Molecular Links between Erythrocyte Adhesion and Vascular Dysfunction in Diabetes Mellitus, Polycythemia Vera, Retinal Vascular Occlusion

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Abstract

Despite clinical and epidemiological studies quantitative and qualitative abnormalities red blood cell (RBC) role in thrombosis has been ignored. Abnormal interaction of RBCs with vascular endothelium has been demonstrated in diabetes mellitus, Polycythemia vera (PV) and retinal vascular occlusion (RVO) after the first description in sickle cell anemia. The molecular basis has been identified. In diabetes mellitus, glycation of RBC band 3 caused the ligation to the receptor for advanced glycation end products (RAGE) present on endothelium. An abnormal Lu/BCAM (CD 239) expression in PV is responsible for the binding to endothelium annexin V. In RVO, RBC phosphatidylserine overexpression mediated the attachment to vascular laminin alpha-5 chain. Red blood cell binding altered the non-thrombocytic properties of endothelium leading to thrombosis.

Keywords: Polycythemia vera; Retinal vascular occlusion; Diabetes mellitus; Thrombosis; Red blood cell adhesion; CD239; RAGE; Annexin V; Laminin alpha-5 chain; NADPH oxidase

Introduction

Vascular dysfunction and thrombosis are predominant causes of death and disability. Arterial thrombosis has been considered to be due to platelet accumulation at vessel site, thrombus formation at damaged endothelium. Red blood cell (RBC) qualitative or quantitative abnormalities have been observed, in ocular and renal complications of diabetes mellitus, in venous thrombosis in Polycythemia Vera (PV) and in Retinal Vein Occlusion (RVO).

Diabetes mellitus

Since diabetes mellitus was defined by Claude Bernard in the nineteen century we know that this is the first risk factor for vascular thrombosis. Increase in blood glucose is often associated with lipid disorders and platelet hyper reactivity. In 1981 we have been able to demonstrate, using the same system as that described by Hebbel [1] that RBCs of diabetic patients have an increased adhesion to endothelium correlated with the severity of vascular injury [2]. RBC adhesion was measured in static conditions using chromium51 labelled erythrocytes and cultured human umbilical vein endothelial cells. Results were expressed as an adhesion ratio (AR) calculated by measuring the radioactivity remaining after washes corresponding to adherent RBCs from patients with diabetes mellitus versus normal subjects. The mean AR percentage of adhering blood cells from diabetic patients versus percentage of adhering control RBCs was 2.33 (range 0.8 to 5.2, p<0.001). The extent of adhesion expressed as an AR was correlated with severity of vascular complications. We have, in a group of diabetic patients followed for 1 year, observed that in the same patient adherence rates evolved in parallel with glycated hemoglobin HbA1c [3]. It took 13 years for us to discover what was the molecule responsible for endothelial adhesion [4]. With antibodies and recombinant molecules we demonstrated that the Receptor for Advanced Glycation End products (RAGE) is the endothelial receptor for RBCs of diabetic patients [5]. In Diabetes Mellitus RBC band 3 protein is glycated and binds to the receptor for Advanced Glycation End products [6]. In diabetes mellitus, one major mechanism of endothelial disturbance is the activation of a cascade reaction leading to NADPH oxidase stimulation, NFkB activation and consequently gene expression of factors implicated in inflammatory reactions [7]. RBC adhesion to endothelium alters several functions leading to an inflammatory reaction: Induction of adhesion molecules Vascular Cell Adhesion Molecule-1 (VCAM-1) Inter Cellular Adhesion Molecule-1 (ICAM-1) cytokine secretion Interleukin-6 (IL6), Vascular Endothelial cell Growth Factor (VEGF), Macrophage-Chemoattractant Protein-1 (MCP-1), tissue factor (TF) expression, augmentation of vascular permeability, alteration of Nitric Oxide (NO) metabolism (Figure 1).

Keywords: RAGE; VEGF; MCP-1

Figure 1: Erythrocyte adhesion in Diabetes mellitus.

