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Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis

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Abstract

Although proteases of the caspase family are essential mediators of apoptosis in nucleated cells, in anucleate cells their presence and potential functions are almost completely unknown. Human erythrocytes are a major cell population that does not contain a cell nucleus or other organelles. However, during senescence they undergo certain morphological alterations resembling apoptosis. In the present study, we found that mature erythrocytes contain considerable amounts of caspase-3 and -8, whereas essential components of the mitochondrial apoptotic cascade such as caspase-9, Apaf-1 and cytochrome *c* were missing. Strikingly, although caspases of erythrocytes were functionally active *in vitro*, they failed to become activated in intact erythrocytes either during prolonged storage or in response to various proapoptotic stimuli. Following an increase of cytosolic calcium, instead the cysteine protease calpain but not caspases became activated and mediated fodrin cleavage and other morphological alterations such as cell shrinkage. Our results therefore suggest that erythrocytes do not have a functional death system. In addition, because of the presence of procaspases and the absence of a cell nucleus and mitochondria erythrocytes may be an attractive system to dissect the role of certain apoptosis-regulatory pathways. *Cell Death and Differentiation* (2001) **8,** 1197 – 1206.

Keywords: apoptosis; erythrocytes; calpain; caspase; spectrin

Abbreviations: Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; Apaf-1, apoptotic protease-activating factor-1; CD95L, CD95 ligand; CHX, cycloheximide; RBC, red blood cell; TNF, tumor necrosis factor; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

Introduction

Apoptosis is the innate mechanism of cell clearance in many physiological and pathological processes including development, tissue remodeling, cell homeostasis and defense processes against various forms of stress. Although the signals capable of inducing apoptosis are very different, alterations such as nuclear condensation, membrane blebbing and formation of apoptotic bodies are stereotypical features common to most, if not all apoptotic cells. Several biochemical changes are associated with these morphological changes; the two best known of them are increases in endonuclease and protease activity.[1–4]

There is increasing evidence that all eukaryotic cells use a similar death program, which in most cases essentially depends on the activation of highly conserved proteases, called caspases.[3,4] Members of the caspase family contain a cysteine residue in their active center and exist as zymogens that need to be activated by proteolytic cleavage adjacent to aspartates. This event liberates a large and a small subunit that subsequently associate to constitute an active tetrameric enzyme. 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), which through the cleavage of several vital proteins finally induce an apoptotic phenotype.[5]

The activation of caspases can be triggered by at least two principal signaling pathways. Induction of apoptosis via death receptors such as CD95 is initiated by oligomerization of the receptor by its cognate ligand, which recruits the adapter protein FADD to the active receptor complex. FADD in turn associates with procaspase-8 that is subsequently activated by autoproteolysis.[6,7] Another death pathway, which is triggered by a number of apoptotic stimuli such as anticancer drugs or irradiation, is initiated by the release of cytochrome *c* from mitochondria. The mitochondrial release of cytochrome *c* is in part under control of pro- and anti-apoptotic Bcl-2 family proteins.[8,9] Once released, cytochrome *c* associates with Apaf-1, another adapter molecule that can bind to and activate procaspase-9 in the presence of dATP.[10–12] Both death pathways converge finally at the sequential activation of effector caspases.

Although caspases are the main executioners, there is evidence that other proteases including calpains may also be involved in apoptosis. Calpains are heterodimeric calcium-dependent cysteine proteases that, similar to caspases, exist as inactive proenzymes.[13,14] Following an increase of cytosolic calcium, calpain translocates from the cytosol to the membrane where it undergoes autoproteolytic activation. The activity of calpains can be further controlled by a specific endogenous inhibitor, called calpastatin. Interestingly, several substrates of caspases

are also cleaved by calpains including structural proteins, such as spectrin, keratins and actin, or proteins involved in signal transduction, such as Bax, protein kinase C and focal adhesion kinase.[15,16] It has been found that caspases and calpains interfere with each other resulting in mutual protease activation.[17–19] However, it is controversial whether calpains function upstream or downstream of caspases in apoptosis. It has also been reported that calpains cleave procaspases to generate a proteolytically inactive caspase fragment.[20–22]

While it is clear that most nucleated cells are capable of undergoing caspase-dependent apoptosis, the role of apoptosis-regulatory molecules in anucleate cells and their possible functions are almost completely unknown. Platelets and erythrocytes comprise the major cellular components devoid of a cell nucleus. Human erythrocytes have a life span of approximately 120 days after which they are removed from the circulation. During this process senescent red blood cells (RBCs) undergo typical morphological changes that enable their recognition and subsequent phagocytosis by macrophages. These alterations include cell shrinkage as the consequence of a progressive release of microvesicles from the cell membrane, as well as shape transformation from a discocyte to a spherocyte form.[23,24] Other changes are the progressive loss of peripheral membrane proteins such as fodrin (spectrin), which constitutes the RBC's cytoskeleton, and the loss of membrane lipid asymmetry with the externalization of phosphatidylserine.[25–27] Such profound alterations in cellular architecture also occur during apoptotic cell death in nucleated cells suggesting that both the senescence of anucleate erythrocytes and apoptosis of nucleated cells might have some morphological similarities.

Recent studies demonstrated that the development and differentiation of erythroid progenitor cells might be regulated through caspase-dependent apoptosis.[28–33] However, whether mature red blood cells contain a functional death machinery is completely unknown. In the present study we investigated the presence of caspases and their potential functions in erythrocytes. Interestingly, we found that RBCs contained considerable amounts of caspase-3 and -8, whereas other essential components of the apoptotic machinery such as caspase-9, Apaf-1 and cytochrome *c* were absent. Strikingly, although caspase-3 and -8 were functionally active *in vitro*, they did not become activated by various proapoptotic stimuli. We rather observed that in response to elevated calcium concentrations morphological alterations similar to senescent erythrocytes involved the activation of calpains, but not a caspase-dependent apoptotic programme. Moreover, since RBCs do not contain a cell nucleus and mitochondria, the erythrocyte cell-free system may be useful to dissect the role of certain apoptotic pathways.

Results

Erythrocytes contain procaspase-8 and -3

In order to determine the presence of caspases and other components of the apoptotic machinery in mature human red blood cells, highly purified erythrocyte fractions from peripheral blood of healthy donors were collected and investigated by immunoblotting with antibodies against different proteins. As shown in Figure 1A, 10×10^6 erythrocytes contained caspase-3 at levels similar to 2×10^6 Jurkat cells. Roughly equivalent amounts of caspase-8 were detected in RBCs (Figure 1B). These high expression levels further suggested that detection of caspase-3 and -8 was not caused by a contamination with leukocytes. However, neither procaspase-2 and -6 (data not shown) nor procaspase-7 and -9 could be detected (Figure 1C,D). Moreover, although expressed in nucleated cells, erythrocytes were devoid of Apaf-1 and cytochrome *c* (Figure 1E,F). In addition, from the Bcl-2 members investigated we could not detect Bcl-2, Bcl-x_L or Bad (data not shown). Thus, erythrocytes express caspase-8 and -3, but none of the components of the mitochondrial apoptosome.

Are caspases activated in intact erythrocytes?

