[Frontiers in Bioscience 14, 2358-2371, January 1, 2009]

Various functions of caspases in hematopoiesis

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1. ABSTRACT

The role of cysteine proteases of the caspase family in apoptosis is well defined. Some caspases were initially shown to be involved in cytokine maturation along inflammatory response. In the recent years, several other non apoptotic functions of caspases were identified. In hematopoietic cells, caspases play a role in specific pathways of differentiation (erythropoiesis, differentiation of monocytes into macrophages, formation of proplatelets by megakaryocytes). These enzymes also play a non-apoptotic and complex role in regulating the maturation and proliferation of specific lymphocytes. Lastly, the apoptotic functions of caspases regulate the life span of several but not all blood cell types. The present review summarizes the current knowledge in these different functions. We show that the nature of involved enzymes, the pathways leading to their activation and the specificity of their cellular target proteins varies strongly from a cell type to another. We indicate also that, in most situations, specific Bcl-2-related proteins are involved in the control of caspase activation. Lastly, we discuss the deregulation of these pathways in hematopoietic diseases, including those in which an excess in caspase activation leads to cell death and those in which a default in caspase activation could block cell differentiation.

2. INTRODUCTION

Homeostasis of the mammalian hematopoietic system, which is critical for normal health, is maintained by balancing stem cell proliferation with commitment to multipotent progenitors and differentiation of hematopoietic lineages. Each mature blood cell has a specialized function and a characteristic life span, from a few days to several years. Blood elements are replaced in response to physiological demands, i.e. when blood cells age and die, and to pathophysiological situations needing functional expansion of one or several hematopoietic cell types (1).

Growth factors control hematopoietic cell production by preventing hematopoietic precursor apoptosis (1). For example, erythropoietin (Epo) stimulates erythrocyte production through activation of Janus kinase 2 (Jak2) and the signal transducer and activator of transcription (Stat) Stat5, which induce expression of Bcl-X_L, an anti-apoptotic protein of the Bcl-2 family (2). Bcl-X_L can bind to and inhibit the proapoptotic BH3-only protein of the Bcl-2 family known as Nix (Nip-like protein) or Bnip3L (3). Any deficiency in this pathway results in apoptosis of erythroid precursors and anemia (4, 5). The number of early hematopoietic progenitors depends on the

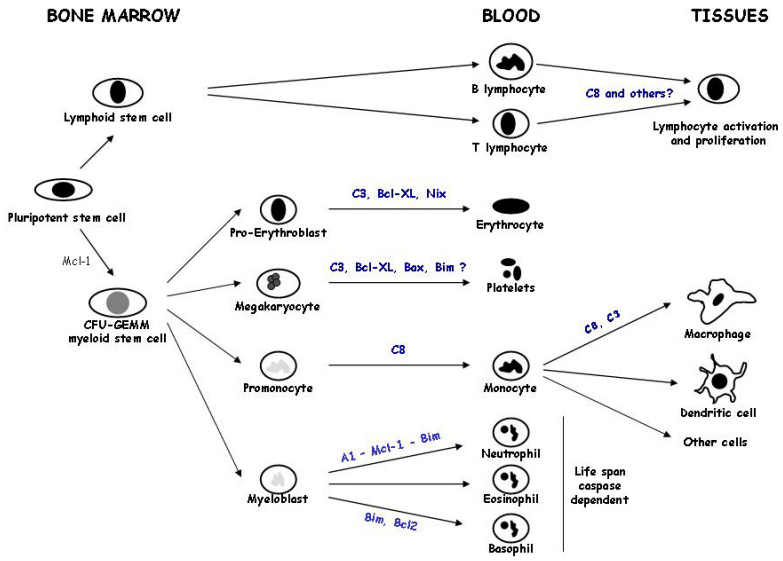

Figure 1. Schematic representation of the main steps of hematopoiesis. Caspases and Bcl-2-related proteins were involved in the control of the differentiation of specific hematopoietic cell types. Caspases also regulate the activation and proliferation of several types of lymphocytes and modulate life span in specific mature blood cells.

expression of another anti-apoptotic Bcl-2 family member, myeloid leukemia-1 (Mcl-1) (6).

One of the hallmarks of apoptotic cell death is the activation of cysteine proteases of the caspase (**c**ysteinyl **asp**artate protein**ases)** family. When activated, these enzymes cleave their substrates on the carboxy-terminal side of an aspartate residue. This cleavage can lead to dismantling of the cell and death when involving a number of intracellular proteins. Caspases are involved in the death of hematopoietic precursors (7, 8). Their role in the death of mature blood cells depends on cell type and death conditions (9, 10).

The activation of caspases does not always trigger apoptosis. These enzymes have demonstrated vital functions in cell proliferation, cell differentiation, cell shaping, lipid metabolism, DNA repair and cell migration (11). Whether caspase activation leads to cell death or not depends on a variety of regulatory mechanisms that include localized activation of these proteases (12), downregulation of downstream nucleases such as CAD (Caspase-Activated DNase) (13) and protection of essential targets from proteolysis by chaperones (14). In the hematopoietic system, controlled activation of caspases has been implicated in the differentiation of erythroid cells as well as that of monocytes into macrophages, the formation of platelets and the clonal expansion of lymphocytes (Figure 1). The aim of the present review is to summarize the current knowledge in the various functions of caspases in hematopoiesis.

