrifuged at room temperature. The supernatant was removed and the cells were washed in phosphate-buffered saline (PBS) at pH 7.4 containing 100 mg l\(^{-1}\) ethylenediaminetetra-acetic acid (EDTA). RBC were then incubated for 2 hr at room temperature in pH 8 PBS-EDTA containing 10 g mg l\(^{-1}\) fluorescein isothiocyanate (FITC, Sigma, France). The supernatant dye was removed by centrifugation and RBC were washed with pH 7.4 PBS-EDTA. The hematocrit was then adjusted to 20% and RBC were kept at 4°C and used within 36 hr. In every case 15 μl of labeled cells was reinjected into littermates of the donor animal (same age and same group). For leukocyte labeling, 20 μl of filtered 1 mg mg ml\(^{-1}\) rhodamine 6G (Sigma, France) was injected intravenously. The vascular network was visualized by subcutaneous injection of 100 μl of sodium fluorescein.
2.3. Intravital microscopy

Intravital epifluorescence microscopy was performed with a modified Leitz® histologic microscope (Leica, Germany). A video camera (Model no. 4510, COHU corporation, San Diego, CA, USA) and an S-VHS video recorder (Blaupunkt, Japan) were used to record the images at 50 frames sec\(^{-1}\). FITC-labeled RBC were injected into the corpus cavernosum of anesthetized and dilated mice, after which there was a rest period of approximately 10 min. Each mouse was placed under the objective, lying on its side. The head was supported so that the iris could be placed in a plane perpendicular to the illumination axis. The refractive power of the cornea was counteracted by placing a glass slide (diameter, 10 mm, weight, 55 mg, Marienfeld, Germany) on a ring surrounding the globe, taking care not to exert pressure on the globe and to maintain the slide in a horizontal plane. Methylcellulose (Goniosol®, Allergan, France) was applied to serve as contact medium between the slide and the cornea. Epi-illumination was delivered by a mercury lamp through appropriate dichroic filters. Focusing was adjusted with a 10\(\times\) objective to the deep capillary layer of a retinal area located about 200 \(\mu\) from the disc on the nasal side. The passage of RBC was recorded for 1 min. Rhodamine 6G was injected into the corpus cavernosum, and a rest period followed of about 1 min. The filter for rhodamine was switched on, and the passage of WBC was again recorded for 1 min. Fluorescein was then injected and the vascular network angiogram was recorded. By switching the filter, WBC was also visible after injection of fluorescein. As a rule, the right eye was examined at 8 weeks and the left eye at 18 weeks.

In preliminary experiments, to determine the magnification factor of the optical system of the mouse eye, the size of the optic disk was measured as visualized in vivo under the microscope after subcutaneous injection of sodium fluorescein. Mice were then sacrificed by a pentobarbital overdose, immediately enucleated, and the anterior segment and vitreous were stripped off. The optic disk was then examined under the same conditions as in vivo, and its size was measured, and compared to the size measured in vivo.

2.4. Data analysis

Using replayed video images obtained with the 10\(\times\) lens (screen magnification \(\times\) 960), the center of the cell was marked on a transparent sheet in different positions. Instantaneous velocity was defined as the velocity calculated from the linear distance between two successive positions of a labeled cell in a capillary segment (separated by an interval of 0.02 sec). For each animal, we also calculated the mean (i.e. time-averaged) velocity of twenty labeled cells circulating in different pathways, the coefficients of variation of the instantaneous velocities of each pathway (temporal coefficients of variation) and the coefficients of variation of the mean velocities of different pathways (spatial coefficients of variation). For the comparison between diabetic and control groups, we compared the statistical distributions of mean (time-averaged) velocities, using the whole set of recorded values (twenty values per mouse in six mice in each group).

Capillary fluorescein angiograms were also analysed to detect any obvious microvascular abnormalities. Capillary densities were then calculated from the angiograms by the following method (modified from Robison et al., 1995): 20–25 frames of the fluorescein angiogram images recorded with a 4\(\times\) lens were captured and digitized with NIH Image 1.62 software (National Institute of Health public domain software). Blurred images due to motion artifacts were discarded one by one. An average image was then constructed and transferred to Adobe Photoshop 4.0 software (Adobe Corporation, San Jose, CA, USA). Capillaries were marked manually. Capillary drawings were then transferred back to NIH software, and skeletonization was performed (i.e. conversion into the one-pixel-wide central axis of each capillary), thus allowing the total length of capillaries to be estimated by counting the residual pixels. The corresponding surface on which the pixels were counted was measured in square pixels.

2.5. Statistical analysis

Results are shown as means \(\pm\) standard deviation. Analysis of variance (ANOVA) or Mann–Whitney’s non-parametric test was used to compare the statistical distribution of the parameters measured.