Because erythrocytes contain caspases, we next examined whether they become activated under certain conditions. First, we analyzed whether caspase processing was increased with the time after collection of erythrocytes. However, neither freshly collected erythrocytes nor RBCs kept for 60 days or longer displayed any detectable levels of the active subunits of caspase-3 or caspase-8 (Figure 2A). Furthermore, in patients with hemolytic anemia only the inactive proforms of both caspases were visible (data not shown). In order to investigate whether caspases can be activated at all in whole cells, we next tested a wide variety of different stimuli that have been shown to induce caspase-dependent apoptosis in various cell types.[34–39] Unexpectedly, none of the known apoptogenic stimuli including agonistic anti-CD95 antibodies, alone or in combination with the protein synthesis inhibitor cycloheximide, the protein kinase inhibitor staurosporine or the anti-cancer drugs etoposide and mitomycin C, was able to elicit signals leading to caspase-8 activation in RBCs (Figure 2B,C). In addition, other proapoptotic conditions, such as ionizing irradiation, exposure to ceramide, prooxidants, TNFα or phorbolester and calcium ionophores, also failed to elicit activation of caspase-3 or caspase-8 in RBCs (Table 1).

In vitro activation of erythrocyte caspases

Because none of the stimuli tested triggered caspase activation in whole cells, we investigated whether erythrocyte caspases could be activated *in vitro*. To accomplish this, we incubated cytosolic extracts of RBCs and Jurkat cells with cytochrome *c* and dATP and then analyzed caspase processing by Western blot analysis. As shown in Figure 3A, in erythrocyte lysates cytochrome *c* and dATP were unable to trigger the processing of procaspase-3 and procaspase-8 to their proteolytically active fragments. Because cytochrome *c*-mediated caspase activation requires Apaf-1 and caspase-9,[40] these results are in line with the previous data showing that RBCs are devoid of both mediators. In contrast to erythrocytes, cytochrome *c* and dATP initiated the rapid processing of both caspases in

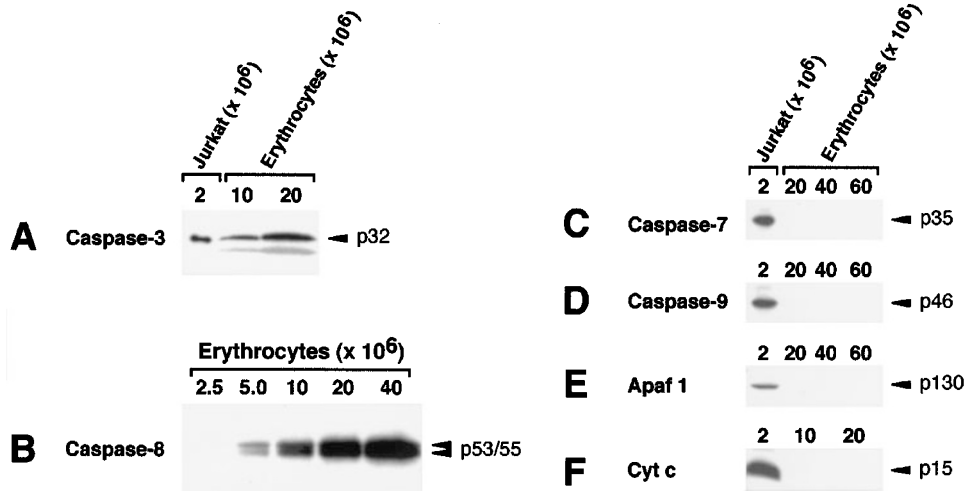

Figure 1 Human mature erythrocytes contain procaspase-3 and procaspase-8. Lysates obtained from the indicated numbers of freshly prepared RBCs or Jurkat cells were separated by SDS–PAGE and analyzed for the expression of caspase-3 (**A**) and caspase-8 (**B**) by immunoblotting. In addition, the expression of procaspase-7 (**C**) and -9 (**D**) as well as of Apaf-1 (**E**) and cytochrome *c* (**F**) was analyzed. In contrast to procaspase-3 and -8, these molecules were only detected in Jurkat cells but not in erythrocytes. The faint protein band detected in (**A**) represents an unspecific protein that comigrates with hemoglobin. The arrowheads indicate the position of procaspase-3, the two different isoforms of procaspase-8, caspase-8/a and -8/b, and the other full-length proteins

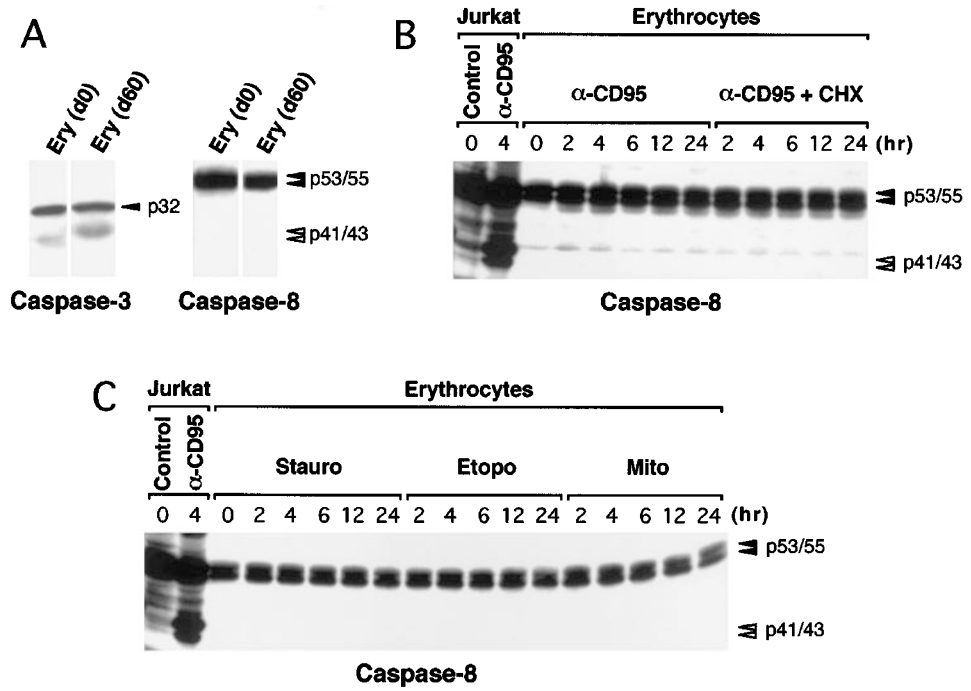

Figure 2 Erythrocyte caspases are activated neither during prolonged storage nor in response to various proapoptotic stimuli. (**A**) Cell lysates from freshly prepared erythrocytes (Ery) or erythrocytes stored for 60 days were subjected to immunoblotting with anti-caspase-3 (left panel) and anti-caspase-8 antibodies (right panel). In both lysates only the proforms of the two caspases were detected. (**B**) Effect of anti-CD95: Erythrocytes were treated for the indicated times with anti-CD95 (α-CD95; 1 μg/ml) in the presence or absence of cycloheximide (CHX; 10 μg/ml). (**C**) Effect of staurosporine and anti-cancer drugs. RBCs were treated for the indicated time with staurosporine (Stauro; 2.5 μM), etoposide (Etopo; 25 μg/ml) or mitomycin C (Mito; 25 μg/ml). As a positive control, lysates from untreated Jurkat cells (Control) or from cells incubated for 4 h with anti-CD95 (α-CD95; 1 μg/ml) were investigated. Following SDS–PAGE, the cleavage of caspase-8 was detected by immunoblot analysis. Filled arrowheads indicate the uncleaved and open arrowheads the cleaved forms of caspase-8

lysates from Jurkat cells (Figure 3B). Since in the mitochondrial cascade caspase-8 cleavage occurs downstream of caspase-3, caspase-8 cleavage was delayed in comparison to the processing of caspase-3.