3. STRUCTURE AND CLASSIFICATION OF CASPASES

Caspases are an evolutionarily conserved family that currently includes 15 enzymes in mammals. These enzymes, which are synthesized as inactive procaspases, are virtually expressed in all cell types. They can be classified according to the length of their N-terminal prodomain into two main groups and function in cell death induction as a proteolytic cascade in which caspases with a long prodomain act upstream of those with a short prodomain (15).

Procaspases with a short prodomain (procaspase-3, -6 and -7) exist in the cells as dimers that require proteolysis at internal aspartate residues to generate two large and two small subunits. Active enzymes result from heterodimerization of these subunits, thus include two active sites. These caspases are the main effectors or executioners of apoptotic cell death by cleaving cellular substrates, either a downstream procaspase or other cellular proteins, on the carboxy-terminal side of an aspartate residue. Other procaspases are characterized by their long prodomain and exist in living cells as monomers that require dimerization or oligomerization for activation, which can occur in the absence of any proteolytic cleavage (15). These initiator enzymes cleave a few specific substrates. These substrates include effector caspases that are activated by this cleavage.

Several caspases synthesized with a long prodomain have rapidly demonstrated non apoptotic functions. For example, the first identified caspase (caspase-1) is the interleukin-1β-converting enzyme (ICE), a cysteine protease that processes the precursor pro-IL1-β into mature cytokine (16, 17). Caspase-1, which also processes IL-18 and IL-33, is activated in a multi-molecular complex called “the inflammasome” (For review, see (18)). Effector caspases have longer been thought to be involved only in apoptosis because of their enzymatic activity with a relatively broad specificity but recent studies have pointed out their non-apoptotic functions.

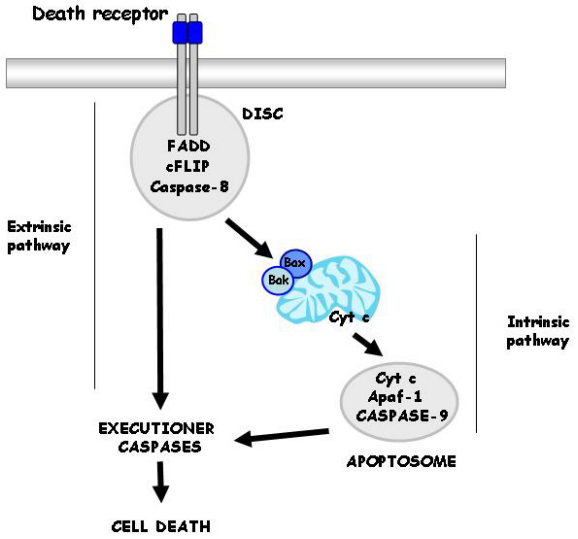

Figure 2. Simplified representation of the two main caspase-dependent apoptotic pathways and their connections. The intrinsic pathway involves the mitochondria and the downstream formation of the multi-molecular platform known as the "apoptosome". The extrinsic pathway involves death receptors at the plasma membrane and the formation of another multi-molecular platform, the death-inducing signaling complex (DISC). Both lead to executioner or effector caspase activation and dismantling of the cell.

4. CASPASE-DEPENDENT APOPTOSIS

Caspases participate in two main pathways leading to apoptosis. Less defined pathways such as dependence receptor induced, caspase mediated apoptosis do exist. The extrinsic pathway is activated when plasma membrane-associated death receptors interact with their specific ligand at the cell surface (19). This ligand/receptor interaction induces the formation of a death-inducing signaling complex (DISC) involving the adaptor molecule FADD (Fas-Associated Death Domain) and procaspase-8 and/or 10. These enzymes are activated and either directly activate a proteolytic caspase cascade or cleave a BH3-only protein of the Bcl-2 family known as Bid to connect the extrinsic to the intrinsic pathway.

This second apoptotic pathway requires mobilization of one or several BH3-only proteins of the Bcl-2 family in response to cellular damage (for review see (20)). These sentinel proteins migrate to the mitochondria to either inhibit antiapoptotic or stimulate multidomain proapoptotic proteins of the Bcl-2 family. This interplay between Bcl-2-related proteins leads to the permeabilization of the outer mitochondrial membrane (21). One of the soluble molecules subsequently released in the cytosol is cytochrome *c* that induces Apaf-1 (apoptotic protease-activating factor 1) oligomerization and procaspase-9 recruitment, which is activated in this complex, the so-called "apoptosome". In turn, active caspase-9 activates downstream effector caspases that cleave a number of intracellular substrates (for review, see (11) and Figure 2).

