Review

The microenvironment can shift erythrocytes from a friendly to a harmful behavior: Pathogenetic implications for vascular diseases

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Received 4 December 2006; received in revised form 2 March 2007; accepted 8 March 2007

Available online 14 March 2007

Time for primary review 22 days

Abstract

Erythrocytes are peculiar cells aimed at the delivery of oxygen and nitric oxide to the periphery and carbon dioxide to the lungs. In addition, they also exert, under physiological conditions, a scavenging activity towards reactive oxygen and nitrogen species often over-produced in morbidity states, e.g. in inflamed tissues. Their deformability is essential for their circulation, specifically in small blood vessels, and this is an important pre-requisite for such vascular “antioxidant” functions. On the other hand, if the erythrocyte undergoes changes in its redox status, i.e. it is not capable of counteracting the pro-oxidant status of the microenvironment, it becomes a source of reactive species and, consequently, its typical structural and functional features are lost. More importantly, the oxidatively modified red cell increases its aggregability and adhesiveness to the endothelium and to other blood cells, thus contributing to vascular damage. In line with recent data from the literature, erythrocytes can be proposed as bioindicators of progression in chronic or acute diseases characterized, as a hallmark, by oxidative alterations.

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Keywords: Erythrocyte; Redox; Biomarkers; Vascular diseases; Vascular complications

1. RBC as a “reporter” of the vascular microenvironment status

During their daily life, red blood cells (RBCs) are exposed to several stress situations. Besides the effects of xenobiotics or pathogens, this stress can be envisioned as (i) physical, occurring for example when RBCs (7 μm) have to squeeze through capillaries which are smaller than themselves (5 μm), (ii) oxidative, when they experience hypoglycemic conditions after a meal, or cross radical-rich tissues with atherosclerotic lesions, or pass more than once a minute the fully oxygenated lung, and (iii) hyperosmotic, when they travel more than once an hour through kidney medulla.

The role of the RBC is generally ascribed to its ability of delivering oxygen and nitric oxide (•NO) [1,2] at the periphery and carbon dioxide to the lungs. To optimize these functions and to survive at the same time the rigors of circulation, RBCs are equipped with extraordinary properties. First, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flexible spectrin-based membrane skeleton supplies the high elasticity to overcome the physical stress [3,4]. Second, a specialized flex

Abbreviations: AGEs, Advanced Glycation End products; NADH, reduced nicotinamide adenin dinucleotide; *NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory drugs; NF-κB, nuclear factor kappa B; RBCs, red blood cells; RONS, reactive oxygen and nitrogen species; SOD, superoxide dismutase.

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protein synthesis, must be equipped with several mechanisms, not yet completely clarified, to counteract cell alterations induced by reactive oxygen and nitrogen species (RONS) or, alternatively, to signal irreversibly damaged cells to the reticulo-endothelial system for their removal [6]. A more complex scenario is however emerging from literature. This brings into play at least three different roles for RBCs: i) as RONS scavenging “devices” they can improve organism’s antioxidant defenses; ii) as pro-oxidant “bullets” they can contribute to the formation of an oxidative microenvironment and, finally, iii) as “signaling mediators” they can provide long distance information [7,8]. These hypothesized roles, scavenging, pro-oxidant and signaling, are sometimes overlapping functions and this is particularly true for signaling.

Fig. 1. (A) Red blood cell structural features and antioxidant defenses. Modified from [38]. (B) Schematic drawing of the modifications occurring in RBCs flowing in an atherosclerotic blood vessel. Note that erythrocytes are structurally and functionally modified by RONS produced within the inflamed microenvironment. \( \cdot \text{O}_2^- \) and \( \cdot \text{NO} \) are relatively harmless whereas peroxides such as \( \text{H}_2\text{O}_2 \) and \( \text{ONOO}^- \) (peroxynitrite) preferentially attack hemoglobin. HOCl (hypochlorite) reacts preferentially with proteins whereas extracellular \( \cdot \text{OH} \) (hydroxyl radical) attacks both surface proteins and lipids.
which is clearly involved in both scavenging and pro-oxidant processes. When RBCs are challenged with pro-oxidant RONS they can provide a pro-oxidant signal to vascular cells. This could be counteracted by providing RBCs with antioxidant drugs that decrease the RONS generation. Of note, RONS normally function to activate RBC physiological metabolism and in this way the RBC can lessen the oxidative stress of other cells. Hence, a critical role in distinguishing among the three different roles is played by the “amount” of reactive species as well as by the success of RBC antioxidant defense machinery. These provocative pathways have been partially detected in experimental and ex vivo studies but, more recently, some new insights also derive from studies carried out in peripheral blood from different human pathological conditions that display biomarkers of oxidative stress as a hallmark. Indeed, some works [8–11] recently proposed that the RBC, sensing the microenvironment found in all tissues, could be considered as a “reporter cell” for the antioxidant status of the whole organism.