RBC band 3 protein is glycated in patients with diabetes mellitus and binds to the receptor for Advanced Glycation End Products (RAGE). Engagement of endothelial RAGE induced Tissue Factor (TF) production, Intercellular Cell Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) expression, and Interleukin-6 (IL-6), Vascular Endothelial Growth Factor (VEGF), Macrophage Chemoattractant Protein-1 (MCP-1) release.
Forty percent of NO released by endothelium cannot penetrate into RBC. Free hemoglobin binds NO and blocks its anti-adhesion properties and vasodilatation. Protein glycation is associated with a high risk of vascular complications, especially in the microcirculation responsible for retinopathy and diabetic nephropathy [8]. Inhibition of AGE formation in animal models prevents or reduces the occurrence of vascular complications [9]. In the Han Chinese population a single nucleotide polymorphism in the myeloperoxidase (MPO) gene was associated with type 2 diabetes mellitus susceptibility. In the same population glutathione peroxidase-1 (Gpx1) rs1050450 and MPO rs2107545 were significantly associated with increased risk of carotid plaques in type 2 diabetes mellitus patients [10]. Current literature has identified several biomarkers that are known to play a key role in cardiovascular disease. Many of these biomarkers are influenced by an increased oxidative stress. The biomarkers stem from seven major pathways: NFkB, Nrf2 antioxidant response element signaling pathway (Keap1-Nrf2), protein kinase-C, macrophage activation, arachidonic acid mobilization, endothelial cell dysfunction and AGE. It appears that RAGE is probably involved in different mechanisms leading to cardiovascular complications [11]. In human the most effective strategies are currently a good balance of diabetes, treatment with anti-free radicals. New strategies are tested including SGLT1 and SGLT2 inhibitors (glifozins), Dipeptidyl-peptidase-4 inhibitor (gliptins), GLP1 analogs (incretins) or polyclonogenic therapy which associated metformin, glifozins, and gliptins [12,13]. Dipeptidyl peptidase-4 (DPP4)-inhibitors, in addition to their effect through improvement of glycemic control, may act directly on the AGE-RAGE axis. Furthermore, while most studies aiming at blocking AGE-RAGE axis have been disappointing, trials investigating the effects on diabetic nephropathy of a AGE inhibitor, pyridoxamine dihydrochloride (Pyridorin®) are presently going on. Blockade of RAGE by antibodies anti-RAGE, RAGE analogs TTP488 (azeliragon) or recombinant rRAGE prevents or limits the deleterious effect of AGE (Figure 2) [14].

**Figure 2:** Schematic representation of receptor for AGE (RAGE) and ligands.

Interaction with ligands Advanced glycation end products (AGE) β-amyloid peptide (βA) S100 calgranulin (S100) and inhibition of this interaction by TTP488 (azeliragon), anti-RAGE antibodies, recombinant RAGE (rRAGE).

**Polycythemia vera**

Polycythemia Vera (PV) is a chronic disorder in which the clonal proliferation of multipotent hematopoietic cells results in an increase in the red cell mass. This expansion is associated with circulatory disturbances mostly related to hyperviscosity. PV is the most common myeloproliferative syndrome, characterized by erythropoietin-independent erythroid colony formation invitro, and was recently shown to be associated in most cases to a somatic point mutation of the JAK2 tyrosine kinase (JAK2 V617F) [15,16], a substitution from valine to phenylalanine at position 617, which renders JAK2 constitutively active, leading to uncontrolled cell proliferation in the erythroid lineage and resulting in increased red cell mass. In addition another JAK2 exon 12 mutation (K539L) was described in patients with PV and idiopathic erythrocytosis [17]. Since the first description of PV in 1892 in a patient with thrombosis, thromboembolism remains a major cause of mortality and morbidity in PV. However the optimal management of patients with PV remains controversial. Phlebotomy and aspirin are still the first line therapy but despite red cell mass reduction, the risk of thrombosis remains higher than in a population of sex and age matched subjects. In addition the use of anti-platelet agents can increase the risk of hemorrhage. The most common vascular events were acute coronary syndrome and transitory ischemic attack [18]. Because adhesion of RBCs to endothelium was shown to be correlated to vascular risk in sickle cell anemia and diabetes mellitus we investigated whether RBCs from patients with PV had abnormal interactions with endothelium by measuring RBC adhesion to Human Umbilical Vein Endothelial Cells (HUVEC) under static conditions (Figure 3) [19].

