Additive effect of red blood cell rigidity and adherence to endothelial cells in inducing vascular resistance


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Red blood cells (RBCs) have special flow-affecting properties that are major determinants of blood flow: deformability, RBC-RBC aggregability, and RBC/endothelial cell (EC) adherence. Deformability is the ability to adapt the shape to the dynamically changing flow conditions to minimize resistance to flow, particularly in capillaries. Aggregability is the ability to form RBC-RBC aggregates in the presence of plasma proteins or other macromolecules. RBC/EC adherence is the ability to adhere to blood vessel wall ECs in pathological conditions. Under normal conditions, flowing RBCs are singly dispersed, their adherence is insignificant, and their deformability is sufficient to enable adequate passage through capillaries. However, in pathological conditions, especially under oxidative stress (OS) states, their adherence is enhanced to various degrees—3–10-fold in the different OS states (25, 30)—and their deformability is reduced, whereas their aggregability might be elevated in some OS states but reduced in others (10, 29, 32, 35).

Multiple studies have suggested that RBCs with impaired flow properties contribute to circulatory disorders (1, 5, 8, 9, 36). However, in vitro studies have usually focused on one RBC flow property at a time, even though it is known that pathological conditions or experimental treatments usually affect multiple RBC properties. Thus the specific contribution of each RBC flow property to circulatory disorders, and its relationship to in vitro measurement, still awaits further documentation. The present study was undertaken to explore the direct differential contribution of RBC flow properties to vascular resistance.

The studies of the effect of RBC aggregation on hemodynamics have not led to unequivocal conclusions. RBC aggregation is associated with increased blood viscosity and the formation of an RBC-free layer near the vessel wall. Accordingly, some studies have suggested that the increased viscosity elevates vascular resistance, whereas others suggested that RBC aggregation facilitates blood flow due to the formation of an RBC-free layer near the vessel wall and/or the viscosity-elevated shear stress, which leads to the production of the vasodilator nitric oxide (1, 5, 8, 9, 36). It thus seems that RBC aggregation has also a physiological hemodynamic role, and this may vary between blood vessels of different size and structure (9, 13, 14).

On the other hand, it is generally accepted that RBC/EC adherence and RBC rigidity induce flow disturbances, whereas their major physiological role is the splenic sequestration of "aged" RBCs (31). For example, it has been suggested that microvessel occlusion by sickle- and malaria-infected RBCs is due to their adherence to ECs of the microvessel wall and/or to their decreased deformability (4, 18). RBC/EC adhesion also decreases blood flow and increases the residence time of RBCs in the microcirculation (19). As to RBC deformability, previous studies have shown that an exchange transfusion of aldehyde-fixed RBCs reduced the flow rate in swine (28) and functional capillary density in hamsters (15). Again, these studies assumed that their treatment affected only the one RBC flow property (either adherence or deformability), whereas their effects on other properties were not addressed.

In the present study we focused on RBC/EC adherence and RBC deformability, using hydrogen peroxide (H2O2)-treated...
human RBCs. In a previous study we have found that treatment of RBCs with H$_2$O$_2$ suppressed their aggregability. However, depending on its concentration, H$_2$O$_2$ differentially elevated their adherence to ECs and their rigidity (reduced deformability) (35). This therefore provides a method for the aggregation-free modulation of RBC deformability and/or adherence and was used in the present study to explore the direct specific effect of RBC deformability and adherence on vascular resistance.

**MATERIALS AND METHODS**

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, and albumin, and trypsin were purchased from Biological Industries. Poly-l-lysine cell culture glass slides were purchased from Sigma (St. Louis, MO). Polylysylene slides were purchased from Electron Microscopy Science (Washington, PA).

**Preparation of RBC samples.** Human blood samples were collected from healthy donors (upon their consent under Helsinki Committee Permit, Hadassah Hospital 20-30/03/01, Israel). RBCs were isolated from blood by centrifugation, washed (3 times) from their plasma by centrifugation in phosphate-buffered saline (PBS, pH 7.4), and resuspended at a hematocrit of 10% in PBS.

