Mechanisms of Suicidal Erythrocyte Death

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Key Words

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Abstract

Erythrocyte injury such as osmotic shock, oxidative stress or energy depletion stimulates the formation of prostaglandin E₂ through activation of cyclooxygenase which in turn activates a Ca²⁺ permeable cation channel. Increasing cytosolic Ca²⁺ concentrations activate Ca²⁺ sensitive K⁺ channels leading to hyperpolarization, subsequent loss of KCl and (further) cell shrinkage. Ca²⁺ further stimulates a scramblase shifting phosphatidylserine from the inner to the outer cell membrane. The scramblase is sensitized for the effects of Ca²⁺ by ceramide which is formed by a sphingomyelinase following several stressors including osmotic shock. The sphingomyelinase is activated by platelet activating factor PAF which is released by activation of phospholipase A₂. Phosphatidylserine at the erythrocyte surface is recognised by macrophages which engulf and degrade the affected cells. Moreover, phosphatidylserine exposing erythrocytes

may adhere to the vascular wall and thus interfere with microcirculation. Erythrocyte shrinkage and phosphatidylserine exposure ("eryptosis") mimic features of apoptosis in nucleated cells which however, involves several mechanisms lacking in erythrocytes. In kidney medulla, exposure time is usually too short to induce eryptosis despite high osmolarity. Beyond that high Cl⁻ concentrations inhibit the cation channel and high urea concentrations the sphingomyelinase. Eryptosis is inhibited by erythropoietin which thus extends the life span of circulating erythrocytes. Several conditions trigger premature eryptosis thus favouring the development of anemia. On the other hand, eryptosis may be a mechanism of defective erythrocytes to escape hemolysis. Beyond their significance for erythrocyte survival and death the mechanisms involved in "eryptosis" may similarly contribute to apoptosis of nucleated cells.

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Introduction

During their daily life, erythrocytes are exposed to several stress situations. In average they pass once a

Fig. 1. Erythrocyte (RBC) phagocytosed by a Kupffer cell in rat liver. Gold particles indicate labelling by the galactose-binding *Ricinus communis* I lectin (By courtesy of Jürgen Roth, Division of Cell and Molecular Pathology, Department of Pathology, University of Zürich).

minute the lung where they are exposed to oxidative stress. More than once an hour they travel through kidney medulla where they face osmotic shock. Erythrocytes have to squeeze through capillaries which are smaller than themselves. Thus, the integrity of erythrocytes is constantly challenged. Rupture of erythrocyte cell membranes releases hemoglobin to extracellular fluid which may be filtered at the glomerula of the kidney, precipitate in the acid lumen of the tubules, obliterate the tubules and thus lead to renal failure. To avoid those complications, erythrocytes, as any other cell, require a mechanism allowing them to be disposed without release of intracellular components.

Abundant, defective or potentially harmful nucleated cells are disposed by apoptosis [1-7]. Apoptosis is triggered either by stimulation of respective receptors such as CD95 [8] or by cell exposure to stressors such as oxidants, radiation or osmotic shock [3, 9, 10]. Typically, apoptosis is paralleled by cell shrinkage which is associated with loss of intracellular potassium [4, 11-21]. Further hallmarks of apoptosis include nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell membrane blebbing, and breakdown of phosphatidylserine asymmetry of the plasma membrane [3, 4]. Cells exposing phosphatidylserine at the cell surface are recognized by macrophages which are equipped with receptors specific for phosphatidylserine [22] and rapidly engulf and degrade the affected cells [23, 24]. Accordingly, apoptosis allows the elimination of those cells without release of intracellular proteins which would otherwise cause inflammation [4]. As illustrated in Fig. 1, erythrocytes are similarly engulfed by macrophages.

Erythrocytes are devoid of nuclei and mitochondria and thus lack crucial elements in the machinery of apoptosis. Thus, until recently, erythrocytes have been considered to be eliminated by mechanisms other than apoptosis. Exposure of erythrocytes to the Ca^{2+} ionophore ionomycin triggers, however, cell shrinkage, membrane

blebbing and phosphatidylserine exposure, all typical features of apoptotic nucleated cells [25-27]. The cell shrinkage results from activation of the Ca^{2+} sensitive "Gardos" K^+ channels [28], the phosphatidylserine exposure from the activation of a Ca^{2+} sensitive scramblase [29-31]. Unlike apoptosis of nucleated cells, the metamorphosis of erythrocytes following ionomycin or hyperosmotic shock does not require the activation of caspases [25, 32, 33]. To distinguish the death of erythrocytes from apoptosis of nucleated cells, we do suggest the term "eryptosis".

