Macromolecular depletion modulates the binding of red blood cells to activated endothelial cells[a)]

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Adhesion of red blood cells (RBCs) to endothelial cells (ECs) is usually insignificant but an enhanced adhesion has been observed in various diseases associated with vascular complications. This abnormal adhesion under pathological conditions such as sickle cell disease has been correlated with increased levels of various plasma proteins but the detailed underlying mechanism(s) remains unclear. Usually it is assumed that the proadhesive effects of plasma proteins originate from ligand interactions cross-linking receptors on adjacent cells, but explicit results detailing binding sites or receptors for some proteins (e.g., fibrinogen) on either RBC or EC surfaces that would support this model are missing. In this study, the authors tested whether there is an alternative mechanism. Their results demonstrate that dextran 2 MDa promotes the adhesion of normal RBCs to thrombin-activated ECs and that this effect becomes more pronounced with increasing thrombin concentration or with prolonged thrombin incubation time. It is concluded that depletion interaction originating from nonadsorbing macromolecules (i.e., dextran) can modulate the adhesion of red blood cells to thrombin-activated EC. This study thereby suggests macromolecular depletion as an alternative mechanism for the adhesion-promoting effects of nonadsorbing plasma proteins. These findings should not only aid in getting a better understanding of diseases associated with vascular complications but should also have many potential applications in biomedical or biotechnological areas that require the control of cell-cell or cell surface interactions. © *2010 American Vacuum Society.* [DOI: 10.1116/1.3460343]

I. INTRODUCTION

Abnormal adhesion of red blood cells (RBCs) to endothelial cells (ECs) has been identified in a wide range of diseases associated with vascular complications.[1,2] For example, in sickle cell anemia, the increased adhesion of RBCs to ECs has been linked to vascular complications associated with this disease, including the episodic occurrence of vaso-occlusive crisis,[3–5] which can ultimately lead to organ failure and even death.[6,7] There is now a general agreement that various receptors on RBCs [e.g., $\alpha_4\beta_1$-integrin[8,9] or CD36 (Ref. 10)] and ECs [e.g., VCAM-1 (Ref. 11) or $\alpha_V\beta_3$-integrin[12]] play a key role for the adhesive interaction between sickle RBCs and ECs.[13,14] Thrombin, an important regulator of coagulation system, has been shown to activate ECs in that ECs treated with this protein exhibit an increased adhesiveness for RBCs via the expression of various adhesion molecules such as ICAM-1 and P-selectin.[15–17] Moreover, EC activated via thrombin contract and thereby expose the subendothelial cell matrix.[18,19] These findings are also consistent with the identification of an increased plasma level of thrombin in patients with sickle cell disease, and further elevated levels during the onset of vaso-occlusive crisis.[20–22]

Plasma proteins are also known to induce or promote RBC adhesion to ECs but the underlying mechanism(s) are often unclear.[6] Whereas some plasma proteins (e.g., thrombospondin or von Willebrand factor[23,24]) have been proposed to act as ligands, cross-linking specific receptors between the adjacent cells, there is still no clear picture on how other plasma proteins such as fibrinogen might be involved.[25,26] For example, in the case of fibrinogen, it has been proposed that this acute phase protein also acts as a ligand,[27] cross-linking receptors on adjacent cells, but a few works have pointed out that specific receptors may not be necessary for the adhesion-promoting effect of this and possibly other plasma proteins.[28,29] Moreover, explicit results detailing binding sites or receptors for fibrinogen on either RBCs or ECs that would support this model have not been reported. Thus, even though it has been known for decades that fibrinogen induces adhesion of normal[30] and pathological RBCs,[25,31] there is still no conclusive picture of the underlying mechanism.

