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Iron-deficiency anaemia enhances red blood cell oxidative stress

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Abstract
Oxidative stress associated with iron deficiency anaemia in a murine model was studied feeding an iron-deficient diet. Anaemia was monitored by a decrease in hematocrit and haemoglobin. For the 9 week study an increase in total iron binding capacity was also demonstrated. Anaemia resulted in an increase in red blood cells (RBC) oxidative stress as indicated by increased levels of fluorescent heme degradation products (1.24-fold after 5 weeks; 2.1-fold after 9 weeks). The increase in oxidative stress was further confirmed by elevated levels of methemoglobin for mice fed an iron-deficient diet. Increased haemoglobin autoxidation and subsequent generation of ROS can account for the shorter RBC lifespan and other pathological changes associated with iron-deficiency anaemia.

Keywords: *Anaemia, red blood cells, oxidative stress, heme degradation, fluorescence*

Introduction

Iron is an essential element in all living cells. The adult human body contains $\sim$ 4 g of iron. Approximately 75% of total body iron is associated with haemoglobin, which is responsible for oxygen transport. Iron deficiency in the body limits the synthesis of heme, a prosthetic group of haemoglobin that in turn limits the synthesis of haemoglobin and decreases the production of red blood cells (RBCs) in the bone marrow, resulting in anaemia. Since cellular energy metabolism is dependent on oxygen, anaemia has a wide range of clinical consequences. One of the consequences of severe iron deficiency is a decrease in the life span of RBCs in circulation [1–6] that further exacerbates the anaemic condition.

The removal of RBCs during anaemia can be attributed to an increase in membrane stiffness and a decrease in deformability [7–9], which decreases the ability of the RBCs to pass through the spleen without being removed. More recently it has been demonstrated that iron deficiency accelerates the suicidal death of RBCs (eryptosis). This process is initiated by an increase in red cell cytosolic calcium, which stimulates phospholipids scrambling and increased exposure of phosphatidylserine to the outer surface of the membrane. Phosphatidylserine on the outer surface of the membrane is recognized by macrophages and results in the removal of erythrocytes from circulation [6,10].

The decrease in deformability, increase in cytosolic calcium and increase in membrane stiffness of RBCs can be attributed to oxidative stress [10–12]. Increased oxidative stress during anaemia is supported by increased lipid peroxidation [5,7,13–17], a decrease in antioxidant defense enzymes including

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glutathione peroxidase [5,13,16] and a greater susceptibility to the addition of pro-oxidants [18,19]. The presence of oxidative stress in RBCs during anaemia has, however, not been consistently observed [20,21].

In earlier studies, the oxidative stress has been assessed by measuring lipid peroxidation products following the reaction with thiobarbituric acid. This method has many pitfalls, particularly in RBCs. Furthermore, RBC lipid peroxidation can be affected by the presence of various oxidants in the circulation and does not specifically assess RBC oxidative stress. We recently developed a specific and sensitive method to measure *in vivo* RBC oxidative stress [22–24]. This method involves measuring the fluorescence (ex. 321 nm:em. 480 nm) of a red cell lysate [24]. This fluorescence is generated from the reaction of hydrogen peroxide with the heme moiety of haemoglobin instead of lipids and is, therefore, specific for RBC oxidative stress [22,23,25]. Our aim in this investigation is to determine whether RBC oxidative stress increases under moderate-to-severe iron-deficiency anaemia in experimental mice.

Materials and methods

Experimental animals

The animal protocol (# 289-LNS-2010) was approved by the Institutional Review Board. Three week old male C57/BL6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and divided into two groups with each group containing eight mice. The control mice were maintained on a standard iron diet of 48 ppm iron (TD.80394, Harlan-Teklad, Madison, WI) for 5 weeks. The experimental mice were maintained on an iron-deficient diet of 4 ppm iron (TD. 80396, Harlan-Teklad, Madison, WI) for 5 weeks. Mice were kept on a 12 h light/dark cycle with the room temperature maintained at $25 \pm 1°C$. At the end of the study, body weights were recorded and the iron status was assessed by measuring hematocrit (HCT) and haemoglobin (Hb). In a second study, the same experiment was repeated under similar conditions with the iron deficient-diet maintained for 9 weeks. For the 9 week study in addition to the HCT and Hb, anaemia was evaluated by measuring the total iron binding capacity (TIBC) at the end of the study.