The following brief review will illustrate some mechanisms involved in the triggering of eryptosis.

Cation channels trigger eryptosis

Erythrocyte cell membranes are usually tight and show little channel activity. Moreover, the erythrocytes are predominantly permeable to Cl^- [34]. Osmotic cell shrinkage, however, opens non-selective cation channels in the erythrocyte cell membrane [35]. The same channels are activated by oxidative stress [36]. The channels are inhibited by intracellular or extracellular Cl^- [35, 36]. Thus, it is necessary to remove Cl^- ions from the medium to observe the cation channels in patch clamp experiments (Fig. 2).

Energy depletion impairs the replenishment of GSH and thus weakens the antioxidative defence of the erythrocytes [37, 38]. Accordingly, energy depletion similarly activates the cation channels [39].

The channels are not only permeable to monovalent cations but as well to Ca^{2+} [36, 40, 41]. Accordingly, exposure to osmotic shock or oxidative stress triggers erythrocyte Ca^{2+} uptake [41]. The Ca^{2+} then stimulates the erythrocyte scramblase [31], thus leading to the breakdown of phosphatidylserine asymmetry [41]. The phosphatidylserine exposure following osmotic shock is

Fig. 2. Erythrocyte cation channels activated by osmotic shock and oxidative stress. A) Activation of cation channels by Cl^- removal either in the presence (middle traces) or absence (right traces) of a permeable cation. B) Schematic representation of erythrocyte cation channel regulation. EIPA, ethylisopropylamiloride and H_2O_2 , hydrogen peroxide.

blunted by amiloride [41] and ethylisopropylamiloride (EIPA) [42], inhibitors of the cation channel [35, 42]. Thus, it appears safe to conclude that activation of the cell volume and oxidant sensitive cation channel and subsequent Ca^{2+} entry contribute to the stimulation of erythrocyte scramblase following osmotic shock or oxidative stress.

Prostaglandins stimulate erythrocyte cation channels and “eryptosis”

Intriguing evidence points to a role of prostaglandins in the regulation of eryptosis. Hyperosmotic shock and Cl^- -removal trigger the release of prostaglandin E_2 (PGE_2) [43]. PGE_2 in turn activates the cation channels [43, 44], increases the cytosolic Ca^{2+} concentration [43, 45], and stimulates phosphatidylserine exposure at the erythrocyte surface [43]. The activation of the cation channels by Cl^- -removal is abolished by the cyclooxygenase inhibitor diclofenac [43]. Moreover, phospholipase- A_2 inhibitors quinacrine and palmitoyl trifluoromethyl ketone and cyclooxygenase inhibitors acetylsalicylic acid and diclofenac blunt the increase of phosphatidylserine exposure following Cl^- removal [43]. PGE_2 further activates the Ca^{2+} dependent cysteine endopeptidase calpain, an effect, however, apparently not required for stimulation of phosphatidylserine exposure [43].

Ca^{2+} sensitive K^+ channels mediate shrinkage in “eryptosis”

Ca^{2+} entering erythrocytes does not only activate the scramblase but in addition stimulates the Ca^{2+} sensitive “Gardos” K^+ channels in erythrocytes [46–48]. The activation of the channels leads to hyperpolarization of the cell membrane driving Cl^- in parallel to K^+ into the extracellular space. The cellular loss of KCl favours cell shrinkage. Moreover, the cellular loss of K^+ presumably participates in the triggering of “eryptosis” [49]. Increase of extracellular K^+ or pharmacological inhibition of the Gardos channels by clotrimazole or charybdotoxin do not only blunt the cell shrinkage but also decrease the phosphatidylserine exposure following exposure to ionomycin [49]. Presumably, cellular loss of K^+ somehow stimulates „eryptosis“ as has been shown for apoptosis of nucleated cells [9, 50]. As PGE_2 increases cytosolic Ca^{2+} activity [43] (see above), it similarly activates the Ca^{2+} sensitive “Gardos” K^+ channels with subsequent cell shrinkage [51, 52].

Cell volume controls sphingomyelinase activity

The effect of osmotic shock on phosphatidylserine exposure is not fully blocked in the absence of calcium

Fig. 3. Activation of sphingomyelinase by cell shrinkage. A) Anti-ceramide FITC labelled erythrocyte. B) Histogram overlay of anti-ceramide FITC labelled erythrocytes following incubation in isosmotic (300 mosM) or hyperosmotic (850 mosM) Ringer solution. C) Schematic representation of sphingomyelinase regulation.