Because caspases form a proteolytic network in which for instance caspase-6 can process and activate caspase-3 and -8,[40,41] we next examined whether procaspases present in erythrocyte lysates could be cleaved by the

Table 1 Effect of various apoptogenic stimuli on caspase activation in erythrocytes. 5×10^6 RBCs were stimulated with the different stimuli for the indicated time. Caspase processing was assessed by immunoblotting

Stimulus	Concentration	Time course	Caspase activation
Anti-CD95	1 μg/ml	2–24 h	none
Anti-CD95+CHX	1 μg/ml	2–24 h	none
	10 μg/ml		
TNFα	20 ng/ml	0.5–72 h	none
PMA	1–8 μM	2–4 h	none
Ionomycin	2–16 μM	2–4 h	none
PMA+Ionomycin	1–8 μM	2–4 h	none
	2–16 μM		
Etoposide	25 μg/ml	2–24 h	none
Mitomycin C	25 μg/ml	2–24 h	none
Fludarabine	1–1000 μM	2–24 h	none
Staurosporine	2.5 μM	2–24 h	none
γ-irradiation	5–40 Gy	16–48 h	none
Hydrogen peroxide	0.5–1 mM	1–5 h	none
C_2-Ceramide	50 μM	4–12 h	none

addition of exogenous caspases. To this end, we added recombinant caspase-6 to lysates from erythrocytes and Jurkat cells and then analyzed endogenous caspase processing by immunoblotting. As shown in Figure 3C, recombinant caspase-6 processed procaspase-8 in both lysates into the typical intermediate cleavage products of 41 and 43 kDa (lanes 3 and 6), which were identical with those observed in intact Jurkat cells after stimulation with anti-CD95 (lane 2). Procaspase-3 of RBCs and Jurkat cells was also cleaved after incubation with exogenous caspase-6, as indicated by the loss of its 32 kDa proform (Figure 3D). In both lysates the processing of endogenous procaspase-3 and -8 was inhibited by the broad-range caspase inhibitor zVAD-fmk.

To provide further evidence that caspases of RBCs can be activated *in vitro*, we measured the catalytic activity of caspase-8 with the fluorogenic substrate DEVD-AMC. This substrate is preferentially cleaved by caspase-3, but is also processed by caspase-8. Since the hemoglobin quenched the fluorescence signals from samples containing erythrocyte extract, caspase-8 was immunoprecipitated after the addition of exogenous caspase-6. Subsequently, the catalytic activity of caspase-8 was determined fluorometrically with DEVD-AMC. As shown in Figure 4A (lanes 2 and 5), DEVDase activity was rapidly induced in caspase-8 immunoprecipitates following incubation of erythrocyte and Jurkat cell lysates with active caspase-6. No caspase activity was observed when the lysates were either not treated with caspase-6 or incubated with zVAD-fmk. In addition, when the same samples were tested for caspase-8 processing by immunoblotting, it was found that DEVDase activity corresponded to the appearance of the characteristic cleavage products (Figure 4B). As a control, caspase-6 alone was not precipitated by the anti-caspase-8 antibody (Figure 4B, lane 7), and therefore did not contribute to any cleavage of DEVD-AMC.

Previously, it has been demonstrated that fodrin, a non-erythrocyte spectrin, is cleaved in nucleated cells by caspase-3.[42] To investigate whether erythrocyte spectrin, a structural protein important for RBC integrity, could also be cleaved, we incubated exogenous caspase-3 with erythrocyte lysates as well as with purified spectrin. Figure 5 shows that full-length spectrin present in erythrocyte lysates was cleaved by caspase-3 into the typical p120/150-fragments. This degradation could be blocked by the addition of zVAD-fmk (Figure 5, lane 7). Similar cleavage products were obtained with purified spectrin, although a 120 kDa immunoreactive protein, which probably represented a degradation product, was already visible in caspase-3 untreated samples. In summary, these experiments demonstrate that, although caspases remain inactive in erythrocytes treated with various proapoptotic stimuli, incubation of lysates with exogenous caspases *in vitro* leads to the activation of erythrocyte caspase-3 and -8 as well as to the degradation of spectrin.

Increase of intracellular calcium induces calpain but not caspase activation

Since neither classical proapoptotic stimuli nor storage of aged erythrocytes led to caspase activation, we investigated other conditions. An increase of intracellular calcium concentration has been reported to induce caspase activation in nucleated cells.[43,44] Treatment with calcium ionophores is also often used as a model for the study of erythrocyte senescence.[45–49] Indeed, incubation with ionomycin rapidly induced cell shrinkage and morphological changes from a normal discocyte to a spherocyte shape of RBCs (Figure 6A). Moreover, erythrocytes rapidly displayed phosphatidylserine exposure as assessed by flow cytometric staining with annexin-V (Figure 6B). These events occurred in less than 60 min, but required more than 5 h to occur in apoptotic Jurkat cells (data not shown). In response to ionomycin, erythrocytes furthermore rapidly activated the calcium-dependent cysteine protease calpain as detected by the proteolytic processing of μ-calpain (Figure 7A). Cleavage of μ-calpain to its active p78- and p76-fragments occurred as early as 10 min after stimulation and was completely blocked by the addition of calpain inhibitors. In contrast to calpain, no processing of caspase-3 (data not shown) or caspase-8 (Figure 7A) occurred in response to ionomycin treatment.

The rapid activation of μ-calpain preceded the degradation of erythrocyte spectrin which occurred 40 min after