2. RBC as a scavenger of RONS

Under physiological conditions, the RBCs serve the important function of a circulating scavenger. This cell is well equipped with non-enzymatic antioxidants such as glutathione, thioredoxin, ascorbic acid and vitamin E. Furthermore, compared with other cell types, RBCs exhibit high activities of the most important antioxidant enzymes, including superoxide dismutase, thioredoxin reductase/peroxiredoxin system, catalase, glutathione peroxidase, glutathione reductase, plasma membrane oxidoreductases to reduce extracellular oxidants and, finally, the methemoglobin reductase/NADH/glycolysis system to maintain hemoglobin in a Fe²⁺-active form [12–14]. Altogether this powerful antioxidant machinery makes the RBC a highly efficient antioxidant system not yet fully appreciated (Fig. 1A). It is conceivable to hypothesize that RBCs crossing inflamed areas can significantly contribute to detoxify RONS and thus to rescue or protect from intense oxidative stress other blood cells [15]. In this respect, recent data also point to RBCs as key regulators of vasodilation in peripheral tissues. For instance, with the advent of the field of *NO biology, RBCs were initially thought to be *NO scavengers, but more recent data demonstrated that *NO scavenging, i.e. vasoconstriction, is observed only at high pO₂, while graded vasodilation was observed at the pO₂ of tissues (5–20 mmHg). The hypothesis that RBCs can indeed generate *NO instead of causing its destruction was first proposed by Jia et al. [1]. These authors proposed that *NO could bind covalently to the hemoglobin β-chain to form S-nitrosated hemoglobin, which could allosterically deliver an S-nitrosothiol during hemoglobin deoxygenation. This hypothesis has been subsequently challenged, and further studies demonstrated that partially deoxygenated RBCs possess vasodilatory ability through the intrinsic nitrite reductase activity of hemoglobin [2,16,17]. More recently, Kleinbongard et al. [18] have expanded this hot field of research with studies suggesting that RBCs possess also an intrinsic and active *NO synthase. A central challenge in this controversial field is to understand how *NO formed within the RBC can “survive” the rapid and irreversible removal by oxyhemoglobin. Anyhow, the scavenging of *NO by hemoglobin may be protective under some circumstances, since it prevents *NO-dependent deleterious reactions such as nitrosation (nitrosative stress) and oxidation/nitration (due to the formation of peroxynitrite, Fig. 1B). On the other hand, vasoconstriction and the formation of intracellular methemoglobin (*NO scavenging produces methemoglobin and nitrate) have been considered as pro-oxidant effects.

Another important scavenging function of RBCs is directed towards immune and endothelial cells. Inflammatory conditions are characterized by lymphocyte activation with the production of a large burst of RONS affecting other blood cells and the endothelium which is particularly susceptible to RONS-dependent signaling [19]. Although conventionally RONS have been considered to function primarily in host defenses, strong evidence supports a role in the regulation of pivotal cellular signaling events (see for example the recent review by Fialkow et al. [20]). Interestingly, it has been reported that RBCs, through RONS scavenging, promote T cell growth and survival and the upregulation of cytoprotective proteins [15,21]. Again, the formation of the atherosclerotic lesion is coupled with a deep alteration of endothelial cells with a considerable production of radicals and other mediators of inflammation and RBCs can unroll a protective effect [22]. In this situation the cross-talk between the vessel and RBCs may trigger a repair process, i.e. a beneficial function yet underestimated.