[42], pointing to the participation of a mechanism other than unselective cation and Ca^{2+} sensitive K^{+} channels. As illustrated in Fig. 3, osmotic shock leads to the appearance of ceramide at the erythrocyte surface [42]. Further experiments revealed that C_6 -ceramide as well as treatment with bacterial sphingomyelinase triggers eryptosis [42]. Moreover, eryptosis induced by osmotic shock can be inhibited by the sphingomyelinase inhibitor 3,4-dichloroisocoumarin. Ceramides have been reported to form large channels in the outer mitochondrial membrane allowing the release of intermembrane space proteins with a molecular weight cut-off of about 60,000 [53]. However, it is shown here that C_6 -ceramide does not enhance Ca^{2+} uptake of erythrocytes. A simple channel-based mechanism of the ceramide effect is therefore rather unlikely. Moreover, the ceramide induced „eryptosis“ is blunted but not abolished in the nominal absence of Ca^{2+} . Thus, C_6 -ceramide induced cell death is probably not secondary to increase of cytosolic Ca^{2+} activity. Instead, the effect of C_6 -ceramide adds to or even potentiates the effects of Ca^{2+} entry on phosphatidylserine exposure. This novel activity of ceramide may be due to ceramide-mediated induction of transbilayer lipid movement as previously demonstrated in large unilamellar vesicles and in erythrocyte ghost

membranes [54]. Accordingly, in the presence of C_6 -ceramide eryptosis following osmotic shock is accelerated.

Platelet activating factor stimulates sphingomyelinase

Cell shrinkage leads to release of platelet activating factor (PAF), a phospholipid mediator involved in the regulation of inflammation, thrombosis, atherogenesis and cardiovascular function [55-61]. PAF in turn stimulates the breakdown of sphingomyelin and release of ceramide from erythrocytes [62]. PAF further triggers cell shrinkage (decrease of forward scatter) and phosphatidylserine exposure (annexin binding) of erythrocytes. The stimulation of phosphatidylserine exposure is blunted by genetic knockout of PAF receptors (PAF receptor knockout mice), and by the PAF receptor antagonist ABT491 [62]. Thus, PAF participates in the stimulation of sphingomyelinase activation and „eryptosis“. PAF further activates Ca^{2+} sensitive K^{+} channels (Gardos channels) in the erythrocyte cell membrane [63] by sensitising them for the stimulating effects of cytosolic Ca^{2+} [64]. Interestingly, PAF is released from erythrocyte progenitor cells upon increase of cytosolic Ca^{2+} activity [65].

Erythrocytes are protected in kidney medulla

During antidiuresis the osmolarity of kidney medulla exceeds the value required to trigger eryptosis *in vitro*. The contact time of erythrocytes *in vivo* is too short, though, to induce eryptosis. Moreover, exposure of erythrocytes to extracellular fluid mimicking the composition of antidiuretic kidney medulla does not trigger eryptosis *in vitro* [66]. In kidney medulla, the erythrocytes are indeed protected by two mechanisms: (i) the cation channel is inhibited by Cl^- and (ii) the sphingomyelinase is blunted by the high urea concentrations prevailing in kidney medulla [66]. Nevertheless, it is noteworthy that during acute renal failure erythrocytes may be trapped in renal medulla [67]. The subsequent eryptosis may then contribute to the derangement of microcirculation [66].

Erythropoietin inhibits eryptosis

Erythropoietin, the most potent stimulator of erythropoiesis, is known to stimulate erythrocyte formation at least in part by inhibiting apoptosis of EryD progenitor cells [68, 69]. However, clinical data suggested that erythropoietin increases the life span of circulating cells [69]. Erythropoietin receptors are hardly detectable at the cell membrane of circulating erythrocytes but their activation is obviously sufficient to blunt the apoptosis following osmotic shock [70]. Patch clamp experiments further revealed that erythropoietin is effective through inhibition of the cell volume sensitive cation channels. Accordingly, the protective effect of erythropoietin is lost in the nominal absence of extracellular Ca^{2+} [70]. The significance of the protective effect of erythropoietin is illustrated by the observation that the percentage of phosphatidylserine exposing erythrocytes in circulating blood of patients with end stage renal disease is significantly decreased within 3 hours of erythropoietin administration.

