

# **Role of apoptotic and anti-apoptotic mechanisms in erythroblast differentiation**

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## SUMMARY

The present study focuses on deciphering the differentiation mechanisms of erythroid cells. In particular, the involvement of apoptotic mechanisms in erythroblast differentiation and enucleation was examined.

A model system of erythroid differentiation was established. The primary culture of human erythroid cells fully recapitulated *in vivo* erythropoiesis as shown by cell morphology and cell surface markers. In addition this culture system was amenable for manipulation.

Using this culture system, caspase activation was found to be associated with and required for erythroblast differentiation. *In vitro* erythropoiesis was blocked by the pan-caspase inhibitor z-VAD.fmk as indicated by the accumulation of early progenitors with concomitant drop in the number of late erythroblasts. We also found that caspase-3 and caspase-9 were specifically activated during erythroid differentiation.

DNA fragmentation has emerged as a second apoptotic mechanism involved in erythropoiesis. DNA degradation occurs at late stages of differentiation as assessed by the detection of TUNEL-positive erythroblasts in cord blood, bone marrow and erythroid culture. The mechanism of DNA degradation was cell-autonomous and resulted in production of high molecular DNA fragments with approximate length of about 50 kb.

During these studies we made the rather unexpected observation that late stage erythroblast did not undergo apoptosis when treated with apoptosis inducers. This observation suggested to us that mature erythroblasts were therefore protected from cell death. We next examined the mechanisms involved in erythroblast protection. The levels of cytochrome c, a potent inducer engaging the mitochondrial pathway in apoptosis, were shown to significantly decrease with the differentiation. In addition, the levels of anti-apoptotic protein Bcl-xL increased, reaching

maxima just before enucleation. Altogether these mechanisms might contribute to the protection from apoptosis in mature erythroblasts, described here for the first time.

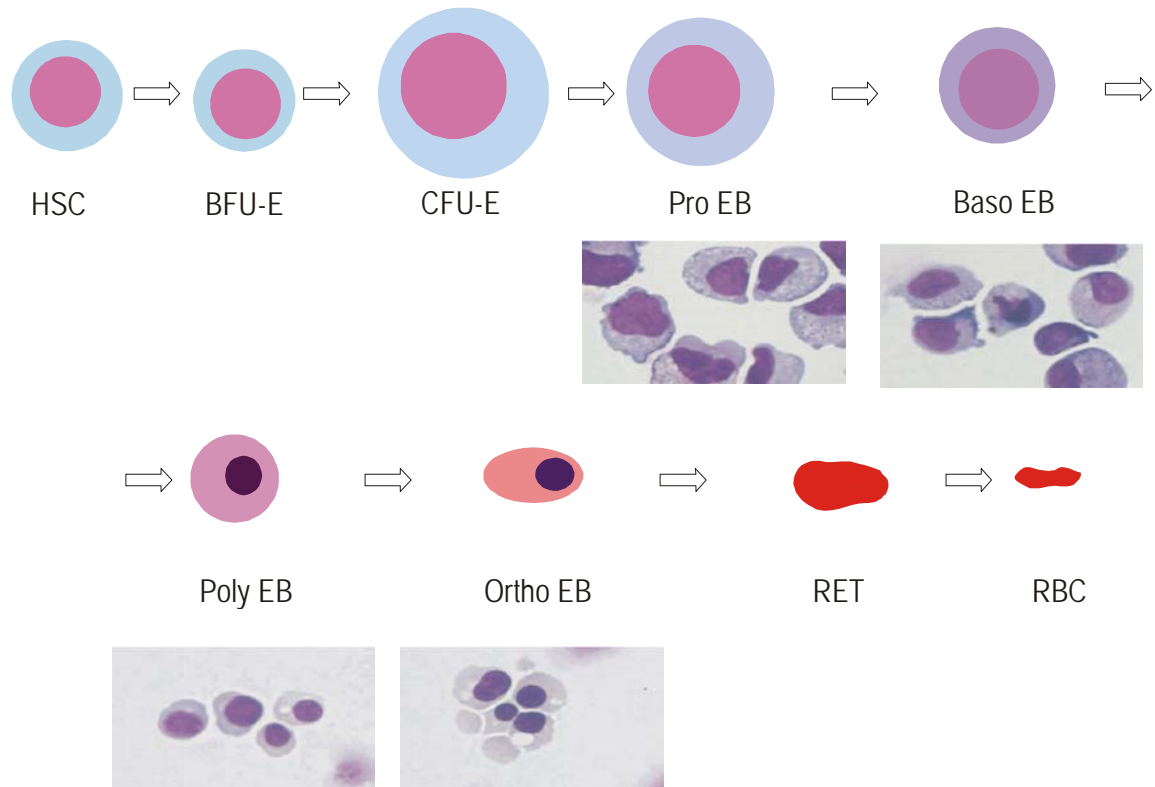


## INTRODUCTION

### 1. Red blood cell differentiation - short background

The production of red blood cells by a process called erythropoiesis, encompasses differentiation from hematopoietic stem cells to the mature enucleated erythrocytes. As stem cells progress through the stages of erythropoiesis, their potential to differentiate into lymphoid or other hematopoietic cell types is restricted and they increasingly commit to differentiation into erythrocytes [1] (refer to Fig.1). The earliest erythroid progenitor, the burst forming unit-erythroid (BFU-E) is a small highly proliferative cell. This cell later develops into colony forming unit-erythroid (CFU-E), which is a larger less proliferative cell, whose survival and differentiation into an erythroblast is highly dependent on erythropoietin. The differentiation of erythroblasts thereafter follows the sequential formation, from proerythroblasts, through basophilic and polychromatophilic erythroblasts to orthochromatic erythroblasts. Then, orthochromatic erythroblasts expulse their nucleus to become reticulocytes [2]. Following this reticulocytes cross the blood barrier to gain access to the blood stream where they mature into erythrocytes [1].

The primary sites of erythropoiesis also change in a temporally and spatially ordered fashion, from yolk sac, followed by the fetal liver and finally the bone marrow.



### Figure 1. Stages of erythroid differentiation

The relative size and the morphological appearance of erythroid cells at various stages of differentiation: pluripotent hematopoietic stem cell (HSC), burst forming unit-erythroid (BFU-E), colony forming unit-erythroid (CFU-E), proerythroblast (Pro EB), basophilic erythroblast (Baso EB), polychromatophilic erythroblast (Poly EB), orthochromatophilic erythroblast (Ortho EB), reticulocyte (RET) and red blood cell (RBC).

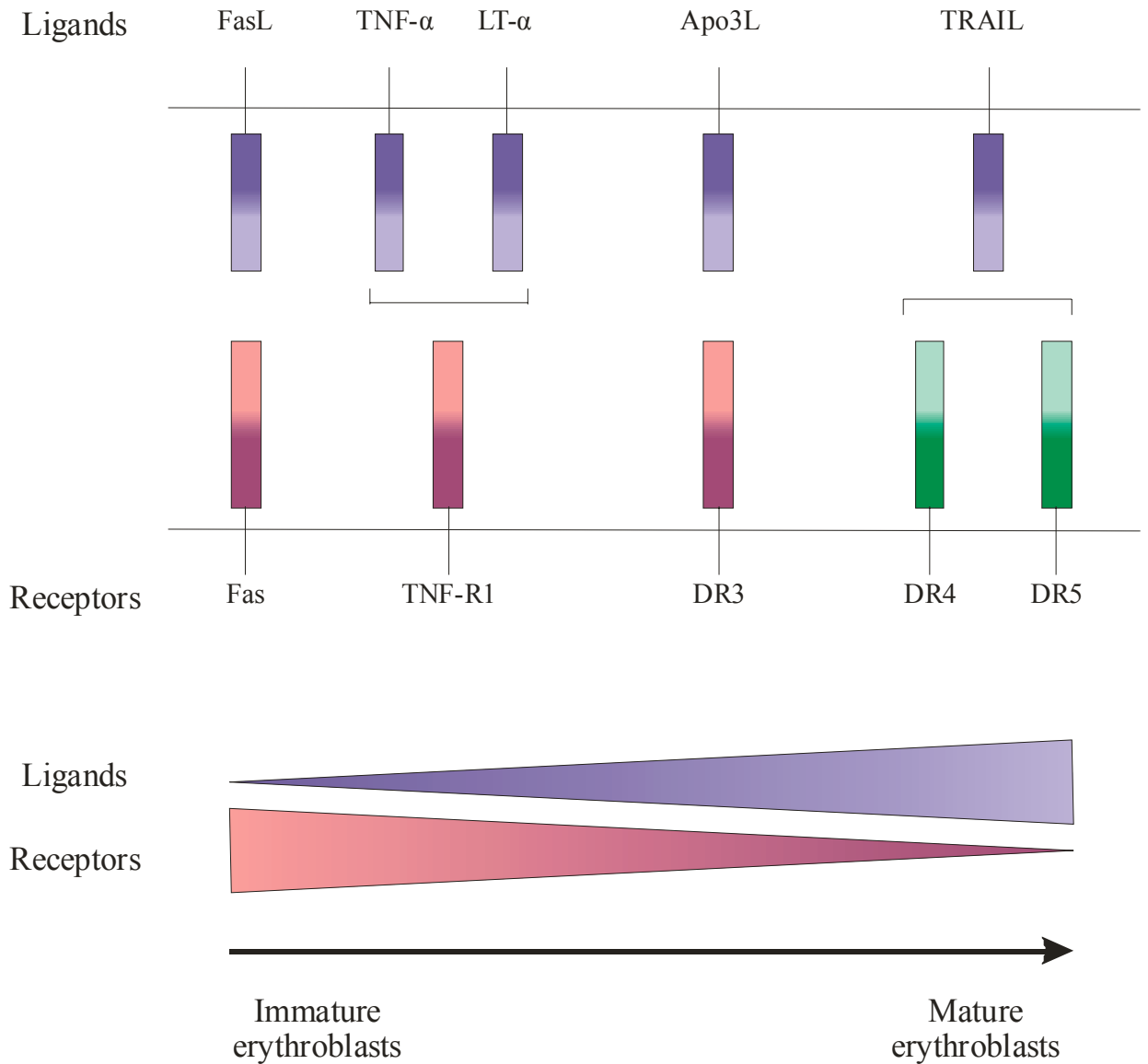
## **2. Apoptosis and red blood cell differentiation**

The morphology of erythroblasts changes dramatically during their differentiation (refer to Fig.1) [3]. Little is known about the molecular mechanisms of these changes, which include nuclear and chromatin condensation, loss of organelles, and finally enucleation. Since some of these morphological changes share similarities with features occurring during apoptosis, apoptotic mechanisms have been proposed to play a role in terminal erythropoiesis. In this introduction

part, the current knowledge about the key apoptotic components involved in erythroblast differentiation is summarized, and compared to those observed in apoptosis.

## **2.1 Death receptors and their ligands**

Death receptors are a family of cell surface molecules that belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily, defined by cystein-rich extracellular domain [4, 5], and a homologous cytoplasmic sequence termed the “death domain”[6]. Mammalian death receptors include Fas, TNF-R1, DR3, and the TRAIL receptors DR4 and DR5 [7] (refer to Fig.2 A). The ligands that activate these receptors are structurally related molecules that belong to the TNF gene superfamily [4, 5]. Fas ligand (FasL) binds to Fas; TNF- $\alpha$  and lymphotoxin- $\alpha$  bind to TNF-R1; Apo3 Ligand (Apo3L) binds to DR3; and TRAIL binds to DR4 and DR5 [7]. Molecular crosslinking of death receptors by their ligands typically results in triggering of apoptosis, but in some instances functions different from and even opposite to apoptosis have also been observed.



**Figure 2. Death receptors and their ligands**

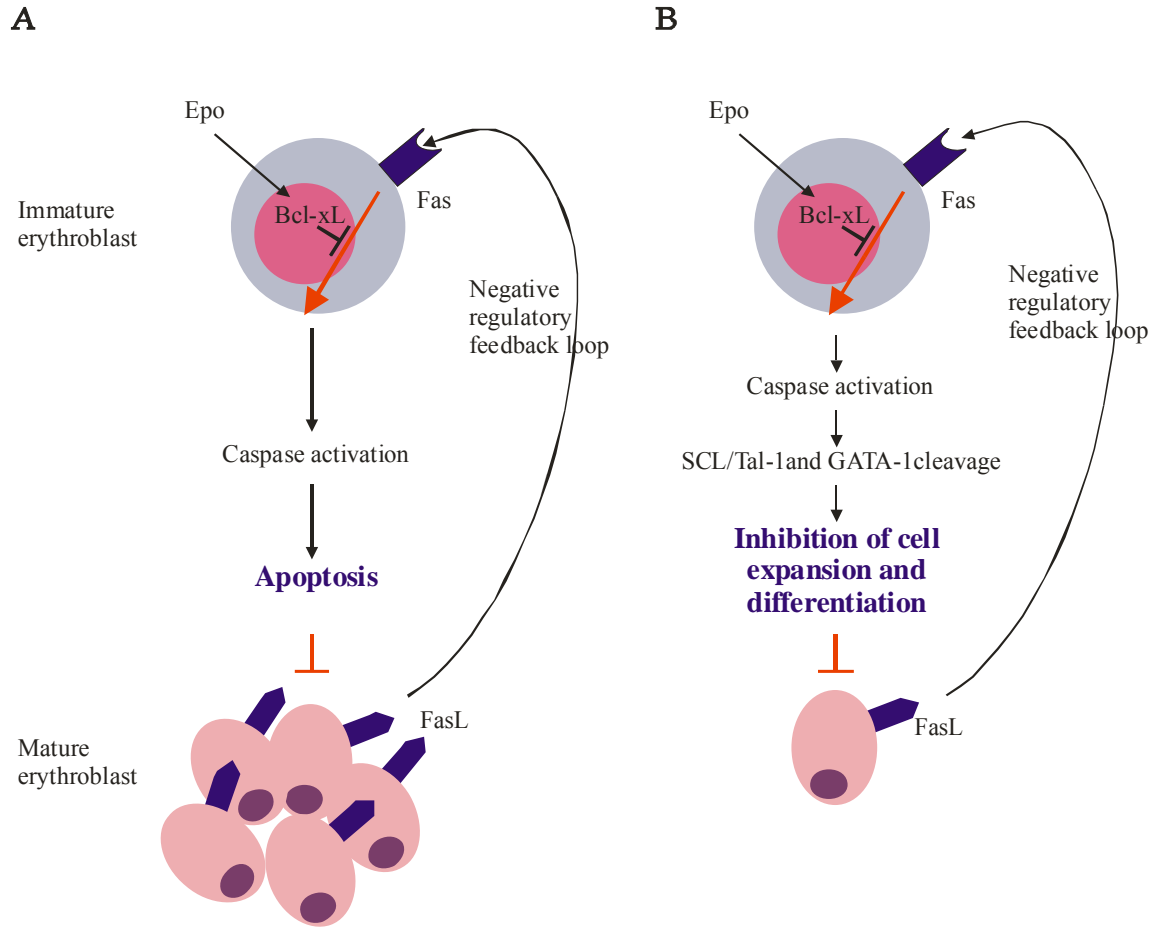
Upper panel - Specific death receptor - death ligand interactions. Lower panel - Differential expression of death receptors and ligands along the differentiation. Fas and TNF-R1 are expressed by immature erythroblasts whereas FasL and TRAIL are expressed by mature erythroblasts. DR4 and DR5 are constantly expressed along the differentiation.

It has been shown that erythroblasts express several members of the “death receptor” family as well as their ligands [8-11]. Moreover, differential expression of both receptors and ligands related to different maturation stages of erythroblasts has been reported [8, 9, 11] (refer to Fig.2 B). These studies have shown that, Fas and TNF-R1 are mainly expressed by immature erythroblast whereas DR4 and DR5 are constantly expressed along the differentiation. On the other hand, FasL and TRAIL, are expressed only by mature erythroblasts. Because of this differential expression it has been suggested that death receptors and their ligands are involved in the regulation of erythropoiesis.

Fas/FasL apoptotic pathway controls the homeostasis of red cell lineage by balancing survival and death of immature erythroblasts (i.e. Pro EB and Baso EB) [8]. Thus, massive FasL production following mature erythroblasts accumulation may act as a negative regulatory feedback loop by inducing apoptosis of immature erythroblasts (see Fig.3 A).

A second mechanism that involves Fas/FasL pathway but differs from apoptosis has also been described. It has been shown recently that in vitro stimulation of immature erythroblasts with low doses of an agonist anti-Fas antibody strongly inhibits erythroblast expansion and differentiation without inducing apoptosis [9, 12] (see Fig.3 B). Therefore, it has been proposed that Fas/FasL pathway triggers different effects in immature erythroblasts depending on both the FasL concentration and Epo concentration. High FasL concentrations induce apoptosis while high Epo concentration protects the cells from apoptosis and can reverse the effects of FasL. In a similar manner, TRAIL and TNF- $\alpha$  also display anti-differentiative effect on immature erythroblasts, indicating that other death receptor ligands may also contribute to the negative regulation of erythropoiesis [9-11]. These findings suggest that death receptors and their ligands mediate a regulatory feedback loop between mature and immature erythroblasts, whereby mature erythroblasts may induce apoptosis or alternatively inhibit expansion and differentiation in

immature erythroblasts. In physiological conditions, the accumulation in the erythroblastic island of mature erythroblasts may thus control the differentiation of immature erythroblasts and hence finely regulate red blood cell production.



**Figure 3. Caspase-mediated negative regulation of erythroblast differentiation**

A - Massive FasL production following mature erythroblast accumulation acts as a negative regulatory feedback loop by inducing apoptosis of immature erythroblasts

B - Low FasL production results in SCL/ Tal-1 and GATA-1 caspase-mediated cleavage thereby inhibiting erythroblast expansion and differentiation

## 2.2 Caspases

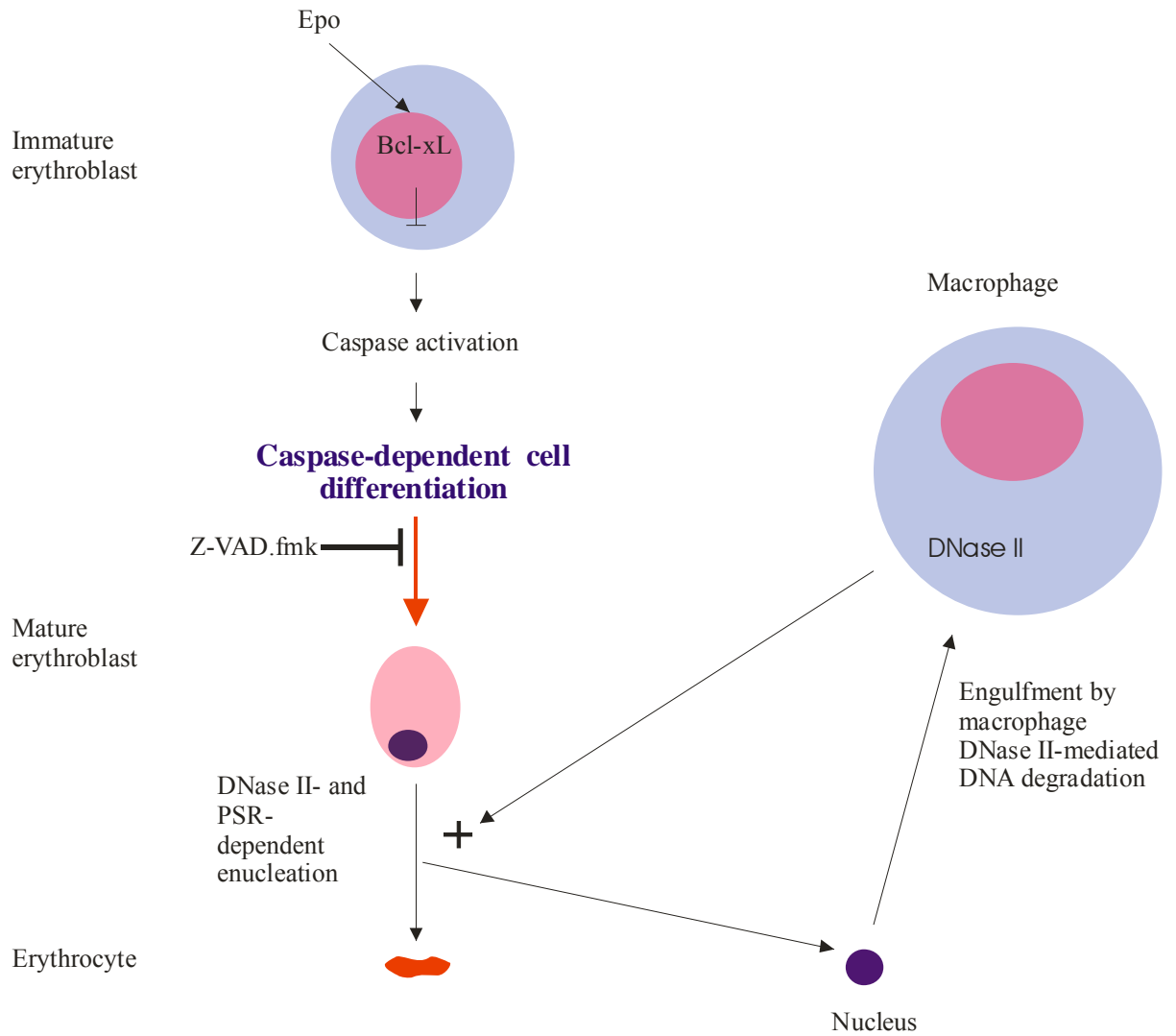
Destruction of key cellular functions by limited proteolysis is central to apoptosis. Members of the caspase family of cystein-containing aspartate-specific proteases play a pivotal role in this process by mediating the majority of proteolytic events. To date, 14 mammalian caspases have been identified, a subset of which participates in apoptosis, with the remainder likely to be involved in the processing of pro-inflammatory cytokines. Caspases participate in the execution of cell death by disabling essential homeostatic and repair processes and by cleaving key structural components. Their targets include cytoskeletal proteins and integrins, nuclear structural proteins, proteins associated with chromatin structure and function and not least caspases themselves which are activated in a proteolytic cascade [13].

