



Editorial

Erythrocytes: Death of a mummy

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Simple spirits consider mammalian erythrocytes as membrane-surrounded hemoglobin-containing vesicles floating in circulation. Indeed, they lack organelles including nuclei and mitochondria and, in a sense, they cannot be considered as true cells. Nonetheless, erythrocytes have been intriguing researchers interested in apoptosis.

The red blood cell (RBC) life cycle can be divided in two phases, the first one, erythropoiesis consisting in the production of mature erythrocytes in hematopoietic organs, the second one concerning the proper existence of the mature erythrocyte in circulation. Erythropoiesis results from the progressive differentiation of erythroid progenitors, from proerythroblasts through polychromatophylic erythroblasts, then orthochromatic erythroblasts to mature erythrocytes. It is now well established that the hormone erythropoietin stimulates erythropoiesis, at least in part, by protecting erythroblasts from apoptosis presumably by upregulating the anti-apoptotic protein Bcl-X_L.^{1,2} Indeed, conditional knock-out of Bcl-X_L suppresses erythropoiesis at a late stage resulting in hemolytic anemia,³ and erythropoietin withdrawal triggers erythroblast death and caspase activation.⁴ A CD95- and caspase-dependent negative regulation of erythropoiesis has been described at the local level. Orthochromatic erythroblasts expressing CD95 ligand would stimulate the CD95 death receptor expressed on immature erythroblasts, thereby creating a negative feedback loop controlling erythropoiesis. Erythropoietin would antagonize this negative control.^{5,6} Thus, not surprisingly, erythrocyte precursors, which are true organelle-containing cells, are susceptible to apoptosis induction, including in pathological stages such as β -thalassemia major,⁷ idiopathic acquired sideroblastic anemia⁸ and parvovirus B19 infection.⁹

Surprisingly, caspase activation has not only negative effects on erythropoiesis. Rather, it appears that the activation of effector caspases is required for RBC formation.¹⁰ *In vitro* erythropoiesis reportedly is blocked by the pan-caspase inhibitor Z-VAD.fmk. During erythroblast differentiation caspases-3, -6 and -7 are proteolytically activated and cleave nuclear proteins (lamin B and acinus) yet leave intact the transcription factor GATA-1 and fail to trigger the exposure of phosphatidylserine on the plasma membrane surface.¹⁰ This suggests that a sort of abortive

apoptosis participates in the differentiation and enucleation of erythroid cells. However, caspase-3^{-/-} mice do not exhibit any obvious defect in erythropoiesis, and transgene-targeted overexpression of Bcl-2 in the erythroid lineage does not induce any erythropoietic disorder *in vivo*.¹¹ Further studies will thus have to confirm the implication of caspase activation and abortive apoptosis in erythroid differentiation. Intriguingly, Nagata and co-workers recently reported that DNase II is required for erythropoiesis in the fetal liver. According to these authors, DNase II present in macrophages would be required for efficient enucleation of erythrocyte precursors. DNase II thus would not participate in a cell-autonomous process and rather act in the heterophagic removal of the nucleus of the differentiating erythroblast,¹² much in the way as this has been described for the macrophage-mediated removal of apoptotic thymocytes.¹³

Mammalian fetal erythrocytes as well as erythrocytes from amphibians, reptiles and birds do contain a nucleus, and these cells can undergo normal apoptosis. Thus, fetal human erythrocytes circulating in maternal blood undergo apoptosis *in situ*.¹⁴ Moreover, chicken erythrocytes can be driven into apoptosis (nuclear and cellular shrinkage, chromatin condensation, TUNEL positivity, staining with Annexin-V) by incubating them either in the absence of serum or in the presence of staurosporin plus cycloheximide.¹⁵ This process is not prevented by Z-VAD.fmk and is not accompanied by caspase activation, suggesting that chicken erythrocyte apoptosis occurs in a caspase-independent fashion.

Two fascinating papers published in this issue of CDD now tackle the question whether mature human erythrocytes can undergo apoptosis-like cell death.^{16,17} In contrast to true cells, human erythrocytes fail to die in the absence of serum or upon addition of staurosporin+cycloheximide, *in vitro*. However, RBC life span is limited to approximately 120 days *in vivo*. It has been widely assumed that RBC aging would lead to an increase in intracellular calcium, modifications of the erythrocyte shape, protein degradation and externalization of phosphatidylserine, thereby activating a clearance mechanism involving heterophagic removal in the reticuloendothelial system.^{18,19} Indeed, splenectomy is accompanied by the occurrence of aged erythrocytes in peripheral blood that have failed to be cleared.

Here, the groups headed by Klaus Schulze-Osthoff¹⁶ and the tandem formed by Jean-Claude Ameisen and Jean Montreuil¹⁷ report the intriguing finding that Ca²⁺ ionophores (A23187 or ionomycin) induce an *in vitro* erythrocyte senescence process characterized by cell shrinkage, membrane microvesiculation, and phosphatidylserine externalization. This process culminates in erythrocyte disintegration, phagocytosis in the presence of macrophages *in vitro*, and in clearance from the circulation *in vivo*.