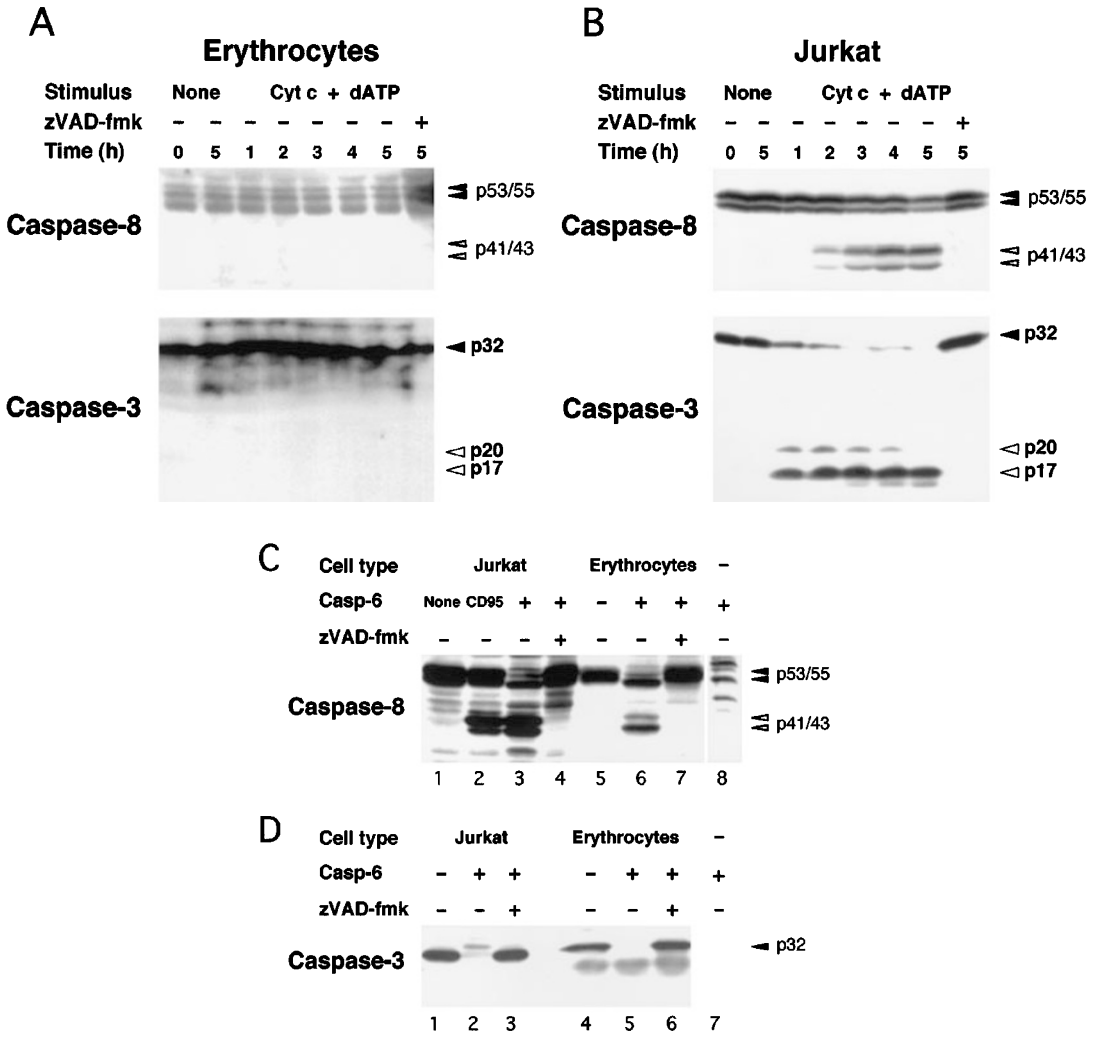

Figure 3 *In vitro* activation of erythrocyte caspases. (**A**) Failure of exogenous cytochrome *c* and dATP to initiate caspase activation in cytosolic extracts from RBCs. Erythrocyte extracts were either left untreated or treated for the indicated time with cytochrome *c* (Cyt c, 1 μM) and dATP (1 mM) in the absence and presence of zVAD-fmk, and then analyzed for caspase-3 and caspase-8 processing by immunoblot analysis. (**B**) Cytosolic extracts from Jurkat cells, which were taken as a positive control, were treated in a similar way. (**C**, **D**) Exogenous caspase-6 is able to process erythrocyte procaspase-3 and -8 *in vitro*. Recombinant caspase-6 was added to lysates of erythrocytes or Jurkat cells and incubated for 3 h at 37°C. Where indicated 100 μM of the caspase inhibitor zVAD-fmk was added prior to treatment with caspase-6. Cellular proteins were separated by SDS–PAGE and the processing of procaspase-8 (**C**) and procaspase-3 (**D**) was detected by immunoblotting. As a control, caspase-8 cleavage in intact cells is shown in (**C**). Here, Jurkat cells were treated with medium (**C**, lane 1) or 1 μg/ml of anti-CD95 (**C**, lane 2). After 3 h the cellular lysates were directly subjected to SDS–PAGE. To demonstrate the specificity of the antibodies, caspase-6 was applied to SDS–PAGE in the absence of cellular lysates (**C**, lane 8; **D**, lane 7). Filled arrowheads indicate the uncleaved and open arrowheads the cleaved form of the respective caspases. In (**C**) the uncleaved isoforms of procaspase-8/a and -8/b (p53/p55) and their intermediate cleavage products (p41/43) are shown

treatment with calcium ionophores and which could be completely prevented by calpain inhibitors (Figure 7A). To address the specificity of calpains in the degradation of erythrocyte spectrin, we further performed an *in vitro* assay in which purified spectrin from erythrocytes was incubated with active μ-calpain. As shown in Figure 7B, μ-calpain was able to degrade spectrin in a calcium-dependent manner and this degradation was prevented by calpain inhibitors. In addition to spectrin, calpain inhibitors prevented also the cell shrinkage of erythrocytes as assessed by forward scatter FACS analysis, whereas phosphatidylserine exposure remained unaffected (Figure 7C). Thus, these data indicate that calpains but not caspases are involved in calcium-mediated degradation of spectrin and alterations such as cell shrinkage of erythrocytes.

Discussion

The purpose of this study was to examine the presence of components of the death machinery in mature red blood cells. Recently, it has been suggested that apoptosis may represent a control mechanism in the regulation of erythropoiesis. These events include the activation of death receptors, which trigger subsequent caspase activation and cell death of erythroid progenitor cells.[32,33] Furthermore, erythropoietin, an essential mediator of erythropoiesis, is able to protect early

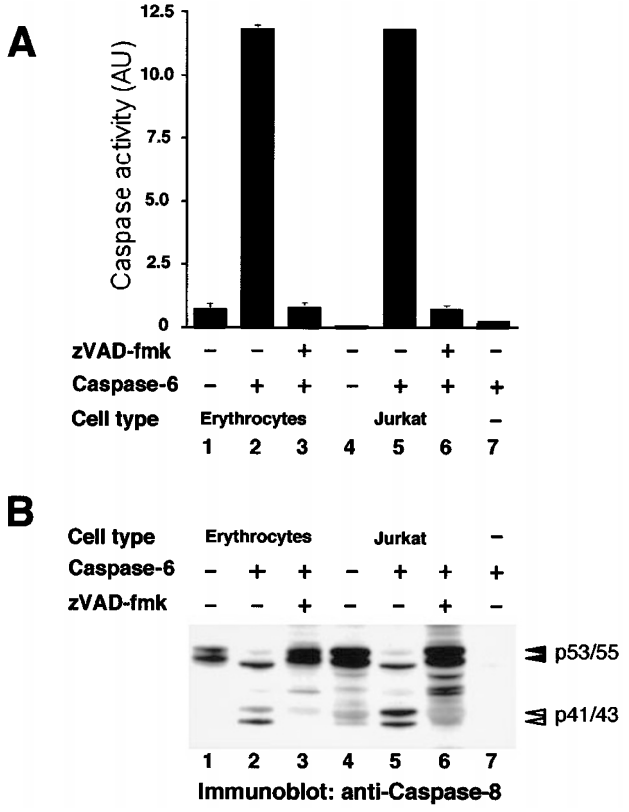

Figure 4 Proteolytically processed procaspase-8 from erythrocytes acquires catalytic activity *in vitro*. Lysates from erythrocytes or Jurkat cells were incubated for 3 h *in vitro* with 2 μg recombinant caspase-6 in the presence or absence of zVAD-fmk, before caspase-8 was immunoprecipitated. As a specificity control the reaction mixture containing caspase-6 was subjected to immunoprecipitation with anti-caspase-8 in the absence of cellular lysates (**A, B**; lanes 7). (**A**) The catalytic activity of precipitated caspase-8 was detected by cleavage of the fluorogenic substrate DEVD-AMC and is given in arbitrary units (AU). (**B**) In parallel, the processing of precipitated caspase-8 was detected by immunoblotting. Filled arrowheads indicate the uncleaved and open arrowheads the cleaved form of caspase-8

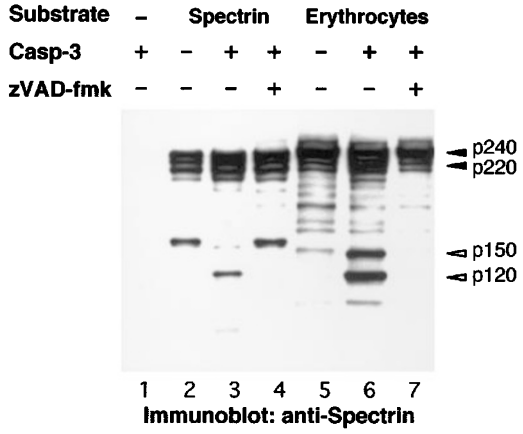

Figure 5 Caspase-3 cleaves purified spectrin and spectrin in lysates from erythrocytes. Purified spectrin (6 μg) or total cellular lysates of RBCs (10×10^6) were incubated with 2 μg of recombinant caspase-3 in the presence or absence of zVAD-fmk for 4 h at 37°C. Subsequently, proteins were resolved by SDS–PAGE and the cleavage of spectrin was determined by immunoblotting. Filled arrowheads indicate the uncleaved and open arrowheads the cleaved forms of spectrin

