Normal and sickle red blood cell dynamics under venular flow.

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Original article

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Abstract

Red blood cell hemodynamics influence blood rheology and thus circulatory function. Different authors showed that red blood cells are heterogeneous in blood flow. This heterogeneity could be explained by physical characteristics such as morphology, aggregability and deformability, but also by cellular and molecular environment. All these notions are found in sickle cell disease where sickle red blood cells become more rigid, leading to vascular occlusions, increased with an inflammatory context.

In a preceding study, our results showed that velocity heterogeneity allow RBC classification, leading to different RBC sub-groups, using a dynamical approach and flow studies. In order to have a better understanding of the physiological meaning of such an heterogeneity, dynamical velocity experiences have been conducted in the case of normal and sickle red blood cells with different endothelial activations by proinflammatory cytokines, under a constant venular flow.

Thus, normal and sickle red blood cells are classified into different sub-groups, showing their heterogeneity. Moreover, sub-population velocities and distribution evolve differently according to vascular state or treatment.

These results show that red blood cell velocities and rheology depend on vascular environment and red blood cell morphology. However, the physiological meaning of this behavior should be explained by cellular and molecular effects of such activation.

Keywords: Adhesion; Blood flow; Hemodynamics; Sickle red blood cell; Velocity.

Abbreviations: EC, endothelial cell; HAC; hierarchical agglomerative clustering; MV, mean velocity; RBC, red blood cell; TrHBMEC, transformed human bone marrow endothelial cells

1. Introduction

Dynamics of the vascular system imply many notions such as fluid dynamic problems, interaction between blood flow, blood elements, vessels and vascular tissues or mathematical modeling (Li, 2006). Blood elements such as red blood cells (RBCs), may play an important role in vascular system dynamics and more generally, on circulatory function. Thus, RBC rheology may influence hemodynamics.

RBC dynamics may be useful to understand this complex phenomenon. Some studies show that RBC population is heterogeneous according to their velocity in the circulatory system, particularly in venules (Rosenblum, 1971; Rosenblum, 1972a; Rosenblum, 1972b; Rosenblum, 1976). In addition, RBC characteristics such as age, density, cellular morphology and bio-chemical environment (Grima, 2007) may influence their behaviors in blood flow.

RBC aggregation, known as ‘‘rouleaux’’, is fully reversible and disrupted by the shear forces that arise as the flow rate increases (Armstrong, Wenby, Meiselman, & Fisher, 2004). It has also been hypothesized that the formation of aggregates of RBCs along the venular centerline (where low shear rates promote aggregation) may also enhance the radial migration of WBCs toward the EC as aggregates exclude WBCs from the axial core of RBCs (Pearson & Lipowsky, 2000). RBC aggregation is often significantly increased in disease states (Armstrong, Wenby, Meiselman, & Fisher, 2004; Bishop, Popel, Intaglietta, & Johnson, 2001; Hardwicke & Squire, 1952; Olshaker & Jerrard, 1997).

Moreover, previous studies have uncovered various findings related to the deformability of RBCs in a steady, uniform shear-flow field, such as RBC membrane changes,

hematocrit and intracellular viscosity (Fischer & Schmid-Schönbein, 1977; Fischer, Schmid-Schönbein, & Stöhr-Liesen, 1978a; Fischer, Stöhr, & Schmid-Schönbein, 1978b; Kon, Maeda, & Shiga, 1987; Pfafferott, Nash, & Meiselman, 1985; Schmid-Schönbein & Wells, 1969; Watanabe, Kataoka, Yasuda, & Takatani, 2006).

It was clearly demonstrated that reductions in RBC deformability may adversely affect capillary perfusion (Driessen, Haest, Heidtmann, Kamp, & Schmid-Schönbein, 1980; Parthasarathi & Lipowsky, 1999). In addition, many diseases manifest reductions in RBC deformability. For example, elevated internal fluid viscosity or abnormal membrane stiffness was found in diabetes mellitus (Parthasarathi & Lipowsky, 1999; Schmid-Schönbein & Volger, 1976), a- and b-thalassemia (Parthasarathi & Lipowsky, 1999; Schrier, Rachmilewitz, & Mohandas, 1989) and sickle cell disease (Parthasarathi & Lipowsky, 1999; Schmalzer, Manning, & Chien, 1989), particularly in microcirculation, where cell size is of the order of the blood vessel diameter (Bransky, Korin, Nemirovski, & Dinnar, 2007). Dynamic response and RBC behavior are also influenced by the nature of shear stress, uniform or reversing flow (Watanabe, Kataoka, Yasuda, & Takatani, 2006).

RBC morphology, combined with cellular environment and vascular state, are two notions commonly found in Sickle Cell Disease, a specific disease. SS disease is a hemoglobinopathy caused by a mutation in the beta-globin gene (Glu6Val) (Ohene-Frempong & Steinberg, 2001). Sickle hemoglobin molecules (HbS) have the property to polymerize when deoxygenated, forming sickle red blood cells (Ohene-Frempong & Steinberg, 2001). Rigid, deformed sickle red blood cells (SS RBCs) promote vascular occlusion, chronic ischemia-reperfusion injury, and episodic painful crises (Kato, Gladwin, & Steinberg, 2007; Ohene-Frempong & Steinberg, 2001; de Ceulaer, Higgs,

Weatherall, Hayes, Serjeant BE, & Serjeant GR, 1983). SS RBCs are proadhesive (Finnegan, Turhan, Golan, & Barabino, 2007; Hebbel, 1997; Wick & Eckman, 1996). Inflammatory mechanisms are thought to play a major role in the pathobiology of SS disease, probably via the endothelial inflammation (Finnegan, Turhan, Golan, & Barabino, 2007; Platt, 2000).

