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Forum Review

Redox Imbalance, Macrocytosis, and RBC Homeostasis

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ABSTRACT

The erythrocyte represents a major component of the antioxidant capacity of the blood through the enzymes contained in the cell, the glutathione system, and the low-molecular-weight antioxidants of the erythrocyte membrane. A further major red blood cell contribution is in regenerating consumed redox equivalents via the oxidative pentose phosphate pathway and glutathione reductase. Moreover, its extracellular antioxidant capacity, its mobility, and the existence of reducing equivalents far in excess of its normal requirements make erythrocytes function as an effective oxidative sink in the organism. That is why red blood cell metabolism and homeostasis strongly affect the antioxidant properties of the whole body. Conversely, the relation between macrocytosis and oxidative stress has not been fully delineated. Reviewing the mechanisms involved in red blood cell homeostasis in cases of redox imbalance is crucial in identification of factors that could potentially improve erythrocyte survival and defense against oxidant damage. *Antioxid. Redox Signal.* 8, 1205–1216.

INTRODUCTION

THE MOST REMARKABLE FEATURE OF THE HUMAN ERYTHROCYTE is its durability, given that it is an anucleated cell without the vital organelles that are considered necessary for the survival and function of most other cell types. They are devoid of mitochondria that ensure efficient oxidative metabolism, ribosomes for generation of damaged proteins, and a nucleus to regulate the regenerative process. *De novo* synthesis of lipids is also precluded, because of a very limited metabolic repertoire.

However, the red blood cell (RBC), through its role as the body's oxygen and carbon dioxide transporter, is under constant exposure to possible sources of oxidative stress and is able to survive by using an exquisitely effective cellular defense to terminate free radical reactions or remove reactive species and their secondary products. Moreover, its extracellular antioxidant capacity and its mobility render the RBC an ideal antioxidant, not only for its own membrane and local environment, but also for other cells and tissues (86). The erythrocyte shows extreme deformability under normal physiologic circumstances, mainly because of the plasticity and viscosity of the membrane. Among the factors that affect membrane deformability and stability are membrane lipid content, cytoskeletal proteins, and transmembrane proteins (29). Abnormalities in both the integrity and function of these factors greatly influence membrane mechanical properties and RBC survival. Thus, when the erythrocyte loses some of the critical enzymes needed for intermediary metabolism and antioxidant capacity, oxidation of critical membrane proteins, lipids, and hemoglobin would then ensue, resulting in distortion and rigidity of the cell membrane and finally in accelerated loss. Macrocytosis after oxidative injury of erythrocytes has also been reported (118).

To review redox imbalance in association with RBC homeostasis and macrocytosis is the major objective of this article.

GENERATION OF OXIDIZING AGENTS

The term *oxidative stress* was introduced in the 1980s (96) and has been used to designate a situation in which the cellu-

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lar redox homoeostasis (*i.e.*, the balance between prooxidants and antioxidants, is altered because of excessive production of reactive oxygen species and/or impairment of cellular antioxidant mechanisms. Because erythrocytes represent an important component of the antioxidant capacity of the blood, oxidative stress is also likely to occur when genetic abnormalities of the hemoglobin molecule affect globin stability or the heme crevice (16).

The production of oxidants and reactive oxygen and nitrogen species (ROS and RNS, respectively) is a sustained event in respiring cells. Oxygen is required for the generation of all ROS, RNS, and reactive chlorine species. The deleterious potential of oxygen is attributed to the formation, *in vivo*, of free radicals, which are defined as chemical species possessing one or several mismatched electrons and are, in general, very reactive (38). As molecular oxygen undergoes successive univalent reductions, a variety of reactive species are produced. The one electron-reduction product of oxygen is the superoxide radical $O_2^{\bullet -}$, whereas two-electrons transfer leads to the generation of hydrogen peroxide, H_2O_2, which is not a radical but it still remains a cytotoxic oxidant because of its eagerness for two more electrons. Certain chelates of ferrous iron and cuprous copper have the capacity to transfer a third electron to hydrogen peroxide, generating the hydroxyl free radical $HO^{\bullet}$, one of the most potent oxidants known. Nitric oxide (NO) and nitrogen dioxide are two nitrogen free radicals. Oxygen and nitrogen free radicals may be converted to other nonradical reactive species, like hydrogen peroxide, hypochlorous acid, and peroxynitrite ($ONOO^-$). NO reacts very rapidly with superoxide, producing peroxynitrite, which may be involved in many of the toxic effects attributed to NO. Nitric oxide is released in the vascular bed by endothelial cell NO synthase and by the inducible form of the enzyme present in activated inflammatory cells (73). Conversely, superoxide is produced by O_2 in biologic tissues from several sources. Currently identified contributors to free radical formation are mitochondria, enzyme cascades such as arachidonic acid or catecholamine catabolism, individual enzymes such as xanthine oxidase or cytochrome P450, and altered iron metabolism.