Determination of hematocrit

Blood was collected by ocular bleeding into EDTA tubes and kept on ice. Hematocrit (HCT) values were determined using a Micro-Hematocrit Reader after centrifugation of blood samples in heparinized microcapillary tubes.

Determination of Hb and metHb

Ten to 20 μl of blood was lysed in 3 ml of milli Q water. The Hb spectrum of the haemolysate was recorded from 490 nm to 640 nm using a Perkin Elmer Lambda 35 spectrophotometer. The concentrations of oxyHb and metHb species were determined by multicomponent analysis of the spectra with known spectra of oxyhaemoglobin (oxyHb) and methemoglobin (metHb) using the 'Spectrum Quant C' software program (Perkin Elmer). The final concentration of oxyHb and metHb in blood samples were determined after multiplying the value obtained by a dilution factor. The total haemoglobin (Hb) concentration was expressed in g/100 ml.

Total iron binding capacity (TIBC)

TIBC was determined utilizing a Roche Cobas Fara II robotic chemical analyser (Bazel, Switzerland). With this analyser unsaturated iron binding capacity (UIBC) and serum iron were measured using the Ferrozine® based method [26] supplied in kits from Equal Diagnostics (Exton, PA); catalogue numbers E306020 and 157-10, respectively. The TIBC of serum samples was calculated by adding the sample values for UIBC and serum iron.

Determination of fluorescent degradation products

The Hb concentration of the haemolysate (see above) was adjusted to 50 μm Hb by dilution. The fluorescent emission (em) spectrum was measured from 400 to 600 nm at an excitation (ex) wavelength of 321 nm using a Perkin Elmer LS50B spectrofluorimeter. The maximum emission at 480 nm was used as a measure of heme degradation products. The ex and em slit widths were kept at 10 nm [24].

Statistical analysis

The data are expressed as means $\pm$ standard deviation. The *t*-test is used to determine the statistical significance of a difference between two groups of mice.

Results

Effect of iron-deficient diet on body weights

The body weights of the mice were measured at the end of each study. Although the body weights for the 5 and 9 week iron-deficient groups were less than the control groups, the differences were not statistically significant (Table I).

Effect of iron-deficient diet on HCT, Hb and TIBC levels

Iron-deficiency anaemia was assessed by measuring the HCT, Hb and TIBC. A decrease in HCT and Hb directly measures anaemia. TIBC is an indirect

Table I. Body weights, HCT% and TIBC of mice after feeding 5 and 9 weeks.

Treatment	Weights (g) 5 weeks	Weights (g) 9 weeks	HCT (%) 5 weeks	HCT (%) 9 weeks	TIBC (µg/dl) 9 weeks
Control	23 ± 2.04	25 ± 1.08	55 ± 1.2	52 ± 4.4	238 ± 70
Anaemia	21 ± 0.94	22 ± 0.94	$40 \pm 12^\star$	$32 \pm 7.6^\star$	$558 \pm 33^\star$

Values are mean $\pm$ SD for eight mice.
*Significantly different from control groups $p < 0.001$.

measure of total transferrin, which increases during anaemia. The HCT values were significantly lower in mice fed an iron-deficient diet as compared to the control group ($p < 0.001$). The magnitude of the decrease in HCT was more pronounced in the 9 week group than the 5 week group (Table I).

Figure 1 shows the haemoglobin values of mice fed with normal and low doses of iron at the end of 5 and 9 weeks. Consistent with the decrease in HCT (Table I), mice fed with an iron-deficient diet had a significantly lower Hb level than the control group. As found for the HCT (Table I), the decrease in Hb
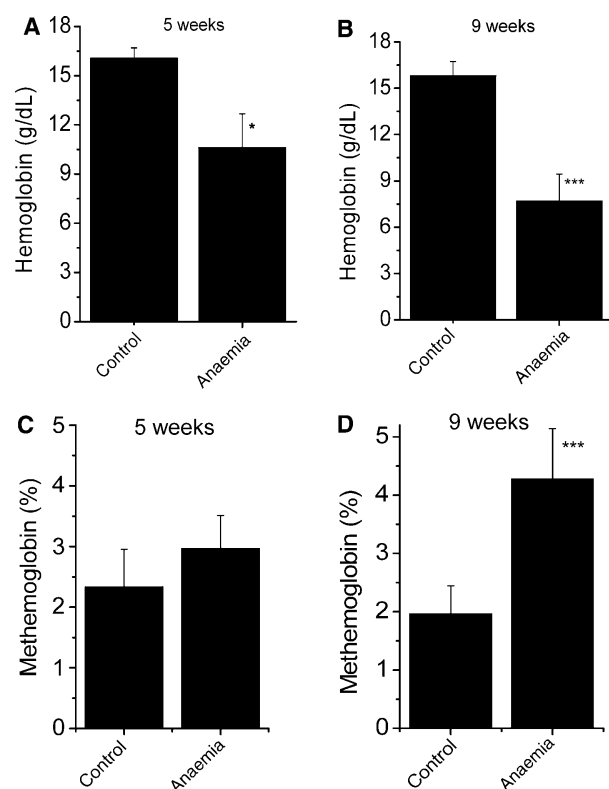

