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# Erythrocyte: Programmed Cell Death

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## 1. Introduction

Erythrocytes are produced by a complex and finely regulated process of erythropoiesis. It starts with a pluripotential stem cell that, in addition of its self replication capacity, can give rise to separate cell lineages. Erythropoiesis passes from the stem cell through the multipotent progenitor CFU-GEMM (colony-forming unit granulocyte erythroid monocyte and megakaryocyte), and then BFU-E (burst-forming unit erythroid) and CFU-E (colony-forming unit eryhtroid), to the first recognizable erythrocyte precursor in the bone marrow, the pronormoblast. This cell gives rise to a series of progressively smaller normoblasts with increasing content of hemoglobin. The nucleus is finally extruded from the late normoblast leading to mature red blood cell through the reticulocyte stage. Erythropoiesis ends with the mature circulating red cell, which is a non-nucleated biconcave disc, performing its function of oxygen delivery. In this process, the glycoprotein hormone erythropoietin has been known as the major humoral regulator of red cell production. It is now well established that erythropoietin stimulates erythropoiesis, at least in part, by protecting erythroblasts from apoptosis.

Human mature erythrocytes are terminally differentiated cells that are devoid of mitochondria, as well as of nucleus and other organelles. In circulation, the red cell is constantly tested for its capacity to undergo marked cellular deformation. This ability to change its shape is essential for optimal cell function, since the resting diameter of the human red cell far exceeds that of the capillaries and splenic endothelial slits through which it must pass (Mohandas & Groner, 1989). A two dimensional network of proteins interacting between transmembrane location and cytoplasmic surface of the plasma membrane gives the red blood cell its properties of elasticity and flexibility that allows the success of this journey.

The mature erythrocyte is unable to self-repair and has no capacity to synthesize proteins. Therefore, its lifespan is finite and is shortened further when the cell's environment becomes hostile or when the erythrocyte's ability to cope with damaging extracellular influences becomes impaired. The erythrocyte limited lifespan implies that, as in other cells, life and death are well regulated for erythrocytes, in spite of their lack of capacity for protein synthesis (Bosman et al., 2005).

In the present review, we aim to show updated information concerning erythrocyte death in order to contribute to the understanding of the physiopathological relationship of this process with the development of anemia.

## 2. Anemia

The term anemia is derived from ancient Greek for "bloodlessness". It is a condition involving abnormal reduction of hemoglobin content. In healthy adults, there is steady-state equilibrium between the rate of release of new red cells from the bone marrow into the circulation and the rate of removal of senescent red cells from the circulation by reticuloendothelial system. Balance disruption appears by decreased cell production, increased destruction or both, leading then to anemia. Different mechanisms which may lead to anemia are blood loss, decreased red cell lifespan, acquired or congenital defects, ineffective erythropoiesis, and impairment of red cell formation.
In this chapter we focus on anemia resulting from accelerated clearance of red blood cells from circulating blood before hemolysis.

## 3. Erythrocyte death

Apoptosis is a regulated process of self-destruction characterized by a series of changes affecting the nucleus, cytoplasm and plasma membrane of the cell, and leading to the rapid capture and ingestion of the dying cell by macrophages. Programmed death allows the elimination of cells without release of intracellular proteins which would otherwise cause inflammation.
It is well known that eukaryotic cells use a similar death program. Moreover, erythrocyte precursors, which are true organelle-containing cells, are susceptible to apoptosis induction. Instead, human mature erythrocytes have been considered as unable to undergo programmed cell death due to their lack of mitochondria, nucleus, and other organelles. Increasing evidence is now available to demonstrate that mature erythrocytes can undergo a rapid self-destruction process sharing several features with apoptosis, including cell shrinkage, plasma membrane microvesiculation, shape changes, cytoskeleton alterations associated with protein degradation, and loss of plasma membrane phospholipid asymmetry leading to the externalization of phosphatidylserine. As described, erythrocyte death is characterized by some features that are shared by apoptosis. To distinguish the death of erythrocytes from apoptosis of nucleated cells, some authors suggest the term “eryptosis” (Lang KS et al., 2005).
Erythrocyte lifespan is limited to approximately 120 days and is ended by a process of senescence during which aging erythrocytes suffer changes that display molecules that are recognized by macrophages leading to their clearance from peripheral blood by reticuloendothelial system (Bratosin et al., 1998). Programmed erythrocyte death prevents intravascular hemolysis and allows the elimination of cells without inflammation. Even though this is one of the processes that regulate effective erythropoiesis, a disturbance of the fine equilibrium between erythrocyte production and cell destruction may be caused by the presence of factors that create a harmful environment.
The knowledge of the mechanism of the erythrocyte death is of the highest importance since, apart from its association with anemia, it could lead to improvements of the storage conditions in blood banks by increasing the time of viability of stored red blood cells (Bratosin et al., 2002).

## 4. Mechanism of suicidal erythrocyte death

As mentioned above, mature erythrocytes can undergo a rapid self-destruction process leading to increased intracellular calcium content, modifications of the erythrocyte

morphology, metabolic disruption, membrane protein modifications, and externalization of phosphatidylserine, thereby activating a clearance mechanism involving heterophagic removal in the reticuloendothelial system.
Data describing cell changes and mechanism involved in erythrocyte premature death are stated below.