Under physiologic conditions, ~1–3% of the O_2 consumed is converted into superoxide and other ROS (100). Hemoglobin (Hb) is involved in a series of redox reactions (87). The ferrous heme of normal functional Hb continuously undergoes autoxidation, producing 0.5–3% methemoglobin [Fe(III)Hb] per day and a superoxide anion radical. This reaction is the source for RBC oxidative stress, because the superoxide and secondary ROS arising from it might lead to cellular damage. The steady-state level of Fe(III) Hb reflects erythrocyte oxidative stress. Hydrogen peroxide generated from superoxide through the reaction of superoxide dismutase in the RBC can react with both Fe(II)Hb and Fe(III)Hb and produce Fe(IV)ferrylHb and oxyferrylhemoglobin, respectively. FerrylHb and oxoferrylHb are potent oxidants capable of oxidatively damaging most biologic substrates and eventually are reduced to metHb. These higher oxidation states (ferryl) of Hb seem to play a significant role in the mechanisms of pathology of various disease conditions, such as those after ischemia/reperfusion injuries or myolytic or hemolytic events (3, 83).

Moreover, globin (peroxyl) radicals, also generated by the reaction of H_2O_2 with Fe(III)Hb, are highly reactive and have the potential to initiate lipid peroxidation reactions directly. These Hb protein radicals form intramolecular cross-links between the heme and amino acids, resulting in irreversible damage to the heme and release of iron (15). Free iron has the capacity to promote oxidative damage *via* classic Fenton chemistry.

During life, there is the potential risk of oxidative stress induced by high rates of oxygen use (intense physical effort), the autoimmune activation of immune system cells (respiratory burst of polymorphonuclear and mononuclear cells), and environmental factors. Several chemical agents can cause oxidative damage to hemoglobin and other cellular components. In some cases, the chemical itself acts as an oxidizing agent, but more frequently, it interacts with oxygen to form free radicals or peroxides. Derivatives of aromatic organic compounds are often involved. An increase in calorie intake also enhances mitochondrial free radical production.

Finally, ROS production can be grossly amplified in response to a variety of other pathophysiologic conditions such as inflammation, hypoxia, hyperoxia, metabolism of drugs, exposure to UV or therapeutic radiation, and deficiency in antioxidant vitamins.

Although oxygen free radicals exert destructive cytotoxic effect on mammalian cells at pathologic levels, they are also involved in neutralization of pathogens by activated macrophages. Moreover, they have been implicated in the activation of a variety of kinases (44, 56) and transcriptional factors (90), resulting in their role as signaling and regulatory molecules (89) in physiologic concentrations. In mammals, many signaling pathways, by being redox sensitive, could be influenced by ROS (24). For instance, the Keap1-Nrf2 system, a basic leucine zipper transcription factor, senses electrophiles and controls the expression of phase II detoxifying enzymes through the antioxidant/electrophile response element (ARE) (26). Hemo-oxygenase 1 gene expression, strongly affected by increased oxidative stress, has also been demonstrated to ameliorate inflammation and mediate potent resistance to oxidative injury (114). NO also affects signal transduction and cellular function in many cases (50). Thus, although oxygen is crucial for aerobic metabolism, it may also become extremely toxic in certain cases.

ERYTHROCYTE HOMEOSTASIS AND REDOX IMBALANCE

All aerobic organisms, including human beings, use a series of primary antioxidant defenses in an attempt to protect against oxidant damage. RBCs play a major role in the antioxidant capacity of the whole body. The strong interrelation between the antioxidant properties of erythrocytes and their metabolism is a crucial factor for their survival. The ability of RBCs to persist in the circulation depends mainly on redox regulation and its ability to maintain hemoglobin in a soluble and nonoxidized state. Erythrocyte enzymes support two important metabolic pathways. Glucose is metabolized anaerobically through the Embden–Meyerhof pathway (glycolysis),

which is the sole source of usable adenosine triphosphate (ATP) in the cell and in parallel generates reduced NADH, a molecule necessary for driving the reduction of methemoglobin to hemoglobin. The anaerobic metabolic pathway produces as one of its intermediates, glucose-6-phosphate, the substrate for glucose-6-phosphate dehydrogenase (G6PD). This enzyme is considered the rate-limiting factor for a linked pathway, which is termed the oxidative hexose monophosphate shunt (pentose shunt), and culminating in the reduction of oxidized glutathione to reduced glutathione (GSH). GSH plays a central role in the overall maintenance of the cellular redox state.

A very important product of the pentose phosphate pathway is reduced NADPH, which serves as a cofactor in the reduction of the oxidized form of glutathione (GSSG). Under physiologic conditions, ~90% of glucose is consumed in the glycolytic pathway, and 10% is used in the pentose shunt. In case of oxidant stress, the contribution of the pentose shunt may be significantly increased. The utilization of NADPH is the main stimulus for this increase. Oxyhemoglobin, as has already been mentioned, undergoes spontaneous autoxidation at a slow rate by the escape of an electron from heme to released oxygen. Methemoglobin cannot reversibly bind oxygen. The formation of methemoglobin may also result from a direct reaction of reduced hemoglobin with endogenous compounds and free radicals (*i.e.*, H_2O_2, O_2^-, NO, $HO^\bullet$). Exogenous agents can oxidize hemoglobin directly, by acting itself as an oxidizing agent, but in most cases by generating H_2O_2 and O_2^- during their metabolism.

The most important pathway in methemoglobin reduction is NADH–cytochrome b_5 reductase for the transfer of an electron from NADH to heme. The NADPH-dependent methemoglobin reductase uses NADPH as a source of electrons to reduce redox dyes like methylene blue and flavin, which, in turn, reduce methemoglobin. Because this system depends on an exogenous electron acceptor, it is not significant under normal conditions. Another mechanism for methemoglobin reduction involves direct transfer of electrons from ascorbic acid, reduced glutathione, and reduced flavin, but occurs slowly and plays a minor role. Hemoglobin is the most abundant and the most prominent target of oxidative assault with naturally occurring oxidants.

