RED BLOOD CELL AGGREGATION: CURRENT STATUS

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INTRODUCTION

The reversible aggregation of human red blood cells (RBC) is of major interest in the field of hemorheology[1,22,28,31,57] in that RBC aggregation is a major determinant of the in vitro rheological properties of blood[26,37]. In addition, in vivo flow dynamics, including RBC distribution at branches and flow resistance of blood, are influenced by RBC aggregation[6,20]. Measures of RBC aggregation such as the erythrocyte sedimentation rate (ESR) are commonly used as diagnostic tests and as one index to the efficacy of therapy (e.g., following drug therapy in rheumatoid arthritis); in diabetes mellitus RBC aggregation is normalized by improved glycemic control[25]. There is now general agreement regarding the positive associations between elevated levels of fibrinogen or other large plasma proteins and enhanced RBC aggregation, and the effects of molecular mass and concentration for neutral polymers such as dextran[4,10,24]. However, the specific mechanisms involved in RBC aggregation have not yet been elucidated, and thus it is not yet possible to fully define the relations between pathology and altered RBC aggregation.

AGGREGATE MORPHOLOGY

During the process of *in vitro* aggregation, RBC suspended in either plasma or solutions containing large polymers (e.g., dextran > 40 kDa) initially form multi-cell linear structures; these linear forms are often termed rouleaux since they resemble a stack of coins. Subsequent to the development of linear structures, three-dimensional aggregates result from orderly or random assembly of rouleaux. The morphology and size of these aggregates depends strongly upon the "strength" of aggregation (i.e., cell-cell affinity), the local hematocrit, and the geometry of the space in which they form: aggregate size and shape may be limited if formed between a slide and cover slip[5]. *In vivo* RBC aggregation occurs at low shear forces or stasis and is a major determinant of low shear blood viscosity and of in vivo flow dynamics[6,12,15]. It is important to note that

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RBC *aggregation is a completely reversible process*, with aggregates dispersed by mechanical or fluid flow forces, and then reforming when the forces are removed[46]. Conversely, RBC agglutination and blood coagulation are irreversible processes due to either protein polymerization or strong antigen-antibody attractive forces. Abnormal increases of RBC aggregation have been observed in several diseases associated with vascular disorders (e.g., diabetes mellitus, hypertension).

RBC aggregation is primarily determined by RBC aggregability (i.e., the intrinsic cell characteristics affecting RBC aggregation) and by the concentration of the inducing macromolecule or the plasma level of large proteins; in many situations enhanced aggregation may be due to elevated aggregability[40,47]. In blood, fibrinogen is one of the most important determinants of blood viscosity due to its strong tendency to increase both plasma viscosity and RBC aggregation[28]. Note that several reports have dealt with the ability of plasma proteins to promote aggregation[24,28,48] and that higher fibrinogen levels have been linked to elevated blood viscosities in hypertension[16].

AGGREGATION MECHANISMS

In general, there is complete agreement regarding the nature of the overall mechanism involved in RBC aggregation: a "force balance" in which cell-cell affinity forces that promote aggregation are opposed by dispersion forces that reduce or prevent aggregation. The extent, rate and strength of aggregation and the morphology of aggregates thus reflect the algebraic sum of these forces. Further, there is general agreement regarding the forces countering aggregation: electrostatic repulsion due to the cell's negative surface charge, membrane strain and fluid shear stress due to mechanical shearing[19,43]. However, this level of agreement does not extend to the details of the process!

At present, there are two co-existing models for the forces promoting RBC aggregation: the "bridging" model and the "depletion" model[10,43]. In the bridging model, red cell aggregation is proposed to occur when the bridging forces due to the adsorption of macromolecules onto adjacent cell surfaces exceed disaggregating forces[19,24]. This model seems to be similar to other cell interactions such as agglutination; with the only difference being that for aggregation the proposed adsorption energy of the macromolecules is much smaller to be consistent with the relative weakness of the forces. In contrast, the depletion model proposes quite the opposite. In this model RBC aggregation occurs as a result of a lower localized protein or polymer concentration near the cell surface compared to the suspending medium (i.e., relative depletion near the cell surface). This exclusion or reduced concentration of macromolecules near the cell surface leads to an osmotic gradient tending to move fluid away from the intercellular gap, thus generating cell-cell attractive forces and depletion interaction[10,11]. As with the bridging model, forces countering aggregation are electrostatic repulsion, membrane strain and mechanical shearing.