5. CASPASES, HEMATOPOIETIC STEM CELLS AND EARLY PROGENITORS

In the fetal liver, then in the bone marrow, hematopoietic stem cells (HSCs) divide slowly and continuously and more HSCs are formed than are necessary to maintain the stem cell pool and provide starting cells for differentiation (22). Therefore, apoptosis is one of the biological events that regulate the HSC pool. Accordingly, transgenic mice ectopically expressing the antiapoptotic protein Bcl-2 in their HSCs demonstrate a two-fold increase in the number of these cells and these transgenic HSCs outcompete wild-type HSCs when transplanted into a lethally irradiated host (23, 24).

HSC apoptosis must be tightly regulated. Bcl-2 is poorly expressed in HSCs in which another antia-poptotic member of the Bcl-2 protein family, Mcl-1, plays a central role in the negative control of apoptosis. Mcl-1 is highly expressed in HSCs and its expression decreases in further differentiated progenitors (25). Inducible deletion of *mcl-1* gene in mice results in bone marrow ablation through the loss of HSCs and early progenitors, indicating that Mcl-1 is essential for promoting the survival of these cells (6).

Whether caspases play a role in the death process that regulates HSC pool has not been explored. The CD95-dependent extrinsic pathway to caspase activation and death does not seem to be involved as CD34-positive HSCs express low levels of CD95 (26-29) and are resistant to CD95-mediated apoptosis (28, 30). Molecular analyses have attributed this resistance to the expression of FLIP (FLICE inhibitory protein), an endogenous inhibitor of CD95 signaling (28, 30) or to the lack of expression of functional isoforms of caspase-8, replaced by a smaller splice variant (30). These observations do not rule out a role for the intrinsic pathway to caspase activation in HSC death.

The cytokine-induced proliferation of downstream early hematopoietic progenitors involves signaling pathways in which FADD and the catalytic activity of caspase-8 play a non apoptotic function. Transgenic expression of a dominant-interfering mutant of FADD or the viral caspase-8 inhibitor cytokine response modifier A (CrmA) negatively interfere with the ability of fetal liver cells to generate myeloid or lymphoid cells upon transplantation into irradiated mice (31). Whether the death receptors are involved in this regulatory effect remains a matter of controversy (31, 32) and FADD, caspase-8 and FLIP could function together in a pathway that is independent of death receptors and downstream effector caspases to promote proliferation during early hematopoiesis.

6. CASPASES AND BONE MARROW MICROENVIRONMENT

The microenvironment in which stem cells reside is essential for their survival, self-renewal, and differentiation. HSCs receive multiple survival signals from this microenvironment, e.g. the signaling molecule PECAM-1 (platelet endothelial cell adhesion molecule-1)

inhibits the intrinsic pathway of apoptosis through Akt activation (33). Bone-marrow architecture involves HSCs in close proximity to the endosteal surface, and osteoblasts, the bone-forming cells, are an essential component of the complex bone marrow cellular network (34). Caspase-3 plays a role here as it is specifically required for osteogenic differentiation of mouse and human bone marrow stromal stem cells by negatively regulating the TGF-β signaling pathway. Caspase-3 disruption also down-regulates the osteogenic master protein Runx2/Cbfa1 (runt-related transcription factor 2 / core binding factor 1 alpha) in pro-osteoblasts (35).

Surprisingly, caspase-8 demonstrated the opposite effect as its activation through CD95 engagement specifically inhibited osteoblast differentiation (36), which could explain the increased bone mass observed in mice that lack a functional CD95-ligand (*gld* phenotype) (37). Whether caspase-dependent regulation of bone formation affects hematopoiesis now requires investigation.

7. CASPASES IN PLATELET FORMATION

The vital function of blood platelets is blood clotting. These anucleate cells adhere to altered endothelial cells or extracellular matrix in the event of overt vascular injury and initiate the formation of a hemostatic plug. They originate from the cytoplasm of bone-marrow precursors known as megakaryocytes (for review, see (38)). These direct platelet precursors share with erythrocyte precurors a common progenitor but their terminal differentiation is very different, e.g. erythroid cells undergo cell-cycle arrest, nuclear condensation, and enucleation whereas megakaryocytes proceed through endomitosis before formation of long, thin, bifurcating cytoplasmic extensions called proplatelets. Platelet formation then occurs into bone marrow sinusoids by fragmentation of these cytoplasmic extensions.

Caspase-3 plays a distinct role in red cell and platelet formation: its transient activation in basophil erythroblasts is required for their maturation into poly-chromatophil erythroblasts, long before enucleation and red cell formation ((39) and see below), whereas its localized activation in the cytoplasm of mature megakaryocytes promotes the formation of proplatelets (12, 40). In addition, active caspases were identified in both the cytoplasm and the nucleus of maturing erythroblasts in which the key transcription factor GATA-1 is protected from caspase-3 mediated proteolysis (as observed in Epo-deprived erythroblasts) by interaction with the chaperone stress protein HSP70 (14) whereas active caspase-9 and caspase-3 remain localized in specific cytoplasm compartments of mature megakayocytes, before proplatelet formation (12). The anti-apoptotic molecule called survivin is also differentially required in erythrocyte and platelet formation (41, 42) but this difference is probably related to its unequivocal role in mitosis rather than to its controversial ability to modulate caspase activity. Interestingly, the platelets generated by a caspase-dependent pathway are functional and retain phosphatidylserine membrane asymmetry and inner mitochondrial membrane potential (40).