It has been suggested that caspases are involved in negative regulation of erythroblast differentiation [8, 9, 12]. As described above, Fas and probably other death receptors negatively regulate erythropoiesis by two mechanisms: apoptosis and inhibition of cell expansion and differentiation [8, 9, 12]. As expected, Fas-induced apoptosis in immature erythroblasts is caspase-mediated and active caspase-3 has been detected in these cells [8] (refer to Fig. 3 A). However, the second mechanism differs from apoptosis in that caspases appear to be activated, but the cells do not die [9, 12]. It has been reported that Fas-induced differentiation arrest is caspase-mediated, as cleavage of caspase-3, caspase-7 and caspase-8 to their active forms has been observed upon Fas engagement. Moreover, active caspase forms are involved in the degradation of the major transcription factors GATA-1 and SCL/Tal-1 [9, 12] (refer to Fig. 3 B). Thus it is possible that, in physiological conditions, accumulation of mature erythroblasts expressing death receptor ligands such as Fas, may temporarily inhibit the expansion and differentiation of immature erythroblasts through caspase-mediated GATA-1 and SCL/Tal-1 down modulation. This pathway is noteworthy in that although the mechanism is caspase-

mediated, it differs from apoptosis as the cells do not die, but their expansion and differentiation are only temporarily inhibited. This, therefore, implies that a novel nonapoptotic role for caspases exists, that is they function to temporarily block cell proliferation and differentiation.

More surprising, recent evidence suggests that caspase activation positively regulate erythroid differentiation [14-16]. In this regard, it has been shown that in an in vitro culture broad spectrum caspase inhibitor (z-VAD.fmk) prevents erythroblasts from differentiating past the basophilic stage (refer to Fig. 4) [14, 15]. Furthermore, during normal erythroid differentiation, activated forms of caspases-3, -7, -6, -2 and -9 have been detected early in the differentiation process [14-16]. A striking feature is that this caspase activation is accompanied by the detection of cleaved nuclear proteins, such as acinus, PARP-1 and Lamin B [14, 15]. Thus, caspase-mediated cleavage of these proteins may account for the nuclear structural changes associated with the maturation of erythroblasts. It is therefore clear that erythroblast differentiation shares similarities with apoptosis, as manifested by caspase activation and subsequent nuclear proteins degradation. In contrast to apoptosis, however, during this positive regulation of erythropoiesis caspases are only transiently activated, and cleave selectively some target proteins, such as acinus and Lamin B, without cleaving other targets, such as GATA-1 and DFF45/ICAD, which are otherwise cleaved during both apoptosis and negative regulation of erythropoiesis.





**Figure 4. Caspase-mediated positive regulation of erythroblast differentiation**

The differentiation of erythroblasts includes a caspase-dependent step as shown by the detection of active forms of caspase-3, -7 and -9. Pan-caspase inhibitor Z-VAD.fmk blocks differentiation at the basophilic stage. Successful enucleation not only requires the involvement of DNase II expressed in macrophages, but also the contribution of phosphatidylserine receptor (PSR).

## 2.3 Mitochondria

Mitochondria, the power-houses of cells, play a crucial role during apoptosis, in that they act both by amplifying extracellular pro-apoptotic stimuli, such as those from death receptors, and reacting to internal distress signals, such as DNA damage or stress mediators. In this regard, mitochondrial membrane permeabilisation (MMP) leading to loss of the mitochondrial transmembrane potential is an important integrating step during the cell death pathway, defining a “point of no return” for the cell [17]. MMP culminates in the complete loss of the outer mitochondrial membrane integrity, thereby leading to the release of potentially apoptogenic proteins into the cytoplasm. Examples of such proteins include nucleases and proteases which act in a caspase-independent manner (Apoptosis inducing factor and Endonuclease G) as well as caspase activators (cytochrome c and Smac/DIABLO) [18]. MMP also disrupts the energy metabolism of the cell, thereby leading to the production of reactive oxygen species which cause further damage to the dying cell. MMP is an early, irreversible event in all cells undergoing apoptosis, irrespective of the apoptosis stimulus. MMP has also been suggested as a key event in the effector phase of apoptosis because it precedes and is sufficient to cause the characteristic changes associated with apoptosis in the nuclear compartment (chromatin condensation, DNA fragmentation), the cytoplasm (cellular condensation, caspase-3 activation) and the plasma membrane (phosphatidylserine exposure) [19].

Since chromatin condensation and caspase-3 activation were observed in differentiating erythroblasts, it appears likely that MMP could be involved in the differentiation process. Evidence supporting this has been obtained from the studies in which erythroblasts were incubated with the potential sensitive dyes DiOC6 and JC-1 [14]. These studies also suggested that MMP is differentiation-dependent, occurring only in late erythroblasts whereas this phenomenon was absent in early erythroblasts. A key difference to apoptosis is that in the

differentiating erythroblasts other apoptotic features such as phosphatidylserine externalization and DNA fragmentation were not observed, although MMP in these cells was associated with caspase activation. Therefore, it has been suggested that MMP is involved in a pathway leading to caspase activation during erythroid differentiation. In contrast to the MMP involvement in apoptosis, there is no evidence so far that mitochondrial pro-apoptotic proteins are also involved in differentiation. It does, however, appear likely that they would be released in the cytoplasm following MMP and could subsequently lead to caspase activation and chromatin condensation, features observed in differentiating erythroblast.

## **2.4 The Bcl-2 family**

Because of its importance in the decision making of a cell, MMP is tightly regulated by a special group of regulatory proteins, namely the family of Bcl-2-related proteins. These proteins act upstream of the caspase cascade and can be divided into two groups: those which inhibit apoptosis (e.g. Bcl-2, Bcl-X<sub>L</sub>) or those which promote it (e.g. Bax, Bak, Bcl-X<sub>S</sub>) [20, 21]. The decision of whether a cell will undergo apoptosis in response to a given stimulus is determined by the ratio between pro- and anti-apoptotic members.

Significant progress has been made in understanding the involvement of the Bcl-2 family members during erythropoiesis. It has been reported that the expression of Bcl-X<sub>L</sub> is greatly increased in mature erythroblasts (Ortho EB), reaching level about 50 fold greater than that in immature erythroblasts (Pro EB) [22]. Further evidence supporting the action of Bcl-xL in erythroblast differentiation is provided by knock out mice having a conditional deletion of the Bcl-x gene. These mice become anemic due to decreased numbers of late erythroblasts and reticulocytes [23]. Altogether these data strongly suggests that Bcl-xL may function as a survival factor not only in nucleated erythroblasts, but also in reticulocytes and erythrocytes [24].

Bcl-X<sub>L</sub> may additionally regulate hemoglobin synthesis [25]. Therefore, Bcl-X<sub>L</sub> could have a dual function during erythropoiesis, on the one hand by protecting from apoptosis thereby facilitating completion of the differentiation program, and on the other hand reacting directly on hemoglobin synthesis.

With regard to the expression of other bcl-2 family members, it has been reported that neither bcl-2 transcript nor protein were detected in erythroblasts. Furthermore, the expression of pro-apoptotic proteins Bax and Bad remains relatively constant throughout erythroblast differentiation, but diminishes at the end of terminal differentiation just prior to enucleation [26]. Collectively these data suggest that some of the bcl-2 family members are involved in erythroblast differentiation, especially in the late stages when the levels of anti-apoptotic members are significantly increased whereas the levels of pro-apoptotic proteins are reduced. These two expression mechanisms may prevent apoptosis thereby ensuring erythroblast differentiation and subsequent enucleation.

## **2.5 Nuclear condensation and DNA fragmentation**

The majority of known targets of the apoptotic executionary machinery are located in the nucleus, and it is this organelle which shows the most dramatic changes both at the morphological and molecular levels during apoptosis. In the nuclei of dying cells chromatin condenses and DNA is fragmented, the entire nucleus shrinks and often fragments into micronuclei [27]. With the exception of nuclear fragmentation into micronuclei, the nucleus in mature erythroblasts (mainly Ortho EB) displays features similar to those in apoptotic cells, indicating that common mechanisms might underlie both erythroblast differentiation and apoptosis.

The first morphologically detectable event of nuclear breakdown during apoptosis is condensation of the chromatin into few large clumps at the periphery of the nucleus [27]. This resembles the situation seen in erythroblasts, where chromatin clumping and nuclear condensation are morphologically distinguishable events which precede enucleation [28, 29]. Chromatin clumping is very distinct in differentiating erythroblasts, starting at the late basophilic stage of differentiation, and reaching maximum at the orthochromatophilic stage. As revealed by spectral imaging analysis, the changes in the nuclear structure during erythroblast differentiation, closely resemble those seen in apoptosis, in that chromatin condensation starts in the periphery and proceeds in circular and windmill-like pattern towards the center [30].

The degradation of DNA during apoptosis is mediated *in vivo* by two systems, one operating cell-autonomously in the dying cells, and the other in phagocytes after the dying cells are engulfed. In cell-autonomous DNA degradation, DNA is first cleaved into fragments of about 50-300 kb with intact nucleosomes, followed by degradation into nucleosomal units [31]. It has been suggested that CAD (caspase activated nuclease) is mainly responsible for the cell-autonomous DNA degradation, although other nucleases (endonuclease G, AIF and others) could also degrade DNA in certain circumstances [32]. In cell non-autonomous DNA fragmentation, DNA of the engulfed apoptotic cells is completely digested into nucleotides by DNase II in lysosomes of the phagocytes [31].

To date, there is little information available regarding DNA fragmentation in differentiating erythroblasts. It has been first proposed that degradation of DNA in erythroblast nuclei does not occur before enucleation [1]. As expelled erythroblastic nuclei are engulfed by macrophages, it has been suggested by analogy to phagocytosis that DNA in the engulfed erythroblastic nuclei would be degraded by the macrophages. This hypothesis was recently confirmed by the study of DNase II knockout mice, where erythropoiesis was severely inhibited, even though the number

of erythroid precursor cells was normal in these mice [33]. Transfer of DNase II-null hematopoietic stem cells into host with normal macrophages led to differentiation to mature enucleated erythrocytes, indicating that DNase II in macrophages is responsible for digesting nuclear DNA expelled from erythroblasts. If the macrophages are not capable of digesting this DNA, erythropoiesis is compromised.

Apart from the DNA degradation in macrophages, a second mode of DNA degradation in erythroblasts, which is cell-autonomous and occurs before enucleation, has also been suggested [3, 16, 34]. This proposal is supported by a report indicating the presence of terminal dUTP nuclear end labeling (TUNEL)-positive Syrian hamster yolk-sac-derived erythroblasts [3]. Notably, only cells that were morphologically identified as late erythroblasts could be labeled by the TUNEL assay whereas early progenitors were TUNEL-negative [3]. These results suggested that DNA fragmentation in erythropoiesis might precede nuclear expulsion prior to the appearance of anucleated mature erythrocytes.

Several proteins have been implicated in chromatin condensation and DNA fragmentation during apoptosis. One of these proteins is Acinus, a precursor of chromatin-condensation factor, shown to be cleaved by caspase-3 [35]. Subsequently, the cleaved Acinus product promotes chromatin condensation without DNA fragmentation. Recently, it has been shown that Acinus is also cleaved during erythroblast differentiation [14]. This mechanism may account for the chromatin condensation observed in these cells. A further important regulator of nuclear condensation is AIF, which has been identified as a protein responsible for caspase-independent chromatin condensation [36]. In addition, AIF has also been implicated in the degradation of DNA into fragments about 50-300 kb in length. Several other enzymes (e.g. caspase-activated DNase (CAD), L-DNase II, cathepsin B and Endo G) have also been implicated in chromatin

condensation and DNA fragmentation during apoptosis [37], none of these has so far been implicated in DNA fragmentation during erythroblast differentiation.

## **2.6 Nuclear envelope**

Along with the nuclear condensation and DNA fragmentation during apoptosis, the nuclear envelope also undergoes dramatic reorganization. The nuclear lamina, a structure that maintains the nuclear envelope, is degraded as a result of the proteolytic cleavage of lamins [38, 39]. This cleavage has been shown to be caspase-mediated [13].

Differentiating erythroblasts express two types of lamins, lamin A/C and lamin B [3]. In a similar manner to that observed in apoptosis, both proteins are cleaved in erythroblast differentiation in parallel with chromatin condensation and DNA fragmentation [3, 14, 15].

## **2.7 Organelle loss in erythrocytes**

The removal of organelles, such as endoplasmic reticulum, Golgi apparatus, and mitochondria from orthochromatophilic erythroblasts and reticulocytes is of fundamental importance for the function of the mature enucleated erythrocytes since it leads to cells comprised mostly of hemoglobin which is therefore very efficient in oxygen transport. The removal of these organelles is tightly regulated and several mechanisms leading to their removal have been described. One such mechanism is mediated by 15-lipoxygenase (15-LOX) [40], whose maximal activity has been described in reticulocytes immediately before organelle degradation [41].

An alternative method of organelle elimination is that of autophagy, where cytoplasmic proteins and organelles are degraded in a lytic compartment. This mechanism may function in erythroblasts and reticulocytes as high numbers of autolysosomes containing mitochondrial fragments have been described in these cells [42, 43]. Since mitochondria are central

components in apoptosis, elimination of these organelles by autophagy in an enclosed compartment, would prevent cytochrome c and other apoptogenic factors release, and hence prevent apoptosis. Therefore, degradation of organelles, especially mitochondria, by autophagy may be a key aspect of mechanisms regulating erythroid differentiation and enucleation.

## **2.8 Phosphatidylserine receptor**

Exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane of apoptotic cells is considered a primary signal which is recognized by phagocytes [44, 45]. Several receptors are implicated in the recognition of PS, including lectin-like oxidized low-density lipoprotein receptor-1,  $\beta_2$ -glycoprotein I,  $\alpha_v\beta_3$  vitronectin and phosphatidylserine receptor (PSR) [46-48]. In vitro, PSR is essential for the engulfment of apoptotic cells by both professional and non-professional phagocytes, including macrophages, fibroblasts, epithelial and endothelial cells [49]. Recently it has been suggested that PSR-mediated cell uptake is required for definitive erythropoiesis [50]. This hypothesis is based on a report indicating that PSR-deficient (PSR<sup>-/-</sup>) mice exhibit severe anemia and hence die during the perinatal period. Moreover, in the PSR<sup>-/-</sup> fetal livers, erythroblast differentiation was blocked at an early erythroblast stage, most likely the proerythroblast or basophilic erythroblast stage. Since the phenotype of PSR<sup>-/-</sup> mice is similar to that of mice lacking DNase II, PSR-mediated cell uptake has been suggested as a major pathway leading to DNA degradation by DNase II in macrophages. However, the precise mechanism by which PSR regulates erythroid differentiation is unclear. It has been shown that PSR-deficiency causes repression of apoptosis in several tissues, such as fetal liver and thymus [50]. This raises the possibility that PSR-uptake of apoptotic cells regulates the cell-death machinery in developing cells through a feedback mechanism between phagocytes and developing cells, probably through a mechanism similar to that described in *C. Elegans* [51, 52]. This feedback



mechanism may play an important role in terminal differentiation of erythroid cells, in which apoptosis-related signaling is required [14].

On the other hand, giving the importance that macrophages engulf the nuclei after they have been expelled from erythroblasts, one can speculate that this process, similarly to the uptake of apoptotic cell by phagocytes, is also PSR-mediated. However, to date there are no reports on PS presence and its exposure on the outer leaflet of the nuclear membrane of erythroblasts.

## **Erythroblasts in fetal blood, cord blood and maternal blood are TUNEL-positive but AnnexinV-negative**

### **Summary of paper 1 and 2 [34, 53]**

The presence of fetal cells, especially fetal nucleated red blood cells (NRBCs), in the blood of pregnant women is now a widely demonstrated phenomenon and is being considered as the basis for a novel noninvasive means for prenatal diagnosis. However, at present little is known about the fate of fetal cells once they enter the maternal circulation.

Fetal cells could be removed from the maternal circulation by the maternal immune system or by the induction of apoptosis by other means [54-56]. These proposals are supported by reports indicating the presence of terminal dUTP nuclear end labeling (TUNEL)-positive fetal NRBCs in the maternal circulation [57], a feature that may be attributable to the increased oxygen concentration in the maternal circulation, as well as by the destruction of apoptotic fetal cells or their remnants in the maternal plasma.

However, elimination of fetal cells by apoptosis from the maternal periphery may not be as widespread as suggested, or it may not affect all fetal cell types equally. Fetal leukocytes or hematopoietic progenitor cells may persist in the maternal circulation for years to decades after delivery [58, 59], and fetal cells with stem cell-like characteristics may even contribute to the regeneration of maternal tissue [60]. Furthermore, microchimerism of the order seen in pregnancy frequently also occurs in solid organ transplant recipients and has, in these instances, been suggested to promote tolerance toward the graft [61]. By analogy, it is therefore conceivable that the passage of fetal cells into the maternal periphery may promote tolerance against the semiallogeneic fetus.

A caveat of previous studies on the status of fetal NRBCs in the maternal periphery is that they investigated only nuclear events (TUNEL positivity) as indicators of the apoptotic status of

trafficking fetal cells. Van Wijk et al. recently reported the presence of a relatively large number of TUNEL-positive cells (which the authors considered as being apoptotic cells) in maternal plasma [54]. Subsequently Sekizawa et al. reported that >40% of the fetal NRBCs detected in blood samples taken immediately after termination were TUNEL positive [55]. Thus, these authors suggested that apoptosis of fetal cells was induced by the maternal immune system and may play a role in the clearance of fetal cells from the maternal circulation without causing inflammatory damage to the mother. Furthermore, it has been suggested that the oxygen concentration in maternal circulation, which is higher than that in fetal circulation, induces apoptosis in fetal NRBCs once they have been transferred to the maternal circulation [55].

Although these reports may indicate that exposure to the maternal environment leads to the induction of apoptosis in fetal cells, they fail to take into account several recent reports suggesting that the terminal differentiation of erythroid cells uses apoptotic mechanisms to facilitate enucleation [14, 42]. The pathways used for erythroid differentiation may involve only nucleus-associated apoptotic features but not membrane associated apoptotic alterations. Indeed data have shown that erythroid differentiation and enucleation may involve chromatin condensation, DNA breakage (detected by the TUNEL assay), and degradation of nuclear components, as well as the apoptotic signals triggering these changes, such as caspase activation and mitochondrial potential reduction. In contrast, membrane-associated apoptotic characteristics that facilitate the rapid engulfment of apoptotic cells, such as phosphatidylserine exposure (PS), are lacking in the maturation process [14]. This appears logical because the erythroblasts need only to eliminate their nuclei and become enucleated erythrocytes intact to perform the important task of oxygen transport.

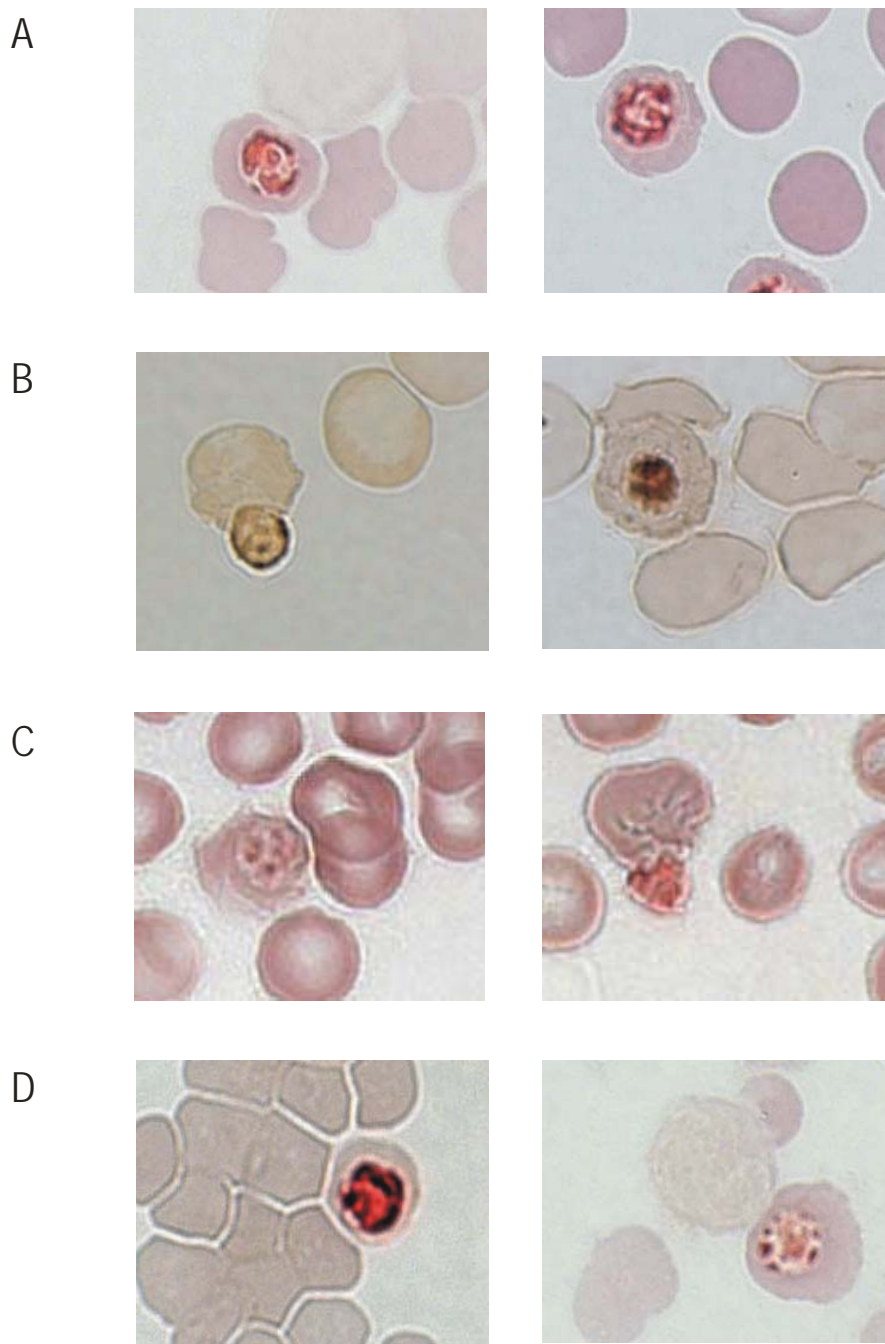
Therefore we addressed the question whether the detection of TUNEL-positive fetal NRBCs is due to apoptosis or alternatively it reflects a normal physiologic stage of erythroid differentiation associated with chromatin condensation and subsequent enucleation.