**Treatment of RBCs with H$_2$O$_2$.** RBC suspension (10% of cells in PBS buffer) was supplemented with H$_2$O$_2$ (0.5 or 5.0 mM final concentration) and 0.4 mM NaN$_3$ and incubated for 1 h at 37°C in a shaking bath. The RBCs were then separated and washed three times with PBS by centrifugation and resuspended at 10% hematocrit in PBS, supplemented with 1 mM CaCl$_2$ and 0.5% albumin. Since H$_2$O$_2$ levels may decrease while mixed in medium, we determined the change in its concentration during treatment of RBCs by monitoring the change in optical density produced by its interaction with potassium thiocyanate as described by Hildebrant et al. (20). It was found that during 1 h treatment of RBC with 0.5 or 5.0 mM H$_2$O$_2$, its concentration decreased by 11% and 13%, respectively.

**EC culture.** Transformed human bone marrow ECs (HBMECs) were grown at 37°C in 5% CO$_2$ and 95% humidity in DMEM supplemented with 10% FCS, 25 mM HEPES, 100 U/ml penicillin, 100 g/ml streptomycin, and 2 mM glutamine. The cell cultures were expanded by trypsinization with 0.25% trypsin in PBS containing 0.025% EDTA. HBMECs were used for all experiments until passage 14. HBMECs were seeded onto poly-l-lysine glass cell culture slides coated with gelatin (1%) and grown to confluence.

**Determination of RBC flow properties in vitro.** In the present research we employed the computerized cell flow properties analyzer (CFA) design, developed in our laboratory (7, 16, 24, 30) for monitoring RBC aggregability, deformability, and adhesiveness as a function of shear stress by direct visualization of their dynamic organization in a narrow-gap flow chamber under controllable flow conditions, resembling those in a microvessel. In this setup, the CFA consists of a thermostated flow chamber with a 200-μm gap, designed by G. M. Artmann (2), and placed under a microscope. RBC suspension (50 μl) was inserted into the flow chamber and subjected to flow induced by a syringe pump (model PHD 2000P, Harvard Apparatus, South Natick, MA). The wall shear stress ($\tau$) is calculated from pressure (P) at the entrance to the flow chamber using a pressure-measuring station (PowerLab 2/20, ADInstruments) according to the following equation: $\tau = 0.5P \times \frac{h}{z}$, where $z$ and $h$ are the channel length and gap, respectively. The RBC images are transmitted by a microscope connected to a camera (model Coolpix995, Nikon) and to a computer equipped with homemade software that provides comprehensive and integrative parameters (some newly defined) for characterization of RBC adherence to ECs (24, 30) and deformability (6, 30).

**Determination of RBC deformability.** RBC suspension (50 μl; 5% of cells in PBS-albumin buffer) were inserted into the flow chamber. The flow chamber consisted of a polystyrene slide that was placed in the base. After a 15-min incubation of RBC suspension over the slide, the flow of buffer was applied, forming a stress of 3.0 Pa, and the deformation of adherent RBCs were monitored at constant shear stress (3.0 Pa). During measurements, 15–20 randomly chosen fields (width area, 0.1 mm$^2$) were collected. The image analysis of the cell shape provides the elongation index (EI) of individual cells and their distribution in the RBC population (6, 30). The average cell count was 1,200 ± 180. For all cells, we estimated the major (a) and minor (b) axes, and EI was calculated by the formula $EI = abh$, ranging from 1 to 3 (30). EI = 1 reflects round RBCs that are not deformed at the shear stress applied in this study (up to 3.0 Pa). In the imaging system used here, the resolution (in pixels per square inch) resulted in a variance of 10% in determining EI. Therefore, we defined undeformable cells as those having $EI \leq 1.1$.