**Figure 3:** RBCs from patients with PV adhere in greater numbers to endothelial cells than RBCs from healthy volunteers under static conditions.

RBC adhesion was measured using 51Cr-labeled RBCs. The nonadherent RBCs were removed by successive washes and the radioactivity was measured in each wash and in the remaining RBCs attached to endothelial cells. The unbroken line within the box represents the median; the dotted line represents the mean. The vertical lines extending beyond the boxes indicate the 25% and 75% percentiles, while the horizontal bars outside the boxes represent the 10% and 90% percentiles. The open circles indicate the values outside this range.

We also evaluated adhesion molecule expression on RBC using specific antibodies and flow cytometry. Blocking experiments indicated that PV RBC adhesion was mediated by CD239, (Lutheran blood group/basal cell adhesion molecule Lu/BCAM), on the RBC side and laminin alpha-5 chain on the endothelial side. It has been shown that the adhesion molecule Lu/BCAM, the unique erythroid receptor for laminin alpha-5 chain, is phosphorylated when sickle RBCs are...
stimulated by epinephrine and that this leads to increased adhesion to laminin alpha-5 chain [20]. We showed in our previous work that PV RBC adhesion to laminin alpha-5 chain was associated with Lu/BCAM phosphorylation (Figure 4).

![Figure 4: Adhesion of RBCs from patients with Polycythemia vera.](image)

Erythrocyte Lu/BCAM (CD 239) from patients with polycythemia vera (PV) is phosphorylated and mediated adhesion to endothelial cell laminin alpha-5 chain (laminin a5).

Lu/BCAM phosphorylation was also strongly increased in a K562 cell line co-expressing Lu and JAK2V617F, suggesting that erythroid activation of Lu/BCAM could result from the presence of JAK2V617F in PV. Growing evidence supports the hypothesis that endothelial dysfunction might contribute to thrombotic events by orchestrating the recruitment of blood cell elements to sites of injury. In PV patients with Budd-Chiari syndrome and portal vein thrombosis, Sozer et al showed that cells with endothelial characteristics lining liver sinusoids and venules were JAK2V617F positive [21]. Using an immunodeficient mouse transplant assay system, the same group showed that JAK2V617F positive CD34+ cells were able to generate endothelial like cells 

Retinal vascular complications

Retinal vein occlusion (RVO) is a common cause of permanent visual loss and is the fifth cause of unilateral blindness. The most frequent and less severe type, Branch Retinal Vein Occlusion (BRVO) is possibly driven by a mechanical factor because it generally occurs at an arteriovenous crossing. Retinal Artery Occlusion (RAO) may also occur. The most rare and sight-threatening form, Central Retinal Vein Occlusion (CRVO) remains of unknown pathophysiology Aging, arterial hypertension, and glaucoma are the only well-established risk factors for RVO. Despite a number of studies, thrombophilic risk factors have not been strongly associated with RVO, which suggests a very limited role of coagulation or anticoagulation factors in the pathophysiology of the disease. In contrast, substantial data exist suggesting that blood hyperviscosity is an important risk factor. Several blood viscosity parameters have been shown to be increased in RVO patients compared with normal subjects, including a higher hematocrit, higher whole blood viscosity, reduced red cell deformability, and enhanced index of erythrocyte aggregation. The laser induced RVO mouse model is characterized by a predominant inflammatory and tissue damage response. Hypoxia was observed in all RVO eyes for up to five days but no significant RVO-dependent changes in gene expression were detected for angiogenesis or hypoxia related genes [24]. Adhesion molecules (CD36, CD47, CD49d, CD239) when tested on RBCs by flow cytometry were similarly expressed on CRVO or RAO RBCs. CRVO patients had a higher number of RBCs expressing membrane phosphatidylserine (PS) compared with RBCs from normal subjects or RAO patients [25]. Under static conditions adhesion of RBCs from patients with CRVO to endothelial cells was significantly increased compared with adhesion of normal RBCs and of patients with RAO (Figure 5).