Our first question was to examine whether:

- NRBCs in the fetal circulation exhibit apoptotic characteristic (TUNEL positivity) similar to that observed in fetal NRBCs in maternal circulation
- fetal NRBCs that have entered the maternal circulation exhibit only nuclear apoptotic phenotypes (TUNEL positivity) or whether they display other membrane-associated apoptotic traits such as PS exposure, which would serve as a signal facilitating rapid cell engulfment.

Our results showed that NRBCs in fetal circulation (prior exposure to the maternal circulation) were TUNEL-positive, which suggest that TUNEL positivity is a feature that could not be attributed to the effect of maternal circulation (refer Figure 5) [34].

We have also shown that the majority of the fetal NRBCs in the maternal circulation do not display PS exposure, although these cells do indeed exhibit nuclear TUNEL positivity [53]. Thus, the discrepancy between the two apoptotic features suggests that the detection of TUNEL-positive/PS-negative NRBCs is probably not associated with apoptosis, but rather is associated with erythroid terminal differentiation and enucleation as the NRBCs mature into erythrocytes.



**Figure 5. DNA fragmentation in late erythroblasts**

A - TUNEL-positive NRBCs in fetal blood; B – ISOL (In situ Oligo Ligation assay)–positive NRBCs in cord blood, note the ISOL-positive enucleating NRBC; C - TUNEL-positive NRBCs in bone marrow

D - TUNEL-positive/ Annexin V-negative NRBCs in maternal blood

## **AIM**

The aim of this work was to examine the involvement of DNA fragmentation during erythroblast differentiation. In particular the exact stage of erythropoiesis at which DNA fragmentation occurs and the nucleases involved in this process were examined. Additionally this project explored whether caspase activation is required for erythroblast differentiation in our model system and whether caspases are activated during differentiation.

This study also focused on deciphering the mechanisms involved in protection of mature erythroblasts from apoptosis. In particular the importance of cytochrome c, Bcl-xL and IAPs was examined.

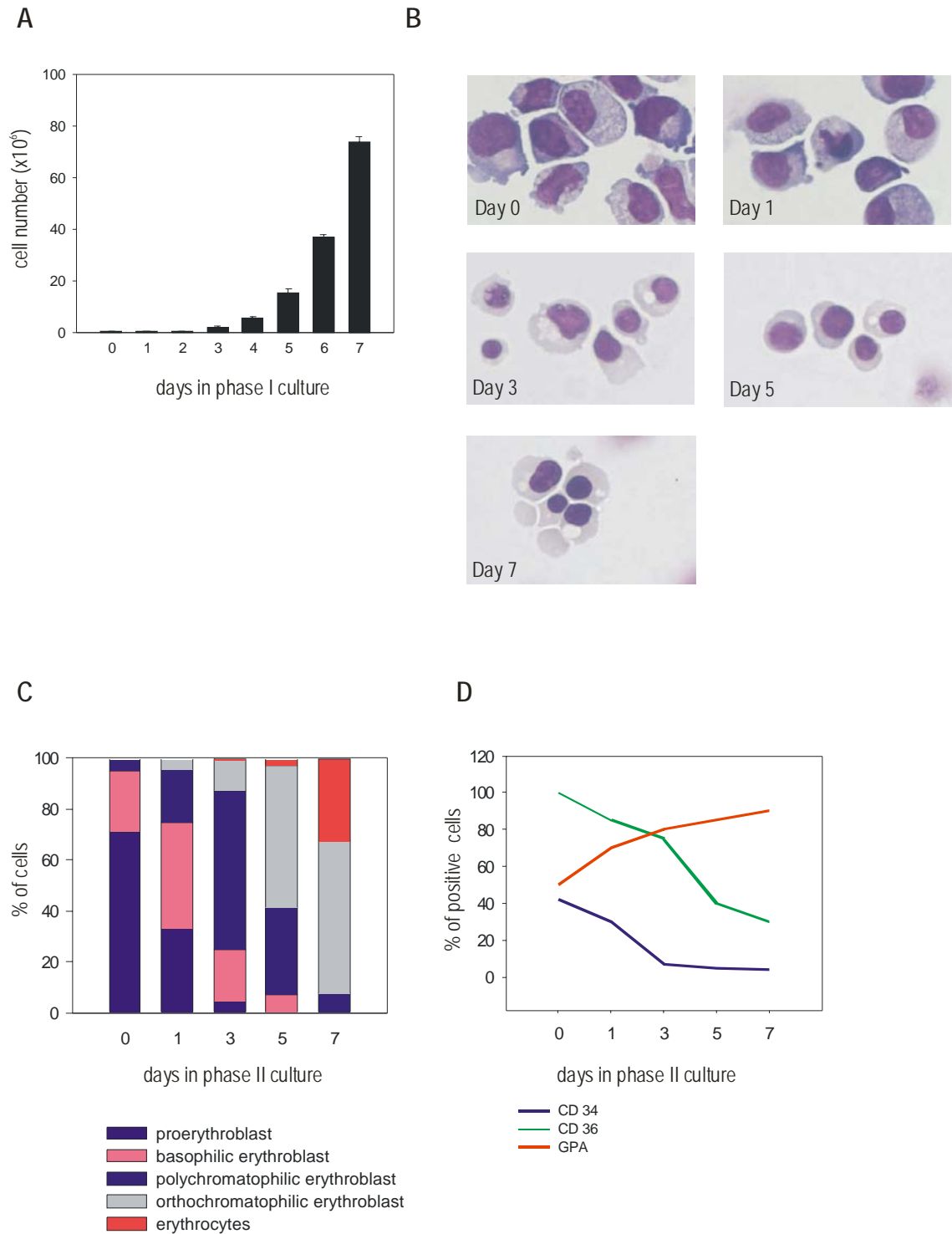
## RESULTS

### 1. Establishment of primary erythroid cell culture

In order to analyze the mechanisms of erythroid differentiation, we developed a culture system for *in vitro* erythropoiesis. CD34<sup>+</sup> progenitor cells were isolated from the mononuclear cell fraction of cord blood to greater than 90% to 95% purity. The mononuclear fraction was subjected to RBCs lysis before CD34 isolation to prevent any contamination of the culture by mature RBCs. The purified CD34<sup>+</sup> cells were then cultured in a two-step culture system:

CD34<sup>+</sup> progenitor cells were amplified during 7 days in the presence of SCF, IL-3, IL-6 and Epo. These culture conditions were found to be optimal for sustained growth of human erythroid progenitor cells. During this amplification step (phase I) cells were counted daily and cumulative cell numbers were determined. A 157-fold mean amplification of the initial cell number (range, 95-280-fold) was observed (Fig. 6 A).

At day 7, CD36<sup>+</sup> erythroid progenitors were isolated and cultured in the presence of SCF, low concentrations of IL-3 and high concentrations of Epo. High concentrations of Epo were used in order to synchronize erythroid culture, increase the percentage of mature erythroblasts and erythrocytes and suppress the number of myeloid cells in the culture. During this differentiation step of culture (phase II, labeled as day 0-7), cells were analyzed by morphological and phenotypic criteria using cyospin preparations stained with May-Grunwald-Giemsa. The majority of the cells in culture (about 95-97%) were erythroid cells that serially recapitulated *in vivo* erythropoiesis while only small portion of cells (about 3 to 5%) morphologically appeared to be monocytes. After the induction of differentiation, cells underwent 3 to 4 cell divisions, began to accumulate hemoglobin and gradually acquired the morphology of normal erythrocytes (Fig.6 B).



**Figure 6. A model system for in vitro erythropoiesis of human CD34+ progenitor cells**

A - Highly purified CD34+ cells were placed in culture and total cell numbers were assessed over time, the graph represents the mean and standard deviation values of 5 independent experiments. B – Representative experiment showing pronormoblasts (day 0), basophilic erythroblasts (1), polychromatophilic erythroblasts (day3),



orthochromatophilic erythroblasts (day 5) and erythrocytes (day7) in phase II culture, cells were stained with May-Grunwald-Giemsa staining. C - Percentage for each erythroblast population at various days in phase II culture; results are expressed as mean values of 5 independent experiments. D - FACS analysis showing the percentage of cells expressing CD34, CD36 or Glycophorin A; results are expressed as mean values of 5 experiments.

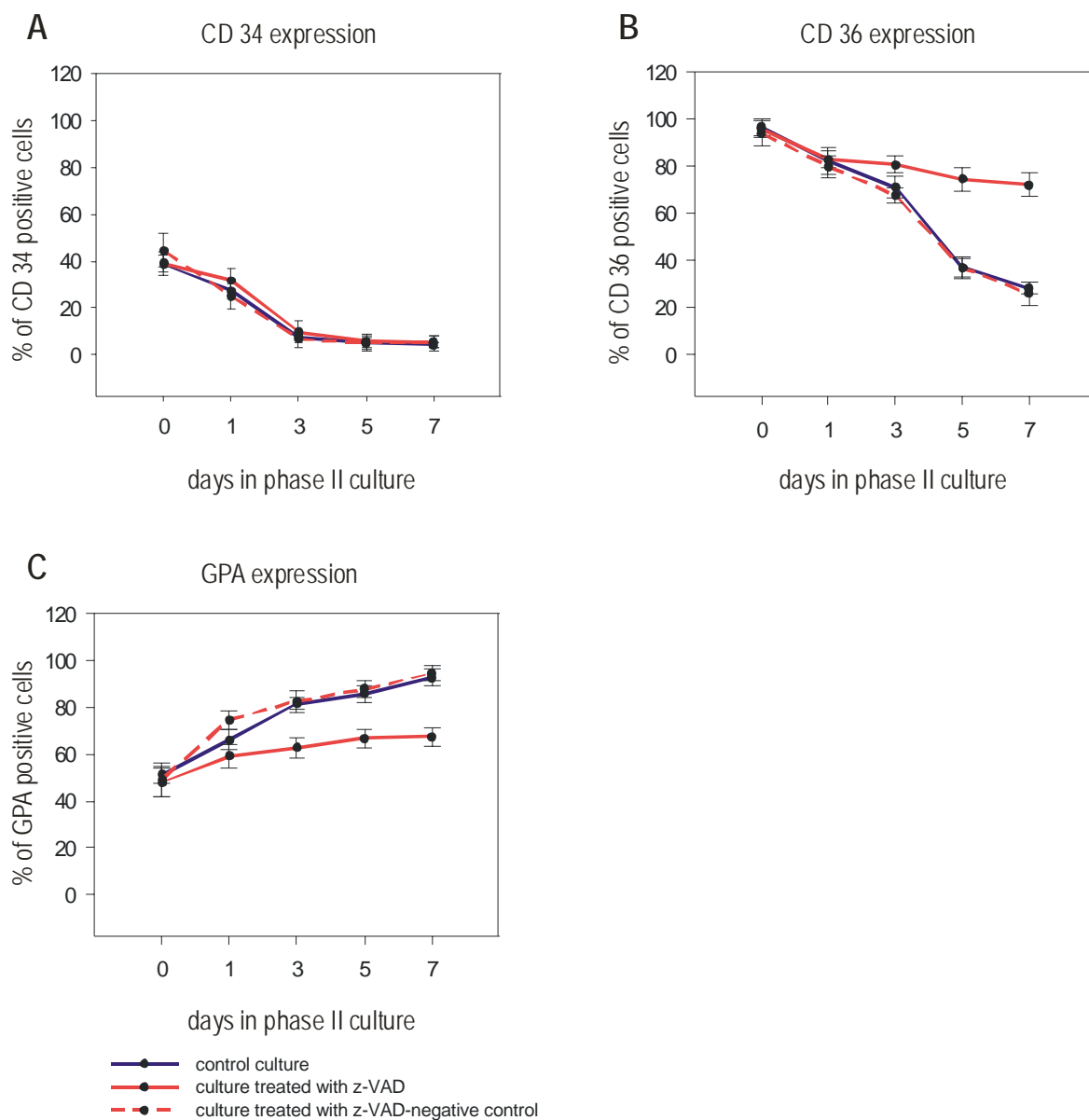
At day 0 of differentiation, proerythroblasts and basophilic erythroblasts (71% and 24% respectively) represented the majority of the cell population (Fig.6 B and C). The proportion of basophilic erythroblast further increased to 42% at day 1 whereas at day 3, the culture consisted mainly of polychromatophilic erythroblast (62.4%). With prolonged time in culture, the differentiation further proceeds, and at day 5 the cell population consisted mainly of orthochromatophilic erythroblasts (56%), while orthochromatophilic erythroblast (60%) and erythrocytes (32%) were the predominant type at day 7. The percentage of cells that enucleate to become erythrocytes is shown in Fig. 6 C.

Analysis of cell surface markers confirmed that the cells in culture were progressing through the stages of normal erythroid development (Fig. 6 D). At day 0, 42% of the cells were CD34+. At day 3, the percentage of CD34+ cells has dropped to 7% and further decline to 5% and 4% at day 5 and 7, respectively. In contrast, at day 0, 100% of the cells were CD36+. At day 3, the percentage of CD36+ cells has fallen to 75%, and further decreases to 40% and 30% at day 5 and 7 respectively. Glycophorin A (GPA) is a marker of more mature erythroid cells. In our culture system, GPA expression increased almost linearly with time until day 7, when about 90% of the cells were GPA positive. These data shows that the majority of the cells in culture were erythroblasts which could reach the late stages of erythropoiesis and more than 30% could undergo enucleation to become erythrocytes.

## **2. In vitro erythropoiesis involves the activation of caspase-3 and caspase-9**

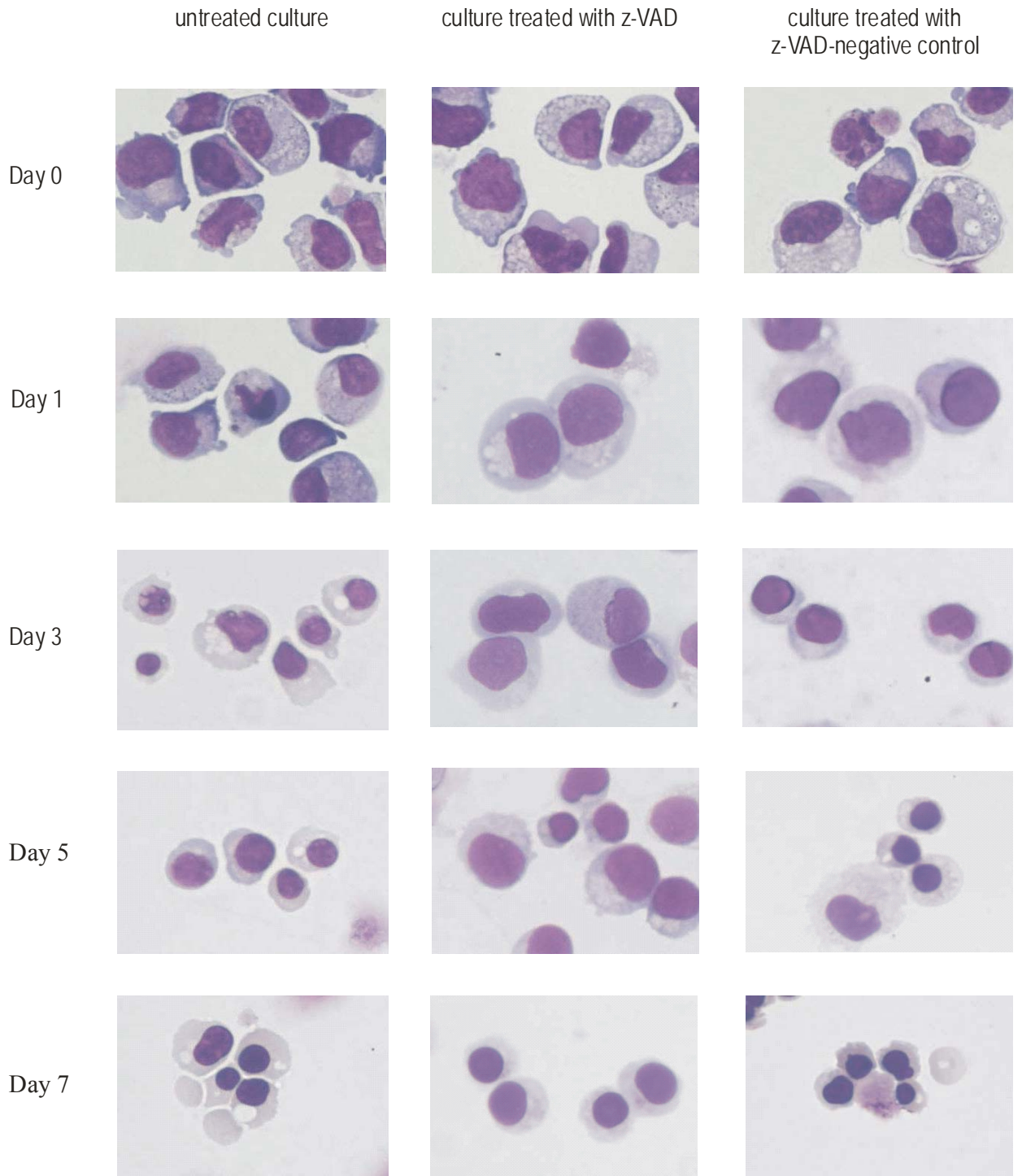
In order to confirm whether caspase activation is required for the differentiation of erythroblasts in our in vitro system a broad spectrum caspase inhibitor (z-VAD) was added to the medium in the beginning (day 0) of phase II in culture and the cells were cultured in presence of the agent thereafter. Z-VAD.fmk induced a significant delay in erythroid differentiation, with accumulation of early progenitors whereas the number of late erythroblasts was significantly reduced .

FACS analysis showed modification of the cell marker profiles. No differences were seen in the expression pattern of the early marker CD34 between the control culture and the cultures treated with z-VAD. Both cultures showed a rapid decline in CD 34 expression, indicating that very early stages of differentiation are not affected by the caspase inhibitor (Fig.7 A). On the other hand, the expression pattern of both CD 36 and GPA differed significantly in the treated and the control cultures. Before day 3 in culture only minimal differences in the CD 36 and GPA expression pattern were observed, whereas after day 3 in culture the expression levels of these markers in the treated and control cultures were easily distinguishable (Fig.7 B and C). Thus, cells treated with caspase inhibitor retain high levels of CD 36, while the levels in the control cultures rapidly decreased (>70 vs 30% at the end of cell culture-day 7). On the other hand, cells treated with caspase inhibitor displayed low levels of GPA, when the levels in the control culture rapidly increased (65 vs 95% at day 7 in culture). Therefore, the expression patterns of the examined markers suggest that addition of caspase inhibitor to the culture medium results in differentiation arrest.