In recent works, we were able to demonstrate that the adhesion-promoting effect of large macromolecules must not necessarily involve binding but that an alternative mechanism could be depletion interaction.[32,33] Depletion interaction is a result of a lower localized protein or polymer concentration near the cell surface as compared to the suspending medium.[34] This exclusion of macromolecules near the cell surface leads to an osmotic gradient and as two cells or surfaces approach; solvent is displaced from the depletion zone into the bulk phase leading to an attractive

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force.[34–37] This work aims to clarify the impact of depletion interaction on the adhesion of RBCs to ECs, which have been activated via thrombin. To mimic the impact of nonadsorbing polymers, RBCs were suspended in solutions containing dextran, a neutral polyglucose, that has been proven to be depleted from the RBC surface[38,39] and that is often used *in vitro* for hemorheological studies.[40]

II. MATERIALS AND METHODS

A. Red blood cells

Blood was drawn into 1.5 mg/ml ethylenediaminetetraacetic acid (EDTA) from the antecubital vein of healthy adult volunteers. RBCs were separated from whole blood by centrifugation (1000 g, 10 min) and then washed thrice with phosphate buffered saline (PBS, 10 mM phosphate, 285 mOsm/kg, pH=7.4) containing 0.2% BSA. RBCs were resuspended in either serum free medium (SFM) or in dextran-SFM containing dextran 40 kDa and dextran 2 MDa (Sigma Aldrich, Singapore) at the desired final concentrations.

B. Endothelial cells

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza, UK. The culture medium consisted of 90% of Ham's F-12 K with 2 mM L-glutamine (Sigma), 1.5 g/l sodium bicarbonate (Sigma), 0.1 mg/ml heparin (Sigma), 0.2 vol % bovine brain extract (BBE, Hammond Cell Tech), 120 U/ml penicillin/streptomycin (Sigma), and 10% fetal bovine serum (FBS). This culture medium without BBE and FBS was used as serum free medium (SFM, s.a) in the adhesion assay. HUVECs were placed in tissue culture flasks precoated with gelatin and cultured at 37 °C in a CO_2 (5%) incubator. Once the cells reached 80% confluence, they were subcultured in 35 mm Petri-dishes (Greiner) precoated with collagen from fish skin (Sigma) and grown to confluence.

C. Flow chamber adhesion assay

The flow system consisted of an acrylic flow deck and a silicone rubber gasket (Glycotech) with the cutout area of the gasket forming the flow channel. Both the gasket and flow deck were placed into 35 mm Petri dishes coated with confluent layers of HUVEC. This flow chamber was then placed on an inverted microscope. The inlet of the chamber was connected by silicone tubing to a miniature low displacement electronic valve that allowed switching between reservoirs containing either RBC suspensions or PBS. The outlet of the chamber was connected to a variable speed withdrawal syringe pump (Harvard Apparatus Co., Millis, MA) that drew either RBC suspension or rinse solution through the flow chamber at a selected volumetric flow rate Q. The wall shear stress τ was calculated via $\tau=6\ \mu Q/a^2b$, where μ is the dynamic viscosity of the solution, a is the channel height (0.254 mm), and b is the channel width (2.5 mm). The density of the solutions was measured by a density meter (Anton Paar DMA35) and the dynamic viscosities were measured by an automated microcapillary viscometer (Anton Paar AMVn). The microscope, valve, inflow tubing, and the reservoirs for the two fluids were maintained at 37 °C via a thermostated enclosure.

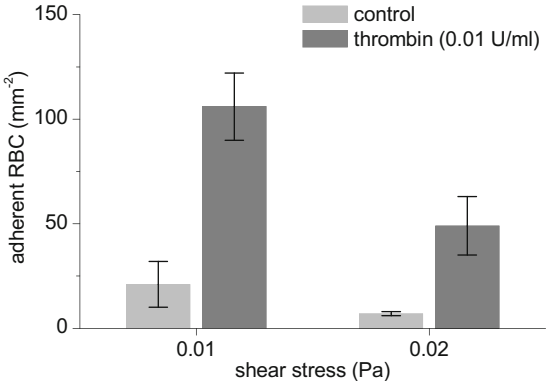

FIG. 1. Adherence of RBCs to normal ECs or thrombin-activated ECs as a function of the applied shear stress. ECs were pretreated with 0.01 U/ml of thrombin for 5 min, followed by the coincubation with RBCs for 15 min before applying a stepwise-increasing shear. Error bars are SDs of the mean adherence values from three individual experiments.