### 4.1 Intracellular calcium content

It is well established that two properties of red blood cells, deformability and elasticity, are dramatically affected by calcium ions. Thus, a rise in internal $Ca^{2+}$ leads to changes in cell shape and volume, increased cellular rigidity and hemolysis (Weed et al., 1969; Palek et al., 1974; Kirkpatrick et al., 1975). Such alterations seem to arise from $Ca^{2+}$ interactions with various molecular targets. These include both low-affinity associations with membrane phospholipids (Chandra et al., 1987) and high-affinity ones with specific membrane proteins, especially the Ca-dependent K channel (Romero, 1976) as well as with some cytoskeletal proteins (Wallis et al., 1993). It was observed that the presence of the bivalent-cation ionophore A23187 did not induce erythrocyte death in the absence of extracellular $Ca^{2+}$, nor in the presence of both $Ca^{2+}$ and the $Ca^{2+}$ chelator EDTA, thus characterizing erythrocyte death as an active process requiring $Ca^{2+}$ entry into the cells (Bratosin et al., 2001).
Since internal $Ca^{2+}$ is subjected to metabolic control via an ATP-dependent extrusion mechanism (Ca pump) (Schatzmann, 1983), it is expected that the decreased ATP content attained during red cell aging should lead to raised cellular $Ca^{2+}$. The homeostasis of $Ca^{2+}$ in these cells is carried out by the concerted action of just two mechanisms: the active extrusion already mentioned and the entry through defined $Ca^{2+}$ channels (Romero & Romero, 1999).
Different factors that may cause cellular stress, such as hyperosmotic shock, oxidative stress, or energy depletion, are capable of $Ca^{2+}$ channel activation in the erythrocyte, including the nonselective cation channel TRPC, with subsequent increased entry of $Ca^{2+}$ (Föller el al., 2008). It has been reported that free $Ca^{2+}$ concentration, cell-shrinkage, and phospholipid scrambling were significantly lower in $Cl^-$-depleted TRPC6 -/- erythrocytes than in wildtype mouse erythrocytes, which let the authors conclude that human and mouse erythrocytes express TRPC6 cation channels which participate in cation leak and $Ca^{2+}$-induced suicidal death (Föller el al., 2008).
The increase in erythrocyte cytosolic $Ca^{2+}$ concentration further stimulates $Ca^{2+}$-sensitive $K^+$ channel (Gardos channel). The subsequent efflux of $K^+$ hyperpolarises the cell membrane, which drives $Cl^-$ exit in parallel to $K^+$. The cellular loss of KCl with osmotically obliged water leads to cell shrinkage. It has been reported that cell shrinkage leads to formation of ceramide. This compound can also contribute to the triggering of cell membrane scrambling (Lang et al., 2004), one of the typical features of suicidal erythrocyte death.
Another important effect caused by a raise in intracellular $Ca^{2+}$ concerns the activation of different enzymes, including calpain. This endogenous protease primarily cleaves the Ca pump, then band 3 protein and finally some cytoskeletal proteins.

### 4.2 Enzyme activity

#### 4.2.1 Enzymes of the glycolytic pathways

Band 3, the anion-exchange protein, also binds various cytoskeletal proteins as well as hemoglobin and cytoplasmic glycolytic enzymes. It has been shown that mild oxidants, such as potassium ferricyanade, diamide, and hydrogen peroxide stimulate red blood cell glycolysis in proportion to the elevation of band 3 tyrosine phophorylation. Band 3

sequences surrounding tyrosine residues have been associated with intracellular binding of several cytosolic proteins, including hemoglobin and the glycolytic enzymes aldolase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase. *In vitro*, the tyrosine phosphorylation of band 3 prevented the binding of these glycolytic enzymes. Since these enzymes are inhibited in their bound state, the functional consequence of N-terminal band 3 tyrosine phosphorylation would be an enhanced rate of glycolysis in the intact cells (Harrison et al., 1991; Mallozi et al., 1995). This mechanism of erythrocyte metabolic regulation can stimulate or reduce energy production in times of special needs, such as during a free radical attack.

### 4.2.2 Enzymes involved in thiol metabolism and protection against oxidative damage

Activities of some cytoplasmic enzymes decline during erythrocyte aging or when they are induced to programmed cell death. Oxidative stress as well as antioxidant depletion cause decreased activity of erythrocyte catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX). However, the enzyme behavior seems to depend on the biological model as well as the oxidant agent, since there are some reports showing activation of CAT, SOD, and GPX which has been associated with the metabolic response to cell injure.

### 4.2.3 Proteases

Members of the caspase family contain a cystein residue in their active center and exist as zymogens that need to be activated by proteolytic cleavage adjacent to aspartates. During apoptosis, caspases function either as initiators (e.g. caspase-8 and -9) in response to proapoptotic signals or as effectors (e.g. caspase-3) (Berg et al., 2001). Mature erythrocytes contain considerable amounts of caspase-3 and caspase-8 whereas other essential components of the mitochondrial apoptotic cascade such as caspase-9, Apaf-1 and cytochrome c are absent. Strikingly, although caspase-3 and -8 were functionally active *in vitro*, they did not become activated by various proapoptotic stimuli. Cysteine protease inhibitors prevented programmed erythrocyte death induced by $Ca^{2+}$ influx, and allowed erythrocyte survival *in vitro* and *in vivo*. However, the cysteine proteases involved seem not to be caspases, since caspase-3, while present in erythrocytes, was not activated during cell death, and cytochrome c, a critical component of the apoptosome, was lacking. Therefore, $Ca^{2+}$-induced erythrocyte death appeared to proceed in the absence of caspase activation (Bratosin et al., 2001). In opposition, pretreatment of red cells with the caspase-8 or the caspase-3 specific inhibitors blocked the oxidative stress-induced inhibition of aminophospholipid translocase activity, leading to the conclusion that caspase-8 dependent caspase-3 activation could play a role in the phosphatidylserine externalization (Mandal et al., 2005). Other authors observed that treatment of erythrocytes with peroxynitrite under conditions in which the oxidant diffuses to the intracellular compartment led to phosphatidylserine translocation in parallel with activation of caspases (Pietraforte et al., 2007). Taking together, the abovementioned results suggest that the role of caspases in the mature human erythrocytes needs to be clarified.

