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Non-adsorbing macromolecules promote endothelial adhesion of erythrocytes with reduced sialic acids☆

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ABSTRACT

Background: Abnormal adhesion of red blood cells (RBCs) to vascular endothelium is often associated with reduced levels of sialic acids on RBC membranes and with elevated levels of pro-adhesive plasma proteins. However, the synergistic effects of these two factors on the adhesion are not clear. In this work, we tested the hypothesis that macromolecular depletion interaction originating from non-adsorbing macromolecules can promote the adhesion of RBCs with reduced sialic acid content to the endothelium.
Methods: RBCs are treated with neuraminidase to specifically remove sialic acids from their surface followed by the evaluation of their deformability, zeta potential and membrane proteins. The adhesion of these enzyme-treated RBCs to cultured human umbilical vein endothelial cells (ECs) is studied in the presence of 70 or 500 kDa dextran with a flow chamber assay.
Results: Our results demonstrate that removal of sialic acids from RBC surface can induce erythrocyte adhesion to endothelial cells and that such adhesion is significantly enhanced in the presence of high-molecular weight dextran. The adhesion-promoting effect of dextran exhibits a strong dependence on dextran concentration and molecular mass, and it is concluded to originate from macromolecular depletion interaction.
Conclusion: These results suggest that elevated levels of non-adsorbing macromolecules in plasma might play a significant role in promoting endothelial adhesion of erythrocytes with reduced sialic acids.
General significance: Our findings should therefore be of great value in understanding abnormal RBC–EC interactions in pathophysiological conditions (e.g., sickle cell disease and diabetes) and after blood transfusions.

1. Introduction

Increased adhesion of red blood cells (RBCs) to endothelial cells (ECs) has been linked to the severity of vascular complications and disorders in various diseases such as sickle cell anemia and diabetes mellitus [1,2]. A number of biochemical factors have been identified to be responsible for pathological RBC–EC adhesions, including unusually activated adhesion pathways [3] and multiple adhesion molecules leading to specific ligand-receptior interactions [4–6]. In addition, biophysical factors such as RBC deformability, density and surface charge have also been found to play a significant role in the adhesion to vascular endothelium [7,8]. Furthermore, markedly increased plasma proteins (e.g. thrombospondin, von Willebrand factor, fibronectin and fibrinogen) are often found to accompany abnormal RBC–EC adhesion [9] and they are usually suggested to act as bridging ligands for the corresponding receptors on either RBC or EC surfaces [10]. Although the underlying mechanisms for abnormal RBC–EC adhesion remain unclear, extensive studies in the past few decades have already indicated that the adhesion is a result of multiple synergistic biochemical and biophysical factors.

Macromolecular depletion interaction is a significant biophysical factor we have recently identified for interpreting the role of pro-adhesive plasma proteins [11,12]. The suggested mechanism has been addressed to the found dependence of cell–cell interaction on the physicochemical properties of proteins (e.g., molecular mass and concentration) [13,14]. Macromolecular depletion interaction is an attractive non-specific force that is generated as a result of the entropy loss of macromolecules near the cell surface [15]. When cells are suspended in solutions containing macromolecules and when the loss of configurational entropy at the cell–fluid interface is not balanced by adsorption energy (i.e., the macromolecules are non-adsorbing [16]), the macromolecules are depleted from the cell surface and a so-called depletion layer develops around the cells. The macromolecular concentration gradient between depletion layer and the bulk phase generates an osmotic pressure that acts as an attractive force bringing two approaching cells into close contact.

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Macromolecular depletion interaction has been shown to govern reversible RBC–RBC aggregation and RBC adhesion to coated surface with different quantitative techniques including micropipette assay [17], electrophoretic mobility measurement [18], interference reflection microscopy [19] and atomic force microscopy [20]. By using dextran molecules to mimic plasma proteins and comparing the effects between dextran and fibrinogen, we have shown that macromolecular depletion interaction can be an applicable model to interpret the adhesion-promoting effects of non-adsorbing plasma proteins/macromolecules [12]. Furthermore, this non-specific force, when linked to biochemical factors capable of inducing specific ligand-receptor interactions, has been shown to promote abnormal RBC adhesion to ECs [21,22].