Overproduction of ROS and RNS or a defect in critical enzymes needed for maintenance of redox status renders erythrocytes vulnerable to oxidative damage. Under these conditions, ROS escape the antioxidant systems, oxidative denaturation continues, and methemoglobin is converted to derivatives, known as hemichromes (46). They are variably denatured Hb intermediates in which the distal histidine unit binds to the oxidized heme. Hemichromes interact with superoxide and hydrogen peroxide to generate hydroxyl radicals, resulting in an increased risk of potential damage to the cell. Once the cell's supply of GSH is insufficient, alterations in globin conformation occur, and normally protected sulfhydryl groups become exposed and are oxidized (46), leading to disruption of the $\alpha_1\beta_2$ contacts. These changes facilitate dissociation of polyptide chains, first into $\alpha\beta$ dimers and finally into monomers. Heme may dissociate from globin, especially in the case of unstable hemoglobins. The end products of these changes are precipitated hemichromes and heme-free globin, which take the form of globular inclusions known as Heinz bodies. In the RBC, only nonenzymatic heme degradation occurs, when the heme iron undergoes redox reactions in the presence of ROS (75).

The erythrocyte does not have a system to remove heme degradation products, which may accumulate on the cell membrane. Similarly, non–heme-bound iron found in sickle erythrocytes and thalassemia can originate from heme degradation. Thus, free iron, hemichromes and Heinz bodies can destroy membrane function directly or by causing oxidation of membrane proteins and lipids (46). Lipid peroxidation might take place, membrane proteins are cross-linked, and adducts between spectrin and denatured globin form (17). Particularly, the formation of membrane-bound, covalently cross-linked spectrin and α-globin chains of Hb seems to occur because of oxidation of Hb (5).

The resultant polymerization of spectrin leads to decreased cellular deformability and increased adherence to and phagocytosis by monocytes. An immune-type mechanism has been proposed to contribute to oxidative damage during normal and pathologic RBC senescence. The cytoplasmic domain of cytoskeletal band 3, a transmembrane anion-transport protein, provides a high-affinity site for hemichrome binding to the membrane in erythrocytes exposed to oxidants (98). This interaction leads to the formation of clusters of band 3 molecules in the membrane, which are recognized by an endogenous isoantibody possessed by all people. This does not occur under normal circumstances, in which band 3 molecules form monomers, dimers, or tetramers. However, the physiologic importance of this mechanism remains controversial.

Other studies suggested that thalassemic and sickle cells bind increased amounts of autologous immunoglobulin (Ig) with α-antigalacytosyl specificity (37), whereas in case of senescent erythrocytes, the autologous anti–protein 3 antibody is predominant (62). Other hypothetical signals from several structural molecules have been involved in marking RBCs for phagocytic removal after oxidation stress, such as lipids in the form of externalized phosphatidylserine (PS) or loss of asymmetrical phospholipids distribution (59). An interesting hypothesis suggested that disruption of normal spectrin tetramer assembly due to hemoglobin oxidation leads to surface decreased deformability, transiently elevated intracellular calcium levels, and finally to PS externalization, which are the signal of macrophage recognition via the CD36 receptor (54). The potential calcium-dependent mechanisms for PS externalization might be activation of the scramblase (12), promotion of the quasi-lipoxygenase activity of the oxidized Hb (52), or destabilization of Hb, allowing direct oxidation of the lipid by free iron (85). Further, recent studies reported that in anucleated cells, the process of PS externalization under oxidative stress is regulated by the activation of caspase 3, which is associated with impairment of aminophospholipid flipase activity (64), whereas peroxynitrite, a milestone of redox-mediated damage in human pathology, induces apoptosis of erythrocytes through the activation of aspartyl and cysteinyl proteases (66). The whole phenomenon continues to be an active topic of investigation.

Alteration of erythrocyte energy metabolism after oxidative stress is another important aspect of RBC homeostasis. Thus, adenosine monophosphate (AMP)-deaminase was found to be

dramatically activated by oxidative stress, resulting in a progressive ATP depletion associated with the deamination of AMP to IMP (impedes mitogenic signal propagation) (108). As a consequence, the energy state of erythrocytes was profoundly imbalanced, as IMP cannot be used by erythrocytes for adenine nucleotide resynthesis (10). Moreover, evidence in the literature suggests that ROS might affect the activity of several other enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (18), glucose-6-phosphate dehydrogenase (18), pyruvate kinase (78), or hexokinase (102), and consequently the RBC energy state. Finally, many *in vitro* and *in vivo* studies indicated that variable parameters of erythrocyte function and integrity are negatively affected by increased oxidative stress. So, besides formation of Hb-spectrin adducts and increased lipid peroxidation, decrease of nonenzymatic antioxidants (109), oxidation of SH groups (99), changes of erythrocyte membrane ionic permeability (106), and activation of proteolysis (23), have all been described after exposure of erythrocytes to different ROS-generating systems.