3. RBC as a pro-oxidant cell

Under physiological, e.g. non-inflammatory conditions, the low levels of RONS generated in the vasculature, can easily be handled by RBC antioxidant machinery. These reactive species also include those generated by hemoglobin inside the RBC, i.e. the superoxide radical generated by partially oxygenated hemoglobin and *NO generated by deoxyhemoglobin [2,16,17]. Conversely, when the RBC crosses a tissue where an intense production of reactive species takes place, e.g. in inflamed tissues with atherosclerotic lesions (Fig. 1B), the cell may accumulate oxidative damage. This reduct imbalance (the disequilibrium between oxidized and reduced compounds inside a cell) may thus reflect the oxidative stress occurring in pathological tissues and organs. For example, accumulating evidence suggests that certain lipid oxidation products, such as oxidized phospholipids [23] and 4-hydroxynonenal [24], an aldehydic oxidative end-product of n-6 polyunsaturated fatty acids, may represent endogenously formed factors that are capable of triggering vascular inflammation. In this situation, if the oxidative insult overcomes the RBC defenses it is conceivable that oxidatively-modified RBCs can act at the periphery
as *pro-oxidant bullets* capable of modifying the behavior and fate of other vascular tissues, e.g. endothelial cells, far away from the primary inflamed/atherosclerotic tissue (Fig. 2). This is confirmed by recent literature data encompassing the pro-oxidant activity exerted by RBCs in vascular diseases including atherosclerosis [25]. One example is that of the heme iron. This actually behaves as an active-redox-metal and the heme of hemoglobin has been demonstrated to repair oxidative insults occurring in the globin chain [26]. Nevertheless, when heme is released from damaged hemoglobin, or hemoglobin is released from lysed RBCs, it can react with peroxides and can be changed into a source of dangerous radicals: an event shifting the behavior of RBCs from a RONS scavenger to a RONS generator. The pro-oxidant nature of cell-free plasma hemoglobin may also contribute to the proinflammatory nature of low density lipoproteins playing a role in the pathogenesis of atherosclerosis [27].

Oxidative stress causes a plethora of RBC changes among which cytoskeleton rearrangement and oxidation and loss of lipid asymmetry. These cells become more rigid and, thus, undergo lysis more easily releasing cytotoxic species in the vasculature. For example, aged and oxidized RBCs release hemoglobin, heme-Fe and iron [28,29]. These molecules are powerful oxidants, sources of radicals and able to modulate certain blood cell functions, e.g. to induce platelet activation [30]. Moreover, oxidized RBCs expose signaling molecules, e.g. phosphatidylserine, which are recognized by monocytes. This cell–cell interaction is sufficient to generate in monocytes an oxidative burst, as demonstrated by Casado et al. [31]. Another example, more relevant in this context, is the appearance in RBCs of a class of glycated proteins termed Advanced Glycation End products (AGEs). These can be detected in oxidized RBCs as well as in RBCs from type 2 diabetic patients [32,33]. In particular, one of the hallmarks of RONS-mediated oxidative reactions as well as of non-enzymatic covalent addition of glucose, is the formation of carbonyl groups. These carbonyls react rapidly with nucleophilic groups on Lys and Arg side chains and N-terminal amino groups of proteins, lipids, and nucleic acids resulting in the formation of AGEs. The interaction of AGEs with their receptors on endothelial cells leads to oxidative

![Fig. 2. Two different functions have been hypothesized for RBCs: their antioxidant role is shown on the left whereas their pro-oxidant role is shown on the right.](image-url)
stress, activation of nuclear factor kappa B (NF-κB), and subsequent expression of NF-κB-regulated genes [34–36]. In this scenario, pro-oxidant RBCs may function as “dangerous bullets” and their cross-talk with vascular cells may contribute, or even trigger a damaging process. Furthermore, the hazard of handling reactive species can also lead, in the long run, to an accumulating damage, a prerequisite for the onset of the majority of degenerative diseases.

4. Removal of damaged RBCs and RBC apoptosis

With regards to oxidative modifications and RBC demise, recent insights come from studies on their senescence suggesting that they can undergo a sort of apoptosis [6,37]. For instance, several works demonstrated that oxidative stress as well as hypertonicity trigger a signaling cascade of events leading to the appearance of biomarkers of senescence. Strikingly, cell changes reminiscent of apoptosis, normally occurring in nucleated cells, have also been detected [38,39]. In particular, the apoptosis of RBCs, was called eryptosis or erythroptosis depending on the injury pathway taken into account. However, the biological meaning and relevance of RBC senescence or apoptosis, characterized by glycophorin A loss or phosphatidylserine externalization respectively, although mainly referred to as critical events responsible for RBC removal at the end of their lifespan, are still a matter of debate [37,40,41]. For instance, the plethora of changes occurring in senescent and apoptotic RBCs under oxidative stress definitely comprises even biophysical changes, e.g. the loss of cell plasticity with impaired deformability associated with changes of cytoskeletal network assembly [3,4]. Finally, oxidative changes leading to senescence or apoptosis of RBCs also include alterations of homotypic (RBC–RBC) and heterotypic (e.g. RBC-endothelial cells) interactions and adhesion patterns. In fact, RBC surface antigens, that can be redox-modulated [39,41], can also significantly affect cell–cell interactions. All these modifications can contribute to the onset of the modified microenvironment detectable in certain vascular pathological conditions, e.g. in inflamed or in structurally modified blood vessels as in atherosclerosis (Figs. 1B and 2).