The pathway leading from thrombopoietin stimulation to caspase-3 activation in mature megakaryocytes and its link with intrinsic and extrinsic pathways of activation identified in apoptosis setting remains a controversial issue. A careful analysis of mitochondria in proplatelet extensions using the fluorescent dye JC-1 did not identify any permeability transition and cytochrome c was present in the mitochondria of platelets generated from these extensions (40). However, immunostaining suggested a transient release of cytochrome c in mature megakaryocytes that demonstrate a localized activation of caspase-3 (12). Overexpression of either Bcl-2 (12) or Bcl-X_L (43) limits proplatelet formation. In accordance with these later observations, transgenic mice overexpressing Bcl-2 in hematopoietic cells demonstrate a 2-fold reduction in their blood platelet number without alteration of their bone marrow megakaryocyte number (24). Interestingly, a similar observation was made in mice with a homozygous deletion of *bim*, which could indicate that shedding from megakaryocyte depends on this BH3-only protein (44).

Staurosporine, which activates the intrinsic pathway to death, induces megakaryocyte apoptosis without stimulating the formation of proplatelets (12) whereas Fas ligation and introduction of active caspase-8 were shown to stimulate the proplatelet extension (40) but this difference could be related to the use of too high doses of staurosporine in the first study. Anyway, heterochromatin condensation typical of early apoptosis was observed by electron microscopy in megakaryocytes undergoing the earliest stage of cytoplasmic extension (40) whereas DNA looked non fragmented (12). Additional studies are needed to determine whether the formation of proplatelets is a consequence of early steps of a classical death pathway that ends with senescence and death of the denuded megakaryocytes (45) or involves a specific pathway in which some actors of cell death such as caspase-8 and the mitochondria could also play a role, as observed in monocytes (46, 47).

Another important question deals with the role of active caspases in proplatelet formation. Proteins such as gelsolin and poly (ADP-ribose)polymerase 1 (PARP1) were suggested to be caspase targets in this context (12) but whether and how their cleavage contributes to the formation of cytoplasmic extensions, e.g. by inducing cytoskeletal changes, remains unknown. Whereas caspase-mediated cleavage of PARP1 impairs the DNA repair capacity of an apoptotic cell, the generated fragments could modulate the activity of DNA-bound NF-κB, as shown in inflammatory cells (48). The status of GATA-1, which is cleaved by caspases in erythropoietin-deprived erythroid precursors (14), has also to be checked in mature megakaryocytes.

Interestingly, murine caspase-12 is a downstream target of NF-E2 (nuclear factor-erythroid 2), a hematopoietic-restricted transcription factor whose deletion prevents platelet formation (49). In mice, caspase-12 is highly expressed in megakaryocytes and participates in the signaling pathway linking some G protein-coupled receptors to activation of the αIIbβ3 integrin (50). In humans, a single nucleotide polymorphism in the *caspase-12* gene results in the synthesis of either a truncated protein (Csp12-S) or a full-length but catalytically inactive enzyme (Csp12-L) (51).

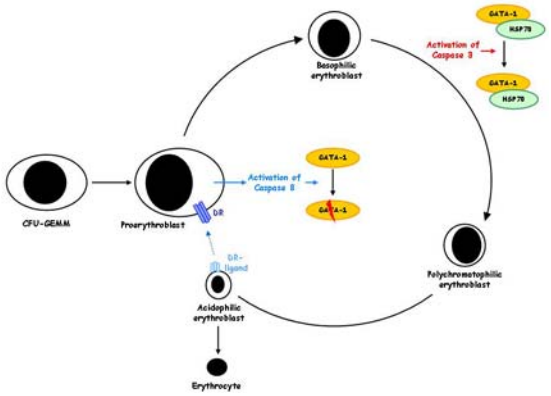

Figure 3. Functions of caspase in the control of red cell production. Two caspase-dependent pathways were involved in the control of erythropoiesis. The first is a negative feedback loop by which mature erythroblast expressing death receptor ligand interact with immature erythroblast and trigger their caspase-8-dependent apoptosis and GATA-1 cleavage. The second is a positive role of caspase-3 in the maturation of basophil into polychromatophil erythroblast and GATA-1 is then protected from caspase-mediated cleavage by interaction with HSP70.

Interestingly, both caspase-12 deficient mice (52) and humans expressing the truncated enzyme (51) are more resistant to sepsis, suggesting a function that does not depend on the catalytic activity of the enzyme. Whether such a non enzymatic function of caspase-12 plays a role in human thrombopoiesis remain to be studied.

Chemotherapy-induced thrombocytopenia involves a caspase-dependent death of megakaryocytes that can be prevented by Stem Cell Factor through activation of the serine/threonine kinase Akt (8). Thrombocytopenia is a frequent cytopenia in myelodysplastic syndromes and is caused mainly by ineffective megakaryocytopoiesis whose mechanism is not elucidated. Although controversial (53), the observed increase in megakaryocyte death could indicate a deregulation of the compartmentalized activation of caspases that precedes proplatelet formation in normal platelet precursors (54).