REDOX AND RBC PRODUCTION

Conversely, ROS are considered part of the signaling chain of the cellular O_2-sensing mechanism regulating erythrocyte production. The glycoprotein hormone erythropoietin (Epo) is the principal regulator of the proliferation and differentiation of erythroid progenitors in bone marrow. The upregulation of Epo gene transcription by hypoxia is mediated by a transcriptional factor, termed hypoxia-inducible factor (HIF-1), which functions as a global regulator of O_2 homeostasis (92). Its activation involves redox-dependent stabilization of its α-subunit, allowing it to form a heterodimer with HIF-1β (Fig. 1). Thus, although at the mRNA level, both HIF-1α and HIF-1β are constitutively expressed, at the protein levels, HIF-1α is found only in hypoxic cells, whereas HIF-1β is constitutively expressed (49). HIF-1 participates in the formation of a large protein complex that binds specifically to a 3′ enhancer of the gene encoding Epo and to promoters/enhancers in other genes important to hypoxia, such as those encoding glycolytic enzymes (34) and glucose transporters (28).

Several mechanisms responsible for HIF activation that include signal transduction via the generation of ROS, either by an NAD(P)H oxidase or the mitochondrial electron transport chain, have been suggested (93). Small diffusible molecules such as NO, H_2O_2, $O_2^{-\bullet}$, and peroxynitrite may also function as messengers between RBCs and sites of HIF-1 regulation (other than O_2) (13), whereas it is likely that extracellular Hb influences HIF-1 expression during conditions of hypoxia through redox mechanisms (116). The generation of ROS in response to hematopoietic growth factors (HGFs) has been reported to contribute to downstream signaling events, resulting in regulation of hematopoiesis (89).

IMPAIRED ERYTHROCYTIC ANTIOXIDANT DEFENSE

Sickle cell anemia, thalassemia, glucose-6-phosphate-dehydrogenase deficiency, and unstable hemoglobinopathies

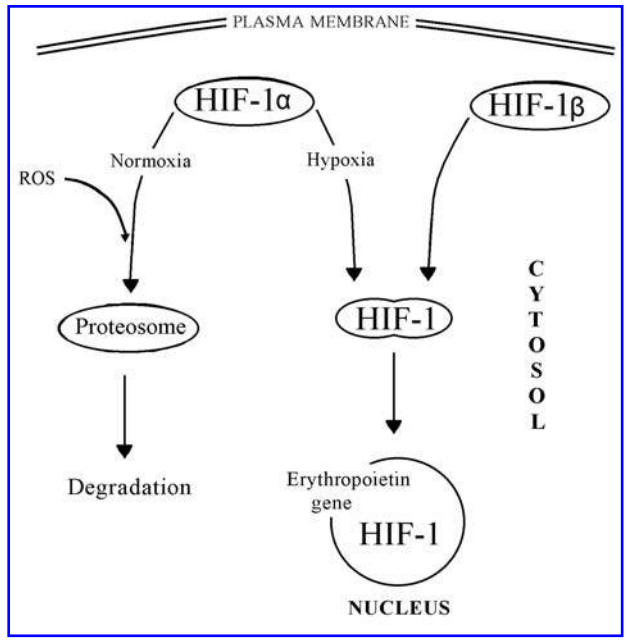

FIG. 1. Pathway leading to the hypoxic induction of the erythropoietin gene, through stabilization of HIF-1α protein and formation of the HIF-1 heterodimer.

are all hereditary disorders with a higher potential for oxidative damage due to chronic redox imbalance in RBCs that often results in mild to severe hemolysis. This imbalance arises from several intrinsic factors, including depletion of enzymes, internal pathologic processes such as denaturation of Hb, the presence of excess unpaired globin chains, the high intracellular content of nonhemoglobin iron, or the low concentration of normal Hb.

Specifically, sickle hemoglobin has been shown to undergo accelerated autoxidation (94), which results in formation of superoxide and met-Hb. Some denatured Hb loses its heme, particularly to the lipid bilayer, where it is easily destroyed to liberate "free" iron. Membrane-associated free iron can form a redox couple with soluble oxyhemoglobin to promote further Hb oxidation (11). Membrane-iron acts as a catalyst and can use cytosolic reducing substances (*e.g.*, ascorbate, superoxide) to redox cycle and generate highly reactive oxidants such as hydroxyl radicals. These mechanisms may account for the spontaneous generation of excessive oxidants by sickle RBCs (47). In sickle RBCs, the oxidation of membrane protein thiols and peroxidation of membrane lipids might be attributed to the coincident membrane location of catalytic iron (11). Moreover, some indications suggest that free iron is nonrandomly associated with the membranes of sickle and thalassemic RBCs (84).

Hbs exhibiting reduced solubility or higher susceptibility to oxidation of amino acid residues within the individual globin chains are called unstable Hbs. The cornerstone in the pathogenesis appears to be derangement of the normal linkages between heme and globin. Destabilization of the heme–globin linkage releases heme from its cleft, leading to the formation of precipitated hemichromes, which cause further denaturation and aggregation of the globin subunits. Heinz bodies are the end product in this process. Free heme

in the erythrocytes may enhance the generation of reactive oxidants (*i.e*., hydrogen peroxide, superoxide, hydroxyl radicals). This results in oxidant damage of the lipids and proteins of the membrane, with consequent premature destruction of the erythrocyte (35). Occasionally, hemolysis may become clinically apparent only in the presence of additional oxidant stress, such as infection, fever, or the ingestion of oxidant agents.