Figure 1. Haemoglobin and methemoglobin values of mice on a control and iron-deficient diet. Blood was collected by ocular bleeding after 5 weeks (A and C) or 9 weeks (B and D) and the haemoglobin (A and B) and methemoglobin (C and D) levels were determined as described in the Materials and methods section. Methemoglobin values are expressed as a percentage of total haemoglobin. Values are mean $\pm$ SD for six-to-eight mice. *Significantly different from control group after 5 weeks, $p < 0.05$. *** Significantly different from control group after 9 weeks, $p < 0.001$.

level was more pronounced in the mice on a reduced iron diet for 9 weeks than for 5 weeks.

TIBC reflects the total plasma transferrin concentration. Transferrin is the protein responsible for the transport of iron and is upregulated in serum during iron-deficiency to maximize the efficiency of iron transport from the intestine to tissues [27]. As expected, TIBC was significantly higher in the iron-deficient group than in the control group (Table I), confirming that the decrease in haemoglobin and HCT is due to iron deficiency.

Effect of iron diet on methemoglobin

In addition to the decrease in the total haemoglobin during anaemia (Figure 1), there is an increase in the percentage of oxidized haemoglobin (methemoglobin) in anaemic rats compared with corresponding controls. This increase of methemoglobin was, however, only significant ($p < 0.001$) for the 9 week study (Figure 1)

Effect of iron-deficient diet on fluorescent heme degradation products

The fluorescence emission spectra of haemolysates from 400 to 600 nm, with an excitation wavelength of 321 nm, are shown in Figure 2 for the controls and for the group on the reduced iron diet for 9 weeks. After 9 weeks, the fluorescence intensity from the haemolysate of all five mice fed an iron-deficient diet was higher than the fluorescence intensity obtained with any of the control mice.

Figure 3 shows the average maximum intensity of the fluorescent peak at 480 nm for each group. The fluorescence intensity of the iron-deficient groups after 5 and 9 weeks feeding were both significantly higher than for the corresponding control group. However, the increase in fluorescence after 9 weeks on an iron-deficient diet was 2.1-fold higher than the control, while the increase in fluorescence after 5 weeks on the iron-deficient diet was only 1.24-fold higher than the control.

Discussion

Feeding an iron-deficient diet to mice for a period of 5 and 9 weeks resulted in a decrease in both the hematocrit and haemoglobin levels, with a greater
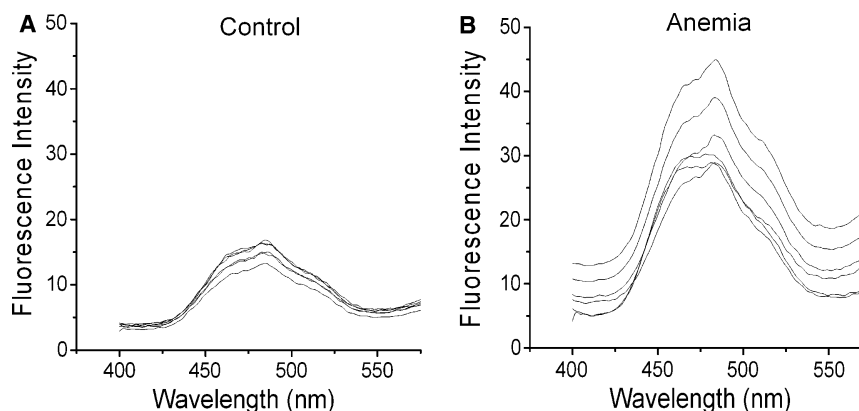
Figure 2.   Fluorescence spectra of red cell lysate of mice on control and iron-deficient diet for 9 weeks. The Hb concentration of the RBC lysate was adjusted to 50 μm Hb. The fluorescent emission (em) spectrum was measured from 400 to 600 nm at an excitation wavelength of 321 nm.

decrease for mice on the iron-deficient diet for a longer period of time. This response is clearly considered to reflect moderate-to-severe anaemia. The increase in TIBC after 9 weeks on the iron-deficient diet establishes the expected physiological response to anaemia involving an increase in transferrin to optimize the uptake of the available iron.