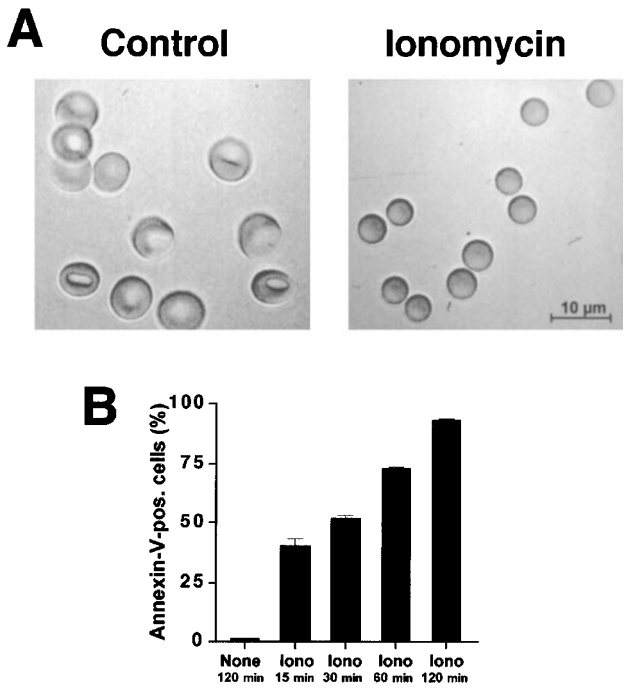

Figure 6 Increase of cellular calcium induces senescence-like morphological alterations in erythrocytes. (**A**) Treatment of erythrocytes with ionomycin induces a transition from the discocyte to a spherocyte shape. Erythrocytes were either left untreated or treated with 8 μM ionomycin. After 1 h cells were analyzed under an inverse microscope for changes in morphology. Note that after treatment with ionomycin RBCs lose the typical discocyte architecture and decrease in size. (**B**) Exposure of phosphatidylserine. RBCs were stimulated for the indicated time with ionomycin and analyzed for phosphatidylserine externalization by flow cytometric staining with annexin-V-FITC

and late erythroblasts from apoptosis through induction of Bcl-x_L, an anti-apoptotic Bcl-2 member.[28–31] In contrast to erythroid progenitor cells, in mature RBCs it is completely unknown whether apoptosis plays a functional role and whether RBCs contain a classical apoptotic machinery. Human erythrocytes comprise a minimal cellular system that is devoid of organelles including a cell nucleus, mitochondria and endoplasmic reticulum. In nucleated cells these organelles are crucial elements in the induction and execution phase of apoptosis. Therefore, it was interesting to investigate whether mature RBCs still possess the potential to activate the apoptotic machinery. Another aspect was to analyze whether caspases or other proteases participate in the degradation of the erythrocyte cytoskeleton and other alterations and thus could be involved in aging or other changes of RBCs.

In the present study, we observed that human mature RBCs indeed express caspase-8, the major initiator caspase of the death receptor pathway, as well as caspase-3, a central executioner caspase. In contrast, RBCs were completely deficient of essential components of the mitochondrial caspase cascade including Apaf-1, cytochrome *c* and caspase-9. Since erythrocytes lose mitochondria and other organelles, it is possible that they concomitantly lose mitochondria-associated components of the apoptotic machinery.

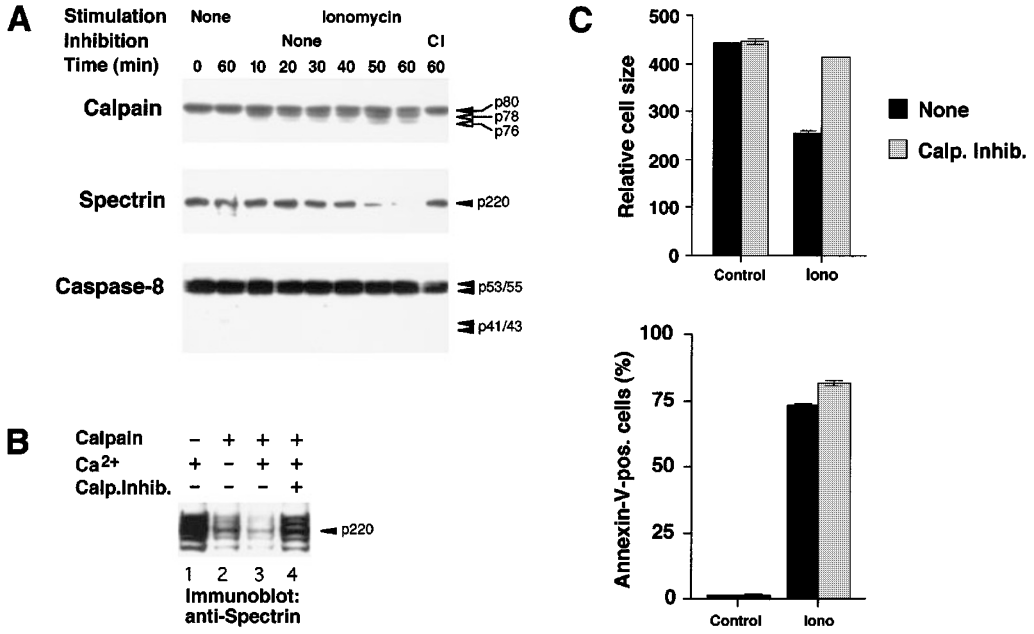

Figure 7 Calpain mediates spectrin degradation and cellular shrinkage of erythrocytes. (**A**) Effects of ionomycin on calpain and caspase processing and spectrin cleavage. RBCs were incubated for the indicated time in the absence (lanes 1, 2) or presence of ionomycin (lanes 3–9) and then subjected to immunoblotting. Where indicated, cells were pretreated for 30 min with calpain inhibitors (CI). The upper panel shows the proteolytic activation of μ-calpain indicated by the processing of its 80 kDa proform (filled arrowhead) to the characteristic p78 and p76 fragments (open arrowheads). The middle panel shows a section of the spectrin immunoblot indicating the degradation of the p220 full-length form of spectrin. In contrast, ionomycin did not induce the proteolytic processing of caspases as shown for caspase-8 (lower panel). (**B**) Degradation of spectrin *in vitro*. Purified erythrocyte spectrin (12 μg per sample) was incubated with 2 μg purified μ-calpain (lanes 2–4) in the absence or presence of calpain inhibitor or 1 mM calcium. After 1 h of incubation at 37°C aliquots of the samples were subjected to immunoblotting with anti-spectrin antibodies. (**C**) Calpain mediates the reduction of cell size, but not phosphatidylserine exposure in response to ionomycin treatment. RBCs were either left untreated (black bars) or pretreated for 30 min with calpain inhibitors (grey bars) and then further incubated in the presence or absence of ionomycin. After 1 h the relative cell size and phosphatidylserine exposure were measured by FACS analysis using the forward scatter profile and staining with annexin-V-FITC, respectively

It is conceivable that caspase-8 and -3 could play a role in erythrocyte dysfunctions including aging, anemias or upon malaria infection with *Plasmodium falciparum*. Strikingly, however, we did not find any activation of caspases either during prolonged storage of erythrocytes, in hemolytic anemia or in response to treatment with a wide variety of established apoptotic stimuli. In erythroid progenitor cells both the CD95 receptor and its ligand are expressed.[32] However, ligation of CD95 did not induce caspase activation in mature RBCs, a finding that might be explained by a loss of death receptors. Indeed, we did not find considerable surface expression of CD95 (data not shown). Since none of the tested proapoptotic conditions induced any activation of caspases, we investigated whether this refractoriness might be due to the expression of endogenous caspase inhibitors. However, we observed neither detectable levels of XIAP, c-IAP1 and c-IAP2 nor any expression of Bcl-2 (data not shown).