**Determination of RBC adhesion to cultured ECs.** A slide of confluent cultured ECs was placed in the flow chamber and covered with RBCs for 20 min at 37°C. The nonadherent RBCs were then washed with 5 ml of Ca$^{2+}$-PBS-albumin solution under controllable increasing $\tau$. According to the shear stress applied, the washing time ranges from 5 min at 0.1 Pa to 10 s at 3.0 Pa. The number of adherent RBCs was continuously counted from images by using original software at each shear stress on 10 randomly chosen fields (with area 0.1 mm$^2$). The number of adherent RBCs remaining was plotted as a function of $1/\tau$, respective with the equation $N = N_0 \times e^{-\alpha/\tau}$, where $N$ is the number of adherent RBCs at a specific $\tau$ ($N_0$ is the number of adherent RBC at extrapolated $\tau \rightarrow \infty$ and $\alpha$ is the adherence coefficient, expressing the strength of intercellular interaction (30).

**Determination of vascular resistance in the ex vivo rat mesocecum preparations.** Blood samples (1 ml) were drawn from healthy volunteers (no patients) upon their consent under a protocol approved by the Institutional Review Board (Albert Einstein College of Medicine, Bronx, NY). All experimental protocols for the use of animals were approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine. Perfusion studies were performed in the isolated, acutely denervated, and artificially perfused ex vivo rat mesocecum vasculature ($n = 8$) according to the method of Baeza et al. (3) as modified by Kaul et al. (22) for the infusion of erythrocytes. Briefly, the perfusion pressure in the mesocecum was maintained at 60 mmHg, and venous outflow pressure was kept at 3.8 mmHg. During perfusion with Ringer-albumin solution containing 3% bovine albumin, a 0.2-ml bolus of a given red blood cell suspension (Hct, 40%) was infused via the arterial injection port over ~5 s. Peripheral resistance units (PRU) were determined as described (2) (expressed in mmHg·ml$^{-1}$·min$^{-1}$·g$^{-1}$). PRU = $\Delta$P/Q, where $\Delta$P is the arteriovenous pressure difference and Q is the rate of venous outflow (in ml/min) per gram of tissue weight. Pressure flow recovery time ($T_{pf}$), defined as the time (in s) required for the arterial pressure and the venous outflow to return to their baseline levels, was determined after the red blood cell infusion. $T_{pf}$ is an estimate of total transit time throughout the isolated vasculature and is influenced by red blood cell deformability and adhesions. Direct intravital microscopy and video recording of the microcirculatory events were carried out using a Nikon microscope (model E400; Nikon, Melville, NY), equipped with Dage-MTI-CCD television camera (model CCD-300T-RC; Dage-MTI, Michigan City, IN) and a Sony U-matic video recorder (model VO5800; Sony, Teaneck, NJ).

**RESULTS**

Effect of H$_2$O$_2$ on RBC adherence and deformability. In search for a method for differentially testing the effect of RBC/EC adherence and RBC rigidity on vascular flow, we have found that RBC oxidation by H$_2$O$_2$ exerts differential effects on RBC adherence and deformability, depending on the H$_2$O$_2$ concentration. Figure 1 shows that treatment with 0.5 mM H$_2$O$_2$ enhanced RBC/EC adherence by ~3.5-fold, and this
was not further increased by a higher H$_2$O$_2$ concentration. Using the same methodology, we have found that RBC/EC adherence might reach a much higher level under different treatments, e.g., a 115-fold elevation by Ca$^{2+}$/H$_{11001}$ ionophore (unpublished data). It thus appears that the H$_2$O$_2$ effect on RBC/EC adherence reaches saturation already at 0.5 mM.

Since cultured cells do not have the surface glycolcalyx that vascular ECs in vivo have, the projection from ECs in vitro behavior to in vivo conditions has been questioned. Therefore, subsequent to determining the adhesion of H$_2$O$_2$-treated RBCs to cultured ECs, we examined their adhesion to vessel wall following their perfusion into rat mesocecum as described in MATERIALS AND METHODS. Figure 2 demonstrates that H$_2$O$_2$-treated RBCs exhibit considerable adhesion to the vessel wall of postcapillary venules in the rat mesocecum preparation.