Vaso-occlusive crisis are initiated by the adhesion reaction between the activated blood vessel endothelium and SS RBCs (Walmet, Eckman, & Wick, 2003). In addition, under flow conditions, reticulocytes were the most adherent of the heterogeneous population of sickle erythrocytes (Hebbel & Mohandas, 2001; Kato, Gladwin, & Steinberg, 2007). HbSS polymerization and adherence of SS RBCs to endothelium are thought to initiate sickle cell vasoocclusive pain episodes (Bunn, 1997; Hebbel, Boogaerts, Eaton, & Steinberg, 1980a; Hofrichter, Ross, & Eaton, 1974; Hoover, Rubin, Wise, & Warren, 1979; Mozzarelli, Hofrichter, & Eaton, 1987; Wagner, Eckman, & Wick, 2004). Reticulocyte adherence provides an additional link between hemolytic anemia and sickle vaso-occlusion (Kato, Gladwin, & Steinberg, 2007). Sickle erythrocytes adhere to cultured endothelial cells and the tenacity of adherence reflects the severity of the disease (Hebbel, Boogaerts, Eaton, & Steinberg, 1980a; Hoover, Rubin, Wise, & Warren, 1979; Kato, Gladwin, & Steinberg, 2007). This abnormal adhesion is directly connected to the severity of the disease (Francis & Johnson, 1991; Hebbel, Boogaerts, Koresawa, Jacob, Eaton, & Steinberg, 1980b; Hebbel, 1991; Setty & Stuart, 1996). Vaso-oclusive process is very complex. This model has evolved from polymerization-based concepts to a complex, wide-ranging schema that involves multistep, heterogeneous, and interdependent interactions among SS RBCs, adherent leukocytes, endothelial cells, plasma proteins, and other factors (Chiang & Frenette, 2005).

Endothelial activation, induced directly or indirectly by the proinflammatory behavior of SS RBCs, is the most likely initiating step toward vaso-occlusion (Chiang & Frenette, 2005). Previous studies demonstrated that sickle RBCs are more adherent to vascular endothelium and that the endothelium in sickle cell patients is inflamed (Duits et al., 1996; Solovey, Lin, Browne, Choong, & Wayner, 1997; Turhan, Jenab, Bruhns, Ravetch, Coller, & Frenette, 2004). Thus, sickle cell vaso-occlusion appears to involve multicellular interactions among RBCs, white blood cells, and the venular endothelium (Frenette, 2002; Turhan, Jenab, Bruhns, Ravetch, Coller, & Frenette, 2004).

Moreover, transient sequestration of sickle RBCs in the low-flow state appears to be dominated by RBC / Endothelial cell (EC) adhesion, which becomes enhanced during crisis (Lipowsky & Williams, 1997). RBC adhesion would be predominant in post-capillary venules (Chaudet, Renard, Seigneur, & Boisseau, 2000; Hebbel, Ney, & Foker, 1989; Hebbel, 1997; Kaul, Fabry, & Nagel, 1989; Montes, Eckman, Hsu, & Wick, 2002; Turitto, 1982). Although aggregation may enhance adhesive contact of RBCs with ECs, it does not increase to the same extent as the rate of sequestration, thus reflecting a greater role of RBC-EC adhesion (Lipowsky & Williams, 1997). In addition, RBC adhesion would be predominant in post-capillary venules with a physiological venular shear stress of 1 dyne/cm² (Kaul, Fabry, & Nagel, 1989; Montes, Eckman, Hsu, & Wick, 2002; Turitto, 1982).

All these different experiments suggest that blood flow studies would be useful to understand vascular disease mechanisms, particularly in sickle cell disease, where RBCs could be implicated in tissular and cellular damages or vascular phenomenon as blood cell adhesion to endothelium.

In a previous study, using a dynamical approach with a flow chamber apparatus to simulate blood flow conditions, it was shown that velocity heterogeneity could be explained by RBC classification, leading to different RBC sub-groups (Allayous, Regis, Bruel, Schoevaert, Emilion, & Marianne-Pepin, 2007). Thus, the present study focuses on this particular RBC dynamic behavior according to vascular state and RBC morphology in order to simulate sickle cell disease inflammation and specific RBC structural dynamics and circulatory behaviors in a constant venular flow of 1 dyne/cm².

2. Materiel and Methods

2.1. TrHMEC culture

Transformed Human Bone Marrow Endothelial Cells (TrHBMEC) were kindly provided by Pr. Elion and Dr. Weksler. They were maintained in humidified air/5% CO_2 at 37°C. TrHBMEC monolayers were grown to confluence in gelatin-coated Thermanox slides (Nalgene Nunc International) as previously described by Schweitzer, et al. (1997). TrHBMEC subcultures at passage 17 were used for flow experiments, and cultures were examined prior to use to ensure confluence.

2.2. Red blood cell (RBC) acquisition

Peripheral venous blood samples were obtained from four healthy normal volunteers (AA) at the Centre National France Transplant (Hôpital Saint-Louis, Paris) and from four individuals with homozygous sickle cell disease (SS) that have not received any

medical treatment within the previous 15 days. RBCs were isolated from whole blood by repeated centrifugation and washed with saline. RBCs were suspended in TrHBMEC culture medium without fetal calf serum and adjusted to 10^6 red cells/ml for the flow assay.