8. CASPASES IN PLATELET LIFE SPAN

Once released in the circulation, platelets rapidly age with a life span of 10-12 days. As in nucleated cells, platelets possess several Bcl-2 family members. By screening mice treated with a mutagen for thrombocytopenia, it was recently demonstrated that Bcl-X_L and Bak were the major components of a molecular clock that determines platelet life span (55). Two mutations that destabilize Bcl-X_L were identified in thrombocytopenic animals that increase the rate of platelet clearance without affecting platelet production. The reduction in blood platelet number without changes in the megakaryocyte aspect observed in mice with a homozygous deletion of bim could indicate that Bim is a third partner in this pathway (44) (Figure 1). In contrast, genetic manipulation of Bcl-2, Bcl-w or Mcl-1 has little effect on platelet life span (55).

Molecules that interfere with proteins of the Bcl-2 family can affect platelet life span (56). The small molecule ABT-737 is a potent antagonist of a subset of antiapoptotic Bcl-2 family proteins that includes Bcl-X_L, Bcl-2 and Bcl-w and induces apoptosis in tumor cells that depend on these proteins for survival (57, 58). In mice and dogs, chronic dosing of ABT-737 induces a rapid thrombocytopenia as the molecule is directly cytotoxic to platelets (59). In contrast to newly synthesized platelets, aged platelets were shown to be highly susceptible to ABT-737-induced caspase-dependent death, as Bcl-X_L degrades with time (55, 60). Actually, Bcl-X_L degrades more rapidly than Bak (9), which might ultimately lead to spontaneous platelet death as Bak appears as the specific killer of platelets. Accordingly, thrombocytopenia could be used as a surrogate marker of the clinical efficacy of some antagonists of the Bcl-2 family (56).

Apoptosis-like events such as cell shrinkage, plasma membrane blebbing and phosphatidylserine redistribution to the cell surface, have been associated also to platelet activation (61) and to the platelet storage lesion that impairs their function before transfusion (62-64). Human platelets contain caspases, the adaptor molecule Apaf-1 and cytochrome c and platelet cytoplasmic extracts can recapitulate apoptotic events upon treatment with cytochrome c. However, depending on the stimulus, the apoptosis-like features that characterize platelet activation and storage lesion can involve either caspases (65-67) or calpains (61, 64).

8. CASPASES IN ERYTHROID DIFFERENTIATION

Erythropoiesis results from the progressive differentiation of HSCs into red cells. The process first involves the progressive differentiation of multipotent into specific progenitors, which progress from proerythroblasts to mature erythrocytes through basophilic, polychromatophilic and orthochromatic erythroblasts. Along this process, red cell precursors become progressively sensitive to the glycoprotein hormone erythropoietin (Epo) that is produced in response to hypoxia and, together with other cytokines, stimulates the expression of erythroid-specific transcription factors such as GATA-1 (68).

At these stages, withdrawal of Epo activates the intrinsic pathway to caspase-dependent apoptosis, indicating that Epo protects erythroblasts from caspase-dependent cell death (69). A critical regulator of erythroid cell apoptosis is the anti-apoptotic protein Bcl-X_L (70) whose expression is dependent on Epo through the transcription factor Stat5 (2, 4, 71, 72). Stem cell factor (SCF), which also protects erythroid precursors from apoptosis (73, 74), up-regulates both Bcl-X_L and Bcl-2 (7, 75, 76) but the Epo/ Bcl-X_L pathway is both necessary and sufficient for terminal erythroid differentiation (77). Bcl-X_L binds to and inhibits Nix (also known as Bnip3L), a proapoptotic BH3-only protein of the Bcl-2 family that behaves as a negative regulator of terminal erythrocyte differentiation (3).

Several observations argue for a regulatory role of the death receptor / death receptor ligand systems in normal erythropoiesis. In zebrafish, morpholino knockdown of the death receptor gene *zDR* increases hemoglobin content in embryos (78) whereas adults expressing a dominant negative *zDR* mutant demonstrate polycythemia (79). In mice, caspase-8 knock-out embryos exhibit congested erythrocyte accumulation in most blood vessels and in various organs (80). In humans, apoptotic erythroblasts surrounding macrophages have been described in erythroblastic islands of the bone marrow (81) and an increased expression of death receptors (82) or a decreased expression of c-FLIP$_{short}$ inhibitory protein (83) could account for the excess in erythroblast apoptosis and the anemia that characterize low grade myelodysplastic syndromes (84).

It has been suggested that CD95-ligand expressing, mature erythroblast killed immature erythroblasts that expressed CD95 in the erythroid island, with macrophages clearing dead cells (Figure 3). CD95-mediated apoptosis of immature erythroblast was shown to involve caspase-mediated GATA-1 cleavage (85). Inflammatory cytokines such as tumor necrosis factor (TNF) and interferon-γ could favor erythroid precursor apoptosis by up-regulating the CD95-ligand/CD95 pathway (86, 87). However, the existence of a spontaneous regulatory feedback involving CD95 was challenged by the observation that CD95-defective mice (*lpr* phenotype) did not accumulate erythroid cells (88).