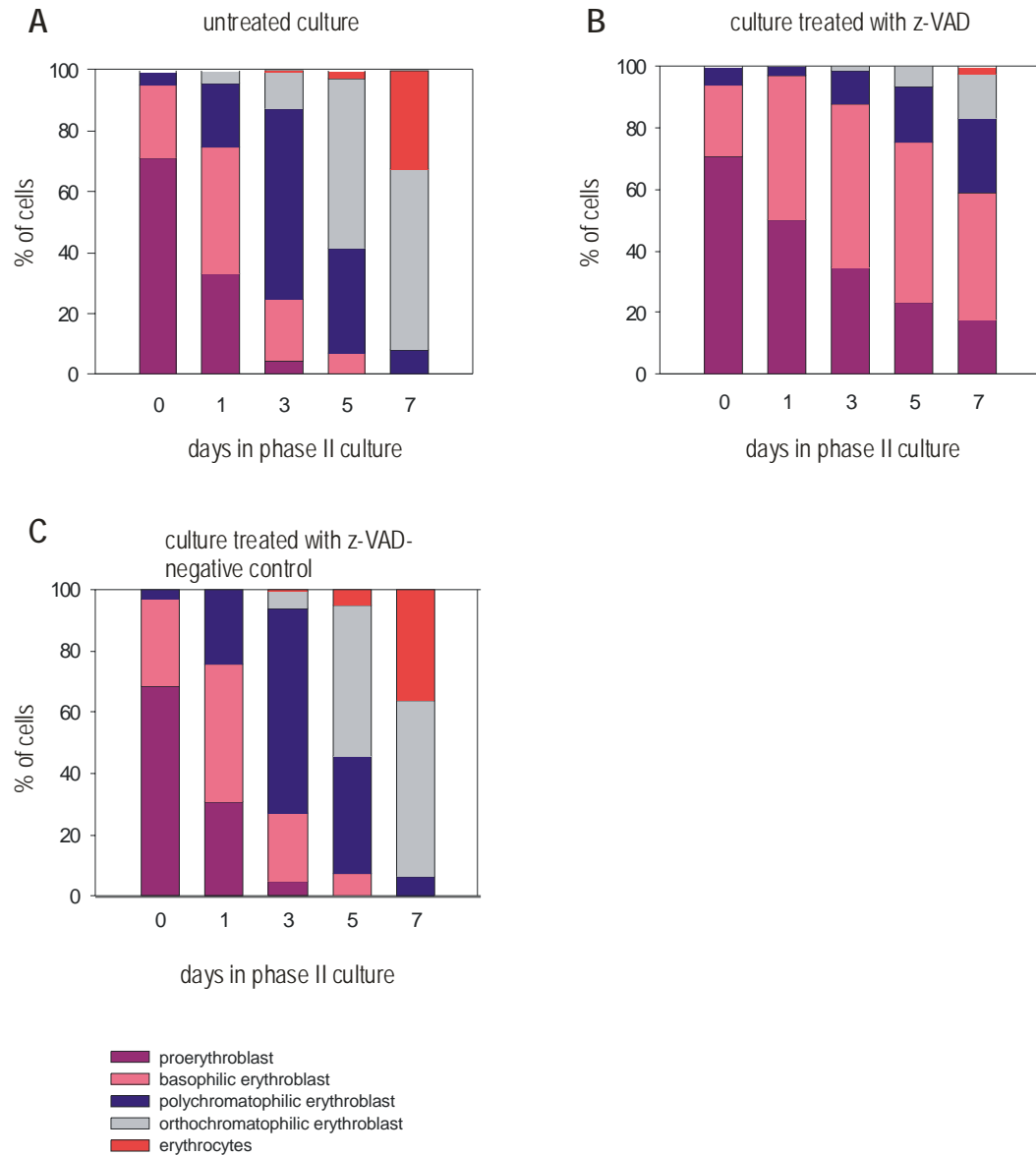
**Figure 7. The caspase inhibitor z-VAD disturbs the expression profile of erythroid cell surface markers.** Cell surface marker expression was examined at various days of differentiation by FACS. A - Shows the expression of the early marker CD34; B - Indicates the expression of the intermediate marker CD 36; C - Depicts the expression of the late erythroid marker GPA. The graphs represent the mean values + Std. of 5 experiments.

To determine when the block in differentiation starts we did a morphological examination of the erythroblasts differentiated in presence or absence of z-VAD.fmk. Starting from the identical pool of CD36<sup>+</sup> erythroid progenitor at day 0, at day 7 the number of proerythroblasts and basophilic erythroblasts in the control culture dropped to 0, while in the z-VAD treated culture the number of proerythroblasts decreased to about 20% but the number of basophilic erythroblasts remained high to about 50% (Fig. 8 and Fig. 9 A and B). In order to control possible unspecific effects of z-VAD, culture treated with caspase inhibitor-negative control (z-FA-fmk) was also included in the experiments. This culture showed cell composition similar to that of the culture grown without inhibitor (control culture) (Fig.9 C). Thus, in the control culture, virtually all of the basophilic erythroblasts were able to progress to polychromatophilic erythroblasts while in the caspase inhibitor-treated culture more than 2/3 of the cells were blocked at the proerythroblast and the basophilic stage. For later erythroblast populations, changes in cell number followed a similar pattern in control and treated cultures, although with much lower numbers of caspase inhibitor treated cells. In both cases, the number of polychromatophilic erythroblasts first increase, then fall to be replaced by later cells. Numbers of orthochromatophilic erythroblasts and erythrocytes increased steadily from day 3. Thus at day 7, orthochromatophilic erythroblasts and erythrocytes were represented with 14,5% and 2% respectively, compare to 60% and 32% in the culture without inhibitor (Fig. 9 A and B). Notably, although the majority of the cells did not proceed beyond the basophilic stage of differentiation, significantly reduced numbers of late erythroblasts were also detected which suggests that erythroid differentiation was not completely inhibited and some cells do manage to differentiate.



**Figure 8. The caspase inhibitor z-VAD inhibits erythroid differentiation**

Cells were stained with May-Grunwald-Giemsa and cell morphology was examined at various days of phase II culture.



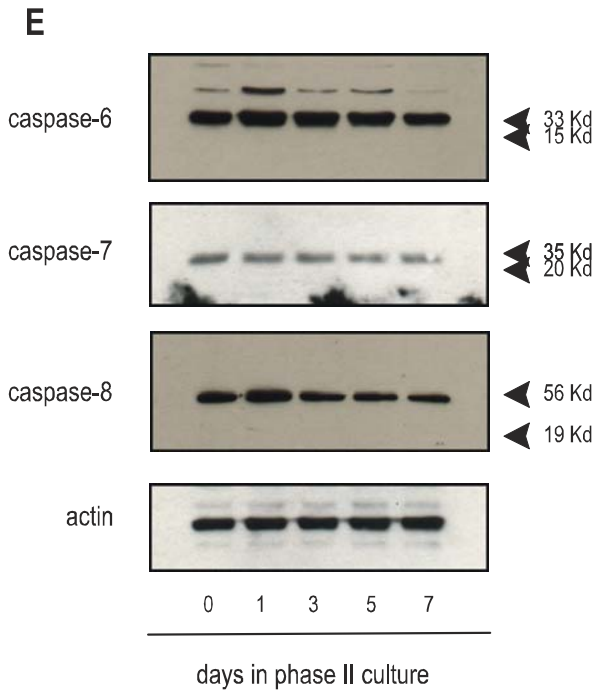
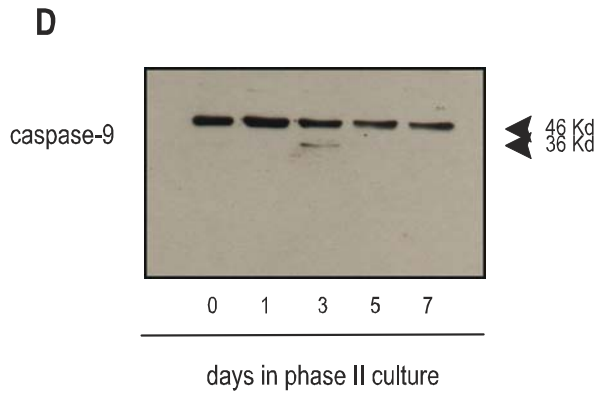
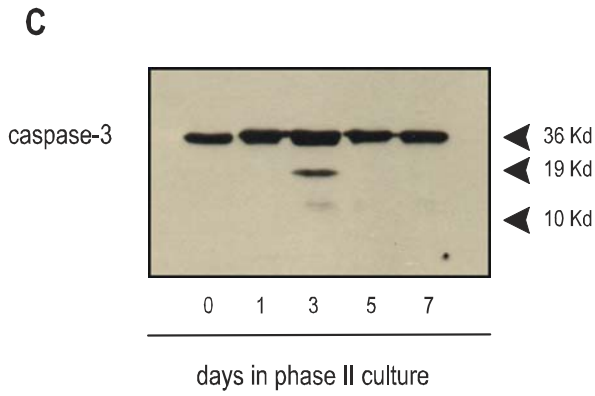
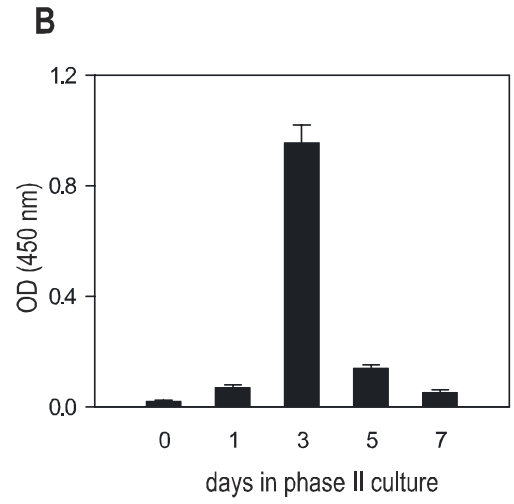
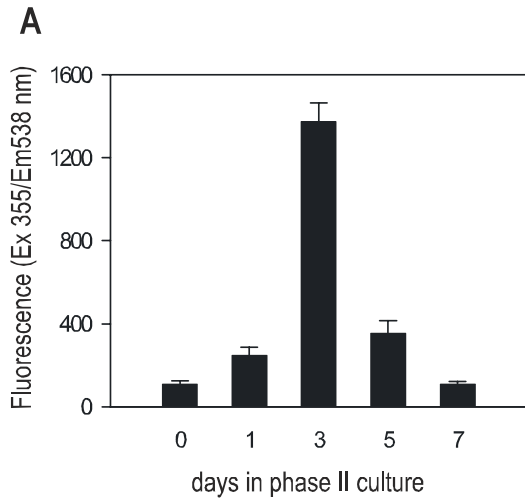
**Figure 9. The caspase inhibitor z-VAD arrests erythroid differentiation at the basophilic stage of differentiation**

Cells were stained with May-Grunwald-Giemsa and cell morphology was examined at various days of differentiation. In each sample 3000 cells were counted. The graphs express percentage for each erythroblast population. A - Control culture; B - Z-VAD treated culture; C - Z-VAD-negative control culture.

To investigate the exact stage of erythroid differentiation when caspases were activated, we studied the ability of cytosolic extracts from erythroid cells at various stages of differentiation to cleave caspase-specific peptide substrates. Ac-DEVD-AFC was used to monitor caspase-3 activity, whereas caspase-9 activity was analyzed by the cleavage of LEND-pNA.

Cytosolic extract of erythroid cells cleaved the peptide substrate Ac-DEVD-AFC, suggesting activation of caspase-3 (Fig.10 A). Caspase-3 activity increased during the first 3 days of culture, reaching maximum at day 3, then decreased at days 5 and 7 of culture. Similar kinetic was observed for the activity of caspase-9, as analyzed by the cleavage of LEHD-pNA (Fig.10 B). These results were confirmed by immunoblot analysis. Procaspase-3 was cleaved to the active forms 19 Kd and 10 Kd at day 3 in culture. Similarly procaspase-9 was cleaved to the active form 36 Kd at day 3 in culture (Fig.10 C and D). There were no cleaved caspase-3 and caspase-9 forms detected at any other days in culture probably because the concentration of these forms was too low to be detected by immunoblotting.

We also examined the activation of caspase-6, -7 and -8 by immunoblot (Fig.10 E). However, our results suggest that none of these caspases were activated during normal erythroid differentiation. We can not rule out however that the concentration of the cleaved forms was under the detection limit of the method.



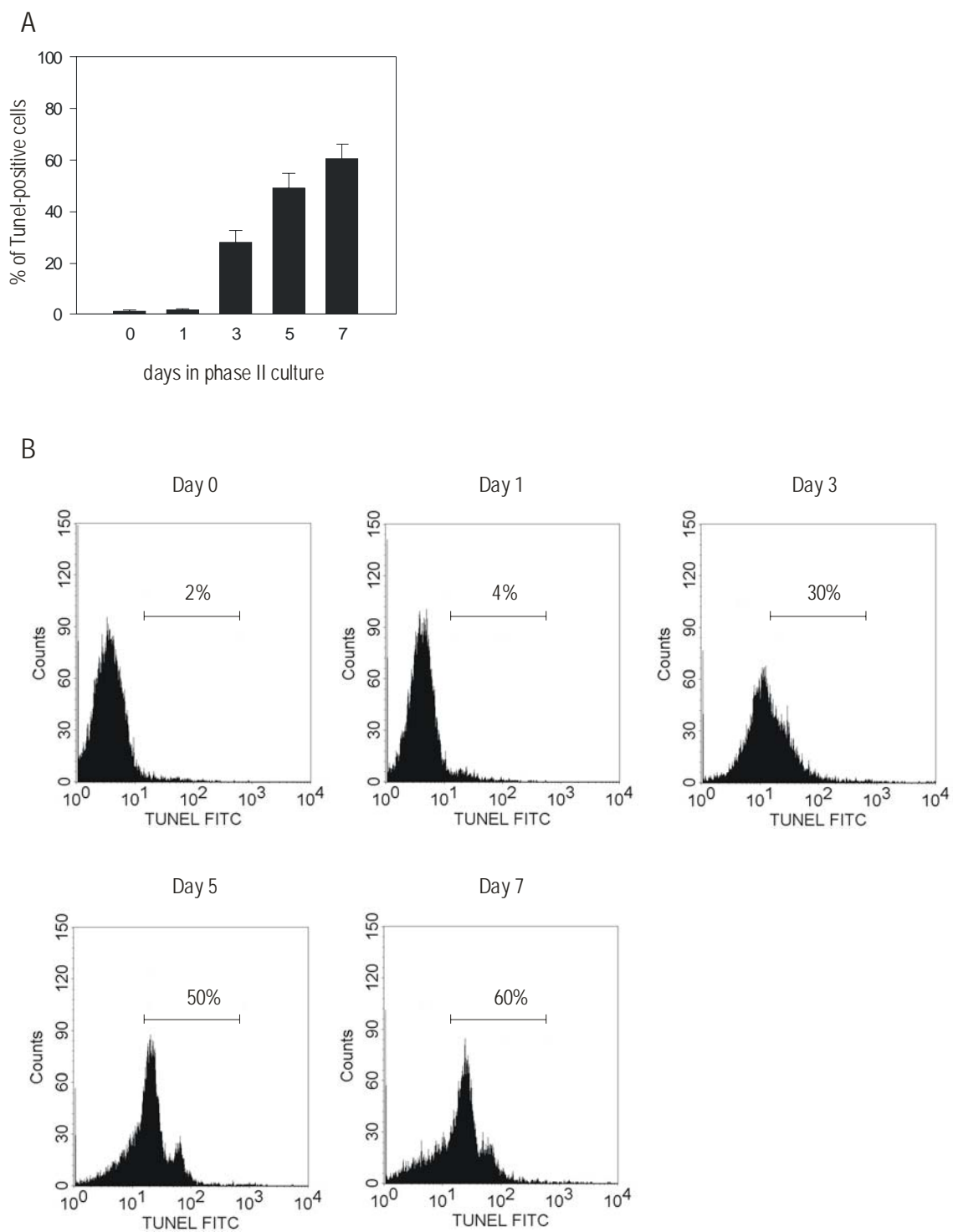


**Figure 10. Caspase-3 and -9 are activated during erythroid differentiation.** A - Caspase-3 activity was monitored in lysates from cells cultured for the indicated times by the cleavage of the DEVD-AFC peptide. Results are expressed as fluorescence measured at Ex355/Em 538 nm. C- Immunoblots were performed on whole cell lysates to detect the 36Kd procaspase-3 and the cleavage products-19Kd and 10Kd; B - Caspase-9 activity was assessed by the cleavage of LEHD-pNA peptide. Results are expressed as relative OD measured at 450nm, D - Immunoblot was used to detect the 46Kd procaspase-9 and the cleavage form 36 Kd; E - Immunoblots for caspase-6, -7 and -8.

### **3. DNA is cleaved in large 50 kb fragments during erythroblast differentiation**

To further explore the mechanism of DNA fragmentation and focus on well-defined controlled steps, we performed TUNEL assay on in vitro cultured erythroid progenitors at various stages of differentiation. We analyzed cells at days 0, 1, 3, 5 and 7 of differentiation. The percentage of TUNEL-positive cells was quantified by FACS performed after the TUNEL labeling. In addition, TUNEL assay in combination with antibody staining for GPA on cells immobilized on slides was also performed. This enabled us to examine cell morphology and to be able to distinguish between late and early erythroblast.

Our results showed that only a few cells (about 2%) were TUNEL positive at day 0 and 1 of differentiation when the culture was mainly represented by early erythroblasts (proerythroblasts and basophilic erythroblasts) (Fig.11 A and B and Fig.12 A). As the differentiation proceeds, at day 3 the proportion of TUNEL-positive cells was about 30% and further increased to 50% at day 5 when the major cell type was orthochromatophilic erythroblasts. At day 7 when the culture consisted mainly of orthochromatophilic erythroblasts and erythrocytes almost all of the erythroblasts were labeled by the TUNEL assay (60% of the total cell number).



**Figure 11. DNA fragmentation in differentiating erythroblasts.** DNA fragmentation was assessed by TUNEL assay performed at various days of differentiation; TUNEL-positive cells were scored by FACS.

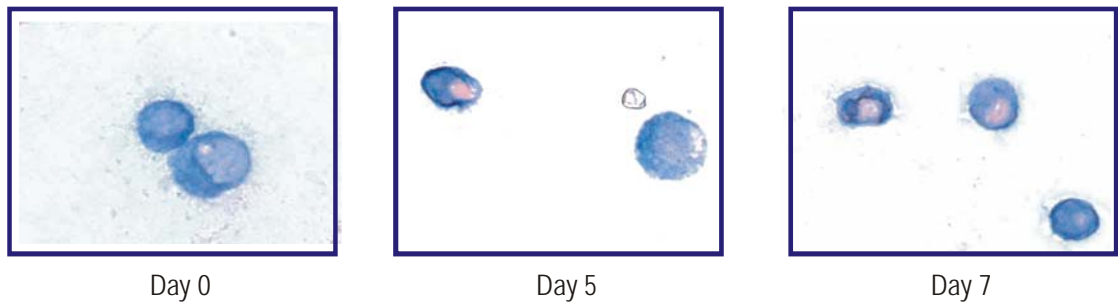
A - Percentage of TUNEL-positive erythroblasts at the indicated days of differentiation; values are indicated as mean + St.d of 5 experiments. B - Histogram blots indicating the % of TUNEL-positive cells in a representative culture.

As TUNEL-positivity is a marker of *in vivo* DNA fragmentation, we next examined the precise nature of the cleavage. DNA fragmentation is in most cases associated with apoptosis, where DNA is fragmented into oligonucleosomal fragments. We next examined whether DNA in differentiating erythroblasts shows similar pattern. However, oligonucleosomal DNA laddering typical for apoptosis was not detected in differentiating erythroblasts (Fig. 12 B).

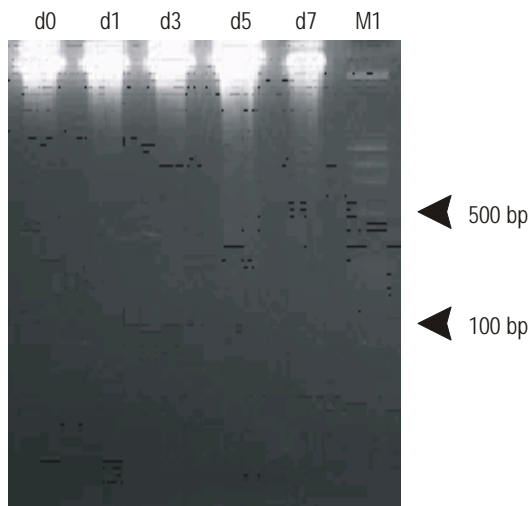
Since it has been shown that the apoptotic fragmentation of DNA into nucleosomal units is preceded by the degradation of chromosomal DNA into high molecular DNA fragments of 50-300 kb, we next examined whether this was the case with DNA fragmentation in erythroid cells.

Pulse field gel electrophoresis experiments showed that indeed DNA in erythroid cells is degraded into high molecular DNA fragments of about 50 kb-200 kb. Furthermore, these experiments have shown that the degree of DNA fragmentation increases as the cells differentiate (Fig.12 C). Taken together our results suggest that DNA degradation represents a defined step in erythroid differentiation that occurs before nuclear expulsion. This process however is distinct from apoptosis since DNA is fragmented in high molecular fragments while nucleosomal DNA fragments are not detected.