Sialic acids are acidic monosaccharides located on various glycoproteins and glycolipids. They do not only contribute to over 90% of the RBC surface charge [23] leading to electrostatic repulsion in cell–cell interactions but also are involved in diverse biological events such as phagocytosis, infection, inflammation, immunity and cancer metastasis [24–26]. A correlation between sialic acids and RBC–EC adhesion has been found. For example, senescent RBCs experience a substantial reduction of sialic acids [27] and also exhibit an increased adhesiveness to ECs [28,29]. In addition, enhanced reduction of sialic acids on RBCs and abnormal RBC–EC adhesion are often found to be concurrent in several diseases including diabetes, β-thalassemia, myocardial infarction and quite likely sickle cell disease [30–33]. One pioneering study in this field has indicated that a reduction of sialic acids is not the single factor responsible for increased RBC adhesiveness to ECs [28]. However, a more detailed understanding is still lacking.

Considering that abnormal RBC–EC adhesion usually occurs in a complex environment with multiple factors involved, we hypothesized that macromolecular depletion interaction may promote endothelial adhesion of RBCs with reduced sialic acids. To test this hypothesis we employed neuraminidase to alter sialic acid content on the RBC surface and studied desialylated RBC adhesion to cultured ECs with a flow chamber system, which allows simultaneous investigation of multiple factors including non-adsorbing macromolecules in solution, flow behavior of desialylated RBCs and the biochemical/biophysical consequence of sialic acid removal in RBC–EC adhesion. Dextran is used as the non-adsorbing macromolecules to induce depletion interaction. This polyglucose is neutral and has been often used for *in vitro* hemorheological studies [34–36].

2. Materials and methods

2.1. Preparation of erythrocytes

Blood was drawn from the antecubital vein of healthy adult volunteers into EDTA (1.5 mg/ml). Each volunteer provided written consent and the study was approved according to the procedures of the institutional review board of Nanyang Technological University. RBCs were separated from whole blood by centrifugation (1000 ×*g*, 10 min) and then washed three times with phosphate buffered saline (PBS, 10 mM phosphate, 285 mOsm/kg, pH = 7.4) containing 0.2% bovine serum albumin (BSA, Sigma-Aldrich). The washed RBCs were re-suspended in the same buffer with the hematocrit adjusted to approximately 20%, and then incubated at 37 °C for 1 h with neuraminidase (type II from vibrio cholerae, Sigma-Aldrich) at different concentrations; following incubation the cells were washed three times with cold PBS to terminate the enzymatic activity. Control cells were prepared in parallel without neuraminidase treatment.

2.2. Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza, UK. The culture medium consisted of 10 vol.% fetal bovine serum (FBS, PAA Laboratories) and 90 vol.% basal medium (Ham's F-12 K with 2 mM L-Glutamine (Sigma-Aldrich), 1.5 g/l sodium bicarbonate (Sigma-Aldrich), 0.1 mg/ml heparin (Sigma-Aldrich), 0.2 vol.% bovine brain extract (BBE, Hammond Cell Tech), and 120 U/ml penicillin/streptomycin (Sigma-Aldrich). The ECs were grown in tissue culture flasks at 37 °C in a CO_2 (5%) incubator until they reached 80% confluence. The cells were then sub-cultured into flat bottom 35 mm petri dishes (Greiner) pre-coated with gelatin from fish skin (Sigma-Aldrich) and grown to confluence.

2.3. Membrane zeta-potential of RBC

Both control and neuraminidase-treated RBCs were washed twice with reduced ionic strength phosphate buffer containing sufficient sucrose to achieve isotonicity (7 mM ionic strength, 285 mOsm/kg, pH 7.4). Thereafter, the cells were suspended in the same buffer at a cell count of 5×10^6/ml and their electrophoretic mobility was measured using a laser-based electrophoresis system (Nanosizer, Malvern Instrument, UK). Measurements were conducted at 37 °C with 20 runs per sample using three separate aliquots of the suspension. The membrane zeta-potential (ζ) was calculated using the Smoluchowski equation $U_E = \frac{\varepsilon\zeta}{\eta}$, where U_E is the cell's electrophoretic mobility, ε the dielectric constant and η the dynamic viscosity of the suspending medium. The final values of mobility are the mean ± standard deviations of the three measurements.