2.3. Flow assay protocol

TrBMEC cultures were activated with culture medium containing pro-inflammatory cytokines: TNFα / IFNγ at 100 U/ml (Schweitzer et al. 1997) for 12, 24 or 48 hours prior to the start of a flow assay. Naïve TrHBMEC were sham-treated in an identical manner with culture medium alone (table 1).

The cultured slides were secured to the flow chamber that is composed of a rectangular plexiglas cavity (0.2 mm height, 29 mm length, 5 mm width). The bottom wall of the chamber is a Thermanox coverslip (0.17 x 60 x 24 mm^3) where endothelial cells were coated and directly in contact with RBCs. The cultured slides were mounted on an inverted-phase contrast microscope (Nikon Eclipse TE300, X20) equipped with a video camera. RBC suspension was then perfused through the chamber at 1 dyne/cm^2, for 10 minutes. The real-time images coming from the flow chamber (Figure 1) are recorded with a video tape recorder (Sony time lapse 168) and analyzed with a specific video analyzer (Pentium with Matrox digitized card). The experimental data obtained such as velocity, acceleration, angular deviation and linearity index, are used to characterize red cells trajectories, cell adhesion and cell-cell interactions. All experiments were performed at room temperature and repeated three times.

2.4. Classification methods

Three classification methods were used, Hierarchical Agglomerative Clustering (HAC), K-Means clustering (K-Means) and Mixture of Gaussian distributions.

2.4.1. HAC

HAC is a hierarchical method based on hierarchical level of aggregation. The method provides increasing levels of classification. The visualization of the classification is made with a binary tree.

2.4.2. K-Means

K-Means is a classical method based on the evaluation of distances between the sample and the provided centers of classes which are given by the user at the beginning of the classification. These centers are then modified during the classification.

The two methods gave different class centers. Number of classes and its centers are fixed beforehand in K-Means method while these parameters depend only on the level of classification chosen by the users in HAC method.

2.4.3. Mixture of Gaussian distributions

In order to classify the RBCs into N classes, with respect to their mean velocity value, we assume that these velocities are distributed as a mixture of Gaussian distributions $p_1 f_1(x) + \ldots + p_N f_N(x)$. Each number $p_i \in [0,1]$ represents the weight of class i, $p_1 + \ldots + p_N = 1$, and the distribution of the velocities within class i, say f_i, is Gaussian with mean

and variance depending on i. The estimation of these parameters is done through a variant of the EM (Expectation Maximization) algorithm implemented in *R* language package *(http://lib.stat.cmu.edu/R/CRAN).* The *R* function *Mclust ( )* yields the results, while the number of classes can be discussed by using a log-likelyhood criteria.

The performance of each of these methods is evaluated by the number of RBCs in each class, the least mean variance and the greater distance between the different classes. The Gaussian mixture method often appears as the best one.

2.5. Statistical analysis

Data are presented as mean ± SE of the values obtained from independent experiments. Statistical analysis was performed using the single-factor analysis of variance (ANOVA) function in XLSTAT software to test for differences in RBC classes. Differences were considered to be statistically significant in cases in which the p value was less than or equal to 0.05 (representing a $\alpha = 0.05$).

3. Results

The present study focuses on RBC mean velocity using a flow chamber associated to an apparatus of video-microscope image analyzer. This method simulates blood circulation in various conditions such as inflammation, medical treatment or vascular diseases where RBC dynamic and cell interactions are modified. The real-time images coming from the flow chamber are recorded and analyzed in order to determine each treatment

effect on RBC dynamic behavior. The purpose of the second part of this paper is to establish different RBC sub-groups using classification methods.

This first part focuses on the general behavior of normal RBCs (AA) and sickle RBCs (SS) in various conditions (tables 1 and 2).

3.1. General population mean velocity

The different RBC mean velocities are summarized in table 2.

3.1.1. Normal and sickle RBC general behavior

AA and SS RBC general mean velocities (MVs) are respectively, 1005.94 ± 299.08 and 1186.27 ± 264.89 µm/ms. In our conditions, SS RBCs seem to be significantly more rapid than AA RBCs.

This preliminary result differs according to TrHBMEC treatment. Indeed, the nature and the time of this treatment both influence RBC evolution and profile in blood flow, as presented in next sections.

3.1.2. Normal and sickle RBC general behavior under proinflammatory conditions

TrHBMEC are activated by proinflammatory cytokines during 12, 24 or 48 hours (table 1).

In case of a 12-hour treatment, AA RBC MV is significantly increased whereas SS RBC MV is similar when compared to basal conditions (table 2; fig. 2).

AA RBC MV significantly decreases to normal MV when TrHBMEC are activated for 24 hours (table 2; fig. 2). In parallel, SS RBC MV is significantly decreased to be similar than AA RBC MV in basal conditions (table 2; fig. 2).

These MVs are not modified with a longer treatment (48-hour stimulation) (table 2; fig. 2).

Thus, proinflammatory treatment has different effects, according to RBC nature and stimulation time.

RBC MV under a constant venular flow seems to depend on various parameters such as RBC nature, normal or sickle in our case and vascular environment, represented here by TrHBMEC treatment. Therefore, it would be interesting to consider each TrHBMEC treatment and its consequences for both RBC type.

