Blood Dynamics in Microfabricated Vessel Networks

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ABSTRACT

Blood displays interesting behavior when flowing through channels with dimensions similar to the blood cells. A well-known example of this is the enhancement of leukocyte (WBC) adhesion in postcapillary venule expansions by erythrocytes (RBCs). Until recently, detailed mechanistic studies of such blood flow anomalies were difficult to perform. To overcome this limitation, we design and microfabricate networks of microchannels cast in a transparent silicone polymer (PDMS). We show that capillary to postcapillary transitions are capable of promoting leukocyte margination. Leukocytes in whole blood solutions marginated more quickly at low shear rates compared to high shear rates. The results indicate the average distance required for a leukocyte to marginate depends on the aggregation level and that normal human blood, with intermediate levels of RBC aggregation, has appropriate rheological properties to facilitate leukocyte margination.

Keywords: microfabrication, microfluidics, hemodynamics, rheology, leukocyte

1 INTRODUCTION

The margination of leukocytes from the center of a blood vessel toward the vascular endothelium is an integral phase of the inflammatory response. Erythrocyte-leukocyte interactions within a vessel determine the frequency of leukocyte-endothelium contact, but once in contact with the endothelium adhesion molecules control leukocyte rolling, firm adhesion and emigration. Previous studies have shown the hematocrit [1, 2], erythrocyte configuration [3], level of erythrocyte aggregation and vessel geometry [2, 4] have a significant effect influencing the overall flux of leukocytes towards the endothelium.

It has been established that leukocytes preferentially roll along and adhere to the endothelium in postcapillary venules. The increased leukocyte activity is a function of decreased shear rates [5] as well as the increased expression of leukocyte adhesion ligands such as P-selectin [6] and ICAM-1 [7] that have been reported in postcapillary venules. The expression of adhesion molecules on leukocytes and endothelial cells controls leukocyte-endothelial interactions once contact has been initiated between the cells. We are interested in identifying the mechanism of margination in postcapillary venules that forces leukocytes towards the vessel wall and endothelial cells to create the initial contact between the cells. Given the physiological importance of the postcapillary venule we will use the postcapillary expansion model, which has been widely used to study leukocyte margination [2-4].

The ability to design, fabricate and utilize microfabricated microchannels has enabled scientists to examine specific vascular geometries under varying rheological conditions in vitro. Recent studies have shown these microchannels can be used to study the microcirculation [8] as well as create a useful device to separate leukocytes from whole blood [9]. In previous studies we have developed the lattice Boltzmann approach to model erythrocyte-leukocyte interactions in postcapillary expansions and demonstrated the effects of erythrocyte configuration on leukocyte-endothelial interactions [3]. In the current study we use microfabricated microchannels to study erythrocyte-leukocyte interactions in a postcapillary expansion model. Our results suggest that erythrocyte aggregation and channel geometry determine the probability that leukocytes marginate in postcapillary venules and validate the microchannel system for further experimentation that accurately reproduces in vivo leukocyte behavior.

2 MATERIALS AND METHODS

2.1 Microchannel Design and Fabrication

The desired network was designed using L-Edit software (Tanner EDA, Tanner Research Inc., Pasadena, CA) and then converted into a machine-specific format using CATS software (Transcription Enterprises Inc., San Jose, CA). A silicon wafer (4" diameter, 475-575 um thickness, 1-20 ohm-cm, <1-0-0>, Silicon Quest International Inc., Santa Clara, CA) was cleaned in oxygen plasma (1 min, 1000W; Branson/IPC P2000 Barrel Etcher), dehydrated on a hot plate (5 min, 170° C), spin-coated with positive resist (XP 9947 Shipley Company, L.L.C., Marlborough, MA) and baked (3 min, 135° C). Direct electron beam writing was used to transfer the network pattern onto the wafer (EBMF-10.5/CS; Cambridge Instruments, UK). After postexposure baking (90s, 135° C) and developing (1 min, 300 MIF; Clariant Corporation, Somerville, NJ), the wafer was cleansed in oxygen plasma

(30 s, 100 W). The pattern was etched with the Bosch fluorine process (Unaxis SLR 770 ICP Deep Silicon Etcher; Unaxis USA Inc., St. Petersburg, FL) and residual resist was removed with oxygen plasma (10 min, 1000W; Branson/IPC P2000 Barrel Etcher). The silicon wafer was now capable of being used as a negative mold for microchannel devices.