2.4. RBC deformability

RBCs were washed with PBS containing 0.2% BSA. The supernatant was removed after centrifugation at 1000 ×*g* for 5 min, and 6 μl of the packed RBCs were added to 0.6 ml of polyvinylpyrrolidone solution (RSD-P01, 30 mPa·s, Sewon Meditech, Korea) for deformability measurements at 37 °C via ektacytometry. The ektacytometer (RheoScan, Sewon Meditech) consists of a parallel plate channel through which the RBC suspension flows due to a known applied pressure gradient; this gradient is allowed to decrease with time and thus allows the applied shear stress to decrease during a measurement. A laser beam passes through the suspension and the flowing deformed cells create an elliptical diffraction pattern that reflects cell shape; analysis of the pattern yields an elongation index (EI) as $EI = \frac{A-B}{A+B}$, where A is the length of the major axis while B is the length of the minor axis. Measurements were conducted on separate aliquots of each cell suspension and are presented graphically.

2.5. RBC membrane ghosts and gel electrophoresis

Membrane ghosts of both control and neuraminidase-treated RBCs were prepared for SDS-polyacrylamide gel electrophoresis (PAGE) as previously described [37]. In brief, 0.5 ml of washed RBCs were added to an equal volume of PBS, following which 14 ml of hypotonic buffer solution (NaH_2PO_4, 21 mOsm/kg, pH = 7.4) was added, yielding a 30:1 ratio of total volume to packed RBCs. The suspension was then centrifuged at 20,000 ×*g* for 40 min at 4 °C, after which the supernatant was decanted. Washing with hypotonic buffer was repeated until the ghost pellets became white and the supernatant became only faintly pink. SDS-PAGE was performed using membrane ghosts of both control and neuraminidase-treated RBCs. The separating gel was 12% and the stacking gel was 5%; the gel was run for 2 h at 120 V and then stained with Coomassie brilliant blue (Bio-Rad Laboratories), following which a 2D proteomic imaging system (PerkinElmer) was used to scan the gel and analyze the membrane proteins.

2.6. Parallel plate flow chamber system

The flow system consists of a circular acrylic flow deck and a silicone rubber gasket (31-001, Glycotech, USA) with the cutout area of the gasket forming the flow channel. Both the gasket and flow deck were placed into a 35 mm petri dish coated with confluent layers of ECs

and hence the EC layer served as the bottom surface of the parallel plate flow chamber. The flow chamber (i.e., flow deck-gasket-EC monolayer system) was then placed on an inverted microscope (IX71, Olympus). 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 cell-free solutions for rinsing. The outlet of the chamber was connected to a syringe pump (Harvard Apparatus Co., Millis, MA) capable of withdrawing either RBC suspensions or rinsing solutions from the two reservoirs through the flow chamber at a selected volumetric flow rate Q. The microscope, valve, inflow tubing and the two reservoirs were maintained at 37 °C via a thermostated enclosure. The wall shear stress τ was calculated via $\tau = 6\mu Q/a^2 b$, 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 (DMA35, Anton Paar) and the dynamic viscosity of different solutions was measured by an automated micro capillary viscometer (AMVn, Anton Paar).

2.7. Experimental protocol

RBC suspensions at 5×10^6/ml were drawn into the chamber and the cells were allowed to settle without flow to the bottom of the chamber (i.e., onto the EC monolayer) for 8 min. The chamber was then rinsed with stepwise increasing flow rates corresponding to wall shear stresses between 0.01 and 0.05 Pa. Each shear stress was maintained for 3 min, at the end of which another three-minute period was used to capture 20 images at random locations in the central flow area. The numbers of adherent RBCs were counted in each image using Image J (V1.37). The adhesion values at each shear stress were calculated as the mean ± standard deviation (SD) of the 20 images and were converted to adherent RBC per mm^2.

