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14. ABSTRACT

Our preliminary data on RBC deformability, Ca++ influx and NO production suggest that the increased complement deposition on RBCs in patients with trauma may lead to the cytoskeletal changes in RBCs. This mechanism may retard the ability of RBCs to travel through capillaries and subsequently affect the delivery of O_2 to the tissues. Thus understanding the pathophysiological relevance of complement activation and red cell dysfunction after trauma will provide a rationale to design pharmacological strategies that can prevent complement-RBC mediated post-traumatic mortality.

15. SUBJECT TERMS
RBC, complement deposition, trauma, red cell dysfunction, post-traumatic mortality

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Table of Contents

W 81XWH-12-1-0001

Introduction

Trauma-induced changes in the red blood cells (RBC) contribute to the reduction of blood flow to distant organs. Complement activation has been implicated to mediate early posttraumatic inflammatory response, which leads to the trauma-induced sequelae and adverse outcome.

Erythrocytes (red blood cells, red cells) are the most abundant specialized cells in the human body. During the life cycle of about 120 days, erythrocytes have to maintain their biconcave shape despite regularly passing through capillaries with diameters (4.5-5µm) about half their size (7.4-8µm) [1]. Erythrocyte are able to do this, because their membrane is highly elastic, being about 100 times softer than a latex membrane of comparable thickness, and yet strong enough to undergo rapid and significant shear stresses without fragmentation [2]. Erythrocyte membrane has there major components: **1)** *membrane proteins*, that are either transmembrane or attached to the plasma membrane through GPI- or lipid-anchors (glycophorins, CD47, CR1, band 3, CD55, CD59, flotillin, stomatin etc.) **2)** *skeletal proteins*, located below the plasma membrane, conferring the erythrocyte its specific biconcave shape (spectrin, protein 4.1R, actin) and **3)** *anchoring proteins*, such as ankyrin, tropomyosin, tropomodulin, protein 4.2, adducin, dematin, that connect the membrane with the skeleton beneath, by linking the cytosolic domain of band 3 and glycophorin C with spectrin skeleton [3]. More recently, adducin and dematin have also been implicated in linking plasma membrane protein Glut-1 (glucose transporter-1) to spectrin [4]. Apart from actin, all skeleton and anchoring proteins are phospho-proteins. Importantly phosphorylation status of several skeletal proteins (adducin, ß-spectrin, protein 4.1) was shown to be altered in pathological situations [5-7] [8]. Spectrin tetramers form a hexagonal network (corrals) connected in junctional complexes by protein band 4.1R, adducin (α and β), dematin, tropomodulin and short actin protofilaments [9,10]. These are dynamic micro-domains in erythrocyte membrane that depend on the phosphorylation status of skeletal proteins (see above) and confine the lateral diffusion of transmembrane proteins by slowing down their movement by forcing them to “hop” from one corral to another [11]. We have recently shown that complement-mediated increased confinement by spectrin skeleton of complement regulatory protein CD55 can adversely affect red cell biological functions [12].

Our group and others have shown that CR1 is part of the membrane-bound complement regulatory protein family and is the receptor for all complement opsonins: C3b, C4b, C1q and MBL [13-19]. CR1 along with soluble factor H, control complement activation by degrading C3b to C3d and C3dg. Importantly, most mammals, notably rodents, do not express CR1 and do not use erythrocytes for binding and transporting complement fragments but rather use platelet-adherent factor H to bind immune complexes, which then will be removed along with the carrying platelet, by macrophages in the liver and spleen. Only humans have a transmembrane form of CR1, which makes the human clearance system and the red cell bound complement regulatory system both unique and difficult to model in rodents. We have shown that upon ligation by complement fragments, CR1 actively clusters on the surface of red cells and interacts with a newly described protein phosphatase, FAP-1[20]. The importance of CR1 presence on stored red cells up

until the moment of transfusion is underscored by the significant improvement (over 2 times) of the half-life of human red cells transfused in mice that expressed high titers of anti- human red cells antibodies when soluble CR1 (sCR1) was present along with transfused cells. In addition, the levels of complement fragments deposited on red cells were over 100 times below those of the control mice that were not treated with sCR1[21].