It is obvious that the two models mentioned above are in conflict: the bridging model predicts increased aggregation consequent to increased protein or polymer concentration at the RBC surface, whereas the depletion model predicts the opposite. Although favored by the authors, it is interesting that depletion forces, which are well known in colloid chemistry, have received little attention in biological systems. One possible reason is that depletion layers are thin and of the same scale as the hydrated size of the depleted macromolecule. Thus, for most plasma proteins and polymers that induce aggregation, it is only a few nanometers thick and hence of the same thickness as the cell's glycocalyx. It is therefore not possible to detect such a layer with direct optical observations. In addition, it is also quite challenging to distinguish between weak absorption and a depletion effect since the latter usually involves some intermixing (i.e., penetration) of the macromolecule and the glycocalyx. In fact, an extensive review of literature absorption values for by Janzen and Brooks[32] has detailed likely technical artifacts (e.g., trapped fluid between RBC) and thus the extremely wide range of reported data for fibrinogen and dextran binding.

Although optical and chemical approaches to detailing the depletion layer are problematic, recent advances in the use of particle micro-electrophoresis have provided valuable information. In solutions of neutral soluble polymers that give rise to depletion layers, particles and RBC have an unexpectedly high mobility[9,11,18]. This effect is due to the reduced viscosity near the particle surface due to depletion[9,31]: electro-osmotic flow decreases rapidly outside the electric double layer, so if the double layer thickness is comparable to or less than that of the depletion layer, the influence of suspending phase viscosity is reduced.

As long as the double layer is significantly thinner than the depletion layer, the mobility will be higher than predicted based upon suspending media viscosity. With increasing double layer thickness the influence of suspending phase viscosity increases, and when the double layer thickness becomes significantly greater than the depletion layer, the measured mobilities or calculated zeta potentials are unaffected by viscosity in the depletion region. Using this approach it has been confirmed that the thickness of such depletion layers is in the same range as expected for the hydrated size of polymer studied, thereby agreeing with the concept of polymer depletion near the RBC surface and lending strong support to a "depletion model" mechanism for reversible RBC aggregation[9,11].

RBC electrophoresis in polymer solutions has also been utilized to investigate specific aspects of red cell aggregation: 1) The depletion model has been shown to be a valid explanation for the concentration dependence of polymer-induced aggregation (i.e., increasing to a maximum then decreasing for higher concentrations)[43]; 2) The 2-fold greater aggregation for older, more-dense cells in plasma and polymer solutions is consistent with a 10-15% decrease of glycocalyx thickness or polymer penetration[41,42,44]; 3) The differing aggregation behavior of RBC from various animal species[7,11]; 4) RBC

surfaces changes subsequent to erythropoietin therapy[17].

HEMODYNAMIC EFFECTS OF RED BLOOD CELL AGGREGATION

Under both *in vitro* and *in vivo* conditions, the extent RBC aggregation is determined by suspending phase and cellular properties and by the shear forces acting on the cells. Aggregates become smaller and contain fewer RBC per aggregate as shear forces increase, and hence RBC aggregates dominate below certain levels of shear stress: as mentioned above, red cell aggregation is major determinant of low shear blood viscosity.

Role of RBC aggregation in venous hemodynamics

At least in some parts of the circulatory system, shear stresses are low enough to allow the formation of RBC aggregates. The venous side of the circulatory system is certainly the mostly likely location since pressure gradients and shear forces are much lower than on the arterial side. Using high speed video recordings, Kim, et al. demonstrated the formation of RBC aggregates in post-capillary venules[34]. Further analysis of these video recordings revealed that aggregate formation reaches a maximum within twenty microns from a bifurcation and is closely related to the collision frequency between red cells[36].

Another study from the same laboratory investigated the effects of RBC aggregation on venous flow resistance using an isolated-perfused muscle preparation[20]. Their main conclusion was that the relationship between venous flow resistance and flow rate is strongly affected by RBC aggregation. Interestingly, the impact of venous flow on flow resistance is most prominent at the normal level of RBC aggregation, with the effects reduced if red cell aggregation tendency is enhanced or decreased. The conclusion from these experiments underlines the importance of physiological levels of red cell aggregation in maintaining the flow dynamics at the microcirculatory level.