We can notice that proinflammatory cytokines treatment increases AA RBC MV after 12 hours and decreases SS RBC MV after 24 hours (table 2; fig. 2). SS RBC behavior is different. Indeed, no effect is observed after a short treatment (12 hours). After a longer treatment (24 hours), proinflammatory cytokines modify this MV by increasing (table 2; fig. 2).

Therefore, in addition to the relation between vascular environment and RBC behavior, this paper focuses on RBC heterogeneous responses and on the heterogeneity of this population. Indeed, those two opposed dynamic behaviors suggest that, in our experimental conditions under a constant venular flow, RBC MV should depend on various parameters such as RBC nature or vascular environment. On the other hand, we

may suppose that treatment effects are too weak to be significant when considering RBC general population. Therefore, it would be interesting to refine these preliminary results and determine whether or not there are different RBC sub-groups for each RBC type. If this hypothesis is true, we will focus on their responses in various vascular conditions and TrHBMEC treatments, in order to better understand the origin of these behaviors. This is the reason why we present, in the second part of this paper, an original idea of RBC classification to determine different RBC classes that would be responsible for population heterogeneity and behaviors already observed. In another way, we will try to see if the different TrHBMEC treatments have same effects on the different RBC sub-populations or classes, to generalize it for RBC dynamic behavior.

3.2. RBC MV classification

This study is based on the hypothesis that RBC velocity distribution should be a Gaussian one. Distribution represented in each condition is a mixture of two, three or even four Gaussian distributions, clearly showed in the different histograms built (data not shown). These observations confirm that RBC population is heterogeneous in each case. The different classification obtained will allow us to establish and characterize the different RBC dynamic behavior, with a constant flow and shear stress in the case of a normal and pathological vascular environment.

3.2.1. Normal and sickle RBC classes

In an earlier article, HAC and K-Means methods lead us to classify AA RBCs into two classes (Allayous et al. 2007). Thanks to the Mixture of Gaussian distributions method, this result is refined in three classes. SS RBCs are divided in three classes also, as presented in table 3 and figure 3.

We can notice that AA and SS are composed of three classes with different MVs. Thus, in class 1 and 2, SS RBC MV is significantly higher than AA RBC MV. In addition, RBC population is not equally distributed among the three classes. In class 1, there are as many AA RBCs as there are SS RBCs. On the contrary, in class 2, RBC number is higher and there are more AA RBCs than SS RBCs. However, there are twice as many SS RBCs as AA RBCs in class 3.

3.2.2. Normal and sickle RBC behavior under proinflammatory conditions

The different results presented in this section are summarized in table 4. AA RBCs are distributed into three classes under proinflammatory conditions, for each treatment time. Consequently, proinflammatory cytokines do not modify AA RBC class number. On the contrary, class distribution is modified according to treatment time. In addition, figure 4 shows that time is an important parameter in AA RBC responses and velocity. We can notice that AA RBC MV significantly increases in each RBC sub-groups, after 12 hours (table 4; fig. 4). MV is significantly reduced after 24 hours in each case, but the biggest decrease is observed only for class 1 and 3, MV in class 2 shows no major differences with MV in basal conditions (table 4; fig. 4). Moreover, a little increase is observed only for class 1 MV after 48 hours, this class MV becomes similar to mean velocity

observed in basal conditions, whereas this value does not change for class 2 and 3 after a longer treatment (table 4; fig. 4).

Proinflammatory conditions modify SS RBC behavior. Indeed, they are distributed into three classes when TrHBMEC are shortly stimulated (12 and 24 hours) and into two classes for longer treatment (48 hours), as presented in table 4. As observed for ASS RBCs, class distribution is modified according to treatment time. Figure 5 shows that SS RBC MV significantly increases after 12 hours only in class 2 and 3 whereas RBCs are more concentrated in class 2. Proinflammatory cytokines have a significant effect after 24 hours. Indeed, MV significantly decreases in the three classes, the most spectacular decrease is observed in class 1. In the same manner, RBC number in class 1 and 3 decreases so as to be concentrated in class 2. This effect is reversed with a longer treatment where SS RBC MV significantly increases in the two classes and RBC are more concentrated in class 2.

To conclude, we can notice that, in our experimental conditions, the same effect is observed on AA and SS RBC MV, after 24 hours. Actually, in each class, MV decreased concurrently with the number of red blood cells. However, we have noticed that class 2 contains the most RBCs and a greater amount of SS RBCs.

4. Discussion

The relation between blood cells, vascular environment and (vascular) tissues are of great importance in the circulatory function, particularly when considering RBC rheology. RBC behavior may depend on many factors such as age, cellular morphology,

density, deformability, blood flow, shear stress or vascular environment (molecules or disease state) (Armstrong, Wenby, Meiselman, & Fisher, 2004; Bishop, Popel, Intaglietta, & Johnson, 2001; Bransky, Korin, Nemirovski, & Dinnar, 2007; Grima, 2007; Hardwicke & Squire, 1952; Kon, Maeda, & Shiga, 1987; Olshaker & Jerrard, 1997; Pfafferott, Nash, & Meiselman, 1985; Watanabe, Kataoka, Yasuda, & Takatani, 2006).

All these notions such as inhibition of RBC deformability, increased RBC adhesion, blood flow abnormalities, vascular inflammation or abnormal cellular interactions are found in sickle cell disease (Bransky, Korin, Nemirovski, & Dinnar, 2007; Embury, Mohandas, Paszty, Cooper, & Cheung, 1999; Fink, Funahashi, Robinson, & Watson, 1961; LaCelle, 1970; Lipowsky, Sheikh, & Katz, 1987; Lipowsky, 2005; Mohandas, Philips, & Bessis, 1979; Parthasarathi & Lipowsky, 1999; Paszty et al. 1997; Rodgers et al. 1984; Schmalzer, Manning, & Chien, 1989).