2.8. Statistics

Adhesion values are reported as mean ± standard deviation of three individual experiments repeated under the same conditions. Wilcoxon signed rank test, a non-parametric method for paired samples, was performed for the comparisons of interest.

3. Results

RBC surface charge has been found to be linearly correlated to sialic acid content and membrane zeta-potential (ζ, mV) is often used as an indicator of RBC surface charge [38,39]. Fig. 1B demonstrates that the absolute value of the ζ potential decreases as the applied neuraminidase concentration increases. Accordingly, in Fig. 1A, the reductions of ζ potential (% decrease) initially increases rapidly to a 40% reduction at 20 mU/ml, and then slows down with increasing neuraminidase concentration with a 60% reduction at 80 mU/ml. These results indicate that a substantial amount of sialic acids are resistant to neuraminidase hydrolysis [40].

RBC deformability can be expected to play a significant role in the flow behavior of RBCs. Thus, initially ektacytometry was employed to examine the effect of neuraminidase treatment on RBC deformability. The elongation index, an indicator of RBC deformability, was measured against shear stresses varying from 0 to 20 Pa. Fig. 2 clearly demonstrates that RBC deformability substantially decreases after neuraminidase treatment, indicating that the cells become more rigid. However, the decrease of deformability is essentially independent of neuraminidase concentrations, as suggested by the overlapping curves of 20, 40, and 80 mU/ml. RBC morphology was also examined with phase contrast microscope and the neuraminidase-treated cells were found to retain the biconcave shape typically seen for normal RBCs (data not shown).

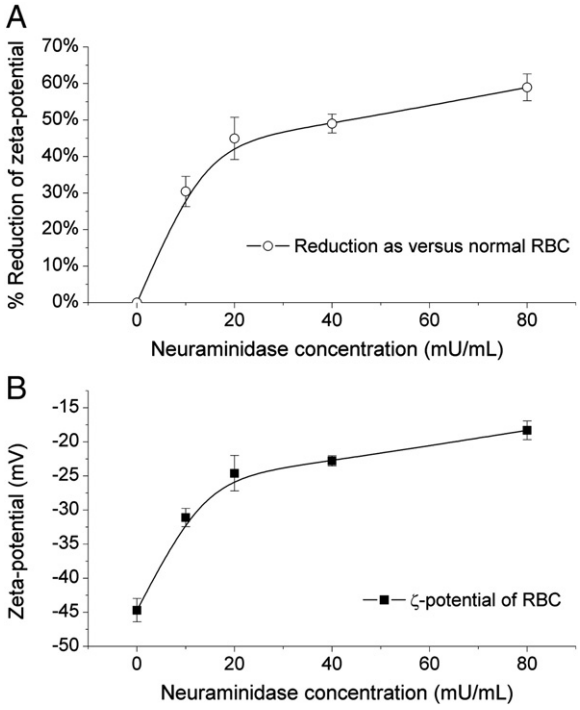

Fig. 1. (A) Percent reduction of RBC membrane ζ-potential and (B) absolute values of RBC membrane ζ-potential as functions of neuraminidase concentrations. ζ-Potential was measured in isotonic sucrose buffer with 7 mM ionic strength at pH 7.4. Error bars are standard deviations of the mean values from three replicates.

To evaluate if neuraminidase specifically removes sialic acids from the glycoproteins and glycolipids of RBC membranes, SDS-PAGE was performed on the membrane ghosts prepared from both normal and neuraminidase-treated RBCs. Fig. 3 presents a representative graph of protein gel electrophoresis in the range of 20–250 kDa. The ladder was determined from the results of protein molecular weight standards. As judged by visual inspection, the bands of normal cells and cells treated with 40 or 60 mU/ml of neuraminidase are essentially identical. These results indicate that neuraminidase specifically removes sialic acids and that in terms of biochemical composition the RBC membranes are most likely staying intact after neuraminidase treatment.