Thalassemia, the most common worldwide genetic disorder, is one more case of enhanced destruction of RBCs due to increased oxidative stress. In β-thalassemia, the relative excess of α-chains in RBC precursors leads to the formation of inclusion bodies consisting of precipitated α-chains (32). Excess α-hemoglobin chains autoxidize, release heme, and generate superoxide at an increased rate compared with that of normal Hb, resulting in generation of membrane-bound hemichromes (91). Their association with the cytoplasmic domain of protein band 3 produces a neoantigen that mediates the immune removal of the cell by macrophages (65). Moreover, protein 4.1 undergoes partial oxidation and becomes unable to participate in the formation of the spectrin–protein 4.1–actin complex, which is crucial for cytoskeleton stability (2). Besides oxidation, free α-chains are degraded to form denatured α-globin protein, heme, and free iron. Free iron, through the Fenton reaction, generates ROS, which cause lipid and protein peroxidation (43), whereas heme and its oxidized form hemin also produce oxidative damage to RBC membranes (82). All these processes contribute to structural and functional alterations, with resultant changes in deformability, stability, and rate of apoptosis (117). *In vivo*, this loss of cellular deformability might account for the impaired extrusion of reticulocytes from the marrow and shortened survival of the peripheral erythrocytes.

Similarly, in α-thalassemia, membrane skeletal-bound β-globins become partially oxidized with consequent membrane damage (65). Unlike α-chains, excess γ- and β-chains have the capacity to form partially soluble γ_4 tetramers (Hb Bart) and β_4 tetramers (HbH). Moreover, in α-thalassemia, the structure and function of protein 4.1 are normal. This might arise from the fact that excess α-globin chains in β-thalassemia are more unstable and dissociate much faster into monomers, with the generation of more free oxygen radicals than the relatively more stable excess of β-globin chains (95). Generally, the timing and pattern of their precipitation seems to be different in α- and β-thalassemia, which might explain some of the differences in the clinical expression of these two entities.

G6PD deficiency is by far the most common enzymopathy worldwide and is caused mainly by diverse point mutations in the G6PD gene. G6PD is a key enzyme for the pentose pathway, which is essential for an adequate supply of NADPH. It is of great importance for protection against oxidant damage for the erythrocyte to maintain a high ratio of NADPH to NADP for reduction of glutathione. Particularly, oxidant damage leads to the oxidation of free –SH groups of Hb, generating disulfide bridges that, in turn, decrease Hb solubility and produce Heinz bodies. The high ratio of reduced-to-oxidized glutathione represents the major defense against the oxidative damage of Hb. The RBC has limited capacity for reducing power, and because it is devoid of intracellular organelles, it depends solely on the pentose pathway for the generation of NADPH needed for glutathione reduction. That is why the production of NADPH in G6PD-deficient erythrocytes is highly compromised, and increased oxidative stress in these cells is well documented (79). Individuals deficient in RBC G6PD or other components of glutathione-dependent detoxification processes are particularly sensitive to the hemolytic effects of oxidant compounds. A continuing depletion of antioxidant nutrients (vitamin E, vitamin C, glutathione) in response to chronic oxidative stress in genetic anemias has been reported, but its role as a trigger to the periodic hemolysis is unclear (16).

Finally, despite the high antioxidant power of human erythrocytes, they may be exposed to a higher risk of increased oxidative injury owing to their very high ferrous iron concentrations, because iron plays a key role in producing harmful oxygen species. Its redox cycling promotes the Fenton reaction, by which the potent oxidant hydroxyl radical is generated (45). Normally iron exists in association with macromolecular complexes, which prevent its reactivity with reduced oxygen metabolites. When erythrocytes are exposed to oxidative stress, iron is released from hemoglobin and its derivatives, resulting in methemoglobin formation. If erythrocyte glutathione is depleted, the nonprotein bound form of iron causes lipid peroxidation and hemolysis (31). Iron has also been implicated in a metal-catalyzed oxidation of membrane proteins, which underlies erythrocyte aging (20). In particular, it is considered that free iron binds to a divalent binding site on the protein, where generating ROS will oxidize adjacent amino acid residues. Other authors report that protein oxidative cross-linking may lead to the formation of senescent cell antigen (SCA) through the clustering of band 3, to which autologous IgGs bind (111). Additional studies showed that iron released from Hb is able to activate oxidative reactions at a distance (30). Iron decompartmentalization can be seen in thalassemic and sickle RBCs, where iron might be associated with the cytoplasmic surface of the membrane in which several discrete iron compartments (denatured Hb, free heme, molecular iron, etc.) can be demonstrated (11). Thus, an increase in extracellular or intracellular iron concentrations, which can result from dietary protein deficiency, dietary iron loading, low levels of iron-binding proteins, or cell injury promotes oxidative stress (21).

ANTIOXIDANT PROPERTIES OF ERYTHROCYTES

In the bloodstream, RBCs encounter a variety of oxidant stressors that can be both endogenous, from cellular generation of superoxide and hydrogen peroxide, and exogenous, in areas of inflammation. Erythrocytes have an important role in the reduction of extracellular oxidants because of their capacity to scavenge exogenous ROS, the permeability of their membranes to oxygen radicals, and their high intracellular antioxidant enzyme activities (86). RBCs also allow redox buffering of both the intra- and extracellular environments through transmembrane electron transport (6). Furthermore, most of the nonenzymic antioxidant capacity of whole blood is localized in erythrocytes.

Erythrocytes possess redundant and overlapping mechanisms for protection against oxygen free radicals, including

enzymes like catalase, superoxide dismutase (SOD), GSH peroxidases, glutathione reductase, and low-molecular-weight antioxidants either produced intracellularly (GSH, NADH/NADPH) or taken up by cells (α-tocopherol, ascorbate, bioflavinoids, selenium).