2.2 Microchannel Preparation

Casts of the microchannel device were made by pouring transparent liquid elastomer poly(dimethylsiloxane) (PDMS, RTV 615 A/B; GE Silicones, Waterford, NY) onto the silicon wafer, baking it (1 h, 100° C), peeling the cast off the wafer and trimming it to the desired size. A glass microscope slide (Micro Slides; VWR Scientific, West Chester, PA) was spin-coated with the same elastomer and used as support for the cast microchannels. The cast microchannels and elastomer covered glass were cleaned with oxygen plasma (100 s, Plasma Cleaner/Sterilizer; Harrick Scientific Corp., Ossining, NY) and the microchannels were placed on the glass slide without using compressive force. The microchannel device was flushed with 1% PEG-Silane (O-methyl-O'[2-(trimethoxysilyl)ethyl)]polyethylene glycol, MW 5000; Shearwater Polymers Inc., Huntsville, AL) for 20 min, followed by perfusion with GASP buffer for 5 min prior to use.

2.3 Blood Preparation

Blood was collected in Vacutainer tubes (10 mL, 17.55 mg (K3) EDTA, BD, Franklin Lakes, NJ). The hematocrit (Hct), leukocyte count and platelet count of each blood sample were measured using a Sysmex K1000 hematology analyzer (Sysmex America Inc., Mundelein, IL). Blood samples were stained using the fluorescent nucleic acid label Syto 13 (Invitrogen, Carlsbad, CA) as per the product instructions. For high and low aggregation studies blood cells were centrifuged and medium recovered three times at 1500 rpm and re-suspended in GASP buffer (9 mM Na_2HPO_4, 1.3 mM Na_2HPO_4, 140 mM NaCl, 5.5 mM glucose and 1% bovine serum albumin at pH~7.4), with 3% Dextran-500 (high aggregation) or without (low aggregation) prior to Syto-13 staining. To adjust hematocrit, erythrocytes were removed during sample washing.

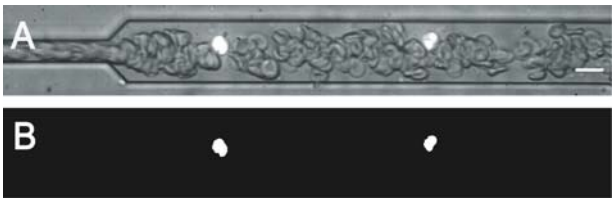

Figure 1. Shown is the postcapillary expansion model. Blood flows through the small capillary (D≈10μm) into the larger postcapillary channel (D≈25μm). Experiments were performed using minimal levels of brightfield trans-illumination concurrently with epi-fluorescence illumination. (A) Blood samples were stained with the nuclear stain Syto-13 which labeled leukocytes and not erythorocytes. (B) Acquired images were binarized and the leukocyte location and velocity were determined using an algorithm in Matlab. The white bar is 10 um in length.

2.4 Experimental Protocol

The prepared blood sample (200 ul) was placed in a small opening at the entry to the device. The output segment of the device was connected to a waste reservoir positioned to provide a pressure drop across the device and create fluid flow. Experiments were performed using the minimum level of trans-illumination to be able to discern the walls of the channels concurrently with epi-fluorescence to visualize the stained leukocytes (Figure 1A).

2.4 Image Analysis

Analysis of images was performed using Matlab (Mathworks, Natick, MA). Images were binarized to track individual leukocytes by recording the location and diameter ($D_{leukocyte}$) of the leukocyte, calculate the instantaneous leukocyte velocity ($V_{leukocyte}$) as well as the distance of the leukocyte from the channel wall (Figure 1B). Leukocytes were deemed to be in contact with the channel wall if the centroid of the leukocyte came within $D_{leukocyte}/2$ of the channel wall. The channel wall was defined by user input prior to image analysis. The erythrocyte velocity ($V_{erythrocyte}$) was determined offline manually with a user tracking 10 individual erythrocytes down the center of the channel for each experimental run. The shear rate in the postcapillary expansion was estimated from the following equation

$$\dot{\gamma} = \frac{2V_{erythrocyte}}{R} \quad (1)$$

where γ is the shear rate and R is the average channel radius (12.6 um). Leukocytes entering the postcapillary expansion or straight channel in close proximity to another leukocyte (within 25 um) were eliminated from analysis.