The adhesiveness of neuraminidase-treated RBCs to cultured ECs was evaluated using an adhesion assay that allowed RBCs to settle onto an EC monolayer, followed by exposing the cells to fluid shear

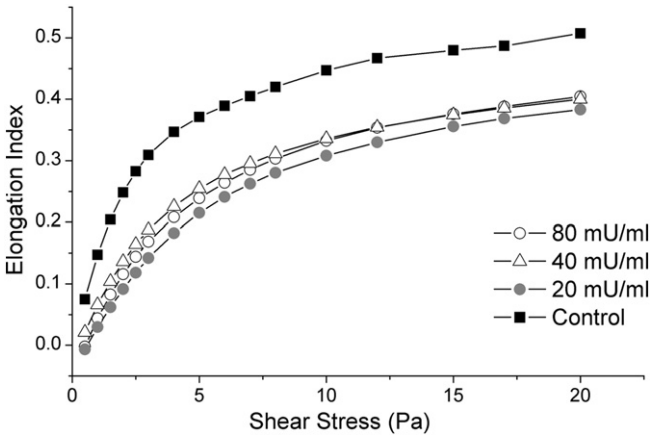

Fig. 2. RBC deformation, indicated by elongation index, as a function of applied shear stress for cells treated with different concentrations of neuraminidase. Elongation index was measured using an ektacytometer; the data presents a representative graph from duplicate measurements.

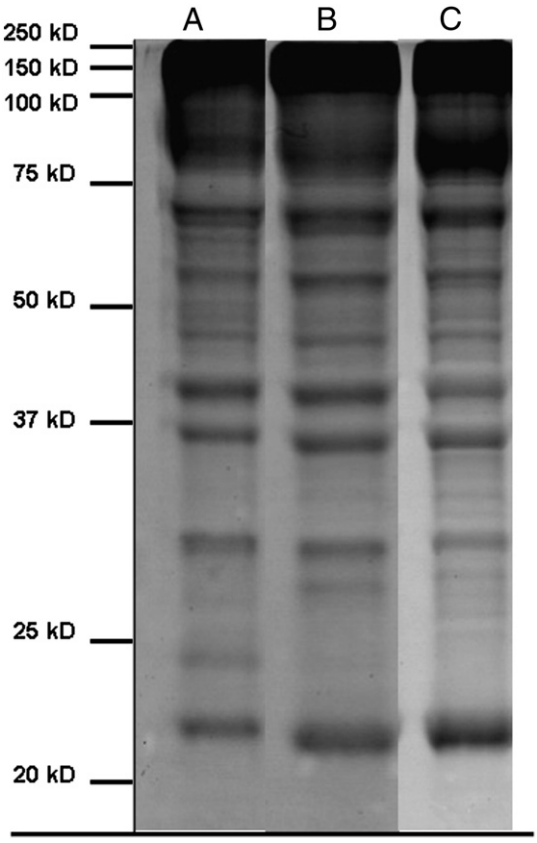

Fig. 3. Ghost membrane proteins analyzed by 2D proteomic imaging system on the result of SDS-PAGE. RBC ghosts were prepared by lysis with hypotonic buffer after the intact cells were treated with neuraminidase at the concentrations of A: 0 (i.e., control); B: 40; and C: 60 mU/ml.

forces tending to detach them. The results of these experiments are presented in Fig. 4 for the adhesion occurring in polymer-free PBS. These data clearly demonstrate an increased adhesiveness of RBCs with reduced sialic acids, as reflected by both increased number of adherent RBCs and greater adhesion strength resisting shear forces. Normal RBCs exhibit minor adhesiveness to EC, with an initial adherence of 33 cells/mm^2 at 0.01 Pa and essentially zero adherence at 0.05 Pa. On the contrary, the adhesiveness significantly increases as RBC surface charge is decreased. RBCs with 45% and 60% reduction of ζ potential adhere to ECs with almost 2-fold and 3-fold increases at 0.01 Pa, and a considerable extent of adhesion remains at even 0.05 Pa. These results indicate that a subpopulation of RBCs can establish relatively strong adhesion to ECs due to the reduction of sialic acids by neuraminidase treatment.