Enzymic activity is a two-step process. In the first step, SOD catalyzes the formation of O_2 from O_2^-. All members of the SOD family use transition metals at their active sites, and the erythrocytic form uses a Cu-Zn–containing SOD. A co-product of SOD is H_2O_2, which is clearly toxic and must be rapidly removed. This is accomplished in a second step, through its reduction to H_2O by catalase and the selenium-dependent GSH peroxidase. Both enzymes detoxify H_2O_2 by reducing it to water and oxygen. GSH peroxidase uses the reducing power of GSH, a tripeptide consisting of L-γ-glutamyl-L-cysteinylglycine, to neutralize hydrogen peroxide. The sulfhydryl moiety of the cysteine residue provides the actual reducing equivalents required for glutathione peroxidase activity. GSSG reductase uses reducing equivalents from NADPH to reconvert GSSG to GSH (Fig. 2). Catalase and GSH peroxidase also eliminate organic peroxides. In higher organisms, glutathione peroxidases seem to have largely supplanted the need for catalase. It appears probable that glutathione peroxidases largely deal with cytoplasmic H_2O_2, and catalases, with peroxisomal H_2O_2 (22).

Conversely, a significant portion of the cellular redox state is governed by key endogenous antioxidants. Once formed from *de novo* synthesis, they are maintained mainly by their corresponding recycling pathways. Because the predominant repairing pathways are enzymic, they need specific cofactors; an adequate supply of these is therefore necessary to restore the cellular redox state.

GSH is considered the major component of the cellular antioxidant system, because it provides the reducing capacity for several reactions: the detoxification of hydrogen peroxide, other peroxides, and free radicals; the formation and maintenance of protein sulfhydryl groups; the regeneration of antioxidants vitamins; and the detoxification of a variety of xenobiotics (80). Glutathione not only protects cell membranes from oxidative damage, but also contributes to the normal function of many proteins through maintenance of their sulphydryl groups in the reduced form. Glutathione radical (GS•) generated from the oxidation of GSH can react with another GS• to produce GS-SG. The antioxidant function

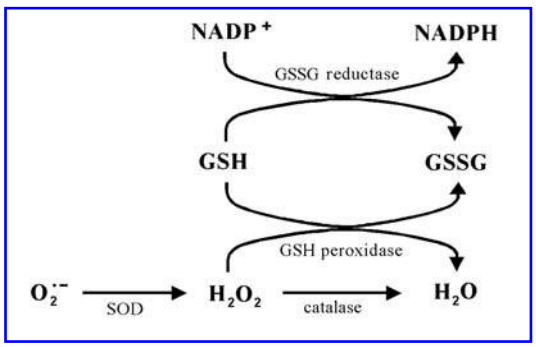

FIG. 2. Mechanisms of ROS detoxification. GSH, glutathione; GSSG, oxidized form of glutathione.

of GSH is directly related to its role as a component of the enzymatic pathway that cells developed against ROS, consisting of GSH peroxidase and GSSG reductase. The role of these enzymes has already been mentioned. Moreover, GSSG reductase contains flavin adenine dinucleotide and uses reducing equivalents from NADPH to regenerate GSH. Thus, adequate intake of riboflavin and glucose metabolism via the pentose cycle play an essential role in providing NADPH, necessary for keeping GSH levels within normal rates. Of glutathione intracellular concentration, 95% is in the reduced sulfhydryl form. The availability of GSH in situations of oxidative stress is ensured by GSH recycling and biosynthetic pathways (42), which can be upregulated in situations of oxidative insult (58). GSH provides a first line of defense against ROS, as it can scavenge free radicals and reduce H_2O_2, whereas GSH-dependent enzymes provide a second line of defense through detoxification of noxious by-products and prevention of the propagation of free radicals. Accumulating evidence suggests that GSH provides a means of regulating protein function by a mechanism called glutathionylation, in which the protein thiol groups are reversibly bound to glutathione. Protein *S*-glutathionylation has been proposed as a mechanism of redox-mediated and NO-mediated signal transduction as well as an adaptive cellular response protecting critical regulatory molecules from permanent loss of function as a consequence of oxidative insult (57).

Defenses against oxidant damage in the plasma membrane are associated with prevention and reversal of peroxidation of polyunsaturated fatty acids (PUFA) in the lipid bilayer. Predominantly α-tocopherol (vitamin E) prevents the peroxidation of PUFA by breaking the radical chain reaction through transfer of its phenolic hydrogen to a peroxyl free radical of the peroxidized PUFA (19). Ubiquinol (107), membrane protein sulfhydryls (99), and a GSH-dependent phospholipid hydroperoxidase (97) may also contribute to the protection of plasma membrane. Further, ascorbic acid within the erythrocyte protects α-tocopherol in the cell membrane by a direct recycling mechanism (68).