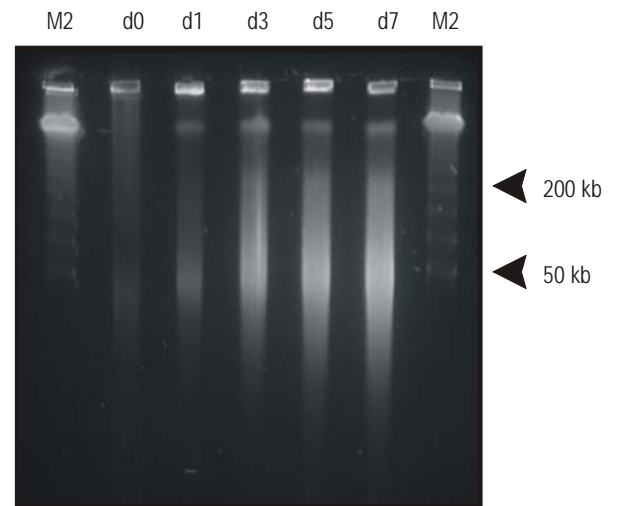
A



B



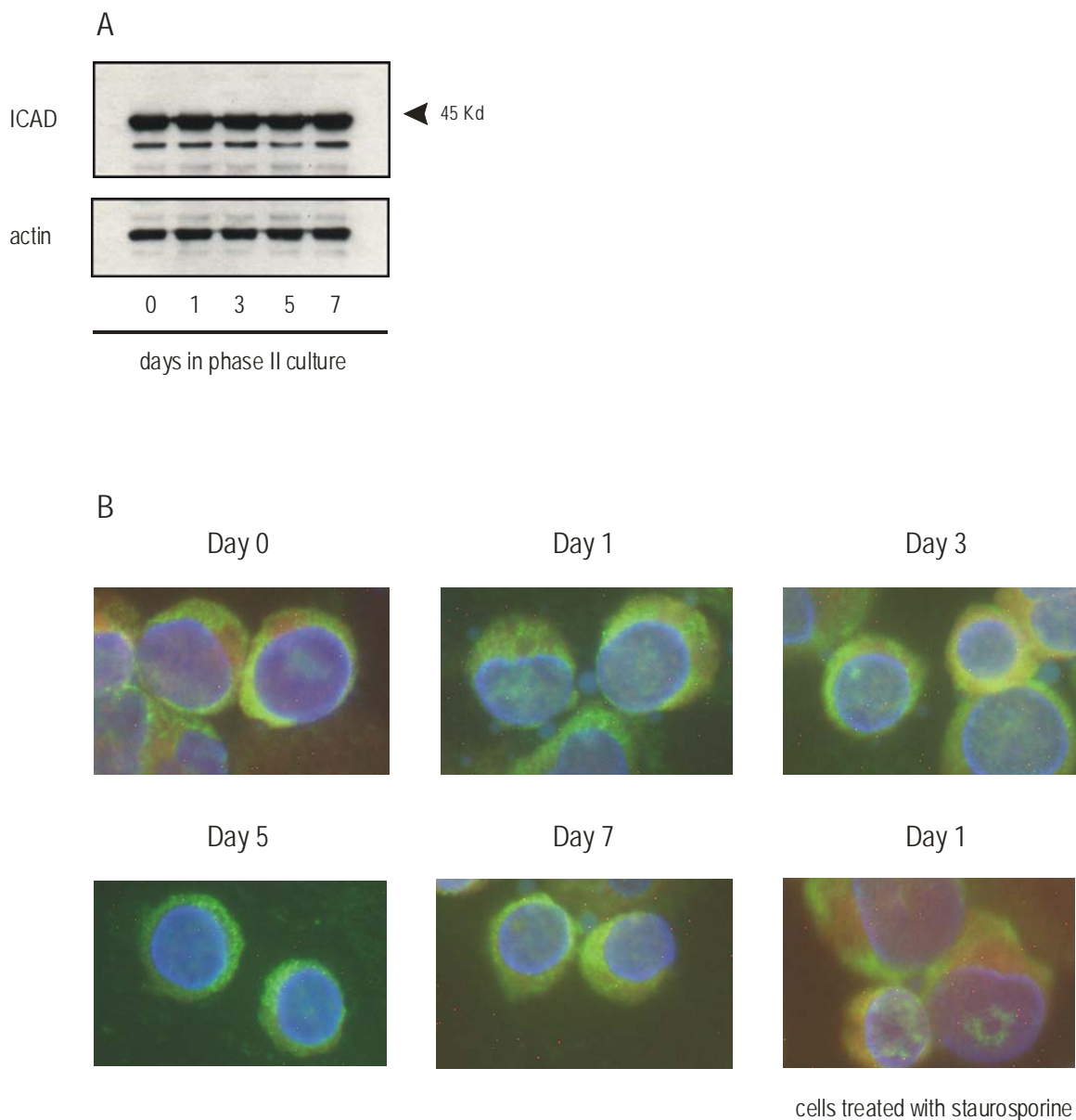
C



**Figure 12. DNA fragmentation in differentiating erythroblasts.** A - TUNEL assay was performed on erythroblast cells at the indicated times. No TUNEL-positive cells were detected at the beginning of the differentiation (day 0). TUNEL-positive cells were easily detectable at the end of differentiation (day 5 and day 7); B - DNA from erythroblasts was separated on an agarose gel. d0-7 DNA from cells at days 0, 1, 3, 5, and 7; M1- DNA marker- DNA mass ladder (Gibco) C - Pulse field gel electrophoresis on DNA from erythroblasts at various times of differentiation (d0-7), M2 -50 kb DNA marker (BioRad).

Next we examined which nucleases could be responsible for the high molecular DNA fragmentation observed in differentiating erythroblasts. One candidate is caspase activated DNase (CAD). CAD interacts and is specifically inhibited by its inhibitor ICAD. During apoptosis, caspase-3 cleaves ICAD, thereby releasing active CAD that digests DNA. The possible role of CAD was explored by assaying the status of ICAD at days 0, 1, 3, 5 and 7 in phase II culture. No cleavage of ICAD was detected by immunoblot at any of the examined stages of differentiation, which suggests that CAD is not involved in the DNA fragmentation during erythroid differentiation (Fig.13 A).

A second nuclease is Apoptosis inducing factor (AIF), a mitochondrial protein known to translocate to the nucleus during apoptosis and there participate in the high molecular DNA fragmentation observed in very early apoptotic stages. We examined the cellular localization of AIF in erythroid preparations at various differentiation stages by immunofluorescent staining with anti-AIF antibody. Our results show that AIF was clearly localized in the mitochondria during all the stages of differentiation and was never detected in the nuclei. In contrast, staurosporine treatment of erythroid progenitors resulted in detection of AIF in the nucleus, suggesting relocalisation during apoptosis. These results therefore suggest that AIF does not participate in the DNA fragmentation during differentiation (Fig.13 B).



**Figure 13. Analysis of possible endonucleases involved in DNA fragmentation during**

**erythroid differentiation.** A - Immunoblot analysis of ICAD at the indicated times of differentiation. Actin was used as a control for equal loading.

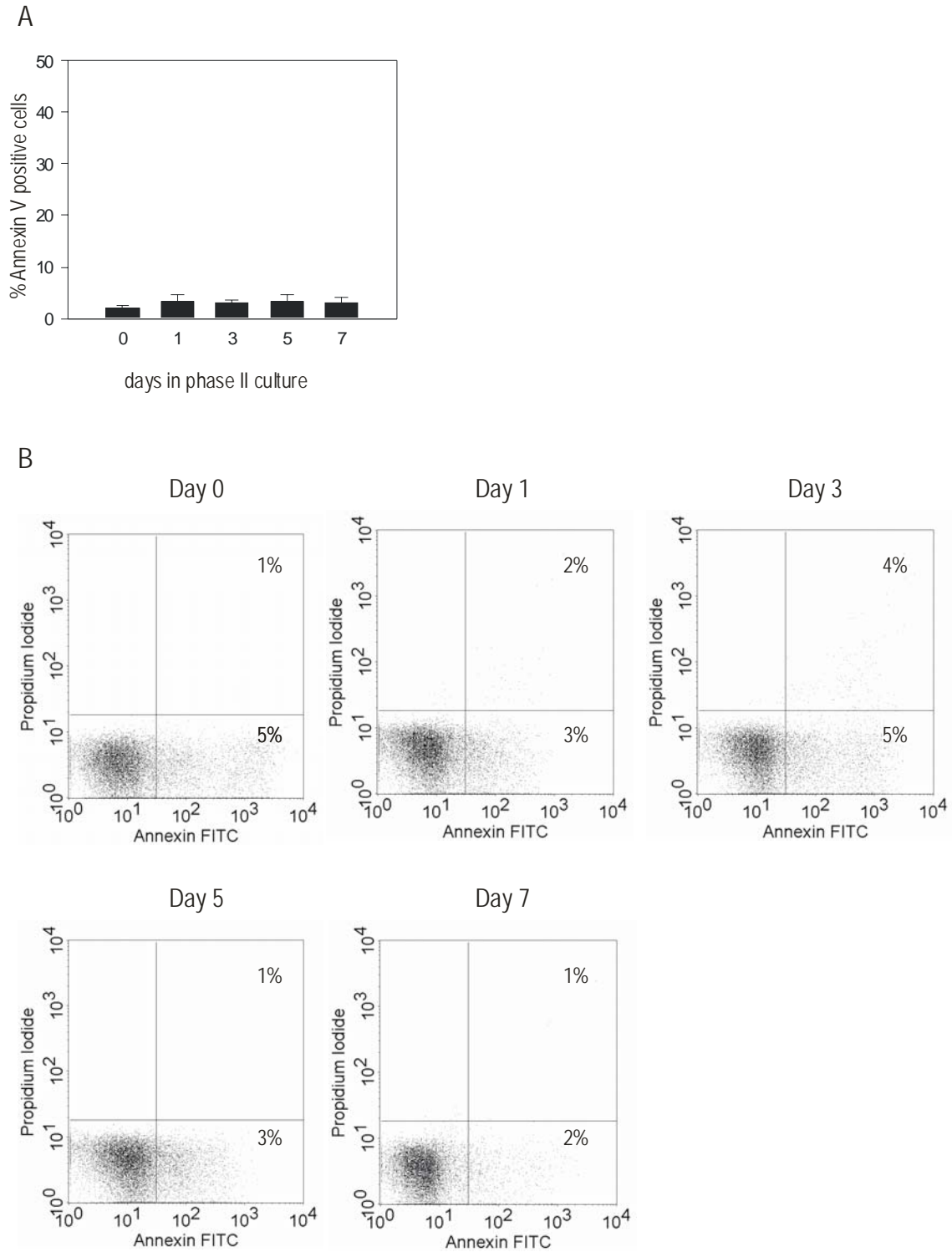
B - Cellular localization of AIF at various times of differentiation. AIF was detected with rabbit anti-AIF antibody followed by detection with anti-mouse FITC labeled antibody. DAPI was used as a nuclear counterstain.

#### **4. TUNEL-positive erythroblasts are Annexin V-negative**

The detection of TUNEL-positive erythroblasts could suggest that these cells are undergoing apoptosis. Therefore it was necessary to find a suitable marker which could be used to distinguish between apoptosis and “apoptosis-like” differentiation.

Externalization of phosphatidylserine (PS) on the outer surface of the plasma membrane has been considered as the most specific marker for apoptosis since it serves as a signal facilitating rapid cell engulfment. Therefore, PS exposure on the cell membrane could be used to confirm that the detection of TUNEL-positive erythroid cells was not associated with apoptosis, rather reflects “apoptosis-like” differentiation process.

Annexin V assay was performed at days 0, 1, 3, 5 and 7 in phase II culture by FACS assay. The percentage of Annexin V+/PI- cells (early apoptotic cells) was between 2% and 5% whereas the percentage of Annexin V+/PI+ cells (late apoptotic or necrotic cells) ranges between 1 and 4% (Figure 14). Together the percentage of Annexin V+ cells did not exceed 9%, which is the typical percentage of apoptotic cells in a vitro erythroid culture. No any dependence of the days in culture, respectively stage of differentiation and percentage of Annexin V+ cells was observed. Therefore these results suggest that despite being TUNEL+ the majority of differentiating erythroblasts in culture do not undergo apoptosis.



**Figure 14. Phosphatidylserine exposure during erythroid differentiation.** Phosphatidylserine exposure along the differentiation was measured by the binding of Annexin V-FITC. A - Percentage of Annexin V-



positive erythroblasts at the indicated days of differentiation. The values are expressed as mean  $\pm$ Std of 5 experiments; B - Histogram blots of representative experiment indicating the % of Annexin V-positive cells

## **5. Protection of mature erythroblasts from apoptosis**

As previously described we have observed that erythroblasts isolated from cord blood could be labeled by the TUNEL assay, which suggest that DNA in these cells is fragmented. However, examining the precise nature of the DNA fragmentation we have observed that DNA in erythroblasts does not show DNA laddering typical for apoptosis. Furthermore, DNA laddering was not detected even when erythroblasts were treated with apoptosis inducing agents. This observation prompted us to investigate whether these cells could in general undergo apoptosis or alternatively they are protected, and if this is the case, which are the mechanisms that protect mature erythroblasts.

### **5.1 Cord blood erythroblasts are resistant to various apoptosis inducing agents**

In order to obtain mature purified erythroblasts, we enriched erythroblasts from cord blood using GPA antibody. The enriched preparations consisted of 90-98 % orthochromatophilic erythroblasts. To examine whether DNA laddering occurs in mature erythroblasts upon apoptosis induction, these preparations were treated with a wide variety of stimuli that have been shown to induce DNA laddering in various cell types (Fig.15 A). Unexpectedly, none of the known apoptogenic stimuli including the protein kinase inhibitor staurosporine, alone or in combination with the protein synthesis inhibitor cycloheximide, the RNA synthesis inhibitor actinomycin D,

or the inhibitors of topoisomerase etoposide and camptothecin, was able to induce DNA laddering (Fig. 15 B). In addition, other proapoptotic conditions, such as exposure to dexamethasone, calcium ionophores and pro-oxidants, also failed to induce DNA laddering. These results prompted us to examine whether mature erythroblasts contain apoptotic components and whether they can be engaged upon apoptosis induction.

## **5.2 Mature erythroblasts contain caspases but they could not be activated**

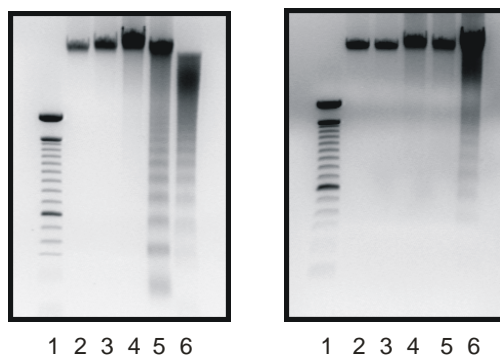
In order to determine whether caspases and other components of the apoptotic machinery are present in late stage erythroblasts, total cell lysates were prepared from purified erythroblasts and investigated by immunoblotting with antibodies against different proteins. Initially, we explored the relative levels of procaspases in erythroblasts versus erythrocytes, HeLa and lymphocytes. As shown in Fig. 15 C erythroblasts contain procaspase-3 in amounts comparable to that observed in erythrocytes, HeLa cells and peripheral lymphocytes. In contrast, the levels of procaspase-6, procaspase-7 and procaspase-8 were relatively lower than those in HeLa cells and peripheral lymphocytes, but similar to those in erythrocytes. While the levels of procaspase-9 were higher in erythroblasts than those in HeLa cell and peripheral lymphocytes, no procaspase-9 was detected in erythrocytes.

We also examined the status of caspases in late stage erythroblasts. No active caspases were detected in mature erythroblasts while procaspases were easily detectable (Fig.15 C). These results therefore suggest that although caspase activation occurs early in the differentiation process, no endogenous activation was observed in mature erythroblasts.

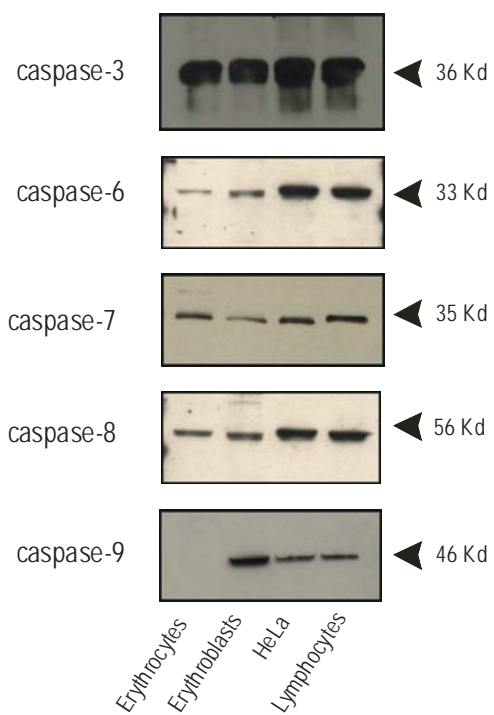
A

Stimulus	Concentration	Time course	DNA laddering
Staurosporine	2.5 $\mu$ M; 10 $\mu$ M	24h-48 h	none
Staurosporine +CHX	2.5 $\mu$ M + 100 $\mu$ M	4-24 h	none
Actinomycine D	10 $\mu$ M	4-24 h	none
Etoposide	100 $\mu$ M	4-24 h	none
Camptothecin	2 $\mu$ M	4-24 h	none
Dexamethasone	10 $\mu$ M	4-24 h	none
$A_{23187} + Ca^{2+}$	0.5 $\mu$ M + 2.5mM	4-24 h	none
Hydrogene peroxide	1mM	4-24 h	none

B

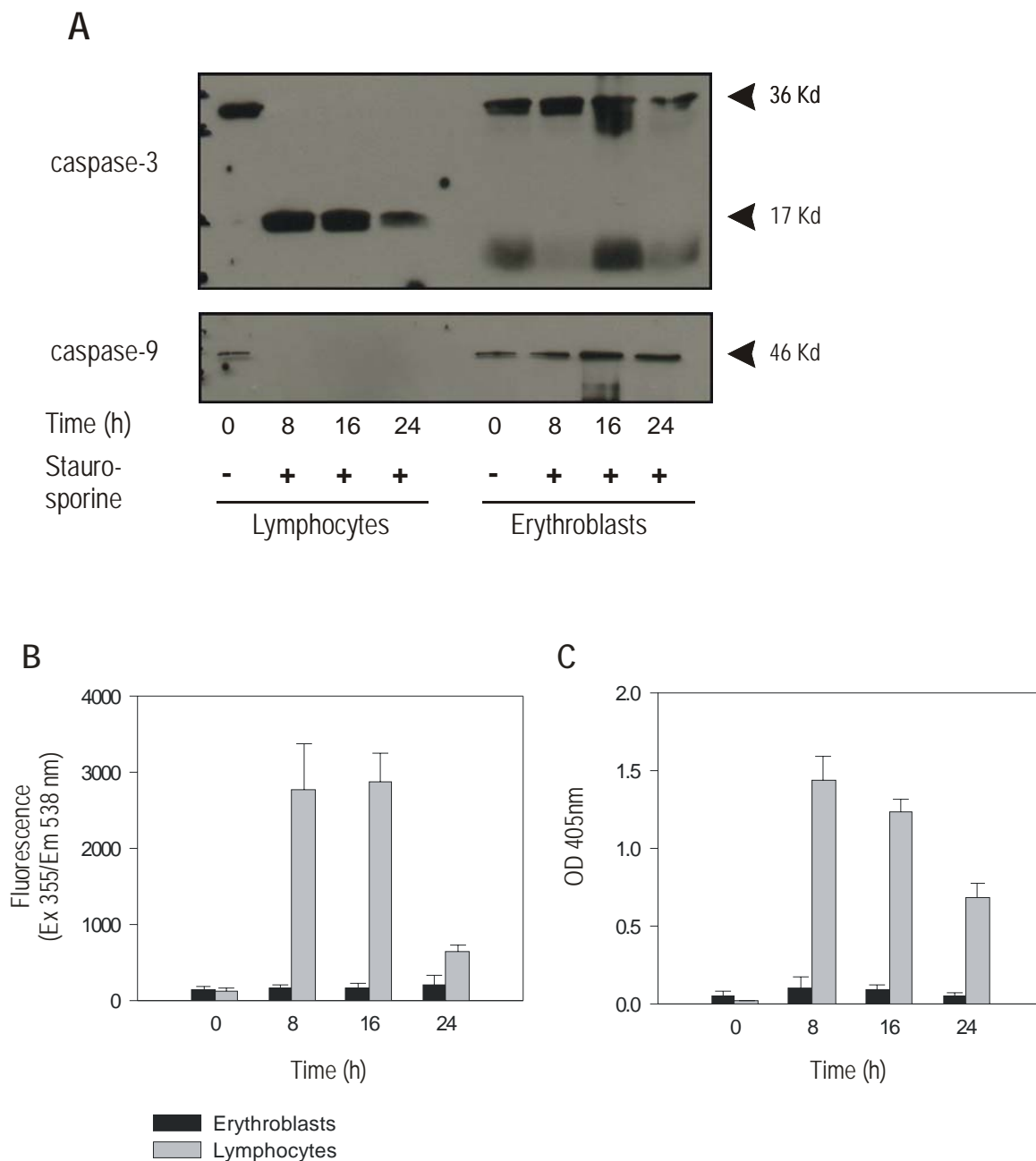


C



**Figure 15. Erythroblasts isolated from cord blood are resistant to apoptotic stimuli.** A - Effect of various apoptotic stimuli on DNA laddering in mature erythroblasts; B - DNA laddering as assessed by agarose gel electrophoresis, lanes 1 and 7 - 100bp DNA ladder marker (Gibco), 2 and 8 - untreated erythroblasts, 3 - staurosporine treated erythroblasts, 4 and 10 - untreated lymphocytes, 5 - staurosporine treated lymphocytes (4h), 6 - staurosporine treated lymphocytes (24h); 9 - etoposide-treated erythroblasts, 11 - etoposide treated lymphocytes (4h), 12 - etoposide treated lymphocytes (24 h). C - Immunoblot detection of procaspases in erythroblasts, erythrocytes, lymphocytes and HeLa cells.

As erythroblasts contain procaspases, we next examined whether they could become activated upon apoptosis induction. We analyzed whether procaspase-3 and procaspase-9 processing occurred after treatment with staurosporine. However, no detectable levels of the active forms of caspase-3 (19 Kd) were observed after incubation with staurosporine for 8, 16 and even 24h while the pro-forms (36 Kd) were easily detectable (Fig.16 A). Similar results were observed when caspase-9 was examined (Fig.16 A). Procaspase-9 forms (46 Kd) were not cleaved at any time following staurosporine incubation. On the contrary, procaspase-3 and procaspase-9 were completely cleaved after 8h in peripheral lymphocytes incubated with staurosporine. To confirm these results in a more quantitative and sensitive assay, we examined the ability of lysates from staurosporine-stimulated erythroblasts and lymphocytes to cleave specific peptide substrates. Cytosolic extracts from erythroblasts induce only minimal and no time-dependent cleavage of the substrate Ac-DEVD-AFC which was not increased after 8, 16 and 24h incubation with staurosporine (Fig. 16 B). Similarly, erythroblast staurosporine-stimulated extracts cleaved very slightly the substrate LEHD-pNA (Fig. 16 C), showing that caspase-9 was also not active. In contrast, extracts from lymphocytes cleaved the substrates Ac-DEVD-AFC and LEHD-pNA very extensively already after 8h of incubation with staurosporine.