The major $Ca^{2+}$-activated cysteine protease found to be involved in the process of cell death is calpain. Following an increase of cytosolic calcium, calpain translocates from the cytosol to the membrane where it undergoes autoproteolytic activation. Although caspases were found inactive in senescent erythrocytes or cells treated with calcium ionophores, activation of the cysteine protease calpain was readily induced in response to elevated calcium levels (Berg et al., 2001). Red blood cells exposed to the oxidative agent peroxynitrite also showed

an increase of the active form of μ-calpain (Matarrese et al., 2005). In contrast, calpain inhibitors did not affect phosphatidylserine exposure suggesting that it is presumably a protease-independent event in erythrocytes. A possible explanation may be that increased intracellular calcium is sufficient to disrupt phospholipid asymmetry by activating an aminophospholipid scramblase and inactivating aminophospholipid translocase.
The activation of calpain in normal human erythrocytes incubated in the presence of $Ca^{2+}$ and ionophore A23187 led to the decline of the $Ca^{2+}$-dependent ATPase activity of the cells, which was prevented by preloading of the erythrocyte with an anticalpain antibody. The decline of the pump activity corresponded to the degradation of the pump protein and was inversely correlated to the amount of the natural inhibitor of calpain, calpastatin, present in the cells. Results suggested that the Ca pump and band 3 were the most sensitive proteins to calpain-induced degradation (Salamino et al., 1994).
Calpain was also responsible for phosphotyrosine phosphatase 1B (PTP1B) cleavage in platelets (Frangioni et al., 1993) and in cell lines with erythroid differentiation ability (Callero et al., 2011), which was accompanied by stimulation of its enzymatic activity. Reversible oxidation of PTP1B *in vitro* strongly facilitated the association with calpain and led to greatly increased calpain-dependent cleavage (Trümpler et al., 2009). Both oxidative environment and increased intracellular $Ca^{2+}$ may account for the altered tyrosyl phosphorylation that may have important implications in the programmed erythrocyte death.

### 4.3 Phosphatydilserine externalization

Alterations in the transbilayer distribution of phospholipids in erythrocyte membrane have significant physiologic consequences. Phospholipids in the plasma membrane of mammalian cells are not randomly distributed between the two leaflets of the membrane bilayer. Choline-containing phospholipids phosphatidylcholine and sphingomyelin dominate the outer leaflet, while the aminophospholipids phosphatidylethanolamine and phosphatidylserine are major components of the inner leaflet (Williamson & Schlegel, 2002). Of these phospholipids, only phosphatidylserine demonstrates an absolute distribution, and the appearance of this lipid on the external surface has significant consequences for the red blood cell (Daleke, 2008). Several functional roles for asymmetric phospholipid distribution in plasma membranes have been suggested. For instance, several regulatory and structural proteins including protein kinase C (Palfrey & Waseem, 1985), annexin (Meers & Mealy, 1994), and membrane skeletal proteins, such as spectrin (O'Toole et al., 1999), appear to localize to the cytoplasmic face of the membrane through their interaction with phosphatidylserine (Manno et al., 2002).
Although asymmetric lipid synthesis and chemical modification make some contribution, ATP-dependent directional lipid transport is the primary mechanism for generation and maintenance of lipid asymmetry. The latter transport is catalyzed by an enzyme called the aminophospholipid translocase, a P-type ATPase that specifically and rapidly transports the aminophospholipids, phosphatidylethanolamine and phosphatidylserine, from the outer to the inner leaflet of the plasma membrane (Tang et al., 1996). At least one membrane protein is required to facilitate a rapid loss of lipid asymmetry. Although no protein mediating this function has been identified they are called "phospholipid scramblases". The bivalent cation $Ca^{2+}$ plays an important role in the regulation of lipid scrambling. In erythrocyte, once activated by $Ca^{2+}$, the scrambling pathway remains active for at least 2 h (Williamson et al., 1992). Scramblase creates a proteinaceous aqueous pore that facilitates migration of the

polar headgroup of the lipids across the hydrophobic core of the bilayer, while keeping the acyl chain moieties in the core of the bilayer (Bevers & Williamson, 2010).
Whether triggered by injury, disease or cell activation, the movement of phosphatidylserine to the surface of the cell alters rheologic and hemostatic properties of the membrane. Erythrocytes with surface-exposed phosphatidylserine adhere to one another and to vascular endothelial cells (Daleke, 2008). Thus, the regulation and control of the distribution of phospholipid asymmetry are essential for maintenance erythrocyte mechanical stability and proper cell function. Furthermore, asymmetric distribution of aminophospholipids has significant effects on cell shape and on membrane mechanical stability. Ghosts that maintained their asymmetric lipid distribution had normal discoid morphology whereas ghosts in which asymmetric lipid distribution was lost exhibited echinocytic morphology (Manno et al., 2002). Understanding the cause of perturbations of transbilayer distribution of phospholipids and the molecular mechanism by which they are regulated is essential for ameliorating some of the consequences of erythrocyte membrane abnormalities.
In summary, phosphatidylserine translocation, now generally accepted as a hallmark of cells in apoptosis, results from the inhibition of aminophospholipid translocase activity and activation of scramblase. Surface exposure of phosphatidylserine on apoptotic cells presents a recognition and engulfment signal for removal by phagocytosis competent cells even before the development of morphological changes usually associated with death (Schlegel & Williamson, 2001).