Vitamins directly scavenge ROS and upregulate the activity of antioxidant enzymes. In particular, vitamin E has been recognized as the most important antioxidant in the cell membrane. By scavenging carbon-based peroxide free radicals, α-tocopherol protects against lipid peroxidation and thus decreases hemolysis (19). A dietary deficiency of vitamin E reduces the activity of GSH peroxidases and glutathione reductase. Several mechanisms have been involved in replacement or regeneration of partially oxidized α-tocopherol in the membrane of erythrocytes, such as transfer from lipoproteins to erythrocytes (7) and regeneration *in situ* in the membrane from ubiquinols (104), a phospholipid hydroperoxide glutathione peroxidase (63), or ascorbic acid (68). Vitamin C exhibits a protective effect against free radical–induced oxidative damage (72). It is an important antioxidant in plasma and in blood. The two-electron oxidation product of ascorbate (DHA) is rapidly taken up by erythrocytes through glucose transporters (112) and rapidly reduced to ascorbate. GSH is the primary electron donor for DHA reduction (71) and is dependent on D-glucose metabolism in the pentose cycle (69). In the absence of D-glucose, NADH-dependent mechanisms also appear to support DHA reduction (69). Moreover, the

NADPH-dependent thioredoxin reductase system can also contribute to DHA reduction (71). More recent evidence suggests that ascorbate recycling depends on reduction of both the ascorbate free radical (AFR) and DHA (67). In particular, AFR reduction (by both NADH- and NADPH-dependent reductases) predominates when oxidant stress is not severe, whereas the rate of GSH-dependent DHA reduction was greater under conditions of more severe oxidant stress.

Levels of pyridine nucleotides, which are major electrons carriers, are determined by adequate niacin intake. By serving as components of $NADP^+$/NADPH, NAD^+/NADH, and $FAD^+/FADH_2$, nicotinamide and riboflavin play an important role against oxidative stress. For instance, as a cofactor for transketolase of the pentose cycle, thiamine is crucial for NADPH generation, whereas NADPH and FAD are cofactors for glutathione reductase, which mediates the regeneration of GSH from GS-GS.

Moreover, because of the crucial role of glucose metabolism via the pentose cycle in providing NADPH, cellular redox cycling is tightly associated with the energy status. Thus, during prolonged periods of energy deficit or deficiencies of niacin or riboflavin, the antioxidant capacity will be reduced.

Although in some cases, flavonoids have been reported to function as prooxidants (14), they generally are considered to protect biologic membranes against oxidation by scavenging ROS (55), inhibiting free-radical–induced membrane lipid oxidation (48) and oxidation of low-density lipoproteins (25), thus exerting beneficial effects under oxidative stress conditions.

Paradoxical observations with regard to certain prooxidant effects of antioxidant compounds (vitamins C, E, and GSH and glucose) have been reported under certain conditions. Conditions necessary to demonstrate the prooxidant effects of reducing agents include the induction of oxidative stress by the presence of either a free radical initiator or the involvement of Fenton metal ions and the severe depletion or absence of one of the key antioxidants. For instance, cellular vitamin E level appears to influence GSH content directly in cells, and under conditions of low or absent vitamin E, GSH and vitamin C can become prooxidants (115).

Besides their ability to use intracellular reducing potential to protect the cell membrane from oxidant stress, erythrocytes have the capacity to reduce extracellular oxidants via the export of electrons across the cell membrane (53). Current evidence suggests that the human erythrocyte membrane contains a number of distinct electron-transfer systems, some of which, at least, involve membrane proteins and NADH or ascorbate as electrons donors. Intracellular flavonoids have also been reported as electrons donors for extacellular ferricyanide reduction in human erythrocytes (33). A superoxide anion channel allows the transport of superoxide and other free radicals into the RBC, where they are deactivated by the erythrocyte antioxidants (86). Finally, erythrocytes are also significant detoxifiers of $-OONO$, generated by the reaction of NO with O_2^- or H_2O_2, due to the rapid membrane crossing of $-OONO$ and high rate constant of its reaction with hemoglobin (105). Much of the NO released into the bloodstream should also be scavenged by Hb in erythrocytes (60) or converted to nitrite in the presence of molecular oxygen (51). Nitrite enters rapidly erythrocytes and reacts with oxyhemoglobin to generate nitrate and methemoglobin, but does not exert a strong oxidant stress (70). Three principal routes of NO interactions with RBCs have been disclosed: NO reacts rapidly with oxy-Hb to form nitrate and methemoglobin. Although it is considered the major pathway for NO elimination in the body (81), other evidence suggests that this assumption may not be valid under all conditions (61). Alternatively, NO may bind to the heme group of deoxy-Hb to form nitrosylhemoglobin (NO-Hb) (27). A third possibility is the reaction of NO, or a higher-oxidation product such as NO_2 or N_2O_3, with β-chain cysteine 93 residue of oxyhemoglobin, leading to formation of *S*-nitrosohemoglobin (SNO-Hb) (41). According to this theory, the conformational transition from the R- to the T-state could promote the allosteric delivery of both oxygen and NO to regions with low oxygen tension. However, other studies report that NO-heme reaction pathways predominate *in vivo*, NO binding to heme groups is a rapidly reversible process, and that *S*-nitrosohemoglobin formation is probably a "salvage" pathway facilitating NO delivery only in regions with significant stress, where hemoglobin-NO scavenging would be deleterious (39). In spite of low levels of NO-Hb and SNO-Hb found *in vivo*, recent findings do not rule out participation of NO-Hb or SNO-Hb in NO-dependent signaling reactions (9).

The fact that erythrocytes are expendable, permeate the entire body via capillary distribution, possess reducing equivalents far in excess of normal requirements, and channels allow the transport of ROS, make them function as an effective oxidative sink in the organism.