D. Experimental protocol

RBCs were resuspended at a concentration of 1×10^7 cells/ml in either SFM or in dextran-SFM. ECs were incubated with thrombin at concentrations between 0 and 0.10 U/ml for 5 min followed by co-incubation with RBC suspension for either 15 min or 30 min.[15,16,19] Thereafter the chamber was rinsed with cell-free SFM or dextran-SFM while applying a stepwise-increasing shear. At the end of each rinse, 20 images were taken at 20 random locations and the absolute RBC attachment per mm^2 was calculated. The final adherence is represented as mean $\pm$ standard deviation (SD) of three individual experiments repeated under the same conditions. Wilcoxon–Mann–Whitney U test, a nonparametric method for two unpaired samples, was performed on adherence values of different samples.

III. RESULTS

A. Thrombin induces adhesion of RBCs to ECs

Initial efforts were directed toward examining the impact of thrombin activation on RBC adhesion to ECs. Confluent EC monolayers were pretreated with 0.01 U/ml of thrombin in dextran-free SFM for 5 min, followed by coincubation with normal RBCs suspended in the same medium for 15 min. Thereafter, nonadherent cells were removed via rinsing the flow chamber with cell-free media applying a stepwise-increasing shear. As shown in Fig. 1 at the lowest applied shear stress of 0.01 Pa, only a few RBCs (21 ± 11) are adherent to normal ECs, whereas ECs treated with 0.01 U/ml of thrombin exhibit a substantially increased adhesion rate. After applying 0.01 Pa, five times as many RBCs remain adherent to activated ECs as compare to untreated ECs. Increasing the shear stress to 0.02 Pa removes about half of the cells bound to activated ECs, whereas two-thirds of cells settled onto untreated ECs are not able to withstand this

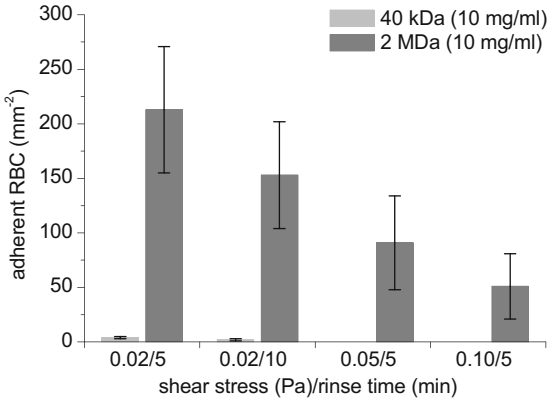

FIG. 2. Adherence of RBCs to normal ECs as a function of the applied shear stress. RBCs were suspended in solutions containing either dextran 40 kDa or 2 MDa at the concentration of 10 mg/ml and were then coincubated with EC for 15 min before applying a stepwise-increasing shear. Error bars are SDs of the mean adherence values from three individual experiments.

shear stress. These results therefore demonstrate that activating ECs with thrombin increases the adhesion efficiency of RBCs to ECs, which is reflected in the number of cells adherent as well as the adhesion strength.

B. Dextran of high Mw induces adhesion of RBCs to ECs

Having confirmed the adhesion-promoting effect of EC activation, studies were conducted to determine the effects of dextran on RBC adhesion to normal ECs. ECs were coincubated for 15 min with RBCs suspended in dextran 40 kDa or 2 MDa at a concentration of 10 mg/ml. Thereafter a stepwise increase of shear stress was applied and the adherent cells were counted. Initially the cells were rinsed at a shear stress of 0.02 Pa and the adherent cells were counted after 5 and 10 min, followed by two subsequent rinsing at 0.05 and 0.10 Pa for 5 min, respectively. Using this stepwise-increasing shear stress allows comparing the number of cells adherent and their respective adhesion strength. As shown in Fig. 2, at the initial shear stress of 0.02 Pa, the adhesion rate in dextran 40 kDa is only minor. No significant difference from the adhesion in polymer free medium to normal EC (Fig. 1) can be detected. The presence of dextran 2 MDa significantly changes this picture. After rinsing the chamber at 0.02 Pa with cell-free medium, on average more than 200 RBCs remain adherent per mm^2 and about a quarter of these adherent cells remained at a shear stress of 0.10 Pa.