Pointing out RBC heterogeneity in blood flow in order to establish velocity profiles, and understanding how these profiles are affected when endothelial cells are activated, are the two aspects that we have developed in this original study.

We have already confirmed that AA RBCs are heterogeneous according to their velocity (Allayous, Regis, Bruel, Schoevaert, Emilion, & Marianne-Pepin, 2007; Rosenblum, 1971; Rosenblum, 1972a; Rosenblum, 1972b; Rosenblum, 1976). This observation is also confirmed with SS RBCs.

In addition, we have noticed that AA and SS are also heterogeneous even when vascular environment is different. Thus, AA and SS RBC classes and dynamic behavior for instance, evolve differently, according to endothelial state represented by nature and

time of TrHBMEC treatment. This heterogeneity seems to reflect RBC position in the flow. Thus, the fastest cells are more into an axial migration in the flow and the slowest ones are closer to the vascular wall (Bishop, Popel, Intaglietta, & Johnson, 2001). However, the flow chamber apparatus used in this study does not allow us to confirm these positions, in a constant venular flow and a constant hematocrit.

These different RBC velocity and class heterogeneities must be explained by RBC nature, endothelial cell activation or state, and also by the different cellular interactions occurring in the blood flow. It is important to notice that in all of the experiments, RBCs were perfused with a particular medium in order to minimize undesirable interactions due to system contamination (plasma proteins, etc…).

RBC nature and dynamic behavior, also known as RBC rheology, includes cellular deformability, intracellular viscosity, flow and shear stress, aggregation, RBC density and age, for example (Armstrong, Wenby, Meiselman, & Fisher, 2004; Fischer & Schmid-Schönbein, 1977; Fischer, Schmid-Schönbein, & Stöhr-Liesen, 1978a; Fischer, Stöhr, & Schmid-Schönbein, 1978b; Grima, 2007; Kon, Maeda, & Shiga, 1987; Pfafferott, Nash, & Meiselman, 1985; Schmid-Schönbein & Wells. 1969; Watanabe, Kataoka, Yasuda, & Takatani, 2006).

Here, RBC heterogeneity, expressed as an increase, a stabilization or a decrease in RBC velocity, could be explained by different notions that will be developed in the following sections.

In normal conditions, RBCs have many cellular properties explaining their particular rheology, like their deformability, their aggregability and their adhesion to endothelial cells (Barshtein, Ben-Ami, & Yedgar, 2007). All experiments were performed with

controlled venular flow and shear stress (1 dyne/cm²) as well as a constant hematocrit, and do not interfere with RBC velocity.

SS RBCs have particular cellular properties such as a decrease in deformability, leading to vascular blockage, a decrease in cellular aggregability and an increase in cellular adhesion (Barshtein, Ben-Ami, & Yedgar, 2007; Bransky, Korin, Nemirovski, & Dinnar, 2007; Dhermy, Simeon, Wautier MP, Boivin, & Wautier JL, 1987; Finnegan, Turhan, Golan, & Barabino, 2007; Hebbel, 1997; Hebbel, 1997; Kaul, Nagel, Chen, & Tsai, 1993; Mchedlishvili, 1998; Mohandas & Chasis, 1993; Parthasarathi & Lipowsky, 1999; Wick & Eckman, 1996). These factors trigger abnormal blood flow (Embury, Mohandas, Paszty, Cooper, & Cheung, 1999; Fink, Funahashi, Robinson, & Watson, 1961; Lipowsky, Sheikh, & Katz, 1987; Paszty et al., 1997; Rodgers, Schechter, Noguchi, Klein, Nienhuis, & Bonner, 1984) and abnormal red blood cell velocities, as observed in the present study.

These RBC properties are combined with vascular environment, represented by endothelial cell activation.

TrHBMEC were activated by proinflammatory cytokines (Schweitzer et al., 1997).

It is well-known that endothelial cells are activated by pro-inflammatory cytokines. This activation is translated by cell-surface adhesion molecule expression and regulation. TrHBMEC is a well-characterized cell line that expresses all endothelial markers such as von Willebrand factor, Vascular Cell 1dhesion Molecule-1 (VCAM-1), InterCellular Adhesion Molecule-1 (ICAM-1), E-selectine or P-selectine (Schweitzer et al., 1997).

It has been recently shown that ICAM-1 expression and release are increased in the presence of cytokines during 48 hours (Brun, Bourdoulous, Couraud, Elion, Krishnamoorty, & Lapouméroulie, 2003; Schweitzer et al., 1997). In addition, VCAM-1

expression is induced by cytokines, the most important expression is observed between 12 and 24 hours, without affecting its release (Brun, Bourdoulous, Couraud, Elion, Krishnamoorty, & Lapouméroulie, 2003; Schweitzer et al., 1997). The level of soluble ICAM-1 reflects the degree of inflammation and endothelial cell activation (Gearing & Newman, 1993). SS RBCs have been shown to induce ICAM-1 expression by endothelial cells in culture and flow studies (Brun, Bourdoulous, Couraud, Elion, Krishnamoorty, & Lapouméroulie, 2003; Shiu, Udden, & McIntire, 2000).
VCAM-1 expression is up-regulated by proinflammatory cytokines and is the receptor of the very late antigen-4 (VLA-4) expressed on SS RBCs (Elion & Labie, 1998; Gee & Platt, 1995; Joneckis, Ackley, Orringer, Wayner, & Parise, 1993; Stuart & Nagel, 2004; Swerlick, Eckman, Kumar, Jeitler, & Wick, 1993).
These molecular modifications could contribute to clinical observations such as a decrease in vascular occlusion in sickle cell patients.