3 RESULTS

Consistency among mean blood cell velocities in the microchannels indicates the ability of the device to produce constant velocities at a given pressure difference imposed across the network. For each experiment a pressure drop of

either 2.5 cmH_2O or 10.0 cmH_2O was used to drive the sample solution through the device.

The average distance required for a leukocyte to marginate from the center of a channel towards the wall reflects the ability of a channel geometry or rheological condition to promote leukocyte margination. The longer the average margination distance the less likely it is that a leukocyte would marginate in vivo given the average length of postcapillary venule segments. In the postcapillary channel the distance until margination was most greatly affected by the level of aggregation in the blood sample (Figure 2). When comparing cells in both plasma and buffer suspensions there was no significant difference between leukocyte margination distances at high shear rates ($p=0.34$), but at low shear rates the margination distance was significantly lower in the plasma suspension ($p=0.035$).

The time until contact (t_c) was defined as the length of time measured between a leukocyte entering the expansion or straight channel and contacting the channel wall. The value of t_c can be indicative of the effects of aggregation when comparing cell populations in similar shear rates. In the postcapillary expansion channels leukocytes in the plasma suspension had an average t_c of 0.92 ± 0.06 s at high shear rates which was not significantly different than the 1.18 ± 0.2 s averaged at lower shear rates ($p=0.57$). On the other hand, in the buffer suspension a t_c of 1.07 ± 0.11 s at higher shear rates was significantly faster than the 3.30 ± 0.29 s averaged at lower shear rates ($p<0.01$).

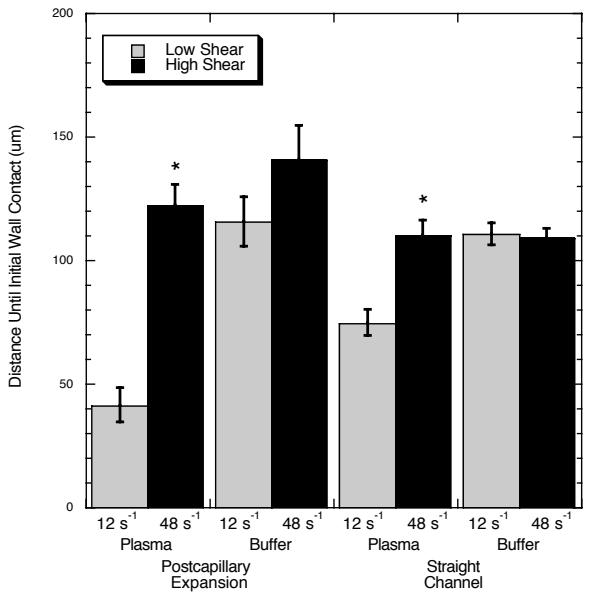

Figure 2. The average length of the expansion or straight channel required for a leukocyte to marginate from the center of the microchannel and make initial contact with the wall. The average distance was significantly affected by the shear rate and erythrocyte aggregation. Low shear rates enabled greater erythrocyte aggregation and led to shorter margination distances. High shear rates and lower aggregation levels led to longer margination distances. All data is shown as the mean ± standard error.

In the straight channels the average t_c of leukocytes in the plasma suspension was significantly longer ($p=0.013$) in low shear rates (2.26 ± 0.16 s) than high shear rates (1.10 ± 0.04 s). Average t_c of leukocytes in the buffer suspension were significantly longer ($p<0.01$) in low shear rates (3.36 ± 0.14 s) than high shear rates (1.11 ± 0.07 s). There was no significant difference between t_c in fast flow in the plasma and buffer suspensions ($p=0.42$).

4 DISCUSSION

The current experiments highlight the physiological advantages of the postcapillary expansion in promoting leukocyte interactions with the endothelium in postcapillary venules. We show that even minute changes in the rheological properties of blood can have a significant effect on the ability of leukocytes to interact with the postcapillary endothelium. The results of these in vitro experiments have been utilized to validate our microchannel system for further iterations and experimental endeavors.