C. Dextran of high molecular mass promotes the adhesion of RBCs to activated ECs

Having separately established the adhesion-promoting effect of EC activation and coincubation in the presence of dextran 2 MDa, further studies were conducted to determine the impact of this macromolecule on the adhesion of RBCs to activated ECs. EC monolayers were initially pretreated with thrombin at the desired concentrations for 5 min, followed by coincubation with RBCs suspended in dextran-SFM for 15 min. Thereafter, nonadherent cells were removed via rinsing the flow chamber with cell-free dextran-SFM, while applying a stepwise-increasing shear as described in the previous paragraphs. The thrombin concentration was varied between 0.005 and 0.10 U/ml. Figure 3 shows the adhesion of RBCs suspended in either polymer-free medium (left panel in Fig. 3) or in solutions containing 10 mg/ml of dextran 2 MDa (right panel in Fig. 3). After applying 0.02 Pa, the maximal adhesion rate in dextran-free SFM is around 50 cells/mm^2. Increasing the shear stress to 0.1 Pa eventually removes all the adherent cells.

The presence of dextran 2 MDa changes this picture dramatically. For all thrombin concentrations, the number of adherent cells as well as the adhesion strength increase significantly, with some cells even withstanding a shear stress of 0.10 Pa. In addition, the adhesion in dextran-SFM shows a strong dependence on the thrombin concentration. For example, after rinsing for 5 min at 0.02 Pa the number of ad-

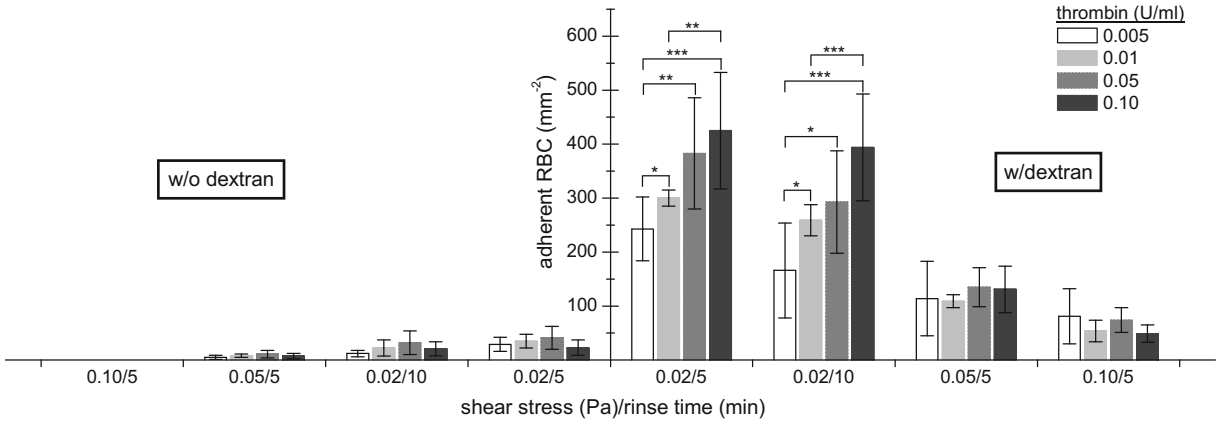

FIG. 3. Impact of the suspending medium on the adhesion of RBCs to thrombin-activated ECs. ECs were incubated with thrombin at concentrations of 0.005–0.1 U/ml for 5 min followed by coincubation with RBCs suspended in polymer-free solution (left panel) or in suspensions containing dextran 2 MDa at concentration of 10 mg/ml (right panel) for 15 min. Error bars are SDs of the mean adherence values from three individual experiments. Wilcoxon–Mann–Whitney U test, a nonparametric method for two unpaired samples, was performed on the adherence values of different samples. $P=0.2$(*); $P=0.1$(**); $P=0.05$(***).