These notions linked to RBC nature could be useful to understand the physiological meaning of the heterogeneity of RBC velocities and the different velocity profiles. Moreover, cellular adhesion, represented by cell-surface molecules and RBC deformability, also participate in this phenomenon. In addition, it has been shown that SS RBC perfusion increases the expression of cell adhesion molecules on endothelial cells, such as ICAM-1 and VCAM-1, and stimulates the release of soluble cell adhesion molecules, which may serve as indicators of injury and/or activation of endothelial cells (Shiu, Udden, & McIntire, 2000). The interactions between sickle red blood flow, inflammatory cytokines, and vascular adhesion events may render sickle cell disease patients vulnerable to vasoocclusive crises (Shiu, Udden, & McIntire, 2000).

Another aspect of this work would consist in linking RBC velocity to RBC trajectory in order to characterize particular RBCs and molecular profiles, according to vascular state (inflammation, drugs, …).

Inflammation state is very important in sickle cell disease. In fact, sickle RBCs are more adherent to vascular endothelium and the endothelium in sickle cell patients is inflamed (Duits et al., 1996; Solovey, Lin, Browne, Choong, Wayner, & Hebbel, 1997; Turhan, Jenab, Bruhns, Ravetch, Coller, & Frenette, 2004).

It would help to have a better understanding of RBC dynamics, particularly by knowing what are the differences between sub-populations at a molecular level, like young RBCs, their density, the expression of cell-surface molecules, etc…. All these questions could be answered thanks to different experiments such as blocking molecules at the surface of endothelial cells, perfusing with different categories of RBCs (young, dense, etc…), so as to understand the physiological meaning of such an heterogeneity better. Moreover, this study would give us a better knowledge of the complex vascular occlusion pathway and also of red blood cell rehology and effects in normal vascular function in order to apply it to pathological mechanisms.

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6. Tables

TrHBMEC treatment	AA RBCs	SS RBCs
None		
TNFα: 100 U/ml / IFNγ: 100 U/ml	12h	12h
	24h	24h
	48h	48h

Table 1: TrHBMEC treatments before flow assay.

RBCs, Red blood cells.

RBCs	Treatment	Time	MV (µm/ms)	RBC N
AA	None		1005.94 ± 299.08	58
	Cytokines	12h	1219.34 ± 421.83	63
		24h	1025.49 ± 357.83	60
		48h	1004.67 ± 308.06	62
SS	None		1186.27 ± 264.89	58
	Cytokines	12h	1167.59 ± 306.55	60
		24h	913.88 ± 307.44	61
		48h	876.97 ± 282.37	60

Table 2: AA and SS RBC general mean velocities after TrHBMEC treatment.

MV, Mean velocity; RBC, Red blood cell; RBC N, Red blood cell number

RBCs	Class	MV (µm/ms)	RBC %
AA	1	709.84 ± 83.98	29
	2	1031.85 ± 153.32	59
	3	1599.22 ± 196.99	12
SS	1	901.95 ± 91.16	31
	2	1175.54 ± 86.61	45
	3	1571.77 ± 100.83	24

Table 3: AA and SS RBC classes when TrHBMEC are not treated.

MV, Mean velocity; RBC, Red blood cell

RBCs	Time	Class	MV (µm/ms)	RBC %
AA	12h	1	868.68 ± 161.12	45
		2	1262.14 ± 92.16	25
		3	1746.05 ± 220.54	30
	24h	1	542.78 ± 117.61	27
		2	947.12 ± 81.58	30
		3	1350.64 ± 198.03	43
	48h	1	774.51 ± 123.85	52
		2	1020.22 ± 12.47	16
		3	1365.14 ± 231.22	32
SS	12h	1	829.33 ± 116.37	35
		2	1272.66 ± 112.67	53
		3	1702.09 ± 143.87	12
	24h	1	336.81 ± 115.58	13
		2	918.49 ± 137.62	71
		3	1355.74 ± 135.35	16
	48h	1	533.79 ± 121.59	32
		2	1036.01 ± 170.56	68

Table 4: AA and SS RBC sub-groups when TrHBMEC are stimulated with proinflammatory cytokines during 12; 24 and 48 hours before flow assay.

MV, Mean velocity; RBC, Red blood cell

7. Figures

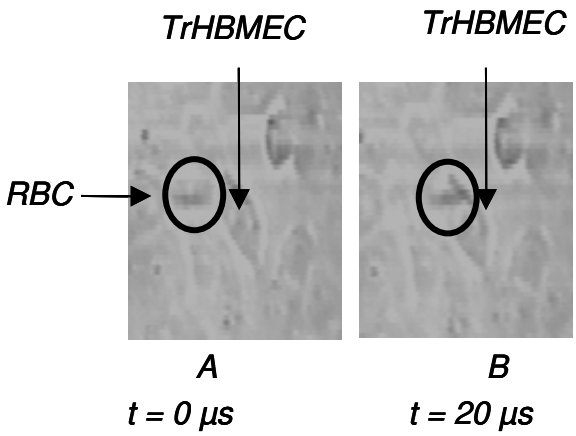

Figure 1: RBC and TrHBMEC interactions under venular blood flow.