Since none of the established apoptotic stimuli could induce caspase activation in whole RBCs, we investigated whether caspases could be activated *in vitro*. Addition of cytochrome *c* and dATP, which induces caspase activation in nucleated cells, completely failed to induce caspase activity in RBCs, which is presumably due to a lack of caspase-9 and Apaf-1. However, caspases could be activated by exogenous caspase-6, suggesting that RBCs principally contain a functional caspase network. It is remarkable that even in chicken erythrocytes that unlike mammalian RBCs contain a cell nucleus, staurosporine can induce apoptosis. However, due to the lack of any effect of caspase inhibitors this cell death is also presumably caspase-independent.[50] Using size exclusion chromatography, so far we have not found a considerable caspase-inhibitory activity in erythrocyte extracts. Thus, whether the failure of erythrocytes to trigger caspase activation is due to the presence of endogenous inhibitors or simply due to the lack of Apaf-1/caspase-9 and cytochrome *c* will have to be addressed in future studies.

During aging, erythrocytes exhibit cell shrinkage, cytoskeletal degradation, plasma membrane microvesiculation and phosphatidylserine externalization.[51] These morphological phenomena are also associated with caspase-dependent apoptosis in nucleated cells. Like in normal cells, also in RBCs the exposure of phosphatidylserine signals the removal of aged RBCs from the circulation by macrophages.[25,26] There is ample evidence in the literature that the morphological changes of aged erythrocytes are mediated by an altered calcium homeostasis.[45–49] Although caspases remained inactive in senescent RBCs or cells treated with calcium ionophores, activation of the cysteine protease calpain was readily induced in response to elevated calcium levels. The activation of calpain preceded the degradation of spectrin. Calpain activation was also involved in cell shrinkage of erythrocytes, an

event that similarly to spectrin cleavage was prevented by calpain inhibitors. By contrast, calpain inhibitors did not affect phosphatidylserine exposure that is presumably a protease-independent event in erythrocytes. It is known that an increase in intracellular calcium is sufficient to disrupt the phospholipid asymmetry in platelets and erythrocytes by activating an aminophospholipid scramblase and inactivating aminophospholipid translocase.[52]

Spectrin plays an important role for the integrity and typical discocyte form of RBCs.[53] Conversely, damage of spectrin and other cytoskeletal proteins has been implicated in the externalization of phosphatidylserine, membrane budding and the characteristic spherical shape of aging erythrocytes. It is interesting to note that calpains but not caspases are also involved in platelet activation which, similar to erythrocyte senescence, displays apoptosis-like morphological alterations including cell shrinkage, microvesiculation and phosphatidylserine exposure.[20] Thus, like platelet activation, aging of erythrocytes may represent a non-apoptotic form of cell death that involves neither caspases nor other classical proapoptotic molecules. Interestingly, unlike erythrocytes, platelets do contain Apaf-1, cytochrome *c* and a functional mitochondrial caspase cascade.

Uncontrolled calpain activity has been observed in several pathological conditions and in many instances of cell death, such as apoptosis of hepatocytes in response to hypoxia and irradiation- and dexamethasone-induced death of thymocytes.[54,55] Furthermore, in erythrocytes of aged and hypertensive individuals the calpain inhibitor calpastatin is partially degraded and diminished resulting in increased calpain activity.[56,57] Interestingly, there are several potential crosstalks between calpain and caspases. Both types of proteases do not only have several common substrates but may also modulate each other's activity. It has been proposed that an increase in calpain activity may be caused by caspase-mediated degradation of calpastatin.[58,59] In other models, it has been found that an upstream calpain activity is required to activate caspases such as caspase-7.[17,18] Finally, in contrast to these studies, very recently it has been found that calpains can also directly cleave caspases to generate a proteolytically inactive caspase fragment.[20–22] In erythrocytes, however, we did not detect any interference between caspases and calpains.

In summary, we demonstrate that human RBCs contain functional proapoptotic caspases but do not seem to have a functional cell death system. What role caspases might play in erythrocyte biology remains unknown. Because erythroid progenitor cells can undergo apoptosis, caspases might simply have been transferred to mature erythrocytes during development. Alternatively, caspases may play a role in erythrocyte production from nucleated precursor cells. Interestingly, while this study was under review, a report appeared suggesting that caspases are required for terminal erythroid differentiation.[60] The authors showed that caspase inhibitors arrest erythroid maturation and, in addition, that caspases are transiently activated and cleave selective proteins during erythroblast differentiation without inducing cell death.

Material and Methods

Cells and reagents

Human blood samples from healthy donors were prepared in EDTA. After centrifugation and removal of plasma, leukocytes and the upper 10% of RBCs, the remaining erythrocytes were washed twice in RPMI-1640 containing 0.4 mM $CaCl_2$ and incubated in culture medium. The human leukemic T-cell line Jurkat was maintained in RPMI-1640 supplemented with 10% FCS, 10 mM HEPES, 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (all from Gibco BRL, Karlsruhe, Germany). The broad-range caspase inhibitor benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Enzyme Systems (Dublin, CA, USA). The chemotherapeutic drugs etoposide and mitomycin C were obtained from the clinical pharmacy (Medical Clinics, Tübingen, Germany). TNFα was obtained from Knoll AG (Ludwigshafen, Germany), cycloheximide (CHX) from Roth (Karlsruhe, Germany), and ionomycin and C_2-ceramide from Calbiochem (Bad Soden, Germany). Fludarabine, phorbolmyristyl acetate (PMA), purified human erythrocyte α/β-spectrin and μ-calpain were from Sigma (Deisenhofen, Germany). Staurosporine and calpain inhibitor-I (N-acetyl-Leu-Leu-norleucinal) and inhibitor-II (N-acetyl-Leu-Leu-methional) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). The agonistic anti-CD95 antibody was obtained from BioCheck (Münster, Germany).

Expression of recombinant caspases

Caspase-3 and caspase-6 were expressed as histidine fusion proteins in *E. coli* BL21. After induction of protein expression with IPTG at room temperature, caspases were purified to near homogeneity on a nickel-chelate affinity resin (His-Bind™-resin, Calbiochem) according to the manufacturer's instructions. The purified fractions were dialysed in 40 mM Tris-HCl pH 7.9, 500 mM NaCl and examined for homogeneity by SDS-polyacrylamide gel electrophoresis (SDS–PAGE). The activity of the caspases was monitored in a fluorimetric assay using the synthetic substrates N-acetyl-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) and N-acetyl-Val-Glu-Ile-Asp-AMC (Ac-VEID-AMC, both from Biomol, Plymouth, PA, USA) as described below.