3. Results

3.1. Systemic parameters

At both 8 and 18 weeks, all db/db mice were hyperglycemic and obese. No difference in systolic blood pressure was found between the diabetic and control groups. Lenses remained clear in all animals. Systemic data are summarized in Table 1.

3.2. RBC and WBC velocities

At onset of hyperglycemia (age 8 weeks) there was no significant difference between the statistical distribution of the RBC velocity of diabetic and control mice (Table 2). In contrast, after 10 weeks of hyperglycemia, mean RBC velocity was significantly higher in diabetic than control mice. WBC velocity was not significantly different in the 2 groups at either age. WBC to RBC mean velocity ratios in the 2 groups was not significantly different at 8 and 18 weeks either. RBC and WBC velocities decreased with age (\(p = 0.0146\) for WBC and \(p < 0.001\) for RBC in the control group and \(p < 0.001\) for both in the diabetic group).
The decrease in RBC velocity was smaller in diabetic than control mice because it was counteracted by the effect of diabetes, leading to a higher RBC velocity in diabetic versus control mice at 18 weeks.

As regards the temporal and spatial heterogeneity of RBC and WBC hemodynamics, as reflected by the temporal and spatial coefficients of variation of velocities, no significant difference was found between diabetic and control mice (Table 3). However, the temporal and spatial coefficients of variation for WBC velocities were significantly higher than those for RBC velocities ($p < 0.001$ for both). In addition, the WBC velocity was always lower than RBC velocity ($p < 0.001$ for both groups at both ages).

### 3.3. Morphometric analysis

No specific vascular abnormality was noted in the diabetic animals, i.e. no microaneurysm, capillary dilation or capillary closure. At 18 weeks, There was no significant difference between the capillary density of diabetic and control mice (respectively, $3.88 \pm 0.4$ versus $3.89 \pm 0.7$, expressed as mean $\pm$ SD in pixels/100 square pixels, $p = 0.9$). No fluorescein leakage was observed, even 1 hr after fluorescein injection (data not shown).

### 4. Discussion

#### 4.1. Model and method

The C57BL/Ks db/db mouse is a model of spontaneous type 2 diabetes, linked to a recessive mutation of an as yet unidentified gene located on chromosome 4 (Hummel et al., 1966; Coleman and Hummel, 1967; Hosokawa et al., 1985). Before the onset of hyperglycemia, hyperphagia and obesity occur, accompanied by an asymptomatic insulin-resistant stage characterized by hyperinsulinemia. This stage is followed by pancreas $\beta$ islet degeneration, which is concomitant with the onset of hyperglycemia (Hummel et al., 1966). Hyperglycemia begins at about 8 weeks (Kodama et al., 1994) and affected mice develop diabetic complications such as neuropathy and nephropathy (Like et al., 1972; Norido, Canella and Gorio, 1982). The heterozygous C57BL/KS db/+ mouse does not develop diabetes, and thus served as control. Histologic studies have also shown that db/db mice develop the early stages of diabetic retinopathy. More advanced stages of diabetic retinopathy have never been reported in mice. Midena et al. (1989) studied db/db mice from age 12 to 64 weeks. They observed loss of pericytes and an increased pericyte/endothelial capillary cell ratio after age 26 weeks. Acellular capillaries were the only evident histologic change observed at 34 and 64 weeks. An increased capillary basement membrane thickness at age 22 weeks was also reported in db/db mice by Clements et al. (1998). These mice have other advantages for the study of the retinal microcirculation: firstly, they are darkly pigmented, and we checked in preliminary experiments that their pigment epithelium screens out the fluorescence of the labeled elements circulating in the choroid. Unlike albino mice, no choroidal vessel was visible in db/db mice, and the pathways of visible fluorescent circulating blood cells were strictly superimposable on the retinal capillaries. Secondly, the appearance of diabetic cataract is delayed in db/db mice, making them suitable for long-term studies. We examined a group of old db/db mice (up to 1 year) and found no cataract (unpublished data). Thirdly, this strain constitutes
a spontaneous model of diabetes, thus reducing the risk of recording physiological changes due to the use of a toxic compound to induce diabetes, or the reactions induced by such a compound (e.g. an inflammatory response to betacell damage). Lastly, it should be noted that we re-injected the labeled RBC into littermates of the donor animal (same age and same group). This was done to take account of possible alterations in the plasma membrane of RBC, and of RBC deformability in diabetic animals (Schmid-Schonbein and Volger, 1976).