MACROCYTOSIS AND REDOX IMBALANCE

The relation between macrocytosis, redox imbalance and RBC destruction has not been clearly established. A review of hematologic data for several types of unstable hemoglobins has reported that most of these subjects with moderate to severe anemia tend to have a macrocytic anemia and decreased mean cell hemoglobin concentration (MCHC) values (118). The elevated mean cell volume (MCV) could not be attributed to reticulocytosis because the degree of macrocytosis appeared to be greater than one would expect in relation with an increased number of circulating reticulocytes. The authors suggested that the combination of macrocytosis and a low MCHC observed in patients with the more severe forms of unstable hemoglobinopathies, in association with the occurrence of cell swelling and lowering of MCHC during thermal denaturation of Hb Zurich *in vitro*, indicates pathophysiologic events underlying cell death.

Conversely, other authors have reported an increase in erythrocytes with high mean corpuscular volumes accompanied by an elevation in HbF expression, in cases of acute erythropoietic response (4, 101, 110). In this case, a sustained or repeated acute erythropoietic stress (*i.e.*, severe hemolysis due to oxidative damage) results in recruitment of erythroid progenitors from the more primitive burst-forming unit (BFUe) pool, instead of the relatively differentiated erythroid

precursors, namely CFUe, which have been depleted. Under these circumstances, acute demands for erythropoiesis are met through shortened mitotic intervals, fewer mitotic divisions, or differentiation without divisions, leading, in turn, to generation of macrocytes.

According to another hypothesis, the number of cell divisions and the ultimate RBC size may be related to intracellular Hb concentration and consequently are dependent on the rate of Hb synthesis. Thus, when an Epo-induced acceleration of Hb synthesis occurs, an earlier onset of nuclear degeneration and a reduced number of cell divisions are found (103). The fact that reticulocytosis is unlikely to increase MCV, except for the first few days after an acute erythropoietic stimulus (110), and that similar hematologic features appear, regardless of the cause of acute erythropoietic stress (hypoxia or anemia), makes it more possible that acute erythropoiesis accounts for the macrocytosis, which may be observed after a severe hemolysis due to redox imbalance. These macrocytes with favorable geometry can persist in the circulation for extended periods without remodeling or reduction in size (76). Conversely, oxidative stress itself has been implicated in the oxygen-dependent activation of the K-Cl cotransporter (KCC) and the Gardos channel, leading to excessive KCl loss and RBC shrinkage (74). The action of various reagents on KCC activity has been correlated with their redox potential. Low reduced glutathione levels in the erythrocyte are considered to be associated with a high activity of the cotransporter. For instance, increasing concentrations of oxidant nitrite decrease GSH, induce MetHb formation, and stimulate K-Cl COT (1). However, it seems that not the absolute GSH levels, but the redox state of the cell determines K-Cl COT activity (36). It is also possible that ROS directly influence the transporter. Thus, H_2O_2 appears to stimulate the phosphatase involved in K-Cl COT activation (8). KCC activity is also inappropriately elevated in certain hemoglobinopathies like HbS and β-thalassemia and in certain enzyme deficiencies (77). All these findings are consistent with the initial hypothesis we put forward to explain the relation between macrocytosis and redox imbalance (*i.e*., that the acute erythropoietic stress accompanying severe hemolysis due to increased oxidative stress might account for the macrocytosis observed in such conditions).

However, several other mechanisms might also contribute to this phenomenon. Hypoxia, a potential cause of oxidative stress, promotes a rapid increase in RBC volume in several species because of a large net uptake of Na^+ and Cl^- through cAMP-dependent Na^+-H^+ exchange (88). Selenium deficiency, implicated in decreased glutathione peroxidase activity and removal of free radicals, and inherited catalase deficiency, leading to oxidative stress with hydrogen peroxide and oxidation of folate, have been reported to be associated with macrocytosis (40, 113), although these cases are not frequently met.

CONCLUSIONS

Imbalance between ROS production and antioxidant cell defenses has been reported to contribute to several pathophysiologic conditions. RBCs are considered important regulators of oxidant reactions in their surroundings. They not only are excellently equipped to handle intracellular oxidative stress through the combined activities of the hexose monophosphate shunt and antioxidant enzymes, but also account for much of the antioxidant capacity of the blood. The low-molecular-weight antioxidants of their membrane, the cell proteins, mobility, membrane permeability, and transmembrane electron transport systems enable erythrocytes to protect against oxidant-mediated cytotoxicity. The antioxidant function is much more complex than simple free radical scavenging. The interactions between oxidative stress and the main parameters representative of erythrocyte homeostasis are highlighted by this review. Such knowledge is important to understand fully the functions of the various components of the antioxidant system, to develop strategies to strengthen the endogenous free radical defenses, and finally to prevent oxidative stress–mediated pathogenesis.

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ABBREVIATIONS

AFR, Ascorbate free radical; ARE, antioxidant/electrophile response element; ATP, adenosine triphosphate; BFUe, burst-forming unit–erythroid; CFUe, colony-forming unit–erythroid; Epo, erythropoietin; GSH, glutathione; GSSG, oxidized form of glutathione; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; HIF-1, hypoxia-inducible factor; H_2O_2, hydrogen peroxide; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; NO, nitric oxide; NO-Hb, nitrosohemoglobin; $ONOO^-$, peroxynitrite; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; RBC, red blood cell; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCA, senescent cell antigen; SNO-Hb, *S*-nitrosohemoglobin; SOD, superoxide dismutase.

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