Cell extracts and immunoblotting

Cleavage of caspases, calpain and spectrin was detected by immunoblotting. Cells were treated with the respective stimuli, and after the indicated times they were washed in cold PBS and extracted in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 3 μg/ml aprotinin, 3 μg/ml leupeptin, 3 μg/ml pepstatin A and 2 mM phenylmethylsulfonylfluoride (PMSF). Subsequently, proteins were separated under reducing conditions by SDS–PAGE and electroblotted onto a polyvinylidene difluoride membrane (Amersham, Freiburg, Germany). Membranes were blocked for 1 h with 5% non-fat dry milk powder in TBS and then immunoblotted for 1 h with mouse monoclonal antibodies directed against caspase-8 (BioCheck), caspase-3 (Transduction Laboratory, Heidelberg, Germany), μ-calpain (Sigma), and spectrin (Sigma). Membranes were washed four times with TBS/0.02% Triton X-100 and incubated with respective peroxidase-conjugated affinity-purified secondary antibody for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using ECL reagents (Amersham).

Protease and cell-free apoptotic assays

For determination of *in vitro* cleavage of caspases and spectrin, 50×10^6 erythrocytes or 2×10^6 Jurkat cells were lysed in 60 μl lysis buffer in the absence or presence of zVAD-fmk. Subsequently, 2 μg of active caspase-3 or caspase-6 were added and the reaction mixture incubated at 37°C for 3 h under shaking conditions. *In vitro* cleavage of purified spectrin was performed in a similar way by adding 2 μg of purified caspase-3 or caspase-6 to 6 μg of purified erythrocyte α/β-spectrin. For *in vitro* cleavage of α/β-spectrin the incubation was performed for 1 h with 2 μg of purified active μ-calpain. When indicated, samples were preincubated with calpain inhibitor-I and -II for 30 min. The cleavage of spectrin was determined by immunoblotting.

To evaluate the ability of cytochrome *c* to activate different caspases in cytosolic extracts, cells were washed twice with PBS, resuspended in buffer A containing 20 mM HEPES, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF, and incubated on ice for 15 min. Cells were then homogenized with 15 strokes in a douncer and centrifuged at 16 000 $\times$ *g*, 4°C for 15 min. For initiating *in vitro* caspase activation, 1 mM dithiothreitol (DTT), 1 μM horse heart cytochrome *c* (Sigma) and 1 mM dATP were added to the supernatants and incubated at 37°C. After different times the reaction mixtures were loaded on a SDS polyacrylamide gel and analyzed for caspase-3 and caspase-8 cleavage as described above.

Immunoprecipitation and fluorimetric assay of caspase activity

For immunoprecipitation of caspase-8, 10^9 freshly prepared erythrocytes or 10^7 Jurkat cells were lysed in 100 μl of a buffer containing 1% NP-40, 20 mM HEPES pH 7.4, 84 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. When indicated, samples were preincubated with 100 μM zVAD-fmk before adding 2 μg of purified caspase-6. Lysates were incubated at 37°C under shaking conditions for 3 h. Subsequently, 2 μg anti-caspase-8 and 20 μl protein-G sepharose (Amersham) were added. As a control, reaction mixtures were incubated in the absence of cellular lysates. Immunoprecipitation was performed at 4°C overnight under shaking conditions. The beads were then washed three times in a buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 0.01% Triton X-100 and used for fluorimetric assays and Western blot analysis. To determine caspase-8 activity, 5 μl of the beads were incubated with 50 μM of the fluorogenic substrate DEVD-AMC in 200 μl of a buffer containing 50 mM HEPES pH 7.4, 10% sucrose, 100 mM NaCl, 0.1% 2-[cyclohexylamino-]1-propanesulfonic acid (CHAPS; Sigma) and 1 mM DTT. The release of aminomethylcoumarin was measured in a fluorometer with an excitation wavelength of 360 nm and an emission wavelength of 475 nm. The remaining 15 μl beads were boiled in Laemmli buffer under reducing conditions and analyzed for caspase-8 cleavage by immunoblotting.

Morphological studies and annexin V-FITC-staining

Freshly prepared erythrocytes (2×10^6 per sample) were equilibrated for 2 h at 37°C in RPMI-1640 containing 0.4 mM $CaCl_2$. Prior to stimulation with 8 μM ionomycin, some samples were preincubated for 30 min in a mixture of calpain inhibitor-I (68 μg/ml) and -II (28 μg/ml). Erythrocyte morphology was examined in an inverse microscope (Axiovert 135, Zeiss, Jena, Germany). Phosphatidylserine externalization was visualized by staining with annexin-V-FITC following the manufacturer's protocol (Roche Molecular Biochemicals) and subsequent flow cytometry using the FL1 profile. The relative cell size was measured using the forward scatter profile. All flow cytometric analyses were performed on a FACScalibur (Becton Dickinson, Heidelberg, Germany) using CellQuest analysis software.

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References

1. Arends MJ, Morris RG and Wyllie AH (1990) Apoptosis. The role of the endonuclease. Am. J. Pathol. 136: 593 – 608
2. Rathmell JC and Thompson CB (1999) The central effectors of cell death in the immune system. Annu. Rev. Immunol. 17: 781 – 828
3. Cohen GM (1997) Caspases: the executioners of apoptosis. Biochem. J. 326: 1 – 16
4. Los M, Wesselborg S and Schulze-Osthoff K (1999) The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. Immunity 10: 629 – 639
5. Stroh C and Schulze-Osthoff K (1998) Death by a thousand cuts: an ever increasing list of caspase substrates. Cell Death Differ. 5: 997 – 1000
6. Muzio M, Chinnaiyan AM, Kishkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME and Dixit VM (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 85: 817 – 827
7. Boldin MP, Goncharov TM, Goltsev YV and Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85: 803 – 815
8. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP and Wang X (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275: 1129 – 1132
9. Kluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275: 1132 – 1136
10. Zou H, Henzel WJ, Liu X, Lutschg A and Wang X (1997) Apaf-1, a human protein, homologous to C. elegans Ced-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90: 405 – 413
11. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell 91: 479 – 489
12. Srinivasula SM, Ahmad M, Fernandes-Alnemri T and Alnemri ES (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol. Cell. 1: 949 – 957
13. Sorimachi H, Ishiura S and Suzuki K (1997) Structure and physiological function of calpains. Biochem. J. 328: 721 – 732
14. Carafoli E and Molinari M (1998) Calpain: a protease in search of a function? Biochem. Biophys. Res. Commun. 247: 193 – 203
15. Chan SL and Mattson MP (1999) Caspase and calpain substrates: roles in synaptic plasticity and cell death. J. Neurosci. Res. 58: 167 – 190
16. Wang KKW (2000) Calpain and caspase: can you tell the difference? Trends Neurosci. 23: 20 – 26
17. Waterhouse NJ, Finucane DM, Green DR, Elce JS, Kumar S, Alnemri ES, Litwack G, Khanna K, Lavin MF and Watters DJ (1998) Calpain activation is upstream of caspases in radiation-induced apoptosis. Cell Death Differ. 5: 1051 – 1061