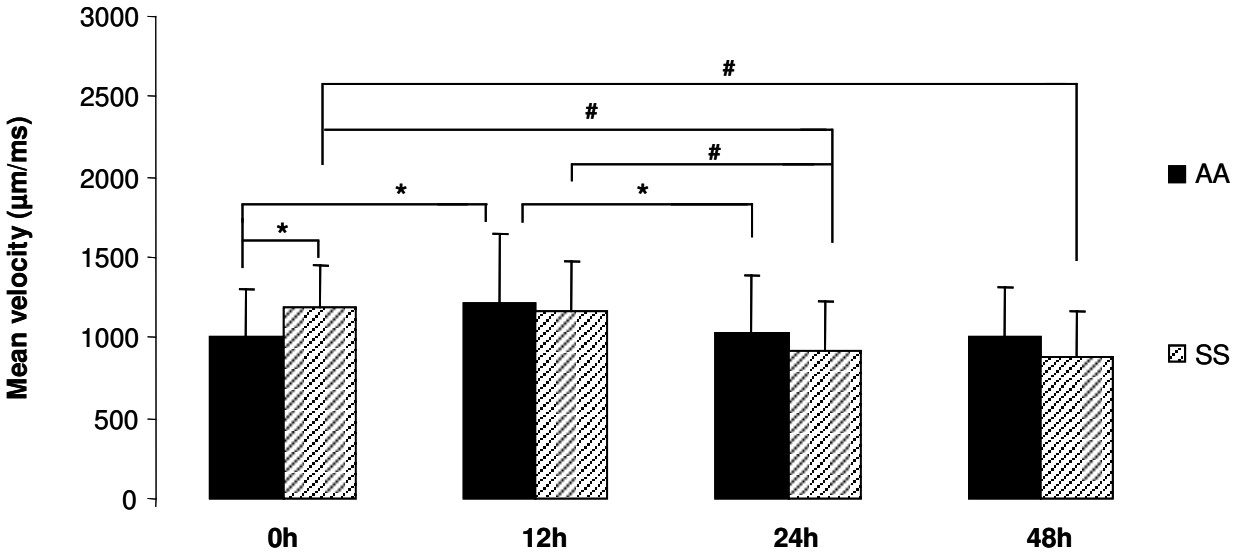

Figure 2: Mean velocity of total AA and SS RBC population when TrHBMEC are stimulated by proinflammatory cytokines during 12, 24 and 48 hours before flow assay. * $p \leq 0.05$ vs. AA (0; 12; 24; 48h); # $p \leq 0.05$ vs. SS (0; 12; 24; 48h).

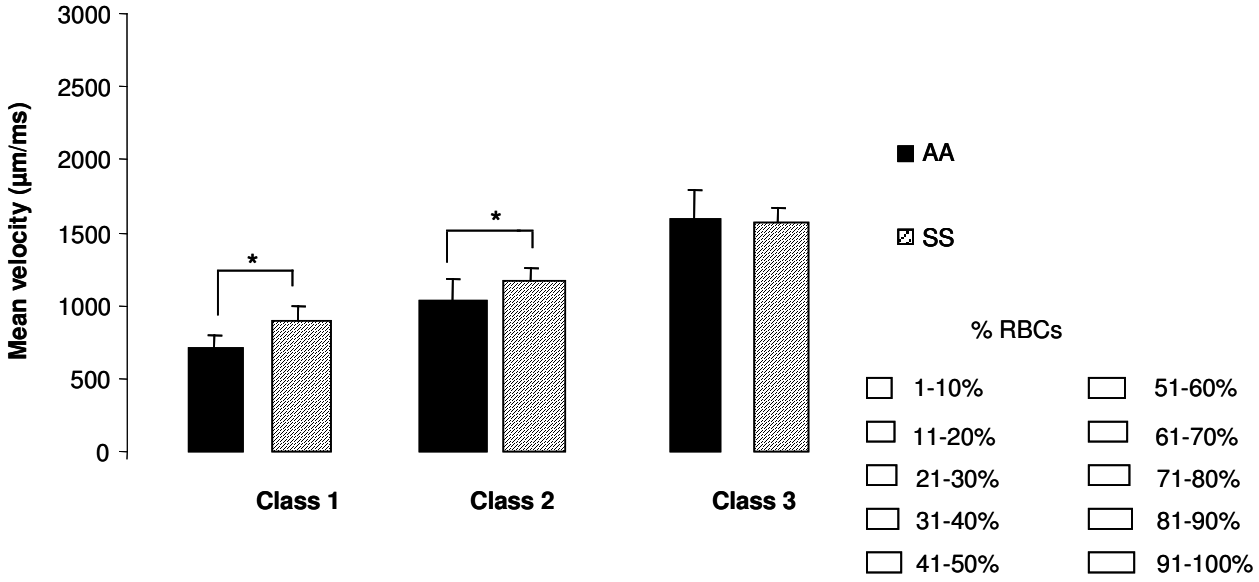

Figure 3 : Evolution of mean velocities of AA and SS RBC classes in basal conditions (TrHBMEC are not activated).

* $p \leq 0.05$ vs. AA. Histogram width reflects the percentage of RBCs in each class.