A different pattern was found with RBC deformability. As shown in Fig. 3, a treatment with 0.5 mM H$_2$O$_2$, sufficient to induce maximal RBC/EC adhesion, did not significantly affect RBC deformability, but this was clearly reduced by a treatment with 5 mM. As shown in Fig. 3, the latter treatment clearly shifted the EI toward that of rigid cells (Fig. 3A). When considering in vivo, circulatory disorders, the increase in the rigid cell portion might be more important than the average change in EI. Figure 3B indeed shows that the H$_2$O$_2$ effect is expressed prominently in the number of rigid undeformable RBCs (cells with EI < 1.1), exhibiting a threefold increase.

Effect of H$_2$O$_2$-treated RBCs on vascular resistance. The results (in Fig. 4) provide a method for examining the flow

![Fig. 1. Red blood cell (RBC) adhesion to endothelial cells (ECs) after treatment with H$_2$O$_2$ (0.5 and 5.0 mM). A: adherence of RBCs as a function of shear stress ($\tau$). The number of RBCs that remained adherent to the cultured ECs under the indicated $\tau$ is determined by cell flow properties analyzer and are plotted vs. $1/\tau$, yielding the function $N = N_0 + \alpha/\tau$, where $N$ is the number of adherent RBCs at a specific $\tau$ and $\alpha$ is the adheriveness coefficient. Data are means ± SE for $n$ blood samples (15 for the control and 9 for the H$_2$O$_2$-treated RBCs). B: relative $\alpha$ for H$_2$O$_2$-treated RBCs. $P < 0.005$ for difference between treated and control, untreated RBCs.](http://ajpheart.physiology.org/)

![Fig. 2. Videomicrographs of artificially perfused mesocecum infused with a bolus of H$_2$O$_2$-treated human RBCs. A: a bolus infused of H$_2$O$_2$-treated RBCs during artificial perfusion with Ringer-albumin solution results in adherence of RBCs in postcapillary venules (arrowheads). Arrows indicate the flow direction from a small-diameter venules to a large-diameter venule. B: scanning of the microvascular revealed adhesion of the treated RBCs in several postcapillary venules. C: higher magnification revealed significant adhesion in many venules.](http://ajpheart.physiology.org/)
functions under the perfusion of RBCs with elevated adhesion to ECs with normal deformability (treatment with 0.5 mM), whereas the effect of RBCs with both elevated adherence and rigidity is determined by the perfusion of RBCs treated with 5 mM H2O2. A subtraction of the first from the latter provided the net effect of RBCs with reduced deformability, all these under aggregation-free conditions.

Figure 4 depicts the changes in vascular resistance, expressed by PRU (Fig. 4A) and $T_{pf}$ (Fig. 4B), occurring following the perfusion of rat mesocecum with H2O2-treated RBCs. As shown in Fig. 4, a considerable elevation of vascular resistance occurs already at 0.5 mM H2O2, and this was further increased at 5 mM H2O2. Analyzing these data of Fig. 4 in light of Figs. 1 and 3 suggests that RBC/EC adherence is responsible for the increased vascular resistance at 0.5 mM H2O2. At 5 mM H2O2, which reduced RBC deformability without a further increase in RBC/EC adherence, the increase in the percentage of undeformable RBCs was associated with an additional increase in vascular resistance (Fig. 4).

**DISCUSSION**

Experimental data and theoretical considerations have suggested that rigid RBCs may attenuate perfusion in peripheral tissues since they can block microvessels and capillaries in particular (27). Adherent RBCs at the vessel wall may impair the flow pattern in large vessels and directly occlude microvessels and capillaries in particular (5, 25).

The results presented above show that the perfusion of the mesocecum vasculature with RBCs with enhanced RBC/EC adherence elevates vascular resistance, as expressed by both PRU and $T_{pf}$. Since this effect is obtained with normal RBC deformability and under aggregation-free conditions, it demonstrates a specific direct effect of RBC/EC adherence on hemodynamics. As shown above, increasing H2O2 concentration (from 0.5 to 5.0 mM) did not further increase RBC/EC adherence but induced a considerable decrease in RBC deformability. The subsequent perfusion of these RBCs further increased vascular resistance in the mesocecum, showing that the additional increase in vascular resistance is a direct consequence of reduced RBC deformability.