18. Ruiz-Vela A, Gonzalez de Buitrago G and Martinez AC (1999) Implication of calpain in caspase activation during B cell clonal deletion. EMBO J. 18: 4988 – 4998
19. Wood DE and Newcomb EW (1999) Caspase-dependent activation of calpain during drug-induced apoptosis. J. Biol. Chem. 274: 8309 – 8315
20. Wolf BB, Goldstein JC, Stennicke HR, Beere H, Amarante-Mendes GP, Salvesen GS and Green DR (1999) Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. Blood 94: 1683 – 1692
21. Chua BT, Guo K and Li P (2000) Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. J. Biol. Chem. 275: 5131 – 5135
22. Lankiewicz S, Luetjens MC, Bui NT, Krohn AJ, Poppe M, Cole GM, Saido TC and Prehn JH (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J. Biol. Chem. 275: 17064 – 17071
23. Waugh RE, Narla M, Jackson CW, Mueller TJ, Suzuki T and Dale GL (1992) Rheologic properties of senescent erythrocytes: loss of surface area and volume with red blood cell age. Blood 79: 1351 – 1358
24. Mohandas N and Groner W (1989) Cell membrane and volume changes during red cell development and aging. Ann. N. Y. Acad. Sci. 554: 217 – 224
25. Connor J, Pak CC and Schroit AJ (1994) Exposure of phosphatidylserine in the outer leaflet of human red blood cells. Relationship to cell density, cell age, and clearance by mononuclear cells. J. Biol. Chem. 269: 2399 – 2404
26. Bratosin D, Mazurier J, Tissier JP, Estaquier J, Huart JJ, Ameisen JC, Aminoff D and Montreuil J (1998) Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. Biochimie 80: 173 – 195
27. Boas FE, Forman L and Beutler E (1998) Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. Proc. Natl. Acad. Sci. USA 95: 3077 – 3081
28. Silva M, Grillot D, Benito A, Richard C, Nunez G and Fernandez-Luna JL (1996) Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-X_L and Bcl-2. Blood 88: 1576 – 1582
29. Gregori PA and Bondurant MC (1997) The roles of Bcl-X(L) and apopain in the control of erythropoiesis by erythropoietin. Blood 90: 630 – 640
30. Gregoli PA and Bondurant MC (1999) Function of caspases in regulating apoptosis caused by erythropoietin deprivation in erythroid progenitors. J. Cell Physiol. 178: 133 – 143
31. Motoyama N, Kimura T, Takahashi T, Watanabe T and Nakano T (1999) Bcl-x prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. J. Exp. Med. 189: 1691 – 1698
32. De Maria R, Testa U, Luchetti L, Zeuner A, Stassi G, Pelosi E, Riccioni R, Felli N, Samoggia P and Peschle C (1999) Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. Blood 93: 796 – 803
33. Zamai L, Secchiero P, Pierpaoli S, Bassini A, Papa S, Alnemri ES, Guidotti L, Vitale M and Zauli G (2000) TNF-related apoptosis-inducing ligand (TRAIL) as a negative regulator of normal human erythropoiesis. Blood 95: 3716 – 3724
34. Wesselborg S, Engels IH, Rossmann E, Los M and Schulze-Osthoff K (1999) Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction. Blood 93: 3053 – 3063
35. Herr I, Wilhelm D, Bohler T, Angel P and Debatin KM (1997) Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. EMBO J. 16: 6200 – 6208
36. Goldkorn T, Balaban N, Shannon M, Chea V, Matsukuma K, Gilchrist D, Wang H and Chan C (1998) H_2O_2 acts on cellular membranes to generate ceramide signaling and initiate apoptosis in tracheobronchial epithelial cells. J. Cell Sci. 111: 3209 – 3220
37. Ojcius DM, Zychlinsky A, Zheng LM and Young JD (1991) Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. Exp. Cell Res. 197: 43 – 49
38. Consoli U, El-Tounsi I, Sandoval A, Snell V, Kleine HD, Brown W, Robinson JR, DiRaimondo F, Plunkett W and Andreeff M (1998) Differential induction of apoptosis by fludarabine monophosphate in leukemic B and normal T cells in chronic lymphocytic leukemia. Blood 91: 1742 – 1748
39. Bertrand R, Solary E, O'Connor P, Kohn KW and Pommier Y (1994) Induction of a common pathway of apoptosis by staurosporine. Exp. Cell Res. 211: 314 – 321
40. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR and Martin SJ (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J. Cell Biol. 144: 281 – 292
41. Van de Craen M, Declercq W, Van den Brande I, Fiers W and Vandenabeele P (1999) The proteolytic procaspase activation network: an in vitro analysis. Cell Death Differ. 6: 1117 – 1124
42. Jänicke RU, Ng P, Sprengart ML and Porter AG (1998) Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. J. Biol. Chem. 273: 15540 – 15545
43. Takadera T and Ohyashiki T (1997) Apoptotic cell death and caspase 3 (CPP32) activation induced by calcium ionophore at low concentrations and their prevention by nerve growth factor in PC12 cells. Eur. J. Biochem. 249: 8 – 12
44. Nakamura K, Bossy-Wetzel E, Burns K, Fadel MP, Lozyk M, Goping IS, Opas M, Bleackley RC, Green DR and Michalak M (2000) Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. J. Cell Biol. 150: 731 – 740
45. Shiga T, Sekiya M, Maeda N, Kon K and Okazaki M (1985) Cell age-dependent changes in deformability and calcium accumulation of human erythrocytes. Biochim. Biophys. Acta 814: 289 – 299
46. Romero PJ and Romero EA (1999) Effect of cell ageing on Ca2+ influx into human red cells. Cell Calcium 26: 131 – 137
47. Allan D and Thomas P (1981) Ca^{2+}-induced biochemical changes in human erythrocytes and their relation to microvesiculation. Biochem. J. 198: 433 – 440
48. Allan D and Thomas P (1981) The effects of Ca^{2+} and Sr^{2+} on Ca^{2+}-sensitive biochemical changes in human erythrocytes and their membranes. Biochem. J. 198: 441 – 445
49. Henseleit U, Plasa G and Haest C (1990) Effects of divalent cations on lipid flip-flop in the human erythrocyte membrane. Biochim. Biophys. Acta 1029: 127 – 135
50. Weil M, Jacobson MD and Raff MC (1998) Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. J. Cell Sci. 111: 2707 – 2715
51. Danon D and Marikovsky Y (1988) The aging of the red blood cell. A multifactor process. Blood Cells 14: 7 – 18
52. Dolis D, Moreau C, Zachowski A and Devaux PF (1997) Aminophospholipid translocase and proteins involved in transmembrane phospholipid traffic. Biophys. Chem. 68: 221 – 231
53. Haest CW, Plasa G, Kamp D and Deuticke B (1978) Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane. Biochim. Biophys. Acta 509: 21 – 32
54. Bronk SF and Gores GJ (1993) pH-dependent nonlysosomal proteolysis contributes to lethal anoxic injury of rat hepatocytes. Am. J. Physiol. 264: G744 – 751
55. Squier MK, Miller AC, Malkinson AM and Cohen JJ (1994) Calpain activation in apoptosis. J. Cell Physiol. 159: 229 – 237
56. Glaser T, Schwarz-Benmeir N, Barnoy S, Barak S, Eshhar Z and Kosower NS (1994) Calpain (Ca(2+)-dependent thiol protease) in erythrocytes of young and old individuals. Proc. Natl. Acad. Sci. USA 91: 7879 – 7883
57. Salamino F, Sparatore B and De Tullio R (1991) The calpastatin defect in hypertension is possibly due to a specific degradation by calpain. Biochim. Biophys. Acta 1096: 265 – 269
58. Porn-Ares MI, Samali A and Orrenius S (1998) Cleavage of the calpain inhibitor, calpastatin, during apoptosis. Cell Death Differ. 5: 1028 – 1033
59. Squier MK, Sehnert AJ, Sellins KS, Malkinson AM, Takano E and Cohen JJ (1999) Calpain and calpastatin regulate neutrophil apoptosis. J. Cell Physiol. 178: 311 – 319
60. Zermati Y, Garrido C, Amsellem S, Fishelson S, Bouscary D, Valensi F, Varet B, Solary E and Hermine O (2001) Caspase activation is required for terminal erythroid differentiation. J. Exp. Med. 193: 247 – 254