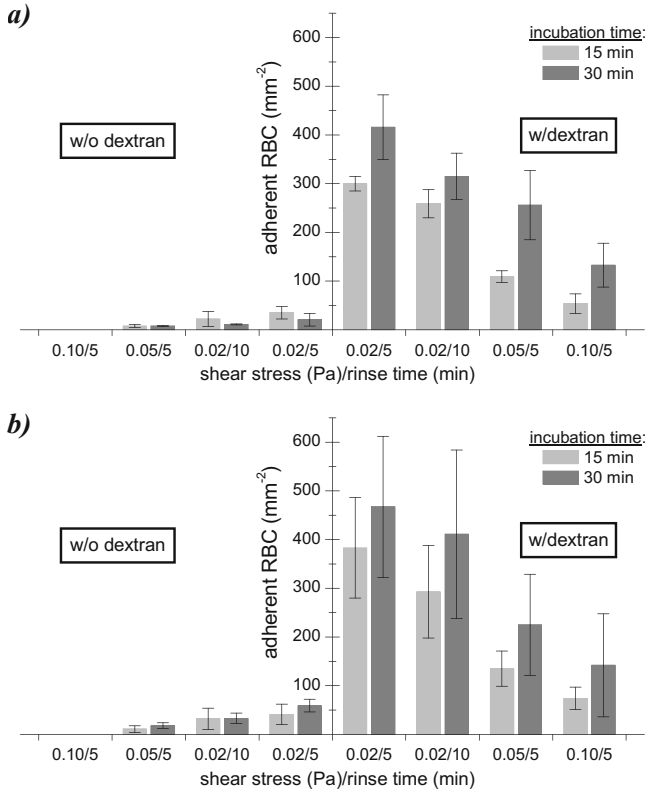

FIG. 4. Impact of the suspending medium on the adhesion of RBCs to thrombin-activated ECs. ECs were incubated with thrombin at concentrations of either (a) 0.01 U/ml or (b) 0.05 U/ml for 5 min followed by coincubation with RBCs suspended in polymer-free solution (left panel) or in suspensions containing dextran 2 MDa at a concentration of 10 mg/ml (right panel) for 15 min or 30 min. Error bars are SDs of the mean adherence values from three individual experiments.

herent cells per mm^2 increases from about 240 at 0.005 U/ml to 425 at 0.10 U/ml and after rinsing for 10 min at 0.02 Pa, the number of adherent cells per unit mm^2 increases from about 166 at 0.005 U/ml to 394 at 0.10 U/ml. These results clearly suggest that dextran of high molecular mass strongly promotes the adhesion of RBCs to thrombin-activated ECs.

Lastly, we investigated the impact of the incubation time on RBC adhesion to ECs. RBCs were coincubated with ECs for 15 and 30 min, followed by a stepwise-increasing shear stress. At a thrombin concentration of 0.01 U/ml, there seems to be no impact of the incubation time on the adhesion efficiency in polymer-free suspensions [Fig. 4(a), left panel]. Independent on the incubation time, fewer than 50 cells/mm^2 are adherent after rinsing for 5 min at a shear stress of 0.02 Pa. However, in the presence of dextran [Fig. 4(a), right panel], the adhesion rate as well as the adhesion strength are not only much higher but increase significantly with increasing incubation time. Doubling the incubation time increases the number of cells withstanding 0.02 Pa (5 min) by almost 40% and more than twice as many cells are able to withstand a shear stress of 0.10 Pa. Increasing the thrombin concentration to 0.05 U/ml leads to similar results [Fig. 4(b)]. In the presence of dextran, the adhesion efficiency increases significantly with increasing incubation time, whereas in the polymer-free suspension, this effect is much less pronounced.

IV. DISCUSSION

The presented results clearly demonstrate that the adhesion efficiency of RBCs to activated ECs increases significantly in the presence of dextran, and that this adhesion-promoting effect becomes more pronounced with increasing thrombin concentration or prolonged incubation time (Figs. 3 and 4). The adhesion-promoting effect of dextran can be ascribed to macromolecular depletion as elucidated in our previous studies.[33] In brief, dextran is a neutral, uncharged polymer without the ability to develop attractive electrostatic interactions and it has been shown repeatedly that dextran is depleted from the RBC surface.[38,39] Thus, it is concluded that the presence of dextran promotes RBC-EC adhesion via depletion interaction. Moreover, past reports have shown that thrombin induces the expression of adhesion molecules on the EC surface, which is time dependent, e.g., P-selectin is expressed within 15 min and ICAM-1 within 30 min.[15] Thus with normal RBCs also bearing receptors for P-selectin,[16,17] this study suggests that nonadsorbing macromolecules (e.g., dextran) can modulate P-selectin mediated RBC-EC adhesion and thus that the thrombin-mediated adhesion in dextran solutions is a synergetic result of both depletion interaction and receptor-mediated interactions.