Although, as stated in the Introduction, the objectives of the present study were to demonstrate the in vivo effect of RBC/EC adherence and RBC deformability; the mechanism of the H2O2 effect is obviously of great interest. To gain an insight into this, we have first considered the possibility that H2O2 induces the phosphatidylserine (PS) translocation to the RBC surface, well known to mediate their adherence to ECs. PS externalization has been shown for some OS states (26, 29,
examined the H2O2 effect of PS externalization and found that, unlike other OS states (26, 29, 30), the treatment of RBCs with H2O2, at any concentration, did not induce PS translocation, suggesting that H2O2-induced RBC/EC adhesion does not involve RBC surface PS. Another candidate proposed as a mediator of RBC/EC interaction is clustered band-3, which has been shown to occur under OS states (5). In the present study we also observed a slight clustering of band-3 after H2O2 treatment (not shown), but the involvement of band-3 in H2O2-induced RBC/EC adhesion (e.g., testing the adherence in the presence of a blocker of band-3 clusters, not yet available), as well as other possible RBC factors (e.g., CD47), is yet to be explored.

The mechanism of RBC interaction with cultured ECs might be different than with vascular ECs in blood vessels since cultured ECs do not exhibit the same glyocalyx structure and level as in vivo (33). It has been further argued that the glyocalyx hinders RBC accessibility to vessel-wall ECs and thus prevents their interaction (33). On the other hand, in a study by Weinbaum et al. (34), it has been concluded that under low-flow rate (velocity < 20 μm/s), the RBCs can penetrate through the endothelial glyocalyx and interact with the ECs. In support of that, Kaul et al. (23) have shown, using ex vivo rat mesocceum preparation, that the adhesion of sickle RBCs to vessel walls increases as the vessel diameter decreases and is highest in postcapillary venules where the flow velocity is the lowest, thus providing “the maximal opportunity for circulating erythrocytes to interact with the endothelium.” In addition, it has been shown that malaria-infected RBCs strongly interact with glyocalyx components, specifically hyaluronic acid and chondroitin sulfate (11, 12). In accordance with these findings, the present study demonstrates that RBCs with elevated adherence to cultured ECs (Fig. 1) also adhere to vessel walls (Fig. 2) and elevate vascular resistance (Fig. 4). It may thus be assumed that EC glyocalyx does not necessarily interfere with RBC/EC interaction and might possibly facilitate it in certain conditions.

When compared with those of pathological RBCs, in the present study we induced a relatively moderate elevation of RBC adherence (up to 3- to 4-fold) and reduction of deformability (up to 2.5% of undeformable cells). For example, using the same system applied here, we have found that the adherence of thalassemic or cold-stored RBCs is enhanced by 10- and 8-fold, respectively (21, 30). Similarly, RBC deformability is markedly reduced during blood banking procedures, since the percentage of undeformable RBCs was increased to 4% during cold storage and up to 40% by γ-irradiation (30). Yet, the moderate changes in RBC/EC adherence and deformability applied in the present study still exerted a considerable increase in vascular resistance.

Of particular interest in this regard is our previous finding that the shear stress required to detach adherent RBCs from ECs is at least an order of magnitude higher than that required to disperse RBC aggregates; whereas RBC aggregates are dispersed at about 0.1–0.2 Pa, some oxidized RBCs remain adherent even at 3 Pa (5). This demonstrates the strength of RBC adhesion to the endothelium, which has been considered in recent years to be a potent catalyst of blood vessel occlusion.

In summary, this study presents specific individual effects of elevated RBC/EC adhesion and reduced RBC deformability on the circulation. Even moderate alterations in these properties are sufficient to induce a considerable increase in vascular resistance, thereby demonstrating the potency of RBC/EC adhesion and RBC rigidity to contribute to circulatory disorders.

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REFERENCES