To evaluate whether increased red cell oxidative stress plays a role in the physiological ramifications of anaemia, we measured the metHb (Figure 1) and the levels of a fluorescent heme degradation product with ex 321 nm and em 480 nm (Figure 2 and 3). Our results clearly indicate an increase in red cell oxidative stress.

It has been demonstrated that hypoxia and the resultant decrease in the oxygenation of RBC haemoglobin destabilizes haemoglobin, increasing the rate of haemoglobin autoxidation [28]. This relationship between hypoxia and red cell oxidative stress explains how anaemia generates red cell oxida-

tive stress. With fewer RBCs and less haemoglobin available the consumption of oxygen by the tissues lowers the partial pressure of oxygen [29,30] and a greater fraction of the RBC oxygen is transferred to the tissues. The increase in partially oxygenated haemoglobin increases autoxidation generating metHb and superoxide. Most of the metHb formed during autoxidation is reduced back to functional Fe(II) haemoglobin by cytochrome b5 reductase. However, the excessive autoxidation of haemoglobin that results from anaemia is not fully reduced and an increase in methemoglobin is observed.

We have shown that the reactive oxygen species, and particularly hydrogen peroxide formed when superoxide released during autoxidation dismutates, results in heme degradation that can be monitored by an increase in the observed fluorescence with an ex of 321 nm and an em of 480 nm. This process not only produces fluorescent products, but also releases iron from the heme and reflects RBC oxidative stress [22,23,25,31].

In evaluating the physiological ramifications of red cell oxidative stress, it is necessary to consider the mechanism by which red cell ROS bypass the highly efficient red cell antioxidant system. Of particular significance for this process is the fact that the increase in haemoglobin deoxygenation that increases the formation of ROS also increases the affinity of haemoglobin for band 3 of the red cell membrane [32,33]. The hydrogen peroxide generated by membrane-bound Hb is not as efficiently scavenged because cytoplasmic catalase does not react with hydrogen peroxide at the membrane surface. The enzymes, which react with hydrogen peroxide and lipid hydroperoxide generated at the membrane, are glutathione peroxidase (GSHPX) and perroxiredoxin 2 [34]. The relationship between these two enzymes and heme degradation on red cells explains the increased heme degradation when cells are treated with iodoacetamide. Iodoacetamide inhibits GSHPX by reacting with GSH and inhibits perroxiredoxin
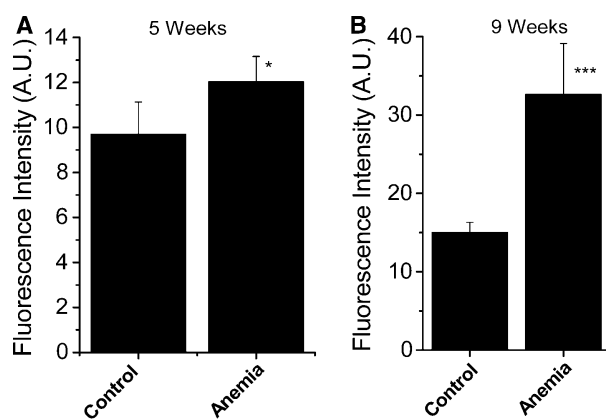

Figure 3.   Fluorescence values of mice on control and iron-deficient diet. The fluorescence spectra of RBC lysate of all animals were determined as mentioned in Figure 2. The maximum intensity of each fluorescent peak at 480 nm was taken. Values are mean±SD for six-to-eight mice. *Significantly increased relative to control after 5 weeks, $p < 0.05$.*** Significantly increased relative to control after 9 weeks, $p < 0.001$.

by reacting with the protein thiol groups [35]. Interestingly, a decrease in GSHPX activity has been reported in iron-deficiency anaemic patients and experimentally-induced iron-deficiency anaemic rabbits [5,13,16,18]. With a reduction of GSHPX activity, there is an increase in the hydrogen peroxide generated at the surface of the membrane that is not neutralized. Heme degradation only utilizes a small fraction of the hydrogen peroxide generated by haemoglobin autoxidation at the membrane surface. This same pool of hydrogen peroxide can damage the red cell membrane, increasing membrane stiffness [7], decreasing deformability [8] and increasing RBC calcium levels [10], which are commonly found in severe anaemic RBCs. In addition, the hydrogen peroxide can also be released from RBCs. The hydrogen peroxide released from RBCs has been shown to enter endothelial cells and causes inflammation [36].