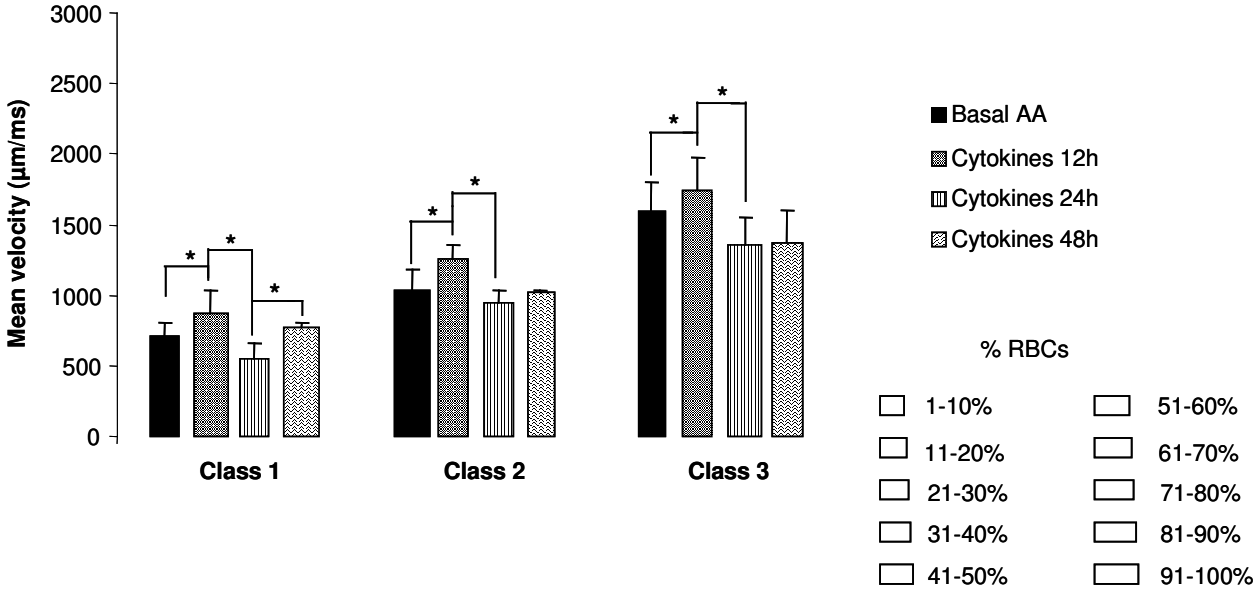

Figure 4 : Evolution of mean velocities of AA RBC classes when TrHBMEC are activated by proinflammatory cytokines during 12, 24 and 48 hours before flow assay. * $p \leq 0.05$ vs. AA (0; 12; 24; 48h). Histogram width reflects the percentage of RBCs in each class.

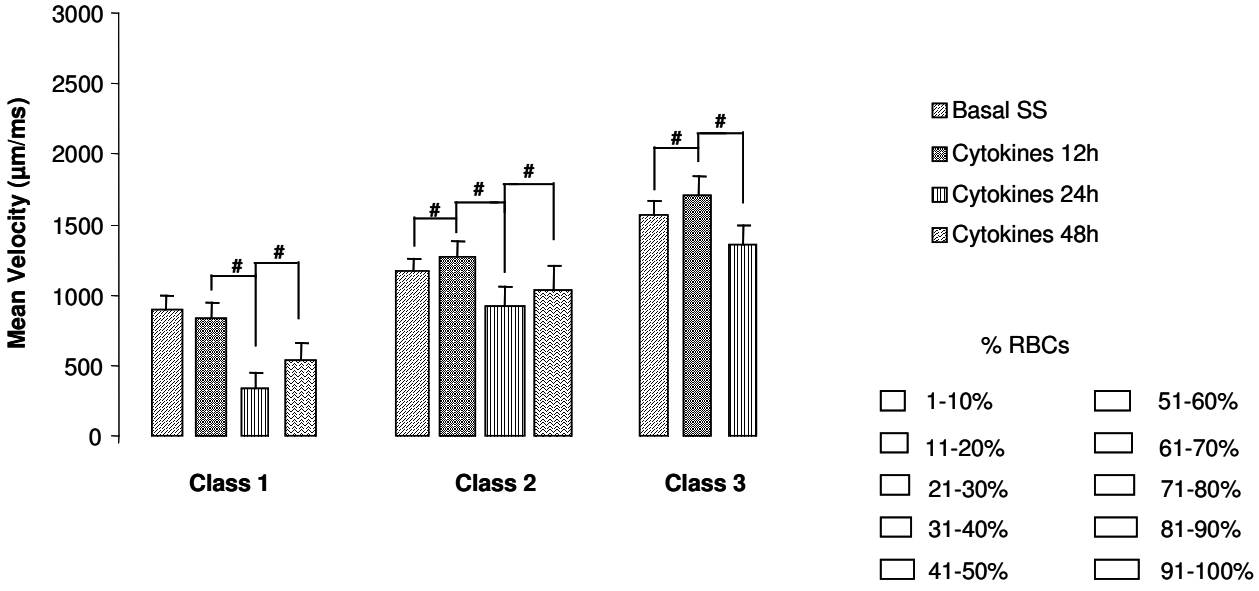

Figure 5 : Evolution of mean velocities of SS RBC classes when TrHBMEC are activated by proinflammatory cytokines during 12, 24 and 48 hours before flow assay. # $p \leq 0.05$ vs. Basal SS (0; 12; 24; 48h). Histogram width reflects the percentage of RBCs in each class.