TNF-related apoptosis-inducing ligand (TRAIL) is an alternative negative regulator of erythropoiesis as it specifically induces apoptosis of erythroblasts showing intermediate levels of glycophorin A when generated by *ex vivo* expansion of human CD34$^+$ HSCs in liquid cultures. Importantly, TRAIL does not trigger apoptosis of other myeloid precursors generated in the same conditions (89). In addition to triggering caspase-dependent apoptosis, TRAIL regulates erythroid differentiation through activation of the extracellular signal-regulated kinases, ERK1 and ERK2 (90). Interestingly, erythroblasts derived by *ex vivo* culture of CD34$^+$ HSCs from polycythemia vera patients harboring the V617F mutation of Jak2 demonstrate an increased resistance to death receptor ligand-induced apoptosis, possibly through the up-regulation of the c-FLIP$_{short}$ inhibitor (91).

Behind this negative function of caspases involving mainly caspase-8, a transient activation of several effector caspases appear to play a positive role in erythroid differentiation, as caspase inhibitors or caspase-3 down regulation arrest erythroblast differentiation at the basophilic stage (39, 92, 93) (Figure 3). Differentiation-associated activation of caspase-3 leads to the proteolytic cleavage of several nuclear protein such as lamin B and acinus but the key transcription factor GATA-1 remains protected from caspase-mediated cleavage by interaction with HSP70. When erythoblasts are deprived from Epo, the chaperone stress protein detaches from GATA-1 that is degraded by caspases, thus inhibiting erythroid differentiation (14). Analyses performed in model cell lines suggested that the MEKK1 kinase could be another caspase-3 target in cells undergoing erythroid differentiation (94) and that *CAD (caspase-activated DNase*) gene down-regulation might prevent DNA cleavage downstream of caspase-3 (13). A central question is now to identify the signaling pathways that lead to caspase activation and GATA-1/Hsp70 interaction in response to Epo (95).

Caspase activation remains transient along erythroblast differentiation and occurs probably too early in the process to account for enucleation, a late and only partially understood event in erythropoiesis. Deletion of the *DNase* II gene in mice is lethal, due to severe anemia, but fetal liver cells from mutants generate normal red cells when transferred in irradiated wild-type animals. This observation was related to a non cell-autonomous mechanism in which macrophage DNase II would trigger DNA degradation in mature erythrocyte nuclei (96). This observation could explain why targeted disruption of the *retinoblastoma* (*Rb*) gene in the mouse, which induces macrophage abnormalities, leads to embryonic death caused by failure of erythroblasts to enucleate (97).

It was shown recently that enucleation somehow reproduces some apoptotic events. Nuclei expelled from the erythroid precursor cells quickly expose phosphatidylserine on their surface and are engulfed by the central macrophage in the anatomical unit known as "erythroblastic island" (98).

9. CASPASES IN ERYTHROCYTE LIFE SPAN

Human mature erythrocytes are devoid of nucleus, as well as mitochondria and other organelles, and their normal life span is approximately 120 days. Aged red cells are cleared from the peripheral blood by the reticuloendothelial cells. These mature erythrocytes contain the essential caspases for apoptosis (99) but survive to conditions that induce apoptosis in nucleated cells such as treatment with staurosporine, serum deprivation (100) and exposure to CD95-ligand (101). Interestingly, staurosporine can induce the death of chicken erythrocytes that, unlike mammalian red blood cells, contain a transcriptionally inactive nucleus, but this death is presumably caspase-independent as caspase inhibitors did not prevent it (102).

Human red blood cell aging induces an increase in intracellular calcium and calcium ionophores recapitulate the red blood cell senescence phenotype that includes cell shrinkage, cytoskeletal degradation, plasma membrane blebbing, microvesiculation and phosphatidylserine externalization, leading to cell disintegration and phagocytosis by macrophages (103). In addition to ion homeostasis disruption and the release of prostaglandin E2 (104), this senescence process involves a proteolytic activity that is sensitive to cysteine protease inhibitors (105). However, neither procaspase-3 nor procaspase-8 is activated in red cells exposed to calcium ionophores. The lack of caspase-3 activity can be related to the absence, in these cells, of several essential components of the intrinsic pathway to death, including cytochrome c containing mitochondria and the cytosolic adaptor molecule Apaf-1 (99, 101). As in activated platelets (61), calpains rather than caspases are the cysteine proteases involved in aging of mature erythrocytes; e.g. through spectrin degradation (101). It remains conceivable that caspase-8 and -3 could play a role in a specific red cell function or dysfunction.