To study the synergistic effects of non-adsorbing macromolecules and sialic acid removal on RBC adhesion to ECs, RBCs with approximately 60% reduction of the ζ potential were used for the same adhesion assay as in Fig. 4. RBCs were allowed to settle for 8 min onto the EC monolayer. Comparisons in adhesion were made between dextran solutions and control (i.e., polymer-free PBS). Fig. 5 demonstrates that 70 kDa dextran at 5 or 10 mg/ml, as compared to the control, results in a slightly increased number of adherent cells at 0.01 Pa. However, this minor adhesion-promoting effect quickly vanishes and in fact at 0.02–0.04 Pa, the adhesion in 70 kDa dextran is slightly less than that of the control. These results indicate that at the selected concentrations (i.e., 5 or 10 mg/ml), 70 kDa dextran does not enhance endothelial adhesion of RBCs with a 60% reduction of the ζ potential.

Fig. 6 demonstrates a set of experiments conducted with the same type of RBCs (i.e., 60% reduction of ζ potential) in 500 kDa dextran solutions. As compared to polymer-free PBS, 500 kDa dextran at 5 mg/ml causes a moderate increase of adhesion. The number of adherent RBCs increase by approximately 50% at 0.01 Pa and 60% at 0.02 Pa. At a higher concentration of 10 mg/ml, 500 kDa dextran causes a significant increase of adhesion as seen from both number of adherent RBCs and adhesion strength. For example, 327 RBCs are adherent at 0.01 Pa in 10 mg/ml dextran 500 kDa, contributing to a 2.7-fold increase compared to control and an 1.5-fold increase compared to 5 mg/ml dextran. With regard to adhesion strength against the increasing shear stress, 40% of the cells adherent at 0.01 Pa remain adherent at 0.05 Pa in 10 mg/ml 500 kDa dextran. These results exhibit a significant promoting effect of 500 kDa dextran on endothelial adhesion of RBCs with reduced sialic acids. In addition, the adhesion-promoting effect of 500 kDa dextran increases with its concentration. Furthermore, comparing Fig. 6 to Fig. 5 indicates that 500 kDa dextran is much more effective than 70 kDa dextran on promoting neuraminidase-treated RBC adhesion to ECs.

Lastly, it should be noted that RBC aggregation (i.e., rouleaux formation) was also found to occur in dextran solutions. During the 8-minute incubation period and right after the beginning of the rinsing period (i.e., 0.01 Pa in Fig. 5) although both single RBC and RBC aggregates

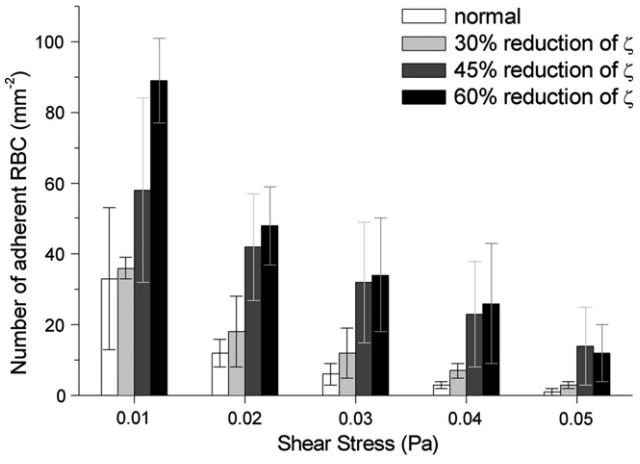

Fig. 4. Number of adherent RBCs to cultured ECs in polymer-free PBS as a function of applied shear stress for 30, 45 and 60% reduction of RBC membrane ζ-potential. Error bars are standard deviations of the mean value from three individual experiments.