Intravital epifluorescence microscopy is widely used to study the microcirculation in different organs. Here, we used this method in an original way which enabled us to measure, for the first time to our knowledge, the absolute values for RBC and WBC velocities in the retinal capillaries of mice, in a robust way, using common devices. This technique provides a comprehensive view of the microcirculation. It allows concomitant visualization of different labeled elements, by switching the filter, thus permitting comparison of RBC and WBC dynamics in the same animals during the same session. For example, when comparing RBC and WBC pathways by this technique, we noticed that there were certain preferential channels for WBC in the retina of mice, as suggested for rats by Nishiwaki et al. (1996). In particular, we observed that some capillary pathways appearing as twinkling after fluorescein injection, in fact represent preferential pathways for WBC: a large proportion of the WBC visible on the screen passed through these pathways, whereas RBC were equally distributed. However, this observation was not quantified and we considered that an extensive description of these pathways was beyond the scope of our study. The device used here provides high magnification together with the possibility of selective focusing on a specific depth of the retina. A deep capillary layer with sinuous vessels was indeed always clearly distinguishable from a superficial layer with straight vessels. In addition, unlike other in vivo methods of studying the retinal microcirculation, this method can be used in mice, which have the advantage of allowing knockout studies. Lastly, it could be used to study not only diabetic retinopathy but also other pathologies, or even the normal retinal microcirculation, which is still to a large extent unknown. For example, a decrease in blood cell velocities in the retinal microcirculation with aging was found in both groups of mice in our experiment. This incidental finding is in line with the results of other experiments, which also suggested a decrease in blood velocities in the retinal vasculature probably due to increased vascular resistivity with aging or impaired autoregulation of the retinal circulation (Grunwald et al., 1993; Groh et al., 1996). Again, what makes this method and our study unique is that the capillary network was examined directly, whereas in most studies, the upstream arteriolar or venular blood flow was measured. One limitation of this technique is the low contrast of the images recorded with usual cameras, which makes data analysis time consuming. Here, four animals in each group had to be excluded from the study because even a slight lack of corneal transparency was enough to prevent us from obtaining sufficiently clear images for proper analysis. This drawback could certainly be overcome by the use of more sensitive cameras or imaging systems such as confocal microscopes or a scanning laser ophthalmoscope.

4.2. Microcirculatory changes during diabetes

The nature and significance of retinal circulatory changes during experimental diabetes remain controversial (Blair et al., 1982; Sinclair et al., 1982; Feke et al., 1985; Small et al., 1987; Bursell et al., 1992, 1996; Patel et al., 1992; Cringle et al., 1993; Arend et al., 1994; Clermont et al., 1994; Miyamoto et al., 1996, 1998, 1999; Kinukawa et al., 1999; Nonaka et al., 2000). The discrepancies between observations may be due to the differences in animal models and/or techniques. Since diabetes may preferentially affect the capillary network, it is more interesting to study microcirculatory changes directly in capillaries than from indirect measurements of upstream arteriole or downstream venule blood flow. In addition, to our knowledge, no previous study of retinal capillary hemodynamics has been performed in the diabetic mouse. The original approach of this study allowed concomitant analysis of the capillary velocities of red and WBCs and of the vascular morphology. Furthermore, the possibility of selective focusing on a specific depth of the retina makes measurements more accurate, thus reducing errors related to the vertical moves of cells in the tridimensional structure of retinal capillaries. We found increased RBC velocity in the retinal capillaries of C57BL/Ks db/db mice compared to controls, after 10 weeks of hyperglycemia (at age 18 weeks). The fact that, as stated in Section 3, this increase was not found at the onset of hyperglycemia (at age 8 weeks) suggests that it is not a direct and immediate effect of hyperglycemia on the retinal circulation, but an effect of persistent hyperglycemia. Several mechanisms could be suggested for this modification.

The first hypothesis might be a decrease in the number of capillaries. Note that to obtain a 20% enhancement of the cell velocity, the number of perfused capillaries must decrease by 20% at constant blood flow (and by even more if there is either an increase in the diameter of the remaining capillaries, or a decrease in blood flow). Permanent capillary occlusion leading to reduced density at this stage of diabetic retinopathy has indeed never been reported as far as we know. However, the authors of recent studies found increased leukocyte entrapment in the retina of diabetic rats (Miyamoto et al., 1998, 1999; Nonaka et al., 2000), which may be associated with temporary capillary non-perfusion. Here, we did not find trapped leukocytes in capillaries, but only a very few leukocytes sticking to postcapillary venules in both diabetic and control mice (note that the present model and methodology were both different.
from those used by others). In addition, no difference was found between groups when we measured the capillary density from fluorescein angiograms, i.e. the density of perfused capillaries at a given time. This density takes into account the reduction due to both permanent and temporary occlusions (caused for example, by leukocyte entrapment). These findings make it unlikely that the changes in capillary velocity are due to changes in capillary density.