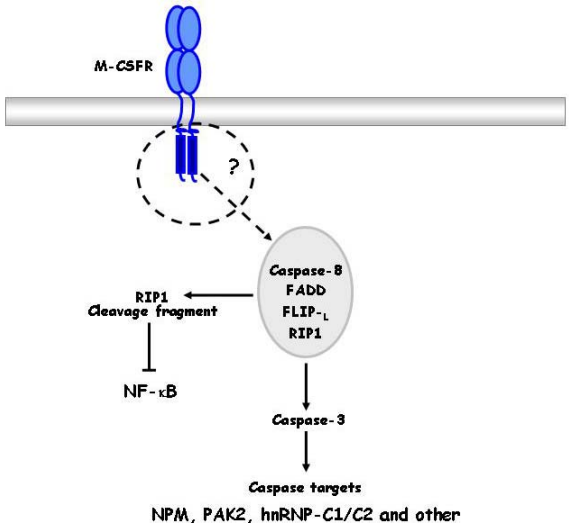

Figure 4. Summary of the current knowledge on the role of caspases in the differentiation of monocytes into macrophages. M-CSF stimulation leads to the formation of a multi-molecular platform in which caspase-8 is activated and cleaves RIP1. In turn, the cleaved kinase down-regulates NF-κB. Caspase-3 is activated downstream of caspase-8 and cleaves other target proteins.

10. CASPASES AND MONOCYTE DIFFERENTIATION INTO MACROPHAGES

Circulating monocytes originate from HSC differentiation in the bone marrow and consist of 5 to 10% of circulating white blood cells in humans. They are heterogeneous in terms of surface markers and functions and behave as committed precursors in transit between the bone marrow and their ultimate sites. Indeed, they rapidly (1-2 days) cross the endothelium to differentiate into a variety of phagocytes, including macrophages and dendritic cells in various tissues, osteoclasts in the bone, microglia in the central nervous system, and Kupffer cells in the liver. The differentiation potential of monocytes is not restricted to cells that function as phagocytes and/or specialized antigen-presenting cells (106), e.g. monocytes expressing the vascular endothelial growth factor receptor-2 (VEGFR-2) could be endothelial progenitor cells (107, 108).

Cytokine conditions that regulate *ex vivo* monocyte differentiation balance between scavenging macrophages and antigen presenting myeloid dendritic cells have been identified, i.e. monocytes differentiate into macrophages in response to macrophage colony-stimulating factor (M-CSF) (109) and into dendritic cells in response to granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) (110-112). At the transcriptional level, dendritic cell differentiation could be instructed by high levels of the myeloid and lymphoid-specific Ets family transcription factor PU.1 together with the down-regulation of the monocyte/macrophage-specific bZip factor MafB (113-115).

When reconstituted *ex vivo*, differentiation of monocytes into macrophages is associated with caspase activation, which is not observed in monocytes undergoing dendritic cell differentiation. Caspase-8 activation is required for *ex vivo* differentiation of monocytes into macrophages and occurs in the absence of any apoptosis (47). This caspase-dependent differentiation, which was also observed in leukemic cell lines whose macrophage differentiation was induced by phorbol esters (47, 116) or by TRAIL (117), was confirmed *in vivo* as conditional deletion of *caspase-8* gene in the mouse myelomonocytic precursors inhibited the formation of macrophages without affecting that of dendritic cells and granulocytes (118). Monocyte activation with lipopolysaccharides or the chemokine CCL8 induces a down-regulation of caspase-8 (119, 120), which might decrease the ability of activated monocytes to form macrophages in response to M-CSF.

Interestingly, deletion of mouse *bid* gene, which encodes a BH3-only protein connecting death receptor-mediated activation of caspase-8 to the intrinsic pathway to death, induces accumulation of monocytes in the peripheral-blood and spleen, thus mimicking the human disease known as chronic myelomonocytic leukemia (121). However, no cleavage of Bid could be detected in peripheral blood monocytes undergoing M-CSF-induced differentiation (unpublished data).

Caspase-8 is the apical enzyme in the caspase cascade leading to macrophage differentiation (Figure 4). In response to M-CSF, caspase-8 interacts with the adaptor FADD, the serine/threonine kinase RIP1 and the long isoform of FLIP in the absence of any death receptors. In macrophages, caspase-8-mediated RIP1 cleavage generated a protein fragment that inhibits the transcription factor NF-κB whose activation is transient along the macrophagic and sustained along the DC differentiation pathways (46).

Active caspase-8 and other caspases activated downstream such a caspase-3 cleave a number of other cellular proteins in monocytes undergoing differentiation into macrophages. These proteins can be either in the cytoplasm or in the nucleus as activated caspases are found in the two cell compartments (47). Identified caspase targets in this setting include several human nuclear RNA binding proteins (hnRNPs), nucleophosmin (NPM), p21-activated kinase-2 (PAK-2), α-tubulin, plasminogen activator-inhibitor-2 (PAI-2), and vinculin (122). Several of these proteins are involved in transcription regulation, cytoskeletal regulation and cell adhesion. In mouse monocytes undergoing differentiation, caspase-3 is also activated, then cleaves and activates the kinase HPK1 (hematopoietic progenitor kinase 1) that in turn activates JNK (c-Jun kinase) (123).