### 4.4 Erythrocyte morphology

The volume of red cells decreases with cell aging and substantial amount of hemoglobin is lost from circulating erythrocytes during total lifespan. This is probably due to loss of potassium and to loss of membrane via microvesiculation, resulting in cellular dehydration, membrane protein removal, and increased density.
Vesicle formation appears to be accompanied by the breakdown of band 3 protein. It has been postulated that removal of senescent erythrocytes by macrophages is mediated by senescent cell-specific autoantigens originated on band 3, the anion exchanger and the major membrane protein of the erythrocyte (Kay, 2005). In accordance, Willekens et al. (2008) confirmed that vesiculation is not only associated with the removal of membrane-bound hemoglobin, but is associated with generation of senescent cell antigen, a neoantigen that originates from band 3 after its breakdown in senescent red blood cells. Based on results from immunological analysis of vesicles and taken into consideration the existence of an efficient body mechanism to remove these vesicles, the authors concluded that vesiculation constitutes a mechanism for the removal of erythrocyte membrane patches containing removal molecules, thereby postponing the elimination of otherwise healthy erythrocytes.
Allan and Thomas (1981) found the importance of a raised intracellular $Ca^{2+}$ concentration in the microvesiculation process. Plasma membrane microvesiculation, induced *in vitro* by $Ca^{2+}$, was found identical to that expressed by the very small subpopulation of *in vivo* senescent erythrocytes purified from peripheral blood of healthy donors (Bratosin et al., 2001). Recent comparative proteomic analysis of erythrocytes and their vesicles provide new clues to the mechanisms involved in erythrocyte death (Bosman et al., 2010).
The structure and molecular interaction of proteins within the complex assembly of the erythrocyte cytoskeleton explain the particular shape and shape transformations of these cells. Spectrin, band 3, actin, ankyrin, and other cytoskeletal proteins play an important role

for membrane integrity, typical discocyte form, and elasticity of red blood cells. Conversely, protein damage has been implicated in altered erythrocyte morphology.

The shape of $Ca^{2+}$-loaded erythrocytes changed from normal discocytes to echinocytes or spheroechinocytes with plasma membrane microvesiculation (Bratosin et al., 2001; Vota et al., 2010) (Figure 1). Morphological changes induced by A23187 in the presence of $Ca^{2+}$ were associated with cell shrinkage, one of the characteristic features of apoptosis that distinguishes this active and regulated self-destruction process from the passive and chaotic event of necrosis induced by plasma membrane damage (Bratosin et al., 2001).

On the other hand, stomatocytes were the main morphological cell transformation associated to *in vitro* (Vota et al., 2010) and *in vivo* (Richards et al., 2007; Antonelou et al., 2011) oxidative stress (Figure 1).