We have also found that higher RBC-heme degradation correlates with increased IgG binding to the RBC membrane [24]. The increased IgG binding is thought to be triggered by the destabilization of the structure of Hb facilitating denaturation. This denatured Hb along with heme degradation products and free iron can induce band 3 protein aggregation and the membrane damage that triggers IgG binding and the subsequent removal of RBCs from circulation [37,38].

In summary, our studies establish unambiguously that severe anaemic RBCs undergo more oxidative stress than normal cells. For this purpose we have quantified the level fluorescent heme degradation products that represent a sensitive reliable method that is not prone to the uncertainties involved in the determination of lipid peroxidation in RBCs. This oxidative stress is primarily formed on the membrane. The reactive oxygen species formed on the membrane can both damage the red cell and release reactive oxygen species to the vasculature [36] and neighbouring tissues contributing to the pathology associated with anaemia. The damage to the red cell can explain the shorter red cell life-span associated with anaemia. Reactive oxygen species on the membrane surface contribute to the changes in deformability and exposure of phosphatidyl serine that have been used to explain the shorter life-span of RBCs during anaemia. In addition, heme degradation has been reported to be coupled to interaction of the RBC membrane with IgG that has also been proposed as a mechanism for the removal of RBCs from circulation.

Acknowledgements

References

[1] Verloop MC, Vuylsteke J, Drogendijk AC. [On a patient with sideroblastic anemia and hemochromatosis.]. Sci Prog 1960;90:1213–1214.

[2] Diez-Ewald M, Layrisse M. Mechanisms of hemolysis in iron deficiency anemia. Further studies. Blood 1968;32:884–894.

[3] Huser HJ, Rieber EE, Berman AR. Experimental evidence of excess hemolysis in the course of chronic iron deficiency anemia. J Lab Clin Med 1967;69:405–414.

[4] Card RT, Weintraub LR. Metabolic abnormalities of erythrocytes in severe iron deficiency. Blood 1971;37:725–732.

[5] Rodvien R, Gillum A, Weintraub LR. Decreased glutathione peroxidase activity secondary to severe iron deficiency: a possible mechanism responsible for the shortened life span of the iron-deficient red cell. Blood 1974;43:281–289.

[6] Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM, Wieder T, Lang F. Enhanced programmed cell death of iron-deficient erythrocytes. Faseb J 2006;20:368–370.

[7] Yip R, Mohandas N, Clark MR, Jain S, Shohet SB, Dallman PR. Red cell membrane stiffness in iron deficiency. Blood 1983;62:99–106.

[8] Tillmann W, Schroter W. Deformability of erythrocytes in iron deficiency anemia. Blut 1980;40:179–186.

[9] Vaya A, Simo M, Santaolaria M, Todoli J, Aznar J. Red blood cell deformability in iron deficiency anaemia. Clin Hemorheol Microcirc 2005;33:75–80.

[10] Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. Antioxid Redox Signal 2006;8:1183–1192.

[11] Pfafferott C, Meiselman HJ, Hochstein P. The effect of malonyldialdehyde on erythrocyte deformability. Blood 1982;59:12–15.

[12] Damonte G, Guida L, Sdraffa A, Benatti U, Melloni E, Forteleoni G, Meloni T, Carafoli E, De Flora A. Mechanisms of perturbation of erythrocyte calcium homeostasis in favism. Cell Calcium 1992;13:649–658.

[13] Kumerova A, Lece A, Skesters A, Silova A, Petuhovs V. Anaemia and antioxidant defence of the red blood cells. Mater Med Pol 1998;30:12–15.

[14] Jain SK, Yip R, Hoesch RM, Pramanik AK, Dallman PR, Shohet SB. Evidence of peroxidative damage to the erythrocyte membrane in iron deficiency. Am J Clin Nutr 1983;37:26–30.

[15] Moshynska OV, Tretiak NN, Anoshina MY, Yagovdick MV. [Hemoglobin-induced lipid peroxidation in anemia]. Lik Sprava 2001;4:39–43.