Past studies aimed at identifying plasma proteins involved in abnormal RBC-EC adhesion were often limited to identifying cell receptors, in that they only considered plasma proteins as ligands cross-linking adjacent cells. A depletion mechanism as suggested by this study proposes quite the opposite. Figure 5 illustrates how nonadsorbing macromolecules might modulate RBC adhesion to the endothelium. Polymer or protein depletion from the cell surface leads to a lower localized protein or polymer concentration near the cell surface as compared to the suspending medium and thus a relative depletion near the cell surface [Fig. 5I]. The size of this depletion layer is in the same range as the size of the depleted macromolecule.[36] This exclusion of macromolecules near the cell surface leads to an osmotic gradient and as two cells approach, solvent is displaced from the depletion zone into the bulk phase leading to an attractive interaction [Fig. 5II], thereby facilitating intimate cell-cell contact and thus receptor-mediated cell interactions [Fig. 5III].

In conclusion our results indicate that not only plasma proteins acting as ligands can induce RBC adhesion to ECs but that an additional mechanism, which needs to be considered, is, depletion interaction, induced by nonadsorbing macromolecules. Thus, future efforts trying to elucidate the mechanisms of abnormal RBC interactions will not only have to consider the various plasma constituents as ligands but will also need to identify nonadsorbing plasma proteins and cellular factors that control depletion interaction.[41,42] Even though direct conclusions regarding the *in vivo* significance of this mechanism are not possible, our results clearly demonstrate the merit of this approach. Moreover, the pre-

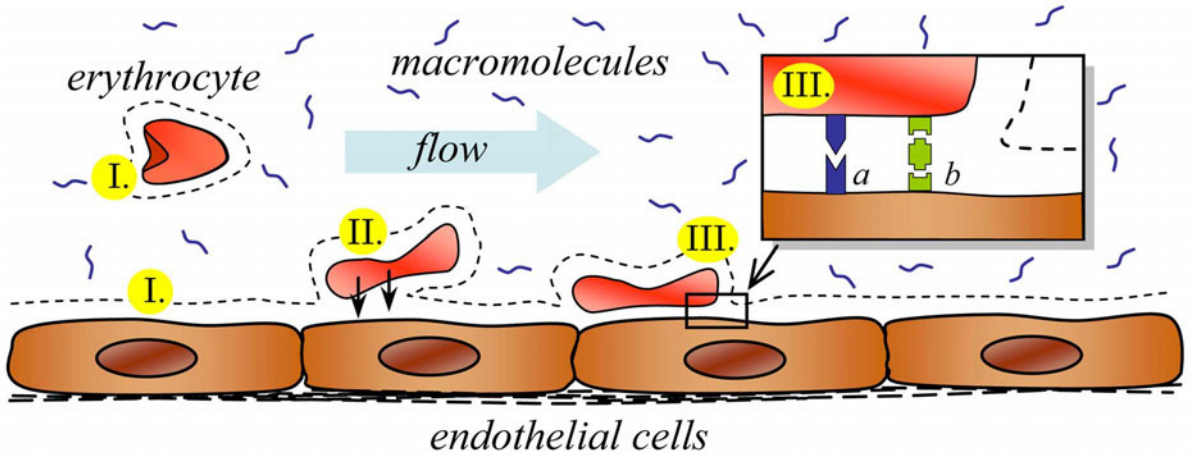

FIG. 5. (Color online) Schematic picture of how macromolecular depletion interaction brings adjacent cells into intimate contact: (I) macromolecules are depleted from both RBC and EC surfaces; (II) attraction develops when depletion layers overlap; (III) the resultant intimate cell-cell contact facilitates (a) receptor-mediated interactions, which might also involve plasma ligands cross-linking adjacent receptors (b).

sented data clearly suggest that depletion interaction allows controlling specific binding of RBCs to ECs or even other cell-cell interactions.[43] Our findings might thus not only be beneficial for a detailed understanding of the pathology of diseases associated with vascular complications but could also potentially be utilized for wide range of *in vivo* and *in vitro* applications that require or may benefit from the control of cell-cell interactions.

ACKNOWLEDGMENTS

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