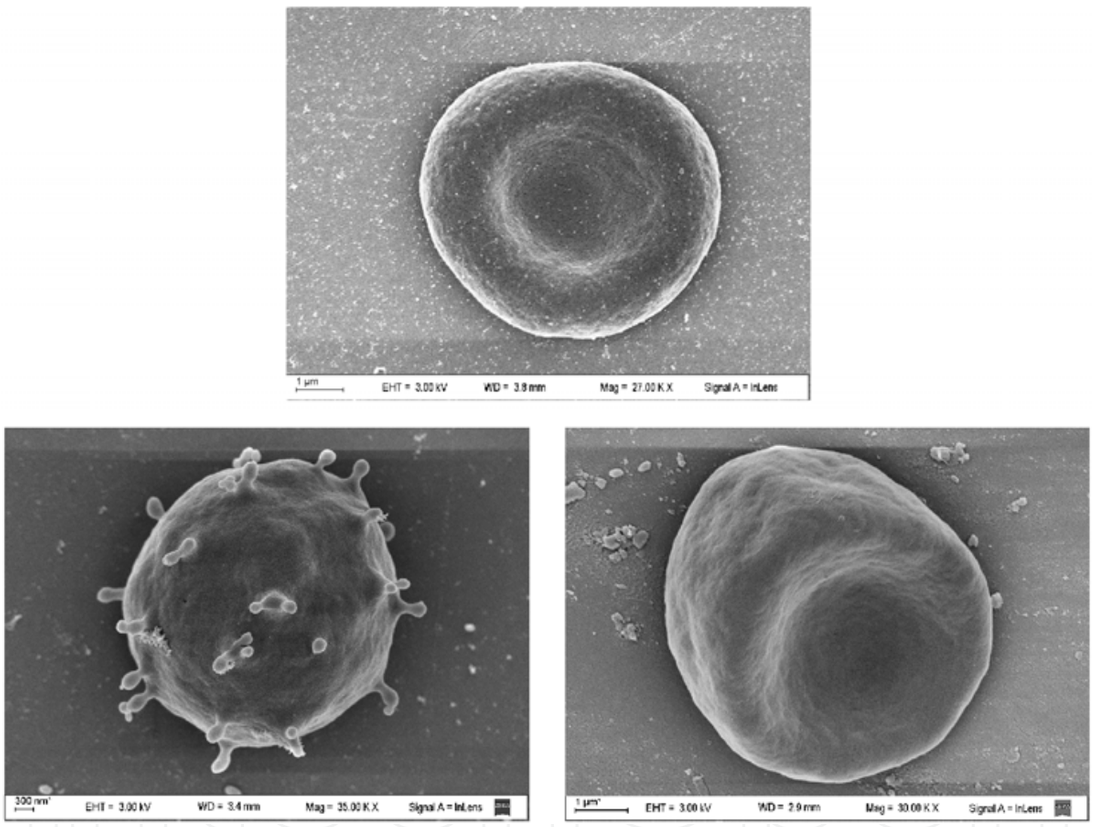


Fig. 1. Altered shapes of erythrocytes subjected to proeryptotic agents. The normal discoid biconcave shape (top) turned to spherocyte with microvesiculation due to increased intracellular calcium concentration (left bottom) or to stomatocyte induced by oxidative stress (right bottom). Results obtained in our laboratory.

### 4.5 Protein modifications

Erythrocyte membrane proteins are susceptible to covalent modification by the lipid peroxidation products generated by an oxygen radical attack. *In vitro* and *in vivo* assays in which erythrocyte metabolic alterations were associated to oxidant environments induced increased lipoperoxidation (Quintanar-Escorza et al., 2010; Calderón-Salinas et al., 2011).

The cytoplasmic domain of band 3 serves as a center of erythrocyte membrane organization and constitutes the major substrate of erythrocyte tyrosine kinases. Tyrosine phosphorylation of band 3 is induced by several stimuli, including malaria parasite invasion, cell shrinkage, normal cell aging, and oxidative stress (Harrison et al., 1991).
Erythrocytes contain protein tyrosine kinase activity, with band 3 protein being the major substrate for the kinases (Brunati et al., 1996). Besides, phosphotyrosine phosphatase was found associated to band 3 protein. This phosphatase is normally highly active and prevents the accumulation of band 3 phosphotyrosine. However, in A23187-treated erythrocytes increased intracellular $Ca^{2+}$ was found to promote band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3 (Zipser et al., 2002).
Tyrosine phosphorylation of band 3 markedly reduced its affinity for ankyrin, leading to release of band 3 from the spectrin/actin membrane skeleton, enhancement of the lateral mobility of band 3 in the bilayer, and progressive vesiculation. Because release of band 3 from its ankyrin and adducin linkages to the cytoskeleton can facilitate changes in multiple membrane properties, the authors suggested that tyrosine phosphorylation of band 3 may produce changes in erythrocyte biology that allow the cell to respond to initial stress (Ferru et al., 2011).
Another marker of red blood cell apoptosis is band 3 clustering, which generates a cell surface epitope identified by autologous IgG antibodies and may act as a signal for the removal of erythrocytes from circulation (Kay et al., 1989).
The nitration of tyrosine residues in proteins occurs through the action of reactive oxygen and nitrogen species such as peroxynitrite, the product of the reaction between nitric oxide and superoxide anion. The nitrated peptides were able to activate *lyn*, an erythrocyte *src* tyrosine kinase. It suggested a mechanism of peroxynitrite-mediated signaling that may be correlated with upregulation of tyrosine phosphorylation (Mallozi et al., 2001).

## 5. Processes that induce premature erythrocyte death

Eryptosis can be triggered by different injuries such as energy depletion, osmotic shock or oxidative stress.

### 5.1 Energy depletion

As mentioned in Section 4.1, the reduced calcium-ATPase activity due to energy depletion leads to decreased calcium efflux and this in turn accelerates the transmembrane movement of potassium and chloride, resulting in cell dehydration.
Energy stress also impairs the replenishment of glutathione and thus weakens the antioxidative defense of erythrocytes (Bilmen et al., 2001). Accordingly, this condition similarly activates cation channels affecting calcium flux (Duranton et al., 2002). On the other hand, phosphatidylserine and phosphatidylethanolamine are maintained in the cell inner leaflet by an ATP-dependent transporter known as flippase (Williamson & Schlegel, 2002). Membrane-bound $Mg^{2+}$-ATPases seem to play a key role in the maintenance of the membrane lipid organization. This subfamily of ATPases has been reported to actively translocate aminophospholipids across membranes. Decreased ATP-dependent transport may be very well one of the consequences of phosphatidylserine exposure (Soupene & Kuypers, 2006). Besides, energy depletion involves activation of PKC and PKC-dependent phosphorylation of membrane proteins with subsequent stimulation of eryptosis (Föller et al., 2008).

### 5.2 Osmotic shock

Osmotic shock is found among the well-known inducers of apoptotic cell death. The cellular mechanisms involved in the triggering of apoptosis following cell exposure to hypertonic extracellular fluid have been deeply studied in nucleated cells. Erythrocytes have similarly been shown to bind annexin following osmotic shock.

Erythrocytes incubated in a hyperosmotic environment released prostaglandin E2 (PGE2), which in turn activated nonselective cation channels (Kaestner & Bernhardt, 2002; Lang PA et al., 2005), and increased the cytosolic $Ca^{2+}$ concentration. Activation of the cell volume- and redox potential-sensitive cation channel and subsequent $Ca^{2+}$ entry contributed to the development of erythrocyte cell membrane scrambling. Osmotic cell shrinkage was involved in the stimulation of sphingomyelinase which caused sphingomyelin degradation with subsequent release of ceramide in erythrocytes (Lang et al., 2004). Ceramide then activated scramblase leading to breakdown of phosphatidylserine asymmetry of the cell membrane. The ability of ceramide to induce this kind of erythrocyte death was somewhat surprising, as erythrocytes lack mitochondria, crucial elements in the ceramide-triggered signaling cascade in nucleated cells (Lang et al., 2004). Thus, at least in erythrocytes, ceramide must trigger annexin binding through a pathway distinct from that described in nucleated cells.