The effect of RBC aggregation on flow resistance in venous vessels is easily understood based on the Poiseuille Equation where blood flow is inversely related to viscosity under a given pressure gradient. As RBC aggregation is a shear rate dependent process, decreased flow and thus decreased shear rate would yield increased aggregation which in turn increases viscosity and flow resistance**.**

Role of RBC aggregation in arterial hemodynamics

Does red cell aggregation only affect blood flow dynamics on the venous side of the circulation? The mean values for shear forces in the arterial vessels are usually not below the levels that would allow RBC aggregates to form. However, there are certain mechanisms which are affected by RBC aggregation tendency which may affect flow dynamics even on the arterial side of the circulatory system.

Axial migration and phase separation

Shear rate in a cylindrical vessel is not constant over the cross-section of the tube, but changes between the central and marginal flow zones. The central fluid zone moves at the highest speed, but shear rate is minimal in this region. However, in the marginal layers of the flowing fluid near the wall, the velocity becomes smaller due to frictional resistance with the vessel wall. Given the requirement that the fluid velocity be zero at the wall, the velocity gradient (i.e., shear rate) is maximal in this region.

As a result of the velocity distribution across the vessel diameter, RBC tend to move towards the central flow zone, resulting in a more stable situation in terms of shear forces. This axial movement is termed axial migration and generates a phase separation in blood vessels, with accumulation of red cells in the central flow zone resulting in a cell-poor layer in the marginal zone. This separation has several important results which affect hemodynamics:

Formation of this marginal, plasma-rich layer results in a significant decrement in the frictional resistance at the vessel wall and a decreased apparent viscosity in small diameter tubes. Alonso, et. al demonstrated that there is an inverse relationship between the thickness of this plasma-rich layer and the apparent viscosity of blood[2].

Phase separation results in a physiological hemodilution as side branches originate from the blood vessels. Daughter branches receive blood from layers close to this plasma-rich, RBC-poor marginal zone. This phenomenon, termed *plasma skimming,* affects the hematocrit and therefore the viscosity of blood in smaller sized blood vessels; microvascular hematocrit values are lower when compared to large vessel hematocrit. Tissue hematocrit, which is the mean value of hematocrit in all sizes of blood vessels in a given tissue, has been reported to be closer to systemic hematocrit but still significantly lower[45,53]. Tissue hematocrit values in guinea pig myocardium have been shown to be markedly affected by enhanced RBC aggregation[54].

Effect of RBC aggregation on axial migration and phase separation

Axial migration of RBC and related mechanisms have been demonstrated to be affected by modifications of RBC aggregation. For example, it has been shown optically that the phase separation resulting from RBC axial migration RBC is affected by their aggregation properties. It has also been observed that the reduction of apparent viscosity or flow resistance in small glass tubes is strongly affected by RBC aggregation[27,30,48].

These findings indicate that changes of RBC aggregation affect mechanisms that play a role in flow dynamics on both the arterial side and the venous side of the circulation. Note that RBC aggregates may exist in the central zone of blood vessels due to both to axial migration and the lower shear rate in this region. Obviously, these red cell aggregates must be dispersed into individual cells in order to be able to pass through microvessels; this disaggregation

process has an energy cost that tends to increase flow resistance.

Effect of RBC aggregation on vascular control mechanisms

Important aspects of vasomotor mechanisms controlling vascular tone are dependent on the wall shear stress acting upon endothelial cells[23]. Wall shear stress regulates nitric oxide synthesizing mechanisms and is determined by the product of fluid velocity near vessel wall and local viscosity[33,38,49]. Red cell aggregation promoting axial migration would tend to reduce local viscosity in the marginal flow zone and hence would lower wall shear stress, thereby down-regulating nitric oxide generation and increasing vascular tone. Using a rat model of chronically enhanced red cell aggregation, it has been shown that flow-mediated dilation in skeletal muscle arterioles is significantly blunted for animals in which enhanced aggregation was maintained for four days[8]; eNOS expression in the same skeletal muscles was also reduced[8].