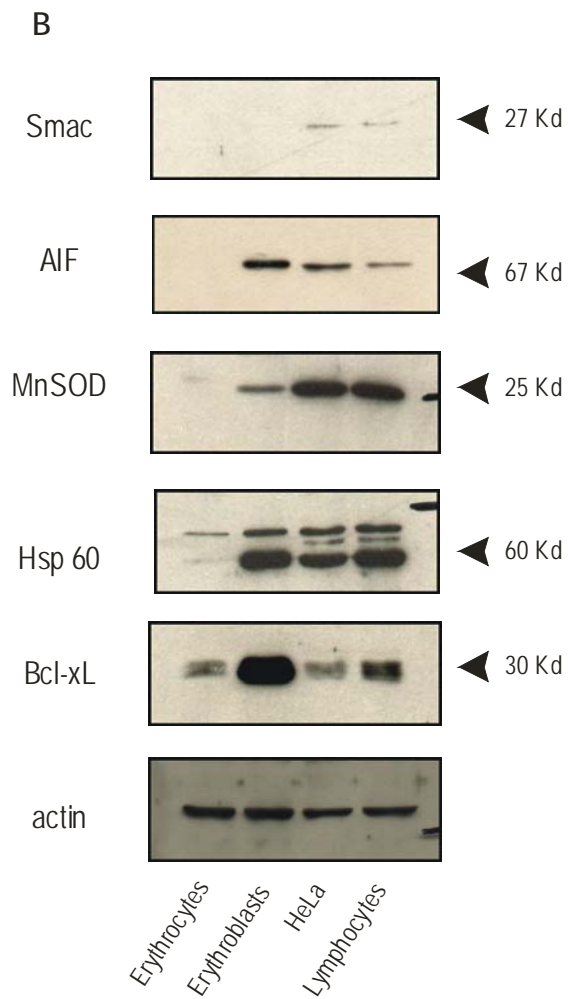
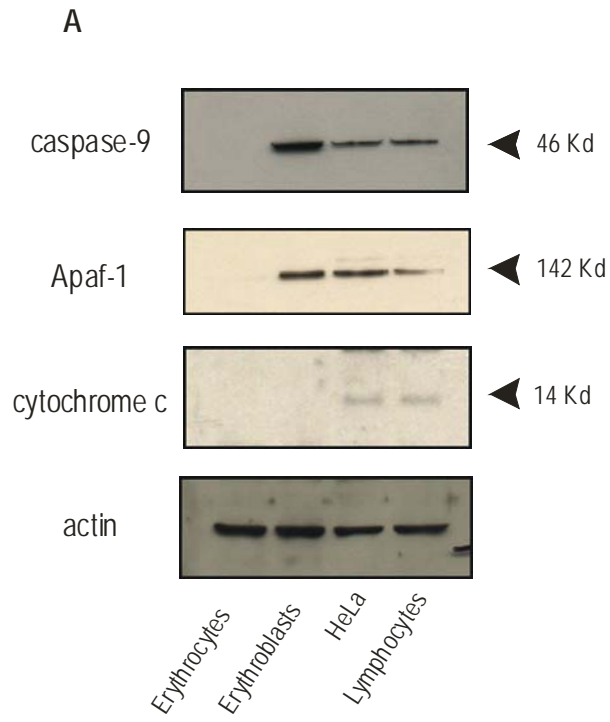
**Figure 16. Failure of endogenous erythroblast procaspase-3 and procaspase-9 to become activated after staurosporine treatment**

A - Immunoblot indicates the presence of procaspase-3 (36 Kd) and procaspase-9 (46 Kd) in erythroblast lysate independent of apoptosis induction with staurosporine. B - Active caspase-3 forms as assessed by the cleavage of the Ac-DEVD-AFC peptide; C - Active caspase-9 forms assessed by the cleavage of LEHD-pNA peptide.

### **5.3 Mature erythroblasts are deficient in cytochrome c**

It is well known that the agents we used to induce apoptosis act via the mitochondrial pathway. However, in the case of mature erythroblasts these agents failed to elicit response leading to caspase activation and DNA laddering, which let us to explore whether the mitochondrial pathway for apoptosis induction is intact in these cells.

Initially we explored the relative levels of Apaf-1, cytochrome c and caspase-9, all known to form complex in the cytoplasm of apoptotic cells called the apoptosome. The formation of the apoptosome is considered as the initial signal for engagement of mitochondrial pathway during apoptosis. As shown in Fig.17 A while procaspase-9 and Apaf-1 were easily detected in erythroblasts, cytochrome c could not be detected in these lysates, suggesting that the apoptosome could not be formed and therefore apoptosis could not be induced via mitochondrial pathway. To ensure that late erythroblasts contain mitochondria and the lack of cytochrome c is not due to lack of mitochondria, we explored the presence of other mitochondrial proteins in mature erythroblasts. Figure 17 B illustrates that, although erythroblasts are devoid of cytochrome c and Smac, they contained readily detectable levels of other mitochondrial proteins, such as MnSOD, Hsp 60, Bcl-xL and AIF, which suggests that mature erythroblasts do contain mitochondria.



**Figure 17. Erythroblasts are deficient in cytochrome c although they contain other**

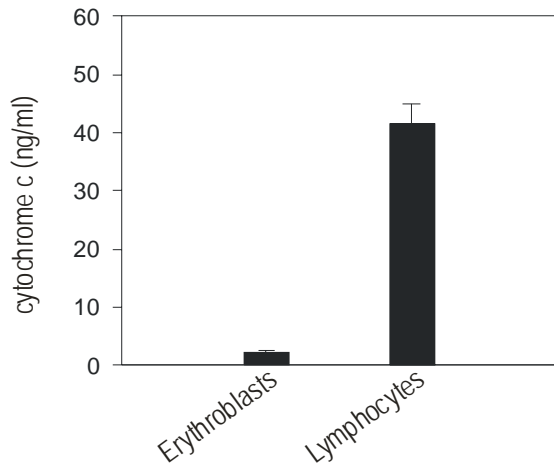
**mitochondrial proteins.** A - Immunoblot showing the presence of Apaf-1 and procaspase-9 but absence of cytochrome c. B – Presence of several mitochondrial proteins in erythroblasts as analyzed by immunoblot. Extracts from HeLa, lymphocytes and erythrocytes were used as control. Extracts were prepared from equal cell number and equal volumes were loaded since the hemoglobin content in erythroblasts and erythrocytes compromises equal protein content loading.

To confirm that erythroblasts are devoid of cytochrome c we conducted a quantitative ELISA assay of erythroblasts lysates in comparison with lysates prepared from peripheral lymphocytes. This assay demonstrates that erythroblasts contain only a very small fraction (<4%) of the cytochrome c contained in lymphocytes (Fig. 18 A).

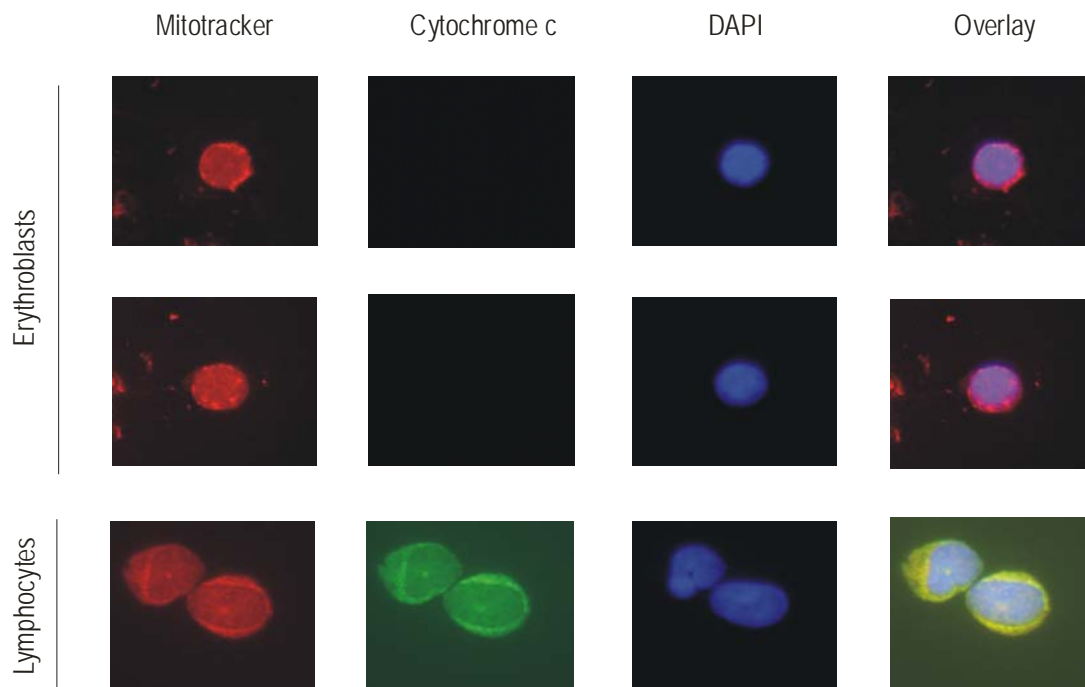
These results were also confirmed by immunofluorescent analysis (Fig. 18 B). Whereas considerable levels of cytochrome c colocalize with the mitochondrial dye MitoTracker into punctuate structures around the nucleus in lymphocytes, cytochrome c was either not detectable or extremely weakly detectable in erythroblasts. On the other hand, erythroblasts stain with MitoTraker, confirming the presence of mitochondria in erythroblasts. Hsp 60 was also used as a control for mitochondrial protein. Hsp 60 was present in erythroblasts mitochondria and colocalize with the MitoTracker dye (Fig. 18 C).



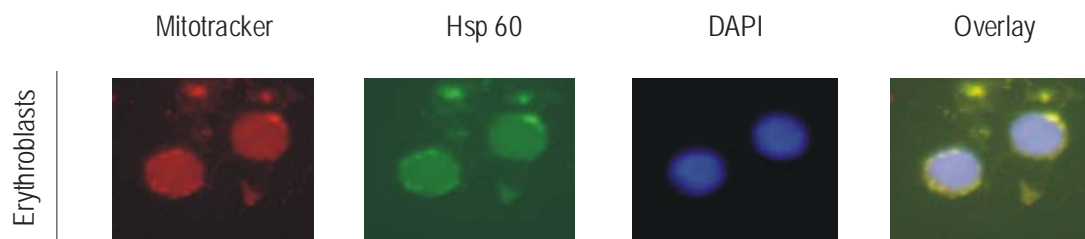
A



B



C



**Figure 18. Lack of cytochrome c in erythroblasts.** A – An ELISA method was used to quantify the level of cytochrome c in erythroblasts. B - Immunolocalization of cytochrome c and mitochondrial protein Hsp 60 in erythroblasts and lymphocytes. Cytochrome c and Hsp 60 were detected with specific rabbit antibodies followed by detection with anti-rabbit FITC labeled antibodies (green). DAPI was used as nuclear counterstain (blue).

#### **5.4 Cytochrome c levels decrease as the erythroblast differentiation proceeds**

The lack of cytochrome c in mature erythroblasts could result from progressive reduction in cytochrome c levels during the differentiation process. To explore this hypothesis we next examined the levels of cytochrome c as the culture proceeds. An ELISA method was used to quantify the amount of cytochrome c in differentiating erythroblast at days 0, 1, 3, 5, and 7 in phase II culture. The experiment illustrates that the amount of cytochrome c slightly decreases during the first 3 days in culture, followed by massive decline at day 5 when the protein dropped by 6 fold and further slightly decreased at day 7 in culture (Fig. 19).

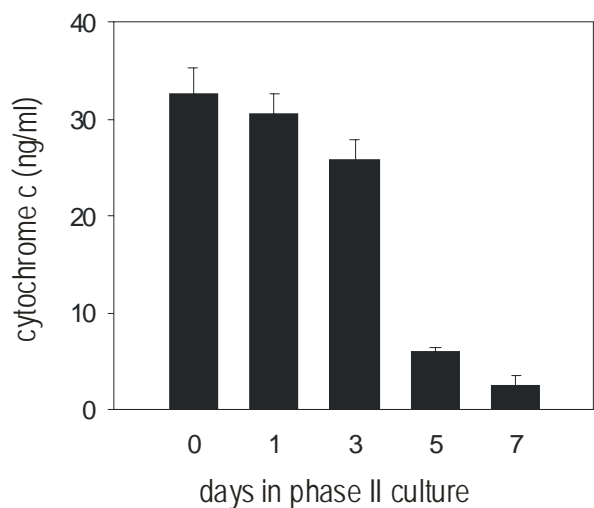


Figure 19. **Cytochrome c levels decrease along the differentiation**

Cytochrome c levels were measured at the indicated times in differentiation. The values are expressed as mean + Std. of 5 independent experiments.

### **5.5 The sensitivity of erythroblasts to apoptosis inducers decreases along the differentiation**

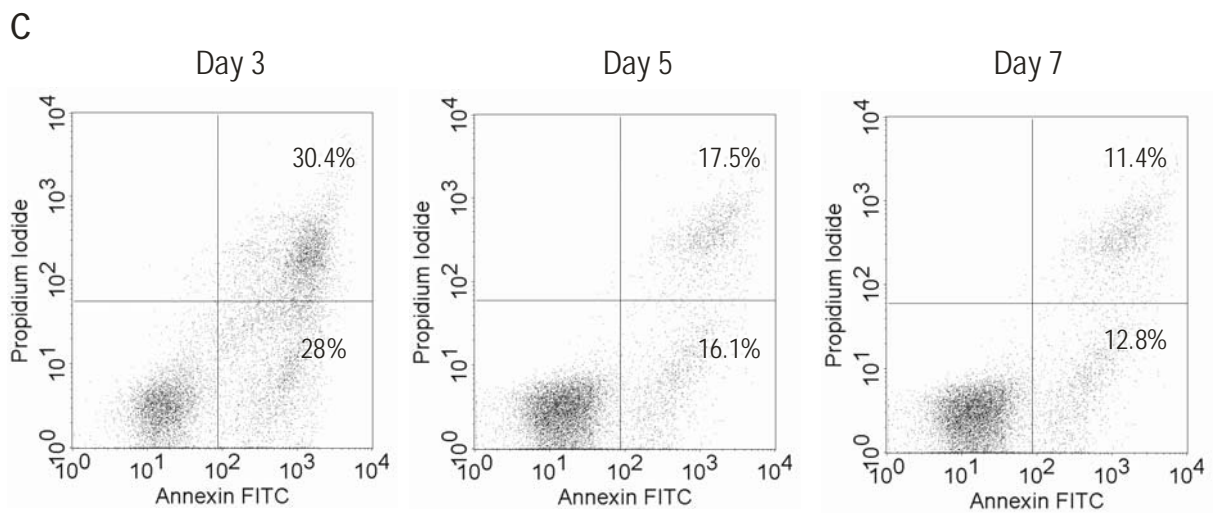
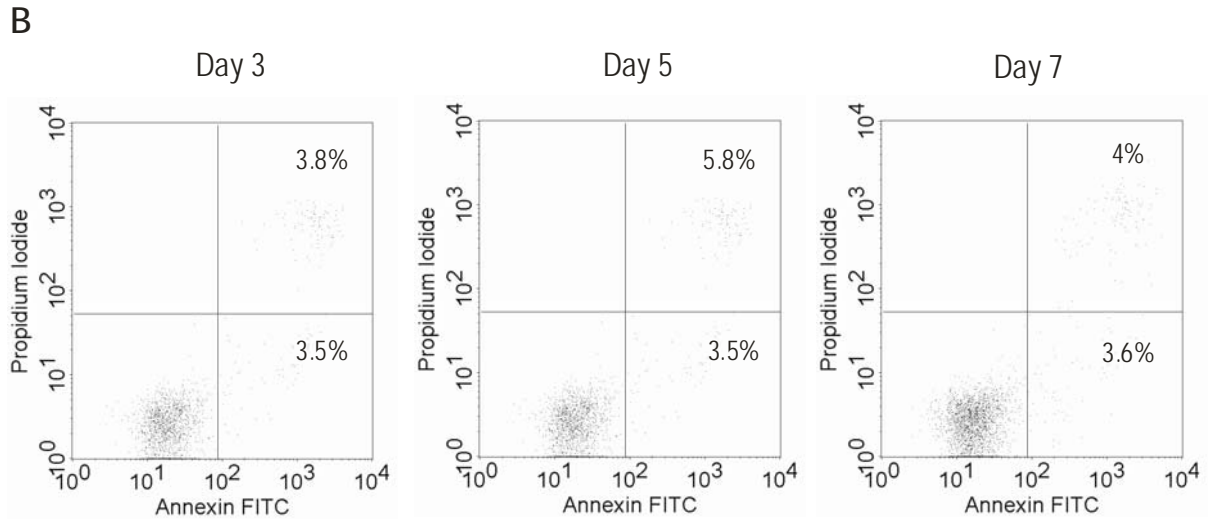
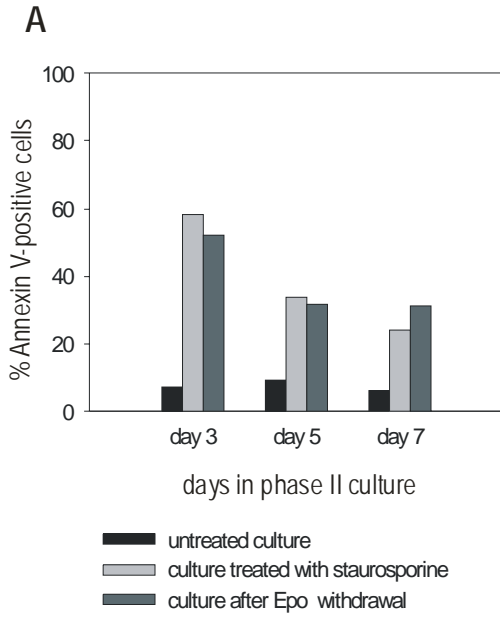
Since it has been shown that cells which are deficient or contain low amounts of cytochrome c are resistant to apoptosis induced via the mitochondrial pathway, we next examined whether by analogy differentiating erythroblasts are protected from apoptosis and whether a correlation between the cytochrome c content in the cells and vulnerability to cell death exist.

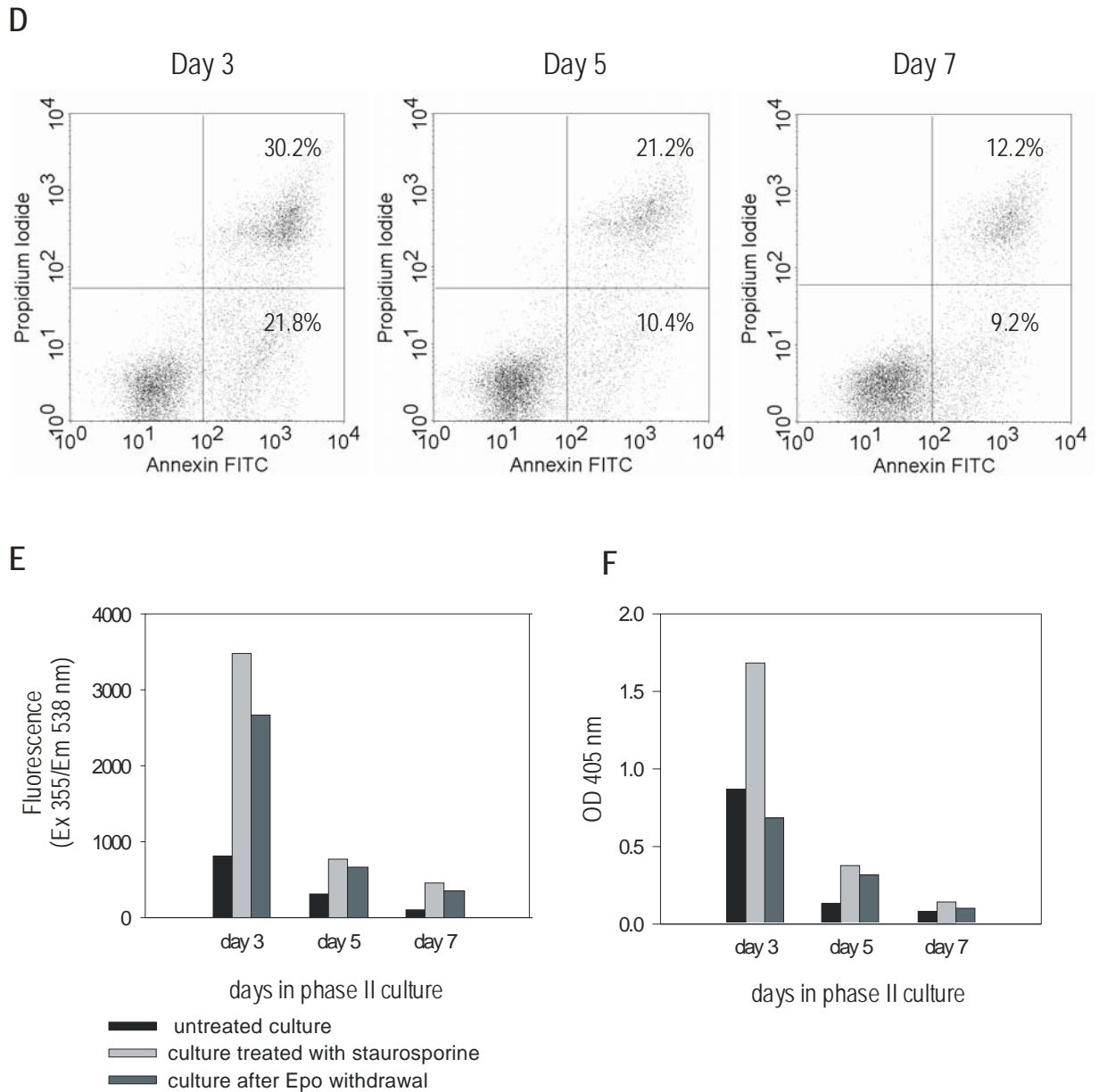
Apoptosis was induced in differentiating erythroblasts at days 3, 5, and 7 in phase II culture by incubation in presence of staurosporine for 6 h or alternatively in absence of the growth factor Epo for 12h. Cells were then examined for PS exposure, caspase-3 and caspase-9 activity.