Erythrocyte deformability or elasticity represents the ability of erythrocytes to change their shape in response to an external force and then to return to the original biconcave shape once the force ceases to act upon them. Erythrocyte membrane deformability is one of the most important rheological factors for controlling microcirculation in organs in both normal and ischemic situations [22-24]. The dynamic, energy-dependent linkage between the membrane and the skeleton is paramount for the ability of erythrocytes to squeeze through capillaries [25,26 27]. During blood storage red cell membrane deformability is progressively lost [28-31].

Reactive oxygen species (ROS) represent a collection of molecules or ions formed by the incomplete one-electron reduction of oxygen. This category includes: singlet oxygen; superoxides; peroxides; hydroxyl radical, nitric oxide and hypochlorous acid. Depending on the amount generated, reactive oxygen species can either: **1)** signal in a variety of cellular processes functioning as mediators or **2)** can have a deleterious effect by interacting with a variety of easily oxidizable cellular targets such DNA (mostly deoxyguanosine), proteins, cholesterol and relevant for our proposed studies, with nitric oxide to generate peroxynitrites [32]. Once generated either intracellulary or extra-cellularly, ROS will affect the red cell membrane significantly decreasing its deformability [33-35].

Body

Red blood cells in trauma patients travel through capillaries with difficulty and display poor gas exchange rate and complement fragments deposited on their surface represents the main culprit. We hypothesize that the activated complement fragments deposit on RBC surface and alters their ability to pass through capillaries and exchange gases. This study aims to determine whether complement activation affects erythrocyte physiology in patients with trauma. To understand red cell dysfunction mediated by complement activation, we used both whole RBCs and sera from trauma patients and compared them with the controls. Specific aims include:

1. To determine the deposition of complement fragments on RBCs from trauma patients
2. To determine the ability of trauma RBCs to travel through capillaries
3. To determine the ability of normal RBCs treated with trauma sera to trigger Ca++ influx and produce NO.

Results to date:

1. Increased C4d deposition on red blood cell (RBC) in Trauma Patients

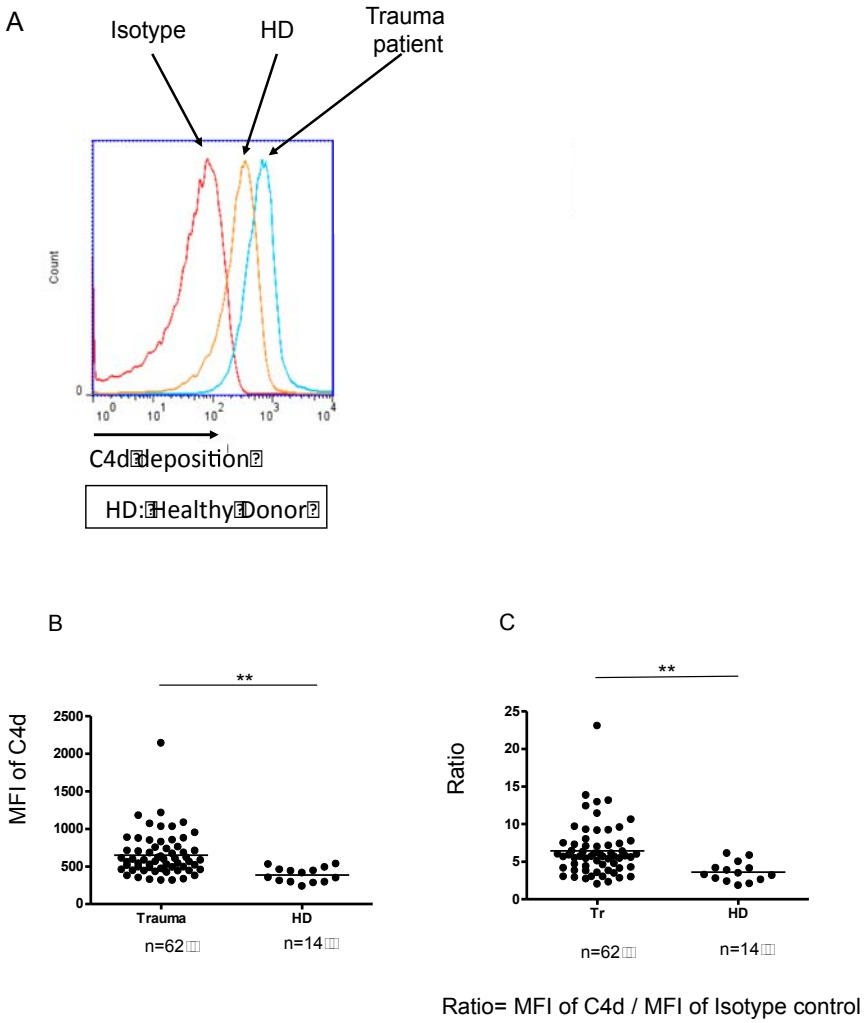

Figure 1: Increased C4d deposition on red blood cell (RBC) in Trauma Patients. The expression of C4d on the surface of RBCs from trauma patients and normal healthy donor were measured by flow cytometry and expressed as mean fluorescence intensity. (A) This is a representative data. Isotype control is shown as a red line. (B and C) shows the cumulative results of C4d deposition on RBC from trauma patients or normal healthy donor. Each dot represents 1 sample data. Horizontal lines indicate the mean. (B) Left graph shows mean fluorescense intensity (MFI) data from trauma patients compared with normal healthy donor. (C) Right shows the increasing ratio of MFI.

2. Trauma sera promote C4d deposition on RBC membranes.

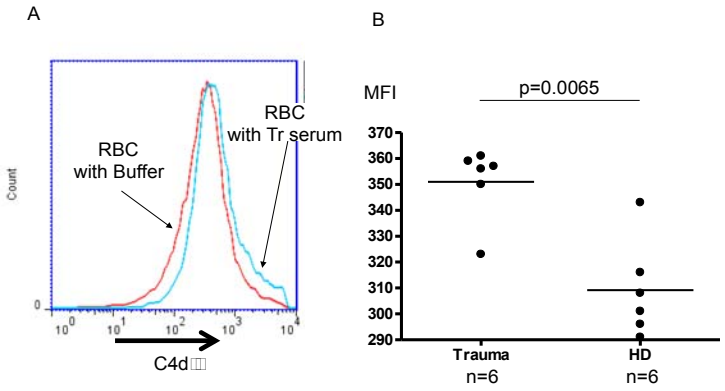

Figure 2: Trauma sera promote C4d deposition on RBC membranes. RBCs from healthy universal donors (type O, Rh negative) were incubated with sera from healthy volunteers of trauma patients. The expression of C4d on the surface of RBCs was measured by flow cytometry and expressed as mean fluorescence intensity. (A) Representative data. (B) Cumulative results from 6 control and 6 Trauma sera. Each dot represents MFI of 1 sample. Horizontal lines indicate the mean.

3. Decreased RBC deformability in patients with trauma.

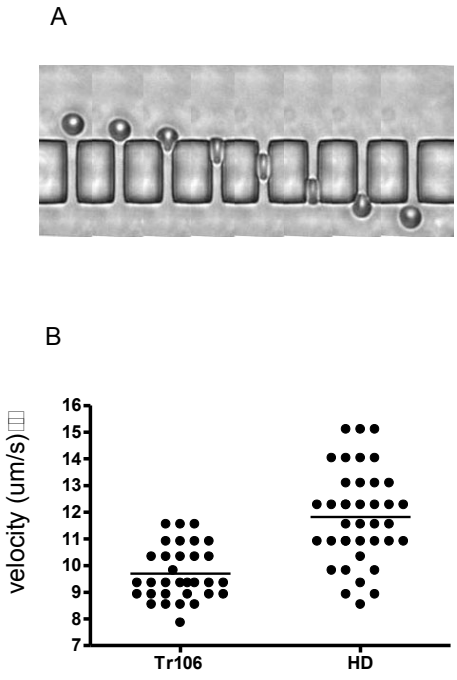

Figure 3: Decreased RBC deformability in patients with trauma. (A) Serial snapshots of RBC passing through a 2-dimensional (2-D) filter. This device has a lot of 5 μm channels as shown. RBCs were loaded into this device and observed under microscopy. The movie of the RBC passage was recorded and evaluated the passage time to pass through the channel. Less deformable RBCs need more time than normal RBC. (B) Representative

data. Each dot represents 1 RBC. RBCs from trauma patients showed lower velocity than normal healthy donor.