The depth of the microchannels (≈10.7 μm) permitted unrestricted leukocyte movement and restricted erythrocytes from passing above and below the leukocytes. There is a possibility that a deeper channel or blood vessel could have a greater amount of margination because the erythrocytes could pass and exert forces on a leukocyte from three dimensions instead of two. The diminished effects of aggregation above shear rates of 250 sec^{-1} have been well established and we felt it unnecessary to perform experiments at high shear rates to reproduce and confirm these results. At very low shear rates the effects of aggregation increase dramatically and the current experiments exploit this increase to show that a small change in shear rate can cause a remarkable change in leukocyte behavior. At $V_{erythrocyte}$ above 667 μm/s ($\gamma \geq 108$ sec^{-1}) a leukocyte would pass through our postcapillary model before erythrocytes have time to form aggregates.

A striking result was that regardless of the blood cell suspension, hematocrit and channel geometry, the lower shear rate samples always had an increased percentage of leukocytes contacting the channel wall when compared to the higher volumetric flow samples. This effect is most likely linked to the ability of erythrocytes to form aggregates more effectively at lower shear rates and therefore leads to increased margination. Previous studies have examined the relationship between shear rate and rolling fraction of leukocytes towards the vessel wall and

have shown that as shear rate decreases the leukocyte rolling fraction increases [10].

A unique aspect of this study was the ability to measure the t_c of leukocytes in the postcapillary expansions and provide insight to the effects of aggregation on leukocyte margination.

The results of t_c indicate the dynamic aggregation of erythrocytes in the expansion takes time, not distance and leukocyte margination is dependent on the time required for erythrocytes to aggregate. A recent in vivo study has suggested that upon entering a postcapillary venule it takes erythrocytes between 15 and 30 um or less than 0.3 s to form aggregates [11]. The current data suggest that with an average t_c less than 0.3 s there is plenty of time for erythrocyte aggregation to begin and influence the margination of leukocytes in the postcapillary expansions. In vitro studies have shown the rate of aggregation increases as hematocrit increases, which corresponds to the current data showing less leukocyte margination and lower hematocrits [12]. An inherent hemodynamic characteristic of the postcapillary expansion is a marked decrease in $V_{erythrocyte}$ at the transition from capillary to venule and it has been shown that a sudden decrease in shear rate can cause an increase in the rate of erythrocyte aggregation to increase [13]. The current results suggest changes in the rate of erythrocyte aggregation in the postcapillary channel affect leukocyte margination.

Given the dependency of erythrocyte aggregation on shear rate, it would appear that given enough time for erythrocyte aggregates to form, a straight channel will have just as much margination as a postcapillary expansion. In a previous study we have shown in extremely long straight channels (>7000 um) over 90% of all leukocytes will marginate [9].

The current experiments we show that both significant increases and decreases in plasma protein levels can lead to drastic changes in the percentages of leukocyte margination. This new and novel discovery suggests that while postcapillary expansions are capable of promoting margination and increasing the amount of leukocytes marginated, straight channels are quite capable of maintaining a constant flux of leukocytes along the channel wall. This may occur when the narrow capillary promotes erythrocyte organization by concentrating them at the center of the channel and when they reach the postcapillary channel they are more capable of pushing the leukocytes towards the sides and causing margination. The system is novel because a decrease in aggregation or an increase in shear rate is all that is needed to disrupt erythrocyte organization and make it difficult for erythrocytes to cause leukocyte margination. Conversely, if the erythrocytes are too ordered and highly aggregated, as is the case with 3% Dextran, the erythrocytes are unable to break their rouleaux and cause margination.

In conclusion, this study shows the importance of erythrocyte aggregation as well as vessel geometry in promoting leukocyte margination as well as their combined effects on how rapidly leukocyte margination may occur. These factors have physiological implications in tissues such as tumors that are capillary deficient as well as clinical significance in patients that may be anemic or receiving large amounts of anti-coagulant treatment that may lower the overall ability of their blood to aggregate. This suggests that current antiangiogenic therapies aimed at normalizing the vasculature of tumors could provide tumors with more postcapillary expansions, increased leukocyte margination and a greater natural immune response.

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