5. RBC as “reporter” cell in cardiovascular diseases

Although the RBCs have been the object of intense studies for more than a century, there are huge gaps in our understanding of the relative pro-oxidant or anti-oxidant contribution of this cell to the vascular patho-physiology. It was hypothesized that they could provide oxidative markers of prognostic value in clinical practice [10]. For instance, it was shown that increased RBC aggregation is associated with a poorer prognosis as a consequence of the unfavorable hemorheologic profile leading to slow capillary flow, tissue deoxygenation and endothelial cell dysfunction [42]. On this basis, cellular biomarkers of oxidatively modified RBCs could be considered as potential candidates for monitoring not only RBC-linked pathologies but, also, for other pathologic conditions associated with oxidative stress and, more in general, to monitor the overall oxidative stress status. It was for instance proposed a reappraisal of the role of RBCs as bioindicators of prognostic value in complications associated with chronic obstructive respiratory disease, a chronic inflammation of lungs [43]. Likewise, membrane remodeling, e.g. phosphatidylserine externalization, has been hypothesized as a key alteration in hemoglobin-linked human diseases such as sickle cell disease [44] and thalassemia [45,46] as well as in other non-hematologic diseases [47,48]. Moreover, RBC aggregation is instructed by a series of extracellular adhesive macromolecules, mainly fibrinogen which contributes to hyper-viscosity and RBC aggregation [49] whereas adherence to the endothelial cells, increased in RBCs exposing phosphatidylserine, appears to be mediated by thrombospondin [50]. Examples of this innovative idea that RBCs can contribute to the pathogenesis of vascular diseases, e.g. as atherosclerosis risk factors, come from recent studies aimed at the comprehension of the role of RBC adhesiveness and aggregation in the onset and progression of such diseases. For instance, it has been shown that an increased C-reactive protein in the blood is associated with increased RBC adhesiveness and aggregation [42]. Further, an altered antioxidant status of RBCs has been described in asymptomatic hypercholesterolemic patients [51]. Accordingly, one of the main effects of statin therapy in hyperlipidemic patients is related to the improvement of hemorheologic-hemostatic parameters [52,53]. Finally, recent data in patients with acute myocardial infarction also showed a direct cardioprotective effect of erythropoietin. This was probably due to a decrease in apoptotic cell death and to an increase in capillary vessels density (neoangiogenesis) resulting in the prevention of left ventricular remodeling and dysfunction [54,55]. However, this drug also exerts beneficial effects on RBC redox alterations of lipids and proteins contributing to the maintenance of RBC deformability and decreasing their microviscosity [56–58]. Furthermore, the finding that recombinant human erythropoietin treatment may be effective in wound healing via an inhibition of lipid peroxidation in wound area [59] could also be of relevance. Accordingly, increased RONS formation during hypoxia was found to induce a variety of genes including erythropoietin [60].

6. Clinical implications

RBCs have been proposed as real-time biomarkers and pathogenetic determinants in the field of atherosclerosis and thrombosis [10,61–72]. In particular, changes of RBC viscosity, adhesivity and aggregability have been detected in a number of human pathologic conditions displaying systemic oxidative stress as a hallmark. For instance, changes of erythrocyte adhesiveness/aggregation and
morphology have been proposed as useful markers to detect inflammatory conditions, plaque instability and atheroma progression in patients with coronary artery disease [62,63,66]. A strong correlation was also found between RBC aggregation and inflammatory state in unstable angina [67]. Moreover, concerning the pathogenetic role, it has been suggested that RBCs can represent a potent atherogenic stimulus contributing to the deposition of cholesterol at the atherosclerotic plaque [68] and the redox changes of RBCs have been hypothesized to play a role in the pathogenesis of hypertension [69,70] and stroke [71,72]. Altogether these findings suggest that the RBCs, thanks to their prompt response to pro-oxidant species, can deeply influence, or be influenced by, an oxidatively modified microenvironment. Interestingly, gender differences have also been detected in this respect, thus suggesting that additional actors can play a role in the complex framework of events that result in vascular damage [65]. On the other hand, the role of RONS in cardiovascular diseases has led to the synthesis of new drugs generated by adding a *NO-releasing moiety to non-steroidal anti-inflammatory drugs (NSAIDs), i.e. adding antioxidant power to the well known anti-inflammatory action of NSAIDs [73]. These new promising drugs are now in human clinical testing and the RBCs could represent a useful tool to investigate their vascular effects. More in general, the improvement of clinical laboratory analyses aimed at the evaluation of RBC integrity and function, e.g. morphological/rheological parameters, expression of surface antigens and, RBC redox state, could provide useful information in the clinical practice in the long run.

Acknowledgements

This work was supported in part by ISS-NIH collaborative project “Peripheral blood determinants of redox changes in human respiratory diseases: biochemical and pathophysiological evaluations” Rif. 0F14.

References