Additional studies of RBC aggregation effects using cylindrical glass tubes lined with human umbilical vein endothelial cells (HUVEC) support these findings[55]. The HUVEC lined tubes were perfused using a pressure servo controlled system at a RBC increased RBC aggregation achieved by two methods: 1) covalent surface coating of RBC with Pluronic F98; 2) adding 0.5% dextran 500 (MW = 500 kDa) to autologous plasma. Following perfusion of tubes with F-98 coated blood cells suspensions the increase of nitrite/nitrate content of HUVEC was found to be blunted compared to suspensions of normal RBC in native plasma. Additionally, phosphorylation of eNOS at serine 1177 position, indicating the activation of the enzyme, was also found to be decreased.

The blunting of the NO-related response in these studies strongly suggests that the effective wall shear stress to which the HUVEC were exposed was lower than the nominal value (i.e., 15 dyn/cm^2), and that this lowering resulted from enhanced axial migration, phase separation and thus decreased local viscosity at the wall. Supporting this suggestion, similar experiments carried out using RBC suspended in plasma plus 0.5% dextran 500 showed eNOS activation and NO synthesis closer to control conditions. Note that the dextran+plasma suspensions had a similar level of enhanced aggregation as the F-98 suspensions, but also had a higher plasma viscosity compared to the F98 coated and control RBC suspensions. These recent findings indicate the need to consider the impact of modified RBC aggregation on NO-related vascular control mechanisms when evaluating the in vivo hemodynamic effects of altered aggregation. Further, it is obvious that the method employed for altering red cell aggregation is also important, with results that depend critically on whether suspending phase viscosity has also been changed.

Multiple effects of RBC aggregation on in vivo hemodynamics

As summarized above, RBC aggregation has multiple, often oppo-

sing effects on *in vivo* blood flow. Therefore, the overall influence of aggregation on flow in the circulatory system is complex and is not easily describe by a simple answer. Several groups have experimentally investigated the role of red cell aggregation on *in vivo* blood flow and report conflicting results. These conflicting results most probably reflect the summed contribution of the various mechanisms discussed above. The overall effect will thus be influenced by, at least, the extent of altered aggregation, the method used for the alteration, whether the altered aggregation causes changed plasma or RBC properties, and the details of the techniques used to evaluate the hemodynamic effect.

In studies employing intravital microscopy it has been observed that intensified RBC aggregation increased microvascular flow resistance[29,39,52]. Functional capillary density (i.e., the number of capillaries having flowing RBC) was also decreased with enhanced RBC aggregation[35,39]. The effect of RBC aggregation in arteriolar and/or capillary microcirculation should therefore reflect the influence of RBC aggregation on the viscosity of blood in larger vessels and the increased energy cost of disaggregation at the entrance of the microcirculation (e.g., bifurcations)[13].

Alternatively, in whole organ preparations, enhanced RBC aggregation has been reported to either decrease, increase or have no effect on flow resistance. Charansonney, et al., used an isolated-perfused rat heart preparation to study various degrees of enhanced RBC aggregation and report that relatively low levels of RBC aggregation may reduce flow resistance, whereas greatly enhanced aggregation increases resistance[21]. Other reports in which whole organ blood flow was used to calculate flow resistance indicate that increased RBC aggregation caused by high molecular weight dextrans either increased blood flow resistance in the liver[50] or had no effect on uteroplacental blood flow[51].

In most of the studies mentioned above, RBC aggregation was modified by adding high molecular weight polymers (e.g., 70 kDa dextran) to blood. However, introducing such macromolecules into plasma increases plasma viscosity as well as cell aggregation, thereby confounding an interpretation specific to the enhanced aggregation. However, a new technique based on the covalent binding of specific polymers to the RBC surface can be used to modify aggregation *without* altering plasma properties[3]. Using this method, RBC aggregation can be enhanced or inhibited by selecting the appropriate molecular size and composition of the polymer, with graded alterations achieved by modifying the polymer concentration during the covalent attachment process. Employing this technique, studies in an isolated-perfused guinea pig hind limb preparation indicate that alterations of flow resistance during perfusion with RBC suspensions having various degrees of increasing aggregation are not monotonic. Rather, both the magnitude and the direction of the effect (i.e., decrement or increment) depend on the degree of RBC aggregation[56]): a significant enhancement of flow resistance at a moderate level of RBC aggregation, a return to control level with a further increase of aggrega-

tion, with enhanced flow resistance again at the highest degree of RBC aggregation. These findings underline the participation of various mechanisms in the relationship between RBC aggregation and hemodynamics[14].

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