### 5.3 Oxidative stress

Increasing intracellular oxidants by altering ambient oxygen concentrations or lowering antioxidant levels accelerates the onset of erythrocyte senescence whereas lowering ambient oxygen or increasing reactive oxygen species (ROS) scavenging appears to delay senescence. In general, conditions that induce senescence often appear to be accompanied by a rise in intracellular ROS levels. Polyunsaturated fatty acids within the membrane, an oxygen rich environment, and iron-rich hemoglobin make red cells susceptible to peroxidative damage. The product of membrane lipid peroxidation can affect the anion transport function and activity of enzymes of the glycolytic pathway associated to band 3 (Dumaswala et al., 1999).

By virtue of its potent oxidant and nitrating ability, peroxynitrite has been proposed as an important mediator of inflammation-induced tissue injury and dysfunction, and it is considered the most efficient nitrating species of biological relevance (Szabó et al., 2007). The red blood cells are, in fact, the major scavengers of peroxynitrite in blood and it has been calculated that at 45% hematocrit about 40–45% of peroxynitrite crosses the cell membrane and quickly reacts with hemoglobin, while the remainder reacts extracellularly with carbon dioxide (Romero & Radi, 2005). In an *in vitro* experimental system mimicking the oxidative imbalance detectable *in vivo,* peroxynitrite acted both extra- and intracellularly as a function of cell density and carbon dioxide concentration, inducing the appearance of distinct cellular biomarkers as well as modulation of metabolism (Pietraforte et al., 2007). Intracellular oxidations, due mostly to direct reactions of peroxynitrite with glutathione and hemoglobin (methemoglobin), lead to decreased ATP and the appearance of apoptotic signs, such as clustering of band 3, externalization of phosphatidylserine, and activation of caspases. Surface/membrane oxidations were principally due to indirect radical reactions causing oxidation of surface thiols, formation of membrane-associated 3-nitrotyrosine, and downregulation of glycophorins A, the latter being considered a senescence biomarker (Matarrese et al., 2005; Pietraforte et al., 2007; Metere et al., 2009).

## 6. *In vivo* erythrocyte death and possible prevention

Oxidative stress is a term used to describe the body's prolonged exposure to oxidative factors that cause more free radicals than the body can neutralize. Under this condition, free

radical formation may play a role in the pathophysiology of several diseases. There is evidence that erythrocytes undergo oxidative changes in conditions where free radical formation is known to be high such as rheumatoid arthritis (Richards et al., 2007), diabetes (Manuel y Keenoy et al., 2001; Calderón-Salinas et al., 2011), and hemodialysis treatment (Zachee et al., 1988). Oxidative damage was also considered the cause of decreased deformability and altered rheology of erythrocytes found in individuals with chronic fatigue syndrome, a condition that may be triggered by certain infectious diseases, multiple nutrient deficiencies, food intolerance, or extreme physical or mental stress (Richards et al., 2007). Erythrocyte alterations would have the physiological effect of reducing oxygen delivery to the tissues. In several models, 2,3-diphosphoglycerate (2,3-DPG) levels were increased and this effect may be explained as a compensation since 2,3-DPG have the effect of decreasing oxygen affinity. Therefore, this would allow more oxygen to be delivered to the tissues.

Any erythrocyte disorder facilitating erythrocyte shrinkage, could, to the extent as it leads to activation of the cell volume regulatory cation channels, trigger premature apoptosis and thus accelerate erythrocyte death. Red blood cells from patients with sepsis (Kempe et al., 2007), sickle cell disease (Wood et al., 1996), thalassemia (Kuypers et al., 1998), glucose-6-phosphate dehydrogenase deficiency (Lang et al., 2002), and phosphate depletion (Birka et al., 2004) are more sensitive to apoptotic stimuli, a property correlating with the shortened erythrocyte lifespan in these disorders. Membrane lipid disorders play an important role in the pathology of hemoglobinopathies, leading to premature removal (anemia) and imbalance in hemostasis (e.g. lipid breakdown products of phosphatidylserine-exposed cells result in vascular dysfunction) (Neidlinger et al., 2006).

Altered phosphorylation of erythrocyte cytoskeletal proteins and increased ROS production result in disruption of cytoskeleton stability in healthy and sickle cell erythrocytes (George et al., 2010).

Significant modifications in red blood cell structure and membrane proteome in end stage renal disease patients were observed in the context of increased ROS accumulation. The intrinsic oxidative stimuli related to the uremic state were closely associated with membrane cytoskeleton instability, loss of surface area through vesiculation, and transformation of normal discocytes. The observed alterations might contribute to premature erythrocyte death and to the progression of anemia (Antonelou et al., 2011).