4. Trauma sera decrease RBC membrane deformability.

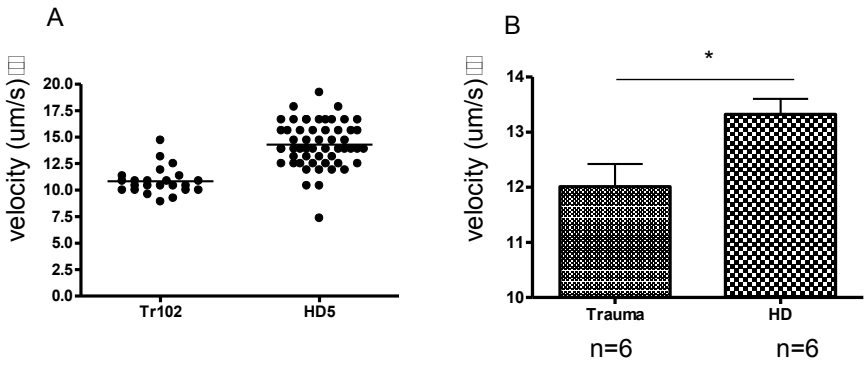

Figure 4: Trauma sera decreases RBC membrane deformability. Using the same system as Figure 2, after incubation with sera, the RBCs deformability were measured. (A) RBCs from healthy universal donors were incubated with sera from healthy individual (HD5) or trauma patient (Tr102). Each dot represents 1 RBC. (B) Shown are cumulative results from 6 control and 6 trauma sera.

5. Sera from trauma patients trigger Ca++ influx in RBCs from healthy donors

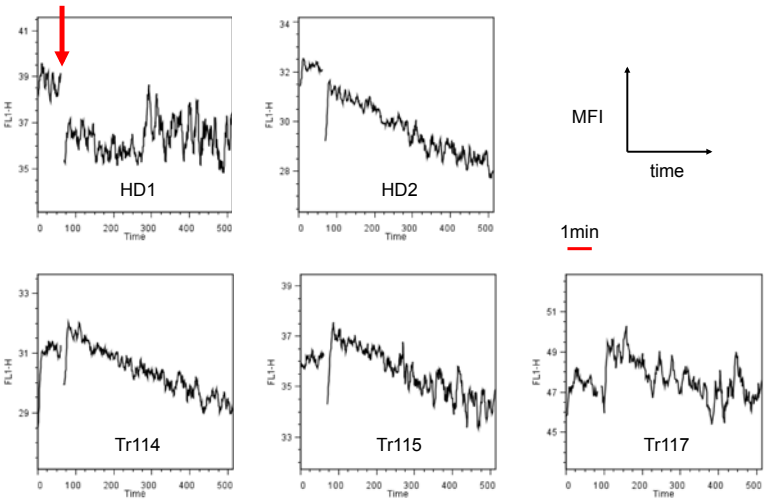

Figure 5: Sera from trauma patients trigger Ca++ influx in RBCs from healthy donors. RBC from healthy donors were preloaded with Fluo-4 AM and incubated with serum from a healthy control (HD1, HD2) or from trauma patients (Tr114, Tr115, Tr116), and Ca++ influx was measured by flow cytometry. Fluorescence levels of RBCs were acquired for 30 seconds using FACScan flow cytometer to establish a baseline for intra-

RBC Ca++ concentration. 100 µl of control or trauma sera (30% of the final volume) was then added to the RBCs, and the fluorescence intensity associated with intra RBC Ca++ concentration was recorded for an additional 6-8 min. These are preliminary and representative data. Red arrow shows the timing of adding serum into RBC solution.

6. Trauma serum induced the production of NO in RBCs from healthy donors

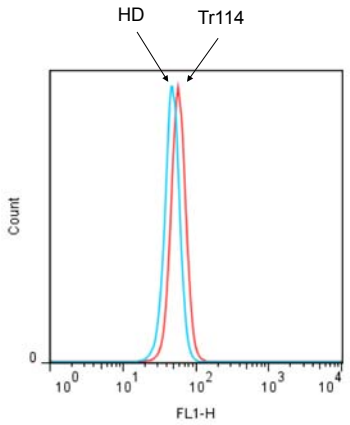

Figure 6: Trauma serum induced the production of NO by RBCs. RBCs preloaded with DAF-FM diacetate were incubated with serum from a normal individual (HD) or from Trauma patient (Tr114) for 15 minutes at 37°C. This is a preliminary and representative data. NO production from RBCs could be increased by Ca++ signal triggered by trauma sera.