Treatment with staurosporine at day 3 in culture resulted in the induction of apoptosis in 58.4% of the cells, of which 30.4% were detected as Annexin V+/PI+ and 28% as Annexin V+/PI-(Fig. 20 A and C). Almost similar results were obtained when the cells were grown in the absence of Epo for 12h (Fig. 20 A and D). In this case apoptosis was induced in 52% of the cells, of which 30.2% were detected as Annexin V+/PI+ and 21.8% as Annexin V+/PI-.

The situation however changes at day 5, when the treatment with staurosporine lead to the detection of 33.6 % apoptotic cells, of which 17.5% were AnnexinV+/PI+ and 16.1% were Annexin V+/PI-. Comparably, Epo withdrawal resulted in apoptosis induction in 31.6 % of the cells in culture. Of them 21.2% were Annexin V+/PI+and 10.4% were Annexin V+/PI-. Further reduction in the number of apoptotic cells was observed at day 7, when 24.2% (11.4% Annexin V+/PI+ and 12.8% Annexin V+/PI-) were detected after treatment with staurosporine. In the case of Epo withdrawal 31.2 % of the cells were apoptotic, of which 12.2% were Annexin V+/PI+ and 9.2% were Annexin V+/PI-.

Similarly, caspase-3 activity as quantified by the cleavage of the specific substrate DEVD-AFC, was almost 5-fold fewer at day 5 compared to day 3, and further decreases almost 2-fold at day 7 in staurosporine treated culture (Fig.20 E). Quite similar pattern of caspase-3 activity was also detected when cell were cultured without Epo. Caspase-9 activity followed very similar pattern upon staurosporine treatment with almost 4-fold reduction at day 5 compared to day 3 and further reduction by almost 3-fold at day 7. Similar reduction was also observed when the cells were cultured without Epo (Fig.20 F).

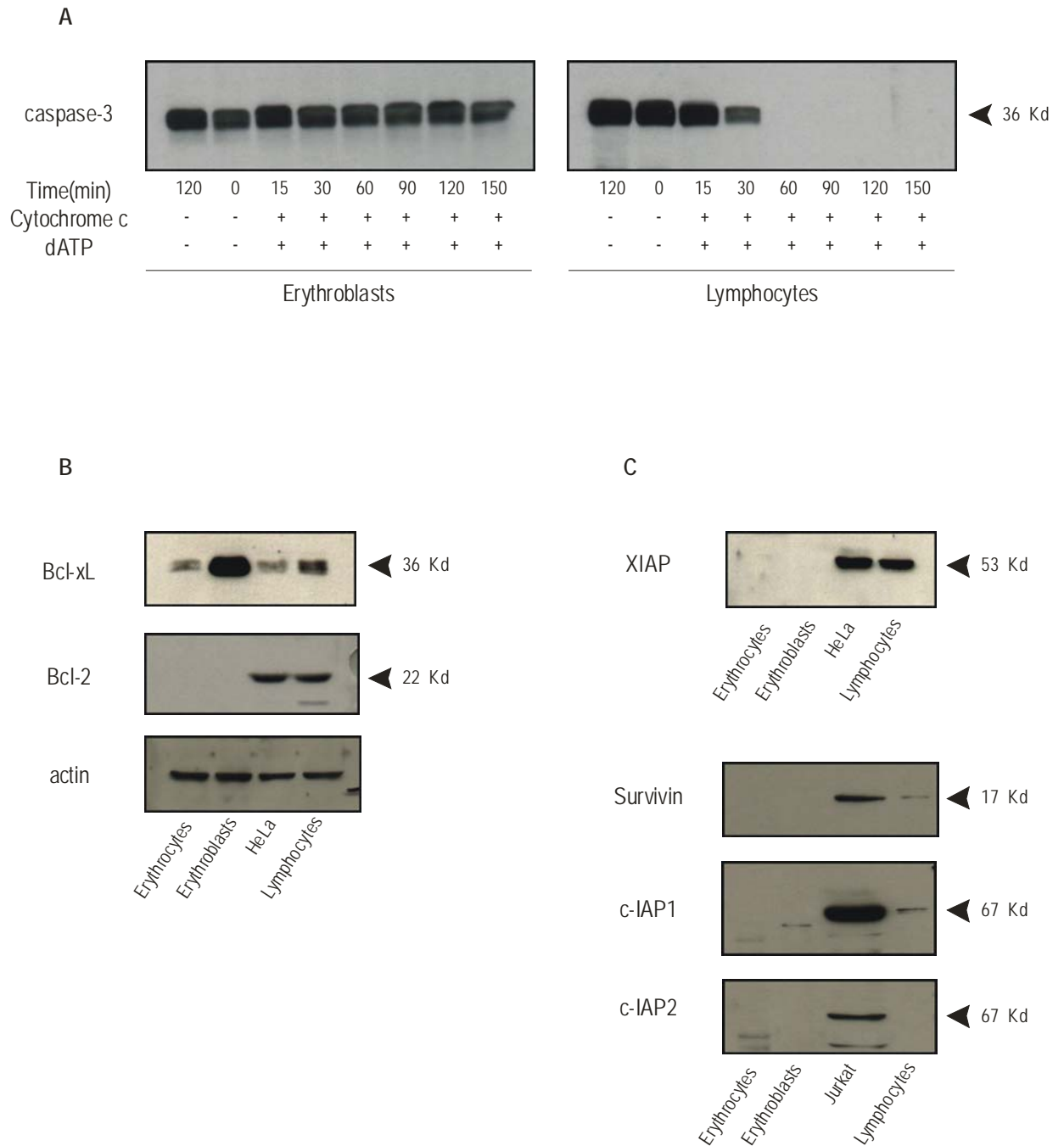




**Figure 20. Late stage erythroblasts are protected from apoptosis.** A - Percentage of apoptotic cells as measured with Annexin V-FITC at the indicated days of differentiation after treatment with staurosporine or Epo withdrawal; B - Dot blots of a representative experiment showing the percentage of apoptotic cells at various stages of differentiation – untreated culture; C – Representative experiment indicating the percentage of apoptotic cells at the indicated stages of differentiation after treatment with staurosporine; D - Dot blots at days 3, 5 and 7 of differentiation after Epo withdrawal; E and F - Caspase-3 and respectively caspase-9 activity at the indicated times of differentiation (representative experiment).

## **5.6 Inability to activate erythroblast caspases in vitro**

To examine whether the absence of cytochrome c is the limiting factor, we next investigated whether erythroblasts caspases can be activated in vitro, with exogenously added cytochrome c. Therefore we incubated cytosolic extracts of erythroblasts and peripheral lymphocytes with cytochrome c and dATP and then analyzed caspase-3 processing by immunoblot. As expected, cytosolic extract from erythroblasts and lymphocytes failed to support caspase activation in the absence of exogenous cytochrome c, even when dATP was present in the system. (Fig. 21 A). However, when cytochrome c was added to the lymphocytes extract caspase-3 processing was detected after 15 minutes and procaspase-3 was completely cleaved after 1h. In contrast, addition of cytochrome c to the erythroblast extract did not result in processing of caspase-3 even after 2,5 h incubation. These results therefore demonstrate that while the absence of cytochrome c might be an important factor preventing caspase activation in erythroblasts, it is certainly not the only mechanism involved in this process.



**Figure 21. Protective mechanisms in late stage erythroblasts**

A – In vitro activation of caspase-3. Failure of exogenous cytochrome c and dATP to initiate caspase activation in cytosolic extracts from erythroblasts. Erythroblast extracts were either left untreated or treated for the indicated time with cytochrome c and dATP, and then analysed for caspase-3 processing. In comparison lymphocyte extract was used. B – Levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL determined by immunoblot. C – Levels of IAPs proteins examined by immunoblot.



## **5.7 Erythroblasts contain elevated bcl-xL levels, but are deficient in IAPs**

Several regulatory mechanisms for prevention of caspase activation and prevention of apoptosis have been described. Although the lack of cytochrome c might participate in preventing caspase activation in erythroblasts, our results showed that it is not the main mechanism involved in this process. Other possible reasons include elevated levels of anti-apoptotic proteins, such as bcl-2 and bcl-xL. To explore this possibility, we assessed the relative levels of Bcl-xL and Bcl-2 in erythroblasts, erythrocytes and lymphocytes. As Fig.21 B illustrates, this analysis revealed that erythroblasts possess highly elevated levels of Bcl-xL but undetectable amounts of bcl-2 protein. A second possible mechanism for protection against apoptosis could be provided by elevated levels of molecules which directly bind and inhibit caspases, such as inhibitor of apoptosis proteins (IAPs). To evaluate the role of IAPs we analyzed the levels of some members of the Inhibitors of apoptosis proteins (IAPs) family, namely XIAP, survivin, c-IAP1 and c-IAP2. Our results show that erythroblasts do not contain detectable amounts of any of the IAP proteins (Fig. 21C).

## DISCUSSION

It has been well accepted that apoptotic mechanisms are involved in differentiation of erythroblasts cells. Apoptosis controls the homeostasis of red blood cell lineage by balancing survival and death of precursor cells. Thus, in physiological conditions, the accumulation in the erythroblastic islands of mature erythroblasts expressing death-receptor ligands may temporarily inhibit the expansion and differentiation of immature erythroblasts or even cause apoptosis of these cells [8, 9, 12].

However, a second and more intriguing role for apoptotic effectors has also recently been proposed. It has been suggested that apoptotic mechanisms may facilitate erythroblast differentiation and enucleation without “killing the cell” [3, 14-16, 34, 53]. This assumption is based on reports confirming the morphological similarities between differentiating erythroblasts and apoptotic cells, but also on reports indicating that several apoptotic mechanisms are activated during erythroblast differentiation. In this regard it has been shown that caspases are activated and mitochondria are permeabilized during differentiation. However, the exact role of these as well as other apoptotic mechanisms in erythropoiesis has not yet been fully elucidated.

Hence we and others have examined the role of several apoptotic mechanisms involved in erythroid differentiation. Although our study was mainly focused on the importance of DNA fragmentation, we have also explored the role of caspase activation. What is more, we have also examined the relationship between these two mechanisms and the order in signaling within they occur during development. Further, we have shown that mature erythroblasts are protected from apoptosis and have also attempted to describe some of the molecular mechanisms, which contribute to this protection.

## 1. Establishment of erythroid cultures

To answer the questions we wanted to address in this study it was first necessary to establish a model system of erythroid differentiation that would fully recapitulate *in vivo* erythropoiesis and would also be amenable for manipulation. Under our culture conditions, almost all of the CD36<sup>+</sup> erythroid progenitors became erythroblasts, as shown by cell morphology and cell surface markers. By day 7 in culture, 60% of the cells were at the stage of orthochromatophilic erythroblasts and over 30% of the cells have proceeded further to become erythrocytes (Fig. 6). Longer times in culture did not result in a significant increase in the percentage of enucleated cells. Thus, only a fraction of the cells that became orthochromatophilic erythroblasts were able to progress to the next stage and become erythrocytes. This might, at least in part, be due to the relatively low number of cell populations, such as macrophages, required for enucleation. Although structures comprising of a central macrophage surrounded by orthochromatophilic erythroblasts, similar to the erythroid islands in bone marrow were detected, their number might not have been sufficient to support the enucleation of higher number of orthochromatophilic erythroblasts. Early erythroblasts transited very efficiently and synchronously through the stages of erythropoiesis, since all of the CD36<sup>+</sup> cells progress through the proerythroblast and basophilic stage to become orthochromatophilic erythroblasts. Therefore we assumed that this culture system provides a valid model of erythropoiesis

## **2. Involvement of apoptotic-like mechanisms in erythropoiesis**

### **2.1 Caspase activation during erythroid differentiation**

Using this culture system, we examined the role of apoptotic-like mechanisms during erythroblast differentiation. Recently, it has been reported that caspase activation is associated with and required for erythroid differentiation in both human and murine culture systems [14-16]. Therefore we tested whether this was also true in our culture system. Treatment of the cultures with a broad spectrum caspase inhibitor (z-VAD) resulted in significant block of erythroid differentiation with accumulation of early progenitors whereas the number of late erythroblasts was significantly reduced (Fig.7 and 8). In particular, caspase inhibitor arrested erythroid differentiation at the basophilic stage of differentiation and greatly reduced the capacity of these cells to complete erythropoiesis (Fig.9). Because of this arrest, these results also suggested that caspase activation was involved in erythroblast maturation after the basophilic stage. This hypothesis was confirmed by experiments indicating that the activity of the two major caspases, caspase-3 and caspase-9, increased during the first 3 days in culture reaching maximal activity at day 3 when the culture consisted mainly of polychromatophilic erythroblasts (Fig. 10 A and B). Moreover, the active forms of caspase-3 and caspase-9, which result from the cleavage of procaspases were also detected at day 3 of differentiation (Fig.10 C and D). Together these results suggest that caspase activation is associated and required for erythroblast differentiation. Thus our results are in a very good agreement with the report of Zermati et al. indicating that both caspase-3 and caspase-9 are activated during erythroblasts differentiation and that caspase activation is required after the basophilic stage of differentiation. In contrast to our data concerning the activation of other caspases (Fig. 10 E), this study also illustrates the activation of caspase-2, caspase-6 and caspase-7 [14].

Our results are also in a good accord to those reported by Carlile et al. where the role of caspase-3 was specifically examined [62]. Similarly to the effect of caspase inhibitors, introduction of caspase-3 siRNA into early erythroid cells impaired their maturation. In this case however cell development was arrested at the proerythroblast stage, which suggests that caspase activation was required after this stage.

An important issue to discuss is how activated caspases could facilitate erythroblast differentiation. A possible role for caspases would be that they cleave nuclear structural proteins. This hypothesis was supported by the fact that activated caspases during erythroblast differentiation cleave nuclear proteins, such as Lamin B and Acinus [3, 14, 15]. This mechanism might be essential for enucleation, since degradation of Lamin B is needed to dissolve the nuclear membrane whereas Acinus fragmentation is required for chromatin condensation. Therefore in the case of erythroid differentiation caspase activation might be regarded as a mechanism which facilitates enucleation.

On the other hand activated caspases might activate nucleases, which are then involved in DNA degradation [31]. Given the importance that DNA is degraded in mature erythroblasts, it seems feasible that caspases might be responsible for nucleases activation. However, we (Fig. 13 A) and others have shown that caspases did not result in CAD activation, and hence argue for a role of caspases in DNA fragmentation [14].

Although the involvement of caspases, especially caspase-3, in erythroblast differentiation is now well established in in vitro system, caspase-3<sup>-/-</sup> mice do not exhibit any obvious defects in erythropoiesis [63, 64]. These contradictory findings suggest that several members of the caspase family can participate in the erythroblast differentiation and thus can substitute each other in different genetic background.

## 2.2 DNA fragmentation during erythroid differentiation

A second apoptotic mechanism involved in erythroblast differentiation is DNA fragmentation. It has been suggested that DNA cleavage probably eases the reorganization of the chromatin structure and subsequent compaction of the nucleus [16].

To date there is little information available regarding the mechanism of DNA fragmentation in differentiating erythroblasts. It has been proposed that degradation of DNA in erythroblast nuclei occurs only after these nuclei have been engulfed by macrophages [1]. It has been suggested that DNA in these nuclei would be possibly degraded by the DNaseII in the macrophages, in a mechanism similar to that observed in phagocytosis. This hypothesis was recently confirmed by DNase II knockout mice, where erythropoiesis was severely inhibited, even though the number of erythroid precursor cells was normal in these mice [33].

On the other hand, a second mode of DNA degradation in erythroblasts, which is cell-autonomous and occurs before enucleation; has also been suggested. This proposal is supported by our initial reports indicating the presence of terminal dUTP nuclear end labeling (TUNEL)-positive erythroblasts in both cord and fetal blood samples as well as in bone marrow samples [34, 53]. Notably, only cells that morphologically could be identified as late erythroblasts were labeled by the TUNEL assay whereas early progenitors were TUNEL-negative (Fig.5). A recent report by Peller et al. has also confirmed our observations and further extended them in that the authors suggested that p53 overexpression in orthochromatophilic erythroblasts triggers “programmed nuclear death” which is associated with DNA fragmentation [16].

Using our well-characterized culture system we have shown here that DNA fragmentation occurs predominantly at the orthochromatophilic stage of differentiation, as the majority of the TUNEL-positive erythroblasts was detected at this stage (Fig.11). Similarly to other reports TUNEL-positivity in our culture samples was not a sign of apoptosis, since the cell viability exceeded 90

% [3, 16]. More detailed analysis looking at the PS exposure have indicated that only small percentage of the cells in culture (up to 9%) were apoptotic (Fig.14). Together these results indicate that DNA in erythroblasts is degraded at late stages of differentiation and the degradation occurs via a cell-autonomous mechanism.

The cell-autonomous mechanism of DNA degradation has been very well examined in the case of apoptosis, where it has been shown that caspase-activated DNase cuts DNA into nucleosomal fragments. These fragments can be then easily detected as a DNA ladder when separated on a gel and thus serve as a sign of apoptosis [32, 65].

However, when DNA samples of erythroblasts at various stages of differentiation were separated on a gel, the pattern did not resemble oligonucleosomal DNA ladder (Fig. 12 B), which suggests that the mode of DNA fragmentation during differentiation may not resemble completely that observed in apoptosis. This finding is in agreement with a study on yolk-sack derived erythroid cells that also failed to detect DNA ladder, although TUNEL staining was detected in the final stages of differentiation [3].

Because it has been shown that in the case of apoptosis the fragmentation of DNA into nucleosomal units is preceded by the degradation of chromosomal DNA into high molecular DNA fragments, we next examined whether this was the case with DNA fragmentation during erythroid differentiation [36]. Indeed, DNA in differentiating erythroblasts was fragmented into high-molecular DNA fragments of about 50-200 kb in length and the degree of fragmentation increased along the cell differentiation, reaching maximum at days 5 and 7 when the culture accumulate terminally differentiated erythroblasts ready to enucleate (Fig.12 C). Therefore these results suggest that DNA degradation in large fragments represents a defined step in the differentiation that occurs before nuclear expulsion. Interestingly, although DNA fragmentation is initiated in a way similar to apoptosis, it does not proceed to oligonucleosomal cleavage. Since

it has been shown that during the DNA fragmentation in large fragments in apoptosis DNA is degraded mainly at the places where it connects to the nuclear matrix it seems likely that this type of fragmentation in erythroblast differentiation would therefore facilitate chromatin condensation and nuclear compaction.

An important issue is to identify the nucleases which are involved in the DNA fragmentation during erythroblasts differentiation. A nuclease that is responsible mainly for the oligonucleosomal DNA fragmentation, but could also contribute to the high molecular DNA fragmentation is the caspase-activated DNase (CAD) described by Nagata [31, 32, 65]. In live cells CAD interacts with and is specifically inhibited by its inhibitor ICAD. During apoptosis, caspase-3 cleaves ICAD, thereby releasing CAD that digests DNA. Because caspase-3 is activated during erythroblast differentiation we checked whether CAD is involved in DNA fragmentation during erythropoiesis. However, by examining ICAD integrity by immunoblot (Fig. 13 A) we have shown that CAD is not activated at any stage of differentiation, suggesting that this protein was not involved in DNA fragmentation.

The second candidate, Apoptosis inducing factor (AIF), is a mitochondrial protein known to translocate to the nucleus during apoptosis and cause there peripheral chromatin condensation [36]. In addition, it has been suggested that AIF itself or alternatively by activating an unknown nuclease can contribute to the high molecular weight DNA fragmentation [36]. Our results however showed that AIF was localized in the mitochondria at any of the stages of differentiation examined and no translocation to the nucleus was observed (Fig.13 B). Therefore these results exclude the involvement of AIF in DNA fragmentation during erythroid differentiation. The suggestion that neither of the two endonucleases examined (CAD and AIF) causes the DNA fragmentation associated with erythroblast differentiation is also confirmed by



the studies examining AIF and ICAD knockout mice [66, 67]. These studies have shown that neither AIF nor ICAD knockout exhibit any obvious defect in erythropoiesis.

### **3. Mature erythroblasts are protected from apoptosis**

While exploring the role of various apoptotic mechanisms during erythroid differentiation, we have also observed that mature erythroblasts, from both culture or cord blood, fail to respond to stimulation with various apoptotic agents (Fig.15 A and B). These observations therefore suggest that these cells are protected from apoptosis. In addition, by examining the sensitivity of differentiating erythroblasts to the apoptotic inducers, we have shown that while early erythroblasts could undergo apoptosis upon chemical triggering, mature erythroblasts are protected. An attractive hypothesis explaining the mechanism(s) of protection against apoptosis, would be that mature erythroblasts being the stage just before erythrocytes are very similar to them. For the latter it is very well established that they are resistant to apoptosis due to lack of mitochondria [68, 69]. In addition, several caspases and other proteins essential for apoptosis induction are also missing.

We have examined the levels of several proteins involved in apoptosis in mature erythroblasts. These proteins include caspases and mitochondrial proteins, namely cytochrome c, Smac and AIF, engaged in mitochondrial pathway of apoptosis induction. We have compared their levels in mature erythroblasts with those in apoptosis sensitive cells (lymphocytes and HeLa cells) and apoptosis resistant cells (erythrocytes).