Under normal conditions, red blood cells are continuously exposed to ROS from both internal and external sources. In healthy erythrocytes, significant oxidative damage is prevented by a very efficient antioxidant system, consisting of enzymatic and nonenzymatic pathways. Enzymes for preventing oxidative denaturation in erythrocytes include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase which sustain glutathione regeneration, and NADH-methemoglobin reductase. In addition to primary antioxidant defense systems that prevent the generation of free radicals or radical chain reactions, secondary systems have been proposed. These include proteases that preferentially degrade proteins damaged by oxidation. Endogenous non-enzymatic antioxidants also provide defense against oxidative damage: they are lipophylic (vitamin E, carotenoids, melatonin) and water soluble compounds (vitamin C, gluthatione, ceruloplasmin, uric acid) (Burak Çimen, 2008).

Free radicals are produced as intermediate products of normal metabolic functions. Thus, antioxidants function as modulators of cellular homeostasis including detoxification of radicals and metals as well as potent free radical scavengers.

Erythropoietin, the hormone that is the principal regulator of red blood cell production, prevents apoptosis of erythroid progenitors, supporting their survival. It is well known that

the target cells for erythropoietin are the progenitors of erythrocytes found in the hematopoietic organs. However, early works have shown prolonged red blood cell survival during treatment with recombinant human erythropoietin (Schwartz et al., 1992; Polenakovic & Sikole, 1996), suggesting a contribution to the maintenance of corrected hematocrit values. Later, other works were performed to elucidate mechanisms of action of erythropoietin upon mature erythrocytes. Myssina et al. (2003) reported that erythropoietin inhibited cell death through a direct effect via erythropoietin receptor on mature erythrocytes. Unlike the general knowledge of the absence of erythropoietin receptors in reticulocytes, the authors detected the expression of about six erythropoietin binding sites per mature red blood cell. In this work, they postulated that erythropoietin bound to erythrocytes inhibited the volume-sensitive cation channel responsible for calcium entry, and thus blocked phosphatidylserine translocation. More investigation is needed to elucidate the erythropoietin effects upon erythrocytes, since it is well known that erythropoietin induces increased intracellular $Ca^{2+}$ concentration in human erythroid progenitors when they are activated via binding of the hormone with its specific receptor.

On the other hand, a direct effect of erythropoietin on mature erythrocytes might be possible, since erythropoietin similar to other proteins would protect red cell membranes from lipid peroxidation by scavenging hydroxyl radicals generated by oxidative stress. Chattopadhyay et al. (2000) reported that the oxidative damage brought about by copper (II) ascorbate upon red blood cells was due to generation of hydroxyl radical and that erythropoietin was able to protect the membrane from oxidative damage.

In a preliminary study, mature erythrocytes from patients with chronic renal insufficiency exhibited higher annexin binding when compared with red blood cells from healthy individuals. Moreover, the number of cells expressing phosphatidylserine externalization decreased after dialysis only when patients received erythropoietin immediately before dialysis (Myssina et al., 2003). Irrespective the erythropoietin mechanism it seems that the hormone does not only inhibit apoptosis of erythroid progenitor cells, but blunts the suicidal death of mature erythrocytes. This protective antieryptotic mechanism may ameliorate erythrocyte death *in vivo*, resulting in increased lifespan of circulating cells.

## 7. Conclusion

The human red blood cell, by lacking nucleus or any other subcellular organelle, represents the final differentiation stage of the erythroid series. After a limited period in circulation, aged cells become sequestered and removed by macrophages from the reticuloendothelial system. This fate implies that erythrocyte life and death should be well regulated.

Senescence of red blood cells occurs along their lifespan in the vascular system. During aging, erythrocytes display molecules that lead to recognition and removal of old damaged cells by the immune system. Current evidence indicates that neoantigens on altered band 3 and phosphatidylserine exposed at the outside of erythrocytes are the main signals for cell removal and phagocytosis. Vesicles, generated as an integral part of the aging process probably to remove damaged membrane patches, disappear rapidly from circulation. The formation of vesicles as well as changes in electrolyte movements lead to decreased cell volume. Disruption of cell metabolism, hemoglobin denaturalization, changes in cytoskeletal protein interaction, protein phosphorylation/dephosphorylation disbalance, and membrane protein modifications are among the factors responsible for the appearance of morphological alterations.

Considering that senescence represents the time-dependent induction of erythrocyte self-destruction process, premature cell death due to proeryptotic factors could greatly contribute to the development of anemia.

Energy depletion, oxidative stress, and osmotic shock are the most common events that can produce erythrocyte damage, leading to premature eryptosis (Fig. 2). The common feature is the increased intracellular calcium concentration due to either calcium channel activation or depressed calcium pump. Calcium accumulation in turn activates the potassium channel favoring this cation efflux, followed by chloride and water exit, which in conjunction generate cell shrinkage. Calpain, activated by calcium, affects cytoskeletal proteins inducing membrane destabilization and blebbling, and is involved in scramblase activation, thus facilitating phosphatydilserine translocation.