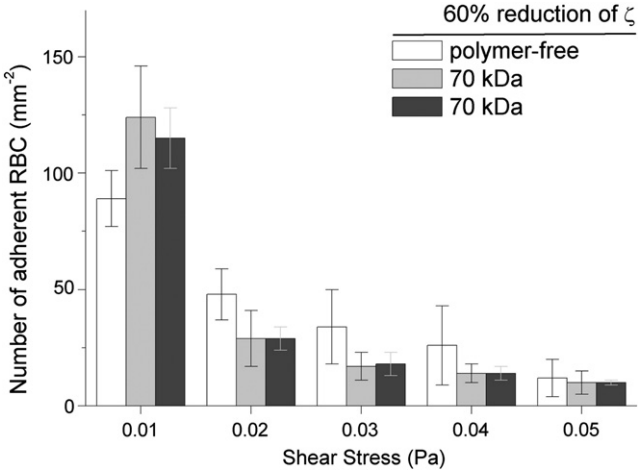

Fig. 5. Number of adherent RBCs to cultured ECs as a function of applied shear stress for studying the effect of 70 kDa dextran on adhesion. RBCs were treated with neuraminidase to achieve a 60% reduction of membrane ζ-potential, and then allowed 8 min to settle onto ECs before rinsing. Error bars are standard deviations of mean value from three individual experiments.

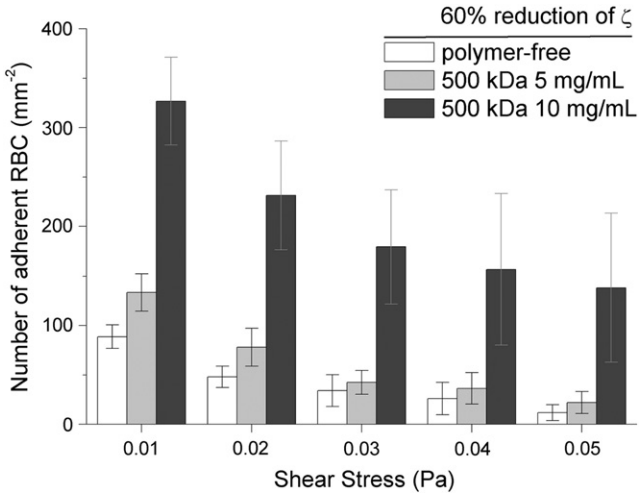

Fig. 6. Number of adherent RBCs to cultured ECs as a function of applied shear stress for studying the effect of 500 kDa dextran on adhesion. RBCs were treated with neuraminidase to achieve a 60% reduction of membrane ζ-potential, and then allowed 8 min to settle onto ECs before rinsing. Error bars are standard deviations of mean value from three individual experiments.

adhered to ECs in 70 kDa dextran, the aggregates were collectively rinsed away. In contrast, the aggregates remained adherent in 500 kDa dextran solutions at low shear stress and then broke at higher shear stress leaving one or two RBCs adherent.

4. Discussion

In the current study neuraminidase was used to remove sialic acids from the RBC surface. Results of the gel electrophoresis and electrophoretic mobility measurement (i.e., zeta-potential) suggest that in terms of biochemical composition, the removal of sialic acids and thus RBC surface charge is rather specific. However, neuraminidase treatment is found to simultaneously reduce RBC deformability, which is in agreement with the previous studies showing that reduction of RBC surface charge is correlated to reduced deformability via: 1) artificial enzymatic treatment [41] and 2) physiological RBC aging [27]. With regard to the effects on RBC–EC adhesion, reduction of surface charge leads to reduced electrostatic repulsion [17,42] and simultaneously a reduced deformability makes it for desialylated RBCs more difficult to establish intimate contact and thus adhesion to ECs under flow. However, the net outcome of neuraminidase treatment, as shown in Fig. 4, is an apparent increase in RBC–EC adhesion. This also implies that the removal of sialic acids by neuraminidase does not only reduce the RBC surface charge, but also exposes sites leading to: 1) recognition for sequestration; 2) activation of endothelial cells; and 3) ligand-receptor interactions [43].