Physiological significance of eryptosis

The mechanisms described here could well participate in the limitation of erythrocyte survival. Erythrocyte ageing is paralleled by increase of cytosolic Ca^{2+} activity [71, 72]. Moreover, according to the present results, oxidative stress or defects of antioxidative defence [73] would enhance Ca^{2+} entry via the cation channels

and thus accelerate erythrocyte death and clearance from circulating blood.

Several disorders may decrease the life span of mature erythrocytes by facilitating eryptosis. As a matter of fact, the sensitivity of sickle cells and of glucose-6-phosphate dehydrogenase deficient cells to osmotic shock and of sickle cells, thalassemic cells and glucose-6-phosphate dehydrogenase deficient cells to oxidative stress and to glucose depletion was significantly higher than that of control cells [39]. This enhanced susceptibility most likely contributes to the decrease of erythrocyte life span in those genetic disorders.

Eryptosis may further be relevant for the intraerythrocyte survival of the malaria pathogen *Plasmodium falciparum*. The parasite invades erythrocytes to escape the immune system. However, transport across the intact erythrocyte cell membrane is not sufficient to meet the excessive demands of the pathogen. Thus, *Plasmodium falciparum* induces novel permeability pathways (NPP) allowing the uptake of nutrients and the disposal of waste products [74]. Most recent experiments revealed that NPP is made up of endogenous host cell channels which are activated by the pathogen through oxidation of the cell membrane [75, 76]. Activation of the cation channel is required for the cellular accumulation of Na^+ and Ca^{2+} which are both needed by the pathogen. By the same token, however, the activation of the cation channel triggers eryptosis [2]. Presently, it is not entirely clear whether phosphatidylserine exposure of infected host cells is favourable for the host or the pathogen. In other model systems, host cell apoptosis has proven to be a crucial defence mechanism of the host [13]. In any case, eryptosis favours the recognition of the erythrocytes by macrophages and thus limits the life span of the infected cell.

Most importantly, eryptosis may serve to prevent hemolysis. Energy depletion, defective Na^+/K^+ ATPase or enhanced leakiness of the cell membrane all lead to gain of Na^+ and Cl^- and osmotically obliged water with subsequent cell swelling [15]. Initially, the entry of Na^+ may be compensated by cellular loss of K^+ , the decrease of the K^+ equilibrium potential will, however, eventually lead to depolarisation which will favour the entry of Cl^- . The increase of cell volume will lead to rupture of the cell membrane with cellular release of hemoglobin (see above). It is intriguing to speculate that an increase of erythrocyte Ca^{2+} activity is indicative for the inability of the cell to maintain its electrolyte gradients. Ca^{2+} stimulates the scramblase which exposes phosphatidylserine at the cell surface, a signal for macrophages to clear the cells

from circulating blood. The activation of the Gardos K^+ channel serves to delay swelling and disruption of defective erythrocytes. Decreasing cytosolic K^+ concentration accelerate the phosphatidylserine exposure. Paradoxically, many of the cell injuries such as oxidative stress and energy depletion lead initially to cell shrinkage. Thus, eryptosis may be an important mechanism to clear erythrocytes prior to detrimental hemolysis.

Conclusions

As summarized in Fig. 4, cellular stress, e. g. osmotic shock, oxidative stress or energy depletion, activate a Ca^{2+} permeable cation channel in the erythrocyte cell membrane presumably via generation of PGE_2 . The subsequent entry of Ca^{2+} leads to activation of a Ca^{2+} sensitive scramblase which exposes phosphatidylserine at the surface of the erythrocyte cell membrane. Moreover, activation of the Ca^{2+} sensitive Gardos K^+ channels counteracts cell swelling and prevents hemolysis. Eryptosis is further stimulated by ceramide which is formed in shrunken erythrocytes by PAF-mediated activation of sphingomyelinase. Those mechanisms serve to safely eliminate aged, defective, leaky and infected erythrocytes before they rupture and thus release cytosolic components including hemoglobin.

The mechanisms underlying erythrocyte apoptosis are apparently important for the tuning of the erythrocyte life span. Moreover, similar mechanisms may be operative in nucleated cells where they may be hidden by the more complex apoptotic machinery. Thus, eryptosis may prove to be a valuable model system to analyse mechanisms which are similarly important for the apoptosis of nucleated cells.

Fig. 4. Mechanisms involved in „eryptosis“. Note that there are Ca^{2+} - and lipid- (ceramide)-mediated mechanisms. AA, arachidonic acid; COX: cyclooxygenase; NSC: nonselective cation channel; PAF, platelet activating factor; PGE_2 : prostaglandin E_2 ; PLA, phospholipase A_2 ; S, scramblase; SM, sphingomyelinase.

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