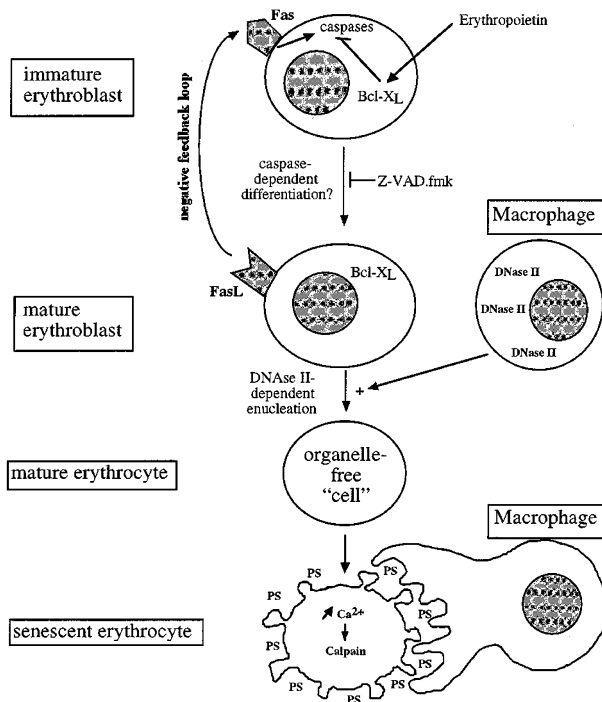


Figure 1 Erythroblasts, the erythrocyte precursors are organelle-containing cells which can undergo full-blown apoptosis. The differentiation process may involve a caspase-dependent step and successful enucleation requires the concurrence of DNase II expressing macrophages. Mature, organelle-free erythrocytes can be driven into *in vitro* senescence by addition of external Ca^{2+} or addition of Ca^{2+} ionophore, which triggers μ -calpain activation and μ -calpain-dependent changes in cellular shape and plasmamembrane structure (in particular PS exposure) reminiscent of apoptosis.

Surprisingly, *in vitro* senescence is not just due to a disruption of ion homeostasis and rather involves the activation of proteolytic enzymes, as indicated by the fact that cysteine protease inhibitors such as Ac-DEVD-CHO or leupeptin prevent all the features of Ca^{2+} ionophore-induced erythrocyte senescence. Erythrocytes were found to contain pro-caspase-3 and -8 as well as μ -calpain. Upon Ca^{2+} exposure, μ -calpain is cleaved to its active fragments which in turn are likely responsible for the degradation of spectrin (erythrocyte fodrin). In contrast, neither pro-caspase-3 nor pro-caspase-8 become activated in erythrocytes, which can be explained by the absence of two essential apoptosome components, Apaf-1 and cytochrome *c*. Thus, calpain but not caspases participate in erythrocyte senescence, and the effect of Ac-DEVD-CHO must be attributed to the inhibition of another protease (presumably μ -calpain) than its classical target, caspase-3.

Calpain has also been involved in apoptotic signaling in true nucleate cells, according to numerous reports. For instance, pharmacological inhibition of calpains can prevent cell death. This applies to excitotoxin-induced neuronal cell death,²⁰ hypoxia-ischemia damage of the brain,²¹ germ cell death in experimental testicular torsion,²² neutrophil apoptosis triggered by cycloheximide,²³ and radiation-induced apoptosis.²⁴ Transfection with the endogenous calpain

inhibitor calpastatin can render B cells resistant to B cell receptor-induced apoptosis.²⁵ Calpains are activated by increased cytosolic Ca^{2+} levels and they trigger apoptosis by proteolytically activating caspases (in particular caspases-3, -9 and -12)^{26,27} and/or by cleaving members of the Bcl-2 family including Bcl-2,²⁸ Bcl-X_L (which convert from an anti-apoptotic to a pro-apoptotic function), Bax²⁹ and Bid³⁰ (which upon truncation increase their apoptogenic potential). Numerous reports underline the existence of a cross-talk of mutual activation between caspases and calpains, and growing evidence supports the notion that either classes of proteases can substitute for each other in apoptotic pathways.³¹ Knock-out of μ -calpain reveals a deficiency in platelet aggregation,³² in accord with the observation that Ca^{2+} ionophore stimulation of platelets induces calpain-dependent and caspase-independent apoptosis-like events including phosphatidylserine exposure.²⁶

A semantic and hence delicate problem arises from the use of erythrocytes for the study of cell death or apoptosis. If we consider Ca^{2+} ionophore-induced RBC senescence as 'apoptotic', then the results reported here^{16,17} may be interpreted to mean that 'apoptosis' can occur in the absence of mitochondria, nuclei, and caspase activation. That apoptosis may occur in the absence of a nucleus (that is in freshly enucleated cells, so-called cytoplasts) or in the absence of caspase activation (that is in the presence of caspase-inhibitors or in Apaf-1 or caspase knock-out cells) has been reported in the past,^{33–35} and the true originality of the present articles^{16,17} would be to show death in the absence of mitochondria.³⁶ However, in a way the erythrocyte must be considered as a post-apoptotic creature, in the sense that transient, presumably mitochondrion-triggered caspase activation¹⁰ has led to the formation of an organelle-free mummy (just as ancient Egyptians removed organs from the corpse before mummification). Seen from this angle, it appears logical that the final implosion of RBC occurs via a non-apoptotic process which we might call erythroptosis.

Irrespective of this semantic problem, the second death of erythrocytes is characterized by some features that are shared by apoptosis. Indeed, classical apoptosis of mammalian cells, *in vitro* erythrocyte senescence,^{16,17} and artificial induction of cell death in monocellular organisms^{37,38} is invariably accompanied by phosphatidylserine exposure and microvesiculation as well as nuclear condensation (when a nucleus is present). Again, two interpretations of this parallelism are possible. Either it hints at the existence of a common biochemical 'program' that has been conserved throughout evolution, or it simply means that an increase in entropy leading to disintegration of a cell or a cell-derived vesicle gives rise to a common phenomenology.

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