Regardless of these possibilities, Fig. 4 has also clearly demonstrated that the interactions between desialylated RBCs and ECs are weak. The result on one hand confirms the previous finding that removal of sialic acids is not the single factor responsible for increased RBC adhesiveness to ECs [28,33]; and on the other hand indicates that significant desialylated RBC adhesion to EC is most likely a result of multiple synergistic factors. Fig. 6, which might be seen as the key result of this study, demonstrates that dextran with high molecular mass significantly promotes endothelial adhesion of RBCs with reduced sialic acids. Macromolecular depletion interaction is the underlying mechanism for the adhesion-promoting effect of dextran observed in this study. This conclusion is based on: 1) dextran is a neutral polyglucose that has been proven to be depleted from RBC surface [34,35]; and 2) the adhesion-promoting effect is dependent on dextran concentration and molecular mass. Figs. 5 and 6 also demonstrate that a molecular mass of 70 kDa is insufficient to result in significant desialylated RBC adhesion to EC at the employed concentrations. Furthermore, for 500 kDa dextran, 10 mg/ml is much more efficient than 5 mg/ml in promoting the adhesion because higher concentration results in an increasing osmotic pressure difference between the depletion layer and the bulk, which is the driving force for the attraction between desialylated RBCs and ECs. It should be noted that these pro-adhesive effects are very similar to those observed in the adhesion of normal RBCs to ECs [11], thereby lending strong support to the suggested mechanism of macromolecular depletion interaction.

It is also interesting to note that at the concentrations used herein, although dextran 70 kDa does promote desialylated RBC adhesion to ECs at 0.01 Pa (i.e., the starting shear stress applied for rinsing), it slightly reduces the adhesion at 0.02–0.04 Pa (Fig. 5). This phenomenon can be explained by dextran-induced RBC–RBC aggregation [44], which is also governed by macromolecular depletion interaction [15] but competes with RBC–EC adhesion in the current experimental setup. Although both 70 kDa and 500 kDa dextran induce significant aggregation of desialylated RBCs, the presence of dextran with 500 kDa leads to the adhesion of desialylated RBCs in the forms of both individual cells and aggregates during rinsing. The fact that 70 kDa dextran results in more pronounced RBC aggregation than RBC–EC adhesion indicates that cellular factors, e.g. the structure and composition of the RBC glycocalyx also affect macromolecular depletion interaction. It has been demonstrated theoretically and experimentally that a thinner RBC glycocalyx leads to stronger macromolecular depletion interactions [45–47]. Since the current study does not rule out the possibility of a collapsed and thus thinner RBC glycocalyx due to the removal of surface charges, the effect of 70 kDa dextran on desialylated RBC aggregation may become enhanced. Furthermore, the glycocalyx of ECs is most likely much thicker than that of RBCs [48], making RBC–EC adhesion more difficult to occur than RBC–RBC aggregation for the same dextran molecule.

Since dextran is used to mimic increased plasma proteins/macromolecules under pathological conditions, our study suggests that desialylated RBC adhesion to ECs *in vivo* might be significantly enhanced by pro-adhesive plasma proteins. These findings might be of pathophysiological relevance, especially when considering that clinical studies have indicated the existence of RBC subpopulations with reduced sialic acid content. For example, during normal physiological aging senescent RBCs, as compared to young RBCs, experience a reduction of total sialic acid of 10–30% [24,27], whereas the difference of sialic acid content in glycophorin can rise to 50% between old and young RBCs in patients with β-thalassemia [30]. In addition, when comparing patients to healthy donors, a reduction of sialic acids in RBC glycophorin has been found in diabetes of 80% [31] and β-thalassemia of 25% [30] (Note that sialic acids are mainly located in glycophorins). Nevertheless, it should also be pointed out that the extent of sialic acid reduction may not be the key determinant in abnormal RBC–EC adhesion. Instead, quite often it is found that sialic acid is a significant regulator in molecular and cellular interactions by masking the recognition sites such as CD44 and galactose residues [43].

In conclusion, we have shown that macromolecular depletion interaction has a significant promoting effect on the adhesion of RBCs with reduced sialic acids to the endothelium. This finding provides a possible mechanism for how sialic acid reduction on the RBC surface, together with an increased level of non-adsorbing macromolecules in plasma, result synergistically in enhanced RBC–EC adhesion under pathophysiological conditions or after blood transfusion [49]. In order to better simulate the *in vivo* conditions, further studies are still required to characterize the alterations of RBC and EC glycocalyx under pathological conditions. In addition, a more specific experimental manipulation of sialic acid instead of the complete removal might also be necessary to gain more insight on how the various molecular factors determine RBC–EC adhesion *in vivo*.

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