Key Research Accomplishments

- Increased C4d deposition on red blood cell (RBC) in Trauma Patients
- Trauma sera promote C4d deposition on RBC membranes.
- Decreased RBC deformability in patients with trauma.
- Trauma sera decrease RBC membrane deformability.
- Sera from trauma patients trigger Ca++ influx in RBCs from healthy donors
- Trauma serum induced the production of NO in RBCs from healthy donors

Reportable Outcome

Manuscript in preparation

Conclusion

Taken together, our preliminary data on RBC deformability, Ca++ influx and NO production suggest that the increased complement deposition on RBCs in patients with

trauma may lead to the cytoskeletal changes in RBCs. This mechanism may retard the ability of RBCs to travel through capillaries and subsequently affect the delivery of O_2 to the tissues. Thus understanding the pathophysiological relevance of complement activation and red cell dysfunction after trauma will provide a rationale to design pharmacological strategies that can prevent complement-RBC mediated post-traumatic mortality.

References

1. Iolascon A, Perrotta S, Stewart GW. Red blood cell membrane defects. Rev Clin Exp Hematol. 2003;7:22-56.
2. An X, Mohandas N. Disorders of red cell membrane. Br J Haematol. 2008;141:367-375.
3. Yawata Y. Cell Membrane: Wiley-VCH Verlag GmbH & Co. KGaA; 2004.
4. Khan AA, Hanada T, Mohseni M, et al. Dematin and adducin provide a novel link between the spectrin cytoskeleton and human erythrocyte membrane by directly interacting with glucose transporter-1. J Biol Chem. 2008;283:14600-14609.
5. Machiedo GW, Powell RJ, Rush BF, Jr., Swislocki NI, Dikdan G. The incidence of decreased red blood cell deformability in sepsis and the association with oxygen free radical damage and multiple-system organ failure. Arch Surg. 1989;124:1386-1389.
6. Powell RJ, Machiedo GW, Rush BF, Jr. Decreased red blood cell deformability and impaired oxygen utilization during human sepsis. Am Surg. 1993;59:65-68.
7. Condon MR, Feketova E, Machiedo GW, Deitch EA, Spolarics Z. Augmented erythrocyte band-3 phosphorylation in septic mice. Biochim Biophys Acta. 2007;1772:580-586.
8. Perrotta S, del Giudice EM, Iolascon A, et al. Reversible erythrocyte skeleton destabilization is modulated by beta-spectrin phosphorylation in childhood leukemia. Leukemia. 2001;15:440-444.
9. Brown FL. Regulation of protein mobility via thermal membrane undulations. Biophys J. 2003;84:842-853.
10. Tsuji A, Ohnishi S. Restriction of the lateral motion of band 3 in the erythrocyte membrane by the cytoskeletal network: dependence on spectrin association state. Biochemistry. 1986;25:6133-6139.
11. Tomishige M, Sako Y, Kusumi A. Regulation mechanism of the lateral diffusion of band 3 in erythrocyte membranes by the membrane skeleton. J Cell Biol. 1998;142:989-1000.
12. Karnchanaphanurach P, Mirchev R, Ghiran I, et al. C3b deposition on human erythrocytes induces the formation of a membrane skeleton-linked protein complex. J Clin Invest. 2009;119:788-801.
13. Fearon DT. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte and monocyte. J Exp Med. 1980;152:20-30.
14. Klickstein LB, Bartow TJ, Miletic B, Rabson LD, Smith JA, Fearon DT. Identification of distinct C3b and C4b recognition sites in the human C3b/C4b receptor (CR1, CD35) by deletion mutagenesis. J Exp Med. 1988;168:1699-1717.
15. Krych M, Hourcade D, Atkinson JP. Sites within the complement C3b/C4b