A second possibility might be an unequal distribution of blood flow in the capillaries, leading to the formation of hypoperfused and hyperperfused areas in the capillary network. However, such a process would have increased the spatial coefficient of variation of RBC velocities. This was not the case, because there was no significant difference between these parameters in diabetic and control mice.

Another possibility might be a change in capillary diameters. However, we observed no such obvious change in diabetic mice on capillary angiograms. Nevertheless, this hypothesis cannot be completely ruled out, because we were unable to measure the diameter of capillaries with satisfactory accuracy, due to the blurring of capillary limits caused by the aberrations of the optic system of the mouse eye.

The last possible explanation for the higher velocities observed in diabetic animals is an increase in capillary perfusion. This could be due to an impairment of the autoregulation of upstream arterioles in diabetic animals, as advocated by some authors (Hill and Larkins, 1989; Su et al., 1995). The effect of such impairment may even be magnified by the alteration of systemic parameters due to general anesthesia. This remains a probable explanation and is in line with the hypothesis that microvascular flow increases in diabetes, first advanced by Parving et al. (1983) and elaborated by others (Tooke, 1986; Zatz and Brenner, 1986).

In the capillaries, blood cells are carried by the blood current and impeded in their motion by their mechanic and specific interactions with capillaries. Here, WBC velocities in capillaries were lower than RBC velocities, in both groups at both ages (see Results and Table 2). Furthermore, we noticed that WBC slow down even more in the parts of the pathway that loop (Fig. 1). Similar observations have also been reported by other authors (Ben-Nun, 1996). This illustrates an already known principle that WBC interact more with capillaries than RBC, which might be due to their larger size and to specific interactions. When diabetic and control mice were compared after 10 weeks of diabetes, WBC velocity in diabetic mice was not significantly different in the two groups, despite the increased RBC velocity in diabetic mice. This may mean that an increase in blood velocity was counterbalanced by increased WBC-capillary interactions in diabetic mice, resulting in equal WBC velocities in the two groups. Increase in continuous and loose interactions between WBC and retinal capillaries and/or increased frequency of WBC slowing down or leukostasis (related to stronger interactions) might explain the phenomenon. Increased WBC adhesion to the endothelium in diabetic animals has been found by other authors (Miyamoto et al., 1999; Barouch et al., 2000). In an attempt to quantifying WBC-capillary interactions, we compared the individual mean WBC to RBC velocity ratios in diabetic and control mice after 10 weeks of diabetes. This ratio was slightly lower in diabetic mice, indicating additional slowing down of WBC, but the difference did not reach statistical significance (Table 3). However, the fact that this comparison did not show a significant difference does not rule the possibility of an increased WBC–capillary interactions in diabetic mice, since the power of the statistical test used is very low. This parameter is indeed calculated as the ratio of RBC to WBC velocity, averaged in each mouse, so that the sampling unit corresponds to the number of animals rather than to the number of velocity measurements. We also noticed that the instantaneous velocity of WBC varied more than that of RBC from one time point to another in the same capillary and same cell, and also from one capillary to another. To quantify these data, we calculated the spatial and temporal coefficients of variation of cell velocities. These coefficients can be considered to be closely dependent on the strong interactions between circulating cells and capillaries, leading to a large slowdown or leukostatis, which can greatly increase the heterogeneity of velocities. An increased spatial coefficient of variation was reported by others for WBC velocities in streptozotocin-induced diabetic rats (Kinukawa et al., 1999). We indeed found significantly higher temporal and spatial coefficients of variation for WBC than RBC, but comparison of diabetic and control mice revealed no significant difference between them in these respects (see Table 3). Moreover, we did not observe, in diabetic mice, the leukostasis reported in rats (Miyamoto et al., 1998, 1999; Kinukawa et al., 1999;
Barouch et al., 2000; Nonaka et al., 2000). The only leukostasis we found was in mice with a large decrease in arterial pressure (thus not selected for the present series of experiments). Consequently, our results demonstrated that leukostasis is not necessarily associated with the early stages of diabetes. Nevertheless, there may be a certain degree of increase in the strength of the continuous weak interactions between leukocytes and retinal capillaries.

In summary, after 10–12 weeks of hyperglycemia, RBC velocity was higher in the retinal capillaries of diabetic than control mice, but WBC velocity was not different between them. This higher RBC velocity was not found at the onset of the hyperglycemia, suggesting that it was not an immediate effect of hyperglycemia, but a consequence of persistent hyperglycemia. This is in line with the hypothesis that microvascular flow increases in diabetes, as one of the first microvascular alterations. In contrast, the present results do not support the hypothesis that leukostasis is necessarily associated with the early stages of diabetes.

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