[16] Cellerino R, Guidi G, Perona G. Plasma iron and erythrocytic glutathione peroxidase activity. A possible mechanism for oxidative haemolysis in iron deficiency anemia. Scand J Haematol 1976;17:111–116.

[17] Perona G, Cellerino R, Guidi GC, Moschini G, Stievano BM, Tregnaghi C. Erythrocytic glutathione peroxidase: its relationship to plasma selenium in man. Scand J Haematol 1977;19:116–120.

[18] Bartal M, Mazor D, Dvilansky A, Meyerstein N. Iron deficiency anemia: recovery from *in vitro* oxidative stress. Acta Haematol 1993;90:94–98.

[19] Melhorn DK, Gross S, Lake GA, Leu JA. The hydrogen peroxide fragility test and serum tocopherol level in anemias of various etiologies. Blood 1971;37:438–446.

[20] Acharya J, Punchard NA, Taylor JA, Thompson RP, Pearson TC. Red cell lipid peroxidation and antioxidant enzymes in iron deficiency. Eur J Haematol 1991;47:287–291.

[21] Ramachandran M, Iyer GY. Erythrocyte membrane lipid peroxidation in iron deficiency anemia. Experientia 1984;40: 173–174.

[22] Nagababu E, Rifkind JM. Formation of fluorescent heme degradation products during the oxidation of hemoglobin by hydrogen peroxide. Biochem Biophys Res Commun 1998;247:592–596.

[23] Nagababu E, Rifkind JM. Heme degradation by reactive oxygen species. Antioxid Redox Signal 2004;6:967–978.

[24] Nagababu E, Fabry ME, Nagel RL, Rifkind JM. Heme degradation and oxidative stress in murine models for hemoglobinopathies: Thalassemia, sickle cell disease and hemoglobin C disease. Blood Cells Mol Dis 2008;41:60–66.

[25] Nagababu E, Chrest FJ, Rifkind JM. The origin of red cell fluorescence caused by hydrogen peroxide treatment. Free Radic Biol Med 2000;29:659–663.

[26] Stookey L. Ferrozine—a new spectrophotometric reagent for iron. Anal Chem 1970;42:779–781.

[27] Chua AC, Graham RM, Trinder D, Olynyk JK. The regulation of cellular iron metabolism. Crit Rev Clin Lab Sci 2007;44:413–459.

[28] Balagopalakrishna C, Manoharan PT, Abugo OO, Rifkind JM. Production of superoxide from hemoglobin-bound oxygen under hypoxic conditions. Biochemistry 1996;35: 6393–6398.

[29] Hebert PC, Van der Linden P, Biro G, Hu LQ. Physiologic aspects of anemia. Crit Care Clin 2004;20:187–212.

[30] Hare GM. Anaemia and the brain. Curr Opin Anaesthesiol 2004;17:363–369.

[31] Nagababu E, Rifkind JM. Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation. Biochemistry 2000;39:12503–12511.

[32] Shaklai N, Yguerabide J, Ranney HM. Classification and localization of hemoglobin binding sites on the red blood cell membrane. Biochemistry 1977;16:5593–5597.

[33] Demehin AA, Abugo OO, Jayakumar R, Lakowicz JR, Rifkind JM. Binding of hemoglobin to red cell membranes with eosin-5-maleimide-labeled band 3: analysis of centrifugation and fluorescence data. Biochemistry 2002;41:8630–8637.

[34] Low FM, Hampton MB, Peskin AV, Winterbourn CC. Peroxiredoxin 2 functions as a noncatalytic scavenger of low-level hydrogen peroxide in the erythrocyte. Blood 2007;109:2611–2617.

[35] Nagababu E, Chrest FJ, Rifkind JM. Hydrogen-peroxide-induced heme degradation in red blood cells: the protective roles of catalase and glutathione peroxidase. Biochim Biophys Acta 2003;1620:211–217.

[36] Kiefmann R, Rifkind JM, Nagababu E, Bhattacharya J. Red blood cells induce hypoxic lung inflammation. Blood 2008;111:5205–5214.

[37] Low PS. Role of hemoglobin denaturation and band 3 clustering in initiating red cell removal. Adv Exp Med Biol 1991;307:173–183.

[38] Turrini F, Arese P, Yuan J, Low PS. Clustering of integral membrane proteins of the human erythrocyte membrane stimulates autologous IgG binding, complement deposition, and phagocytosis. J Biol Chem 1991;266:23611–23617.