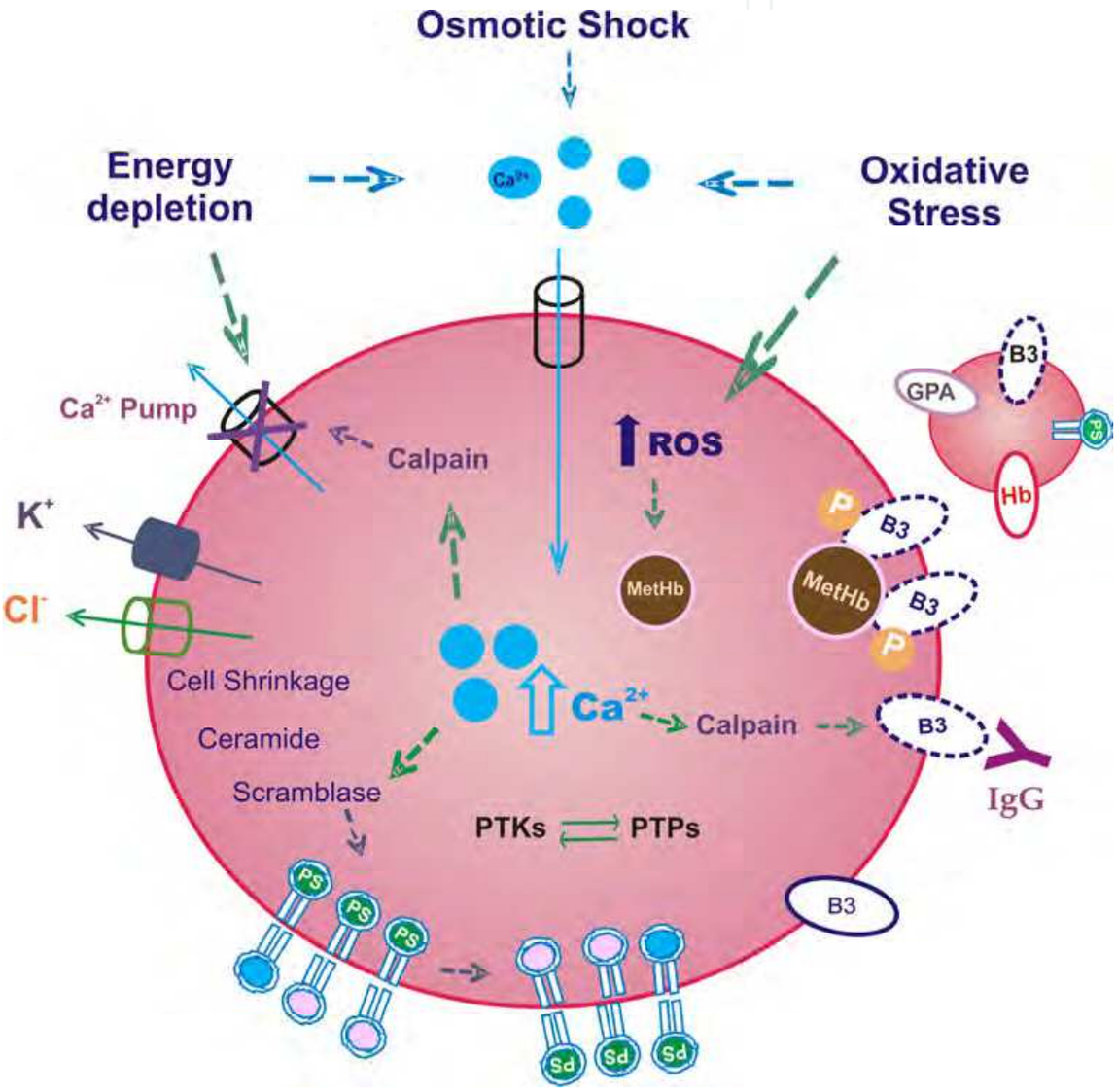


Fig. 2. Mechanisms involved in programmed erythrocyte death. B3: band 3; ROS: reactive oxygen species; Hb: hemoglobin; MetHb: methemoglobin; GPA: glycophorin A; PTKs: phosphotyrosine kinases; PTPs: phosphotyrosine phosphateses; PS: phosphatidylserine.

Additional increase in intracellular oxidants by altering ambient oxygen concentrations or lowering antioxidant levels also accelerates the onset of senescence. Concurrent effects mediated by oxidized hemoglobin and by protein phosphorylation due to disbalance in the kinase/phosphatase ratio are directed towards erythrocyte damage, and consequently to eryptosis.

It is evident that elucidation of mechanisms that regulate eryptosis is a complex issue because of technical problems in obtaining purified cell fractions of a well-defined cell age or in the correct manipulation of erythrocytes *in vitro*, and especially due to the low probability to test hypothesis of programmed erythrocyte death *in vivo*. However, to get an insight into this mechanism is essential for understanding the pathological circumstances surrounding anemia associated to many different diseases.

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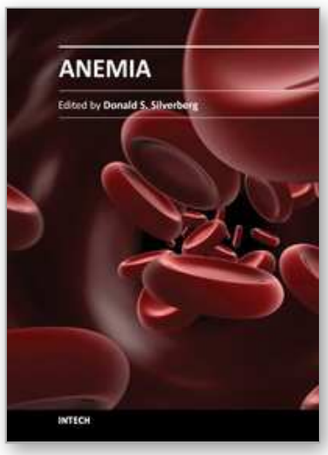


**Anemia**

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This book provides an up- to- date summary of many advances in our understanding of anemia, including its causes and pathogenesis, methods of diagnosis, and the morbidity and mortality associated with it. Special attention is paid to the anemia of chronic disease. Nutritional causes of anemia, especially in developing countries, are discussed. Also presented are anemias related to pregnancy, the fetus and the newborn infant. Two common infections that cause anemia in developing countries, malaria and trypanosomiasis are discussed. The genetic diseases sickle cell disease and thalassemia are reviewed as are Paroxysmal Nocturnal Hemoglobinuria, Fanconi anemia and some anemias caused by toxins. Thus this book provides a wide coverage of anemia which should be useful to those involved in many fields of anemia from basic researchers to epidemiologists to clinical practitioners.

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