receptor important for the specificity of ligand binding. Proc Natl Acad Sci U S A. 1991;88:4353-4357.
16. Makrides SC, Scesney SM, Ford PJ, Evans KS, Carson GR, Marsh HCJ. Cell surface expression of the C3b/C4b receptor (CR1) protects Chinese hamster ovary cells from lysis by human complement. J Biol Chem. 1992;267:24754-24761.
17. Klickstein LB, Barbashov S, Liu T, Jack RM, Nicholson-Weller A. Complement Receptor Type 1 (CR1, CD35) Is a Receptor for C1q. Immunity. 1997;7:345-355.
18. Tas SW, Klickstein LB, Nicholson-Weller A. C1q and C4b are additive ligands for CR1 and mediate erythrocyte adhesion. J Immunol. 1999;163:5056-5063.
19. Ghiran I, Barbashov SF, Klickstein LB, Tas SW, Jensenius JC, Nicholson-Weller. Complement Receptor 1/CD35 is a receptor for mannan-binding lectin. J Exp Med. 2000;192:1797-1808.
20. Costa M, Ghiran I, Peng C-K, Nicholson-Weller A, L. GA. Complex dynamics of human red blood cell flickering: Alterations with in vivo aging. Physical Review E. 2008;78:020901.
21. Yazdanbakhsh K, Kang S, Tamasauskas D, Sung D, Scaradavou A. Complement receptor 1 inhibitors for prevention of immune-mediated red cell destruction: potential use in transfusion therapy. Blood. 2003;101:5046-5052.
22. Vaya A, Lopez JM, Contreras MT, et al. Erythrocyte deformability in survivors of acute myocardial infarction measured by two different methodologies. Clin Hemorheol Microcirc. 2002;27:17-25.
23. Szapary L, Horvath B, Marton Z, et al. Hemorheological disturbances in patients with chronic cerebrovascular diseases. Clin Hemorheol Microcirc. 2004;31:1-9.
24. Koksal C, Ercan M, Bozkurt AK. Hemorrheological variables in critical limb ischemia. Int Angiol. 2002;21:355-359.
25. Chasis JA, Mohandas N, Shohet SB. Erythrocyte membrane rigidity induced by glycophorin A-ligand interaction. Evidence for a ligand-induced association between glycophorin A and skeletal proteins. J Clin Invest. 1985;75:1919-1926.
26. Van Dort HM, Knowles DW, Chasis JA, Lee G, Mohandas N, Low PS. Analysis of integral membrane protein contributions to the deformability and stability of the human erythrocyte membrane. J Biol Chem. 2001;276:46968-46974.
27. Kalfa TA, Pushkaran S, Mohandas N, et al. Rac GTPases regulate the morphology and deformability of the erythrocyte cytoskeleton. Blood. 2006;108:3637-3645.
28. Relevy H, Koshkaryev A, Manny N, Yedgar S, Barshtein G. Blood banking-induced alteration of red blood cell flow properties. Transfusion. 2008;48:136-146.
29. Panzera P, Rotelli MT, Salerno AM, et al. Solutions for organ perfusion and storage: haemorheologic aspects. Transplant Proc. 2005;37:2456-2458.
30. Card RT. Red cell membrane changes during storage. Transfus Med Rev. 1988;2:40-47.
31. Nagaprasad V, Singh M. Sequential analysis of the influence of blood storage on aggregation, deformability and shape parameters of erythrocytes. Clin Hemorheol Microcirc. 1998;18:273-284.
32. Mallozzi C, Di Stasi AM, Minetti M. Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. Faseb J. 1997;11:1281-1290.
33. Rifkind JM, Abugo OO. Alterations in erythrocyte deformability under hypoxia: implications for impaired oxygen transport. Adv Exp Med Biol. 1994;361:345-351.

34. Spolarics Z, Condon MR, Siddiqi M, Machiedo GW, Deitch EA. Red blood cell dysfunction in septic glucose-6-phosphate dehydrogenase-deficient mice. Am J Physiol Heart Circ Physiol. 2004;286:H2118-2126.
35. Rifkind JM, Ajmani RS, Heim J. Impaired hemorheology in the aged associated with oxidative stress. Adv Exp Med Biol. 1997;428:7-13.

Appendices

None.

Supporting data

Included in body.