Similarly to apoptosis sensitive cells the main caspases were present in mature erythroblasts, suggesting that lack of caspases is not the mechanism by which these cells are protected (Fig.15 C). However, in contrast to the apoptosis sensitive cells treatment with apoptosis inducers did not

result in caspase activation, indicating that other component(s) involved in the caspase activation might be missing (Fig.16).

Since the apoptosis inducers used engage apoptosis via mitochondrial pathway it was tempting to speculate that the protection from apoptosis observed in mature erythroblasts is due to lack of mitochondria. In addition, it has been shown that mitochondria gradually decreased along the erythroblast differentiation, which makes the assumption even more attractive [42]. However, mitochondria although present in small numbers are not completely absent in mature erythroblasts, since they could be easily visualized by staining with mitochondria specific dye MitoTracker (Fig. 18 B). In addition, mitochondrial proteins, including MnSOD, Hsp 60 and Bcl-xL (Fig. 17 B and 18 C), were detected in the samples, thus further implying that mature erythroblasts do contain mitochondria.

Surprisingly, examining the levels of mitochondrial components we were able to show that cytochrome c content decreases during the differentiation, reaching minimum at the final stage of differentiation as seen in culture or even completely absent as seen in cord blood erythroblasts (Fig.18 A). We also observed that decrease of cytochrome c follows a specific pattern, in that the levels did not change significantly for the first 3 days followed by a massive decline at day 5 in culture (Fig.19). Together these results therefore suggest that the absence of cytochrome c in the final stages of differentiation is not due to the lack of mitochondria. Instead, the disappearance of cytochrome c could result from its release from the mitochondria into the cytoplasm where it is eliminated. Since mitochondrial permeabilization occurs early in the differentiation, corresponding to day 2-3 in culture, when caspases are also activated [14], it seems feasible that cytochrome c is indeed released from mitochondria in order to “endogenously” activate caspases during the differentiation process. This event is then followed by the rapid disappearance of cytochrome c from the cytoplasm. Similar mechanism has been also described in differentiating

lens fibre cells [70]. However, the mechanism by which cytochrome c disappears from the cytoplasm has not yet been elucidated.

Given the importance that cytochrome c is a crucial factor for apoptosis induction via the mitochondria pathway it was tempting to conclude that the extremely low levels/absence of cytochrome c in mature erythroblasts is the mechanism which protects these cells from apoptosis. However, addition of cytochrome c to erythroblast extracts in an in-vitro system did not result in caspase activation (Fig.21 A), which suggests that although the absence of cytochrome c might be an important factor in protecting these cells from apoptosis, it is certainly not the only mechanism involved in this process.

The resistance of mature erythroblasts to apoptotic stimuli could be due to multiple factors. In addition to the lack of cytochrome c, which as already discussed has a limiting effect, other possible mechanisms include elevated levels of anti-apoptotic proteins, such as bcl-2 and bcl-xL. As shown in several reports, while the expression of bcl-xL increases gradually along the differentiation, reaching a maximum level immediately prior the enucleation, bcl-2 was not detected in erythroblasts [22]. In a similar way, our results have shown that mature erythroblasts contain high levels of Bcl-xL whereas bcl-2 was not detected (Fig.21 B). Therefore this suggests that the protection of late stage erythroblasts might at least in part be due to the high levels of Bcl-xL.

A second possible mechanism could be provided by elevated levels of molecules which directly bind active caspases and target them to the proteasome or alternatively bind procaspases and thereby prevent their activation [71]. In both mechanisms these proteins called inhibitor of apoptosis proteins (IAPs) inhibit caspases and thus protect from apoptosis. The role of IAPs during erythroid differentiation was examined for the first time in the present study. However, no

detectable levels of these proteins were observed in mature erythroblasts, implying that IAPs are not involved in the protection of these cells (Fig.21 C).

Based on our present findings, a key aspect of the mechanisms allowing the mature erythroblasts to escape apoptosis is suggested to be the combination of decrease in cytochrome c levels along the differentiation and extremely high expression of Bcl-xL anti-apoptotic protein.

It remains, however, unknown why it is necessary for mature erythroblasts to be protected and what is the physiological role of this phenomenon. One suggestion would be that since the differentiation of erythroblasts is time and energy consuming process, it would be disadvantageous for the organism to allow killing of cells which are then needed to perform an essential physiological function, namely oxygen transport. Another reason is provided by the studies, indicating that DNA in mature erythroblasts is fragmented in order to facilitate enucleation. Altered DNA structure and function is a signal for a cell to undergo apoptosis. However if this would be the case in mature erythroblasts, it would mean that the organism would be never able to make erythrocytes. Therefore it is clear that in order to produce functional erythrocytes, protective mechanisms allowing mature erythroblasts to escape apoptosis should be developed.

In addition to DNA fragmentation other apoptotic mechanisms, such as caspase activation and mitochondria permeabilization, are also activated during erythroblast differentiation. Hence erythroblasts differentiation is considered as a specialized kind of attenuated apoptotic process, in which the cell degrades its organelles including the nucleus but falls short of disintegrating entirely. It is therefore likely that the protective mechanisms in mature erythroblasts might assure avoidance of cell demise and thereby facilitating completion of the differentiation program.

Based on the available data and our own results we would like to propose a model for erythroid differentiation, whereby yet unknown signal(s) trigger caspase activation at early stages of

differentiation via cytochrome c-dependent pathway. Activated caspases cleave then nuclear structural proteins, therefore facilitating nuclear compaction and chromatin condensation.

These nuclear changes or alternative stimuli could induce p53 expression to execute “nuclear cell death”, which is associated with DNA fragmentation at the final stage of differentiation. This last step further eases chromatin condensation and therefore assists enucleation.

In addition, to escape cell demolition and thereby complete erythroblast differentiation several protective mechanisms are also functional at the final stage of differentiation. Together the above mentioned mechanisms result in enucleation to produce mature erythrocytes.

## **METHODS**

### **1. Cell culture and erythroblast isolation**

#### **1.1 In vitro generation of erythroid progenitor cells**

CD34<sup>+</sup> progenitor cells were obtained from umbilical cord blood (CB) collected after normal full-term deliveries. Use of all samples was approved by the institutional board committee.

Light-density mononuclear cells were separated by Ficoll-Histopaque centrifugation at 680g for 20 minutes. The accompanying RBCs were lysed by suspending the mononuclear cell pellet in Amonium Chloride solution (Stem Cell Technologies) and enriched for CD34<sup>+</sup> cells (typically of >90-95% purity) by two cycles of positive selection using anti-CD34 antibody and magnetic cell sorting on Midi-MACS then Mini-MACS columns ( CD34 Progenitor Cell Isolation kit, Miltenyi Biotech, Glodbach, Germany). CD34<sup>+</sup>-enriched cells were counted and viability (typically of 95-98%) determined by trypan blue exclusion. Cells were suspended in serum-free medium (StemSpan SFEM, StemCell Technologies) at a final concentration of 10<sup>5</sup> cells/mL in the presence of 10ng/mL recombinant human (rh) IL-3, 10ng/ml rhIL-6, 100ng/mL rh SCF and 2U/mL hrEpo.(all cytokines were purchased from Stem Cell Technologies). The cultures were incubated at 37°C in 5%CO<sub>2</sub> in air during a 7-day period. To ensure good cell proliferation, an equal volume of fresh medium containing the cytokines was added on days 3 and 5 of culture. At day 7, cells were pelleted by centrifugation, counted and resuspended in phosphate-buffered saline (PBS) with 1% BSA. Monoclonal CD36 IgG1 antibody (Immunotech) was added to the cell pellet at a final concentration of 1 µg/10<sup>6</sup> cells and incubated for 30 minutes at 4°C. Cells were washed in PBS-BSA, then incubated with rat anti-mouse IgG1 antibody coupled to magnetic microbeads (Miltenyi Biotech). Immunomagnetic separation was performed on either Midi-MACS or Mini-MACS columns, depending on the numbers of cells treated. CD36<sup>+</sup> at concentration of cells 10<sup>5</sup>/mL were cultured again for 7 days in the presence of 5 ng/mL rh IL-3,

100 ng/mL rhSCF and 10 U/mL rhEpo. To ensure good cell proliferation and avoid cell growth arrest cells were diluted every 2 days with fresh medium containing the cytokine mixture to a concentration of  $10^5$  cells/ mL. Samples were collected at day 0, 1, 3, 5 and 7.

To assess the effect of caspases on erythroid differentiation and enucleation the permanent broad spectrum caspase inhibitor z-VAD-fmk (Alexis) was resuspended in methanol according to the manufactures' instructions and added to the culture at concentration of  $150\mu\text{M}$  at day 0. Thereafter the culture medium was always supplemented with  $150\mu\text{M}$  z-VAD.

### **1.2 Erythroblast isolation**

Magnetic activated cell sorting (MACS) was used to isolate late (ortochromatic) erythroblasts from cord blood samples found to contain mainly this erythroblastic population. In brief, mononuclear cells were separated by Ficoll-Histopaque centrifugation at 680g for 20 minutes. Cells were then washed twice with PBS containing 1% BSA and incubated with  $10\mu\text{l}$  of anti-glycophorin A antibody (Mylteniy Biotec) per each  $10^7$  cells for 15 minutes at  $4^\circ\text{C}$ . The antibody labeled cells were then separated over a Mini MACS column (Mylteniy Biotec) according to the manufactures' instructions. GPA+ and GPA- fractions were stored for further assays.

### **1.3 Cell proliferation and differentiation assay**

Cell proliferation was assessed by counting cell number every day during the proliferation phase after trypan blue dye exclusion staining. Cell differentiation was assessed by morphologic analysis of cells after cytocentrifugation and May-Grünwald-Gimsa staining at days 0, 1, 3, 5 and 7 during the differentiation phase. The composition of the cell population was determined by assessing and counting cells in 5 different fields of view, for a total of 5000 cells. In addition,

cells were analyzed for cell surface marker expression by flow cytometry. In brief, approximately  $1.10^6$  cells were collected, washed with PBS containing 1% BSA and 0.1% sodium azide and incubated with monoclonal antibodies against CD34, CD36, CD71 and Glycophorin A (Pharmingen). Cells were then washed and stained with appropriate FITC-conjugated secondary antibody (Pharmingen).

## **2.Preparation of cell protein extracts, subcellular fractions and immunoblotting**

### **2.1. Preparation of cell protein extracts and immunoblotting**

Total cells lysats were prepared by lysing the cells in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 3 $\mu$ g/ml aprotinin, 3 $\mu$ g/ml leupeptin, 3 $\mu$ g/ml pepstatin A and 2 mM phenylmethylsulfonylfluoride (PMSF). Subsequently proteins were separated under reducing conditions by SDS-PAGE and electroblotted onto Transfer-Blot nitrocellulose membrane (Bio-RAD laboratories). Immunoblot analysis used specific antibodies and enhanced chemoluminescence-based detection (SuperSignal West Pico Chemiluminescent Substrate, Pierce)Antibodies used were: polyclonal anti-human cytochrome c, caspase-3, caspase-9, caspase-6, caspase-7, Bcl-xL, XIAP and anti-rabbit IgG HRP-linked antibody (Cell Signaling); polyclonal anti-human cIAP-1, cIAP-2, survivin(MBL); polyclonal anti-human actin, AIF, Smac, ICAD, Hsp 60(Santa Cruz Biotechnology); polyclonal anti-human caspase-8 (Oncoge research products), polyclonal anti-human Apaf-1, Mn SOD (StessGen).

### **2.2 Preparation of subcellular fractions**

To obtain cytosolic and mitochondrial extracts, freshly isolated erythroblasts were fractionated with Mitochondrial/Cytosol Fractionation kit (Biovision) according to the manufactures



instructions. In brief,  $5 \times 10^7$  cells were washed with ice-cold PBS, resuspended in 1ml Cytosol Extraction Buffer Mix containing DTT and protease inhibitor, and incubated on ice for 10 min. Cells were then homogenized with manual pestle. Plasma membrane permeabilization of cells was confirmed by staining in a 0.2% Trypan blue solution. The homogenate was then centrifuged at 700g for 10 minutes at 4°C and the supernatant was centrifuged at 10 000g for 30 minutes at 4°C. Then the supernatant was saved as cytosolic extract. The pellet was solubilized in 0.1ml Mitochondrial Extraction Buffer mix containing DTT and protease inhibitor, vortex for 10 seconds and saved as mitochondrial extracts.

### **3. Assays for apoptosis**

#### **3.1 Measurement of caspase activity**

In order to avoid Hb interference on fluorometric and colorimetric assays, the active caspase-3 or 9 forms from the cells lysats were first immobilized on ELISA plates with anti-caspase 3 or 9 antibodies and then incubated with the specific substrates.

Caspase-3 activity was assessed by the cleavage of the fluorometric substrate Ac-DEVD-AFC (Caspase-3 activity assay, Roche). AFC released from the substrates was excited at 355 nm. Emission was measured at 538 nm.

Caspase-9 active forms were captured by anti-caspase-9 antibody (cat.N 9502, Cell Signaling) and caspase-9 activity was determined by cleavage of the colorimetric substrate LEHD-pNA(R&D Systems). pNA released from the substrate was measured at 405nm.

#### **3.2 Terminal dUTP nuclear end labeling (TUNEL)**

TUNEL staining was performed using a commercially available in situ cell death detection kit (Roche Molecular Biochemicals) according to the manufactures' instructions. In brief, the cells

were cytopspined on a glass slide and fixed with 4% paraformaldehyde/PBS solution at room temperature for 30 minutes. After washing with PBS the slides were permeabilized with 0.1% Triton X-100/0.1% sodium citrate for 5 minutes on ice and washed twice with PBS. Cells were then incubated with 50 $\mu$ l TUNEL mixture at 37° C in a humidified chamber. After washing twice, the slides were incubated with 50  $\mu$ l Converter-AP followed by incubation with the DAKO Fuchsin Substrate-Chromogen (DAKO). As positive and negative controls, the samples were incubated with DNase I and label solution devoid of terminal deoxynucleotidyl transferase (TdT), respectively. The TUNEL staining was confirmed by a light microscope (Zeiss Axioskop, Germany).

### **3.3 Apoptag Peroxidase In situ Oligo (ISOL) Apoptosis Detection kit**

In situ staining of double strand DNA breaks was performed by the ApopTag in situ oligo ligation (ISOL) kit using hairpin oligonucleotide probes(Paper Didenko, Biotechniques). ISOL analysis was carried out on erythroblasts cells at various stages of differentiation using oligo A and oligo B nucleotides according to the manufacturer's instructions (Intergen, Purchase, NY). In brief, the cells were cytopspined on a glass slide and fixed with 1% paraformaldehyde/PBS solution at room temperature for 10 minutes. After washing with PBS the cells were post-fixed in ethanol: acetic acid (2:1) for 5 min at -20° C and the endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in PBS for 5 min at room temperature. Following washing the cells were incubated in 1x Equilibration buffer for 20 min at room temperature. Then Working Strength DNA ligase enzyme solution, containing Oligo A or Oligo B nucleotides and DNA ligase, was applied and the cells were incubated for 16 h at RT. After washing and incubation with Streptavidin-Peroxidase conjugate for 30 minutes at RT, the color reaction was

developed with DAB peroxidase substrate. The ISOL staining was confirmed by a light microscope (Zeiss Axioskop, Germany)

### **3.4 DNA gel electrophoresis**

Oligonucleosomal DNA fragmentation was detected by agarose gel electrophoresis (ref).

DNA samples for pulse field gel electrophoresis (PFGE) were prepared by lysis of cells embedded in agarose plugs (1.106 cells/plug) with the CHEF genomic DNA plug kit (Bio-Rad Laboratories) followed by digestion of proteins with proteinase K (25U/ml final concentration) in Proteinase K reaction buffer, according to the manufactures' protocol. PFGE was carried out in a Bio-Rad CHEF DR II system (Bio-Rad Laboratories) at 14°C for 18h at a voltage gradient of 6V/cm with the switch time ramped linearly from 0.5-60s. After electrophoresis the gel was stained with SYBR gold nucleic acid gel stain (Molecular Probes).

### **3.5 Cell-free apoptotic assay**

Cell-free reactions were set up in 200µl reaction volume, where 100µl cell extract was brought to a final volume of 200µl in Cytosol Extraction Buffer Mix. Caspase activation was initiated by the addition of 50µg/ml horse heart cytochrome c (Sigma) and 1mM dATP. 25µl samples of cell-free reactions were collected at various times and frozen at -20°C for subsequent analysis of caspase activation.

## **4. Cytochrome c assays**

### **4.1 Immunostaining**

Freshly enriched erythroblasts and lymphocytes were cytopsin on microscope slides at a density of 1x10<sup>5</sup> cells/spot followed by immediate fixation in 4% paraformaldehyde/PBS for 20

minutes. Fixed cells were then washed three times in PBS and permeabilized in 0,1% saponin in PBS for 10 minutes followed by incubation with 200nM Mitotracker Red (Molecular Probes) for 30 minutes. Cells were blocked for 30 minutes in 1%BSA/PBS and probed with a 1:50 dilution of anti-cytochrome c (Santa Cruz) for 2 h at room temperature. After incubation with primary antibody, cells were washed three times in 0.1% saponin/PBS followed by probing for 1h with 1:500 dilution (3µg/ml final concentration) of Cy2-conjugated goat anti-rabbit antibodies (Jackson Immunoresearch Laboratories). Immunostained cells were washed three times with 0.1% saponin/PBS, stained with 5µg/ml DAPI in PBS, and mounted in Prolong antifade mounting medium (Molecular Probes).

## **5.2 Cytochrome c ELISA**

For a quantitative assay of cytochrome c in the samples, a cytochrome c ELISA kit was used according to the manufactures' instructions (Oncogene Research Products). Briefly, cells were washed twice with ice-cold PBS, resuspended in cell lysis buffer to a concentration of 1,5x10<sup>6</sup> cells/ml and incubated for 1h at room temperature with gentle mixing. Following centrifugation at 1000g for 15 minutes, the supernatant was collected, diluted 5-fold with Calibrater diluent and 100µl cell lysat was transferred to the microtiter plate wells containing immobilized anti-cytochrome c antibody. Following a two-hour incubation the wells were washed with Wash buffer, 200µl of cytochrome c conjugate was added to each well and incubated for 30 minutes at room temperature. After washing 200µl of Substrate Solution was added, incubated for 30 minutes at room temperature and the optical density was measured at 450 nm. Cytochrome c concentration in each sample was calculated with a standard curve of the calibrators supplied with the kit.

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 Nationality            Bulgarian  
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 Languages            English, German, Russian, Bulgarian

## EDUCATION AND RESEARCH EXPERIENCE

- 2000-present            **University of Basel, Switzerland**  
 Ph.D. candidate, Biomedical Research and Pharmacology-graduation in August
- University Women's Hospital, Laboratory for Prenatal Medicine  
**Graduate Research Assistant**
- Studied the involvement of apoptotic mechanisms in red blood cells differentiation. Demonstrated their influence on the analysis of fetal nucleated red blood cells in maternal blood.
  - Analyzed the correlation of anti-DNA and antiphospholipid antibodies with the circulatory DNA levels in pregnancies affected by preeclampsia.
- 2000                      University of Basel, Biozentrum, Department of Biochemistry  
**Visiting scholar**
- Studied the mechanisms for generation and maintenance of polarity in endothelial cells
- 1997-2000              Bulgarian Academy of Sciences, Institute for Immunology-Sofia, Bulgaria  
**Staff Scientist**
- Developed quantitative ELISA assays for digoxin. Demonstrated possible applications of these assays for drug monitoring
  - Demonstrated the importance of autoantibody testing for clinical correlations and prognosis in patients with recurrent pregnancy loss and preeclampsia
- 1992-1997              **Sofia University "St Kliment Ochridski"- Sofia, Bulgaria**  
 M.S., Molecular Biology and Clinical Chemistry

## AWARDS

2000	Boehringer Ingelheim Travel fellowship, Boehringer Ingelheim Fonds, Heidesheim, Germany
1996-1997	Fellowship for Academic Excellence, Sofia University

## PUBLICATIONS

### Peer reviewed publications

- **Hristoskova S**, Holzgreve W, Hahn S. More than one-half of the erythroblasts in the fetal circulation and cord blood are TUNEL positive. **Clin. Chem.** **2001** Oct; 47 (10): 1870-1.
- **Hristoskova S**, Holzgreve W, Hahn S. Fetal nucleated red blood cells in maternal circulation do not display a classical membrane associated apoptotic characteristic (phosphatidylserine exposure) despite being TUNEL positive. **Clin Chem** **2003** Nov; 49(11):1934-7.
- **Hristoskova S**, Holzgreve W, Hahn S. Anti-phospholipid and anti-DNA antibody levels do not correlate with circulatory DNA levels in pregnancies affected by preeclampsia. **Hypertens Pregnancy** (in press)

### Publications in preparation

- **Hristoskova S**, Holzgreve W, Hahn S. Erythroblast differentiation and enucleation – a link with apoptosis?
- **Hristoskova S**, Rusterholz C, Holzgreve W, Hahn S. Working title: Role of caspase activation and DNA fragmentation during erythroblast differentiation and enucleation
- Babochkina T, **Hristoskova S**, Mergenthaler S, Tercanli S, Holzgreve W, Hahn S. Fetal nucleated red blood cells in maternal blood are not amenable to analysis by FISH: a feature related to their nuclear phenotype.

### Book contributions

- Hahn S, Zhong XY, **Hristoskova S**, Zimmermann B et al. Prenatal Diagnosis using fetal cells and free fetal DNA in maternal blood: Current status in Basel. Early prenatal diagnosis, fetal cells and DNA in the mother

## PRESENTATIONS

- **Gordon Research Conference on Cell Death**, Colby College, Waterville, MA. **2002**

- **Cold Spring Harbor Laboratory Research Conference on Cell Death,**  
Cold Spring Harbor Laboratory, NY, **2003**