![Figure 5: Adhesion of RBCs from patients with retinal vascular occlusion (**p<0.01, ***p<0.001).](image)

RBCs from patients with Central Retinal Vein Occlusion (CRVO) adhere in greater numbers on human microvascular endothelial cells than do RBCs from patients with retinal artery occlusion (RAO) or RBCs from healthy volunteers under static conditions. The unbroken line within the box represents the median; the dotted line represents the mean. The vertical lines extending beyond the boxes indicate the 25% and 75% percentiles, while the horizontal bars outside the boxes represent the 10% and 90% percentiles. The open circles indicate the values outside this range.

In a flow based adhesion assay RBCs from patients with CRVO adhered in a greater number and were more resistant to washout than control RBCs (p<0.001). The number of RBCs remaining adherent after washout at the highest shear stress (0.3Pa) was 20 ± 8/mm² for...
the patients and <1/mm² for the controls. The percentage of PS-positive RBCs was significantly higher in CRVO patients and was correlated with the extent of RBC adhesion. The anti-PS receptor antibodies inhibited 55% of RBC adhesion in static conditions. Annexin V, which binds to PS, was the most efficient RBC adhesion blocker (Figure 6) [25].

**Figure 6:** Schematic representation of adhesion of RBCs from patients with Central Retinal Vein Occlusion.

Blockade of Phosphatidylserine (PS) RBCs and endothelial PS receptor by annexin V or anti-PS receptor indicated that the couple PS-PS receptor is responsible for increased adhesion of RBCs from patients with CRVO. PS Receptor (PSR), Brain-specific angiogenesis inhibitor-1 (BAI-1), Stabilin-2 Stab (Stab) and CD36, potential ligands for PS are present on endothelial cells.

These results reinforce the hypothesis that PS exposure on CRVO RBCs is an important parameter for the increased RBC adhesion. The possible use of recombinant annexin V or derived peptides as a treatment for CRVO and similar disorders was patented in USA (US patent number 9463217, October 2016).

**Conclusion**

In a recent review article Byrnes and Wolberg [26] reactivate the concept that red blood cell could be a trigger for vascular thrombosis. The authors quoted numerous articles dealing with different routes by which RBCs may be involved in thrombosis, via platelet activation, rheological mechanisms and interaction with endothelium. Since the description of a link between increased erythrocyte adhesion and vascular complications in diabetes, several publications have described the consequences of RBC interaction with endothelium, mostly in sickle cell anemia. Different RBC molecules and different endothelial cell receptors have been identified to be responsible for the abnormal RBC-Endothelium interactions. In Diabetes Mellitus RBC band 3 protein is glycated and binds to RAGE. In PV the mutation of the kinase JAK2 results in phosphorylation of Lu/BCAM (CD239) which is a ligand for endothelial laminin alpha-5 chain. In CRVO phosphatidylserine overexpression mediated the attachment to vascular laminin alpha 5-chain. These different examples indicated that RBC adhesion to endothelium may participate in the development of vascular complications in several diseases through different adhesion mechanisms. The role of RBCs in vascular complications suggests that new therapies might target the involvement of RBCs in vascular thrombosis. Indeed, previous experimental studies or ongoing clinical trials demonstrated that infusion of antibodies against adhesion molecules or peptides derived from adhesion molecules, may change the dramatic consequences of vascular occlusion and prevent in diabetic rats the occurrence of blindness [27].

**References**