The functional importance of the cleavage of these proteins, that occurs relatively late in the differentiation process, remains to be established. Further studies will shed the light also on how some proteins such as poly (ADP-ribose) polymerase 1 and lamin B are protected from caspase-mediated proteolysis during macrophagic differentiation (47) while being cleaved by caspases during erythroid differentiation (39). More importantly, deciphering the molecular pathway leading to caspase-8 recruitment in a multiprotein complex and activation in monocytes undergoing

macrophagic differentiation could help to understand the pathophysiology of chronic myelomonocytic leukemia.

11. CASPASES IN NEUTROPHIL LIFE SPAN

Neutrophils or granulocytes are polymorphonuclear leukocytes (approximately 60% of all human leukocytes) with a very short life span in the circulation (half-life ~6-18 hours). It is vital that, once these inflammatory cells with a potentially histotoxic content have achieved their physiological function, i.e. to destroy and digest invading microorganisms, they are cleared rapidly. Caspases did not demonstrate any non apoptotic function in granulocytes, e.g. these enzymes were not involved in granulocyte precursor differentiation, and the main function of caspases in these cells is apoptosis. Caspase-dependent spontaneous apoptosis facilitates their dramatic turnover (~10^{11} per day), which is required for immune system homeostasis (124, 125). Once apoptosis has been engaged, the neutrophil secretory activity is shutdown. If this process is impaired, chronic inflammation may ensue (126).

Neutrophils constitutively express a number of proapoptotic and antiapoptotic proteins of the Bcl-2 family (127). Among these proteins, the BH3 only protein Bim and the antiapoptotic proteins Mcl-1 and A1 (Bfl1) play a central role. Mice lacking Bim have a 2-fold increase in blood granulocytes, due to decreased spontaneous apoptosis (44, 128). In these animals, both A1- (129) and Mcl-1 (130) deficient neutrophils exhibit a severely impaired survival, due to enhanced spontaneous apoptosis. The transcription factor NF-κB is also a critical regulator of spontaneous neutrophil apoptosis (131) that leads to recognition and removal by macrophages (125, 132).

Most inflammation-related processes alter neutrophil apoptosis. Several proinflammatory cytokines, chemokines, and growth factors delay neutrophil cell death (124, 133), which is likely important for effective clearance of invading microorganisms. Cytokines such as GM-CSF and granulocyte-colony stimulating factor (G-CSF) increase neutrophil survival and phagocytic functions by increasing the stability of Mcl-1 (134-139). More specifically, GM-CSF prolongs neutrophil survival through activation of JAK/STAT pathway and phosphatidylinositol-3 kinase (PI-3K) that modulate Mcl-1 (140, 141) and Bad (142). Conversely, phagocytosis of bacteria can accelerate apoptosis of neutrophils, presumably to facilitate clearance of these cells when containing dead bacteria (143).

Spontaneous apoptosis of neutrophils is dependent on caspase activation through the intrinsic pathway. This process is temporally associated with an increase in caspase-9 and caspase-3 activity and is attenuated by pan-caspase inhibitors (144, 145). Caspase-8 may be required independently of death receptors but the pathway leading to its activation remains unexplored (146, 147). Caspase-independent mechanisms of apoptosis do exist in these cells, as identified by inhibiting caspases in TNF-α-stimulated neutrophils (148). Calpains are also present in neutrophils and were suggested to play a role in their spontaneous apoptosis (149). Interestingly, cyclin-dependent kinases (CDK) control caspase activation in neutrophils and small molecules inhibitors of CDK such as *R*-roscovitine (Seliciclib or CYC202) facilitates the resolution of inflammation in mouse models through stimulating caspase-mediated apoptosis of neutrophils (150). They molecules developed as anti-cancer drugs may have potential in treatment of inflammatory diseases (151).

12. CASPASES IN EOSINOPHIL LIFE SPAN

Caspases are also involved in spontaneous and drug-induced, e.g. glucocorticoid-induced, apoptosis of eosinophils, which are important effectors in parasitic infections and play a pathogenic role in atopic diseases such as asthma (For review see (152)). Their apoptosis involves the intrinsic pathway to caspase activation, can be induced by ligation of the death receptor CD95 (153) and is potently suppressed by interleukin-5 (154-156), interleukin-3 and GM-CSF. Eosinophils undergoing apoptosis overexpress the TNF receptor family member CD30 at their surface, which could be used to eliminate unwanted eosinophils in atopic diseases as eosinophils become sensitive to CD30-mediated death that is not inhibited by interleukin-5 (157). Similarly, ligation of sialic acid binding immunoglobulin-like lectin 8 (Siglec-8) expressed on eosinophils activates a caspase-dependent pathway that resist to the protective effect of IL-5 and GM-CSF (158). Interestingly, caspases cleave and activate the mammalian sterile 20-like 1 and 2 (Mst1/Mst2) kinases in eosinophils, not in neutrophils (159). A similar caspase-dependent cleavage of Mst1 was shown to be involved in skeletal muscle cell differentiation (160) but how the cleavage of this kinase contributes to eosinophil clearance remains to be explored.

13. CASPASES AND MAST CELL LIFE SPAN

Mast cells, which are other important players in allergy and certain infections, are also dependent on caspases for undergoing apoptosis upon kit-ligand/SCF or serum starvation (161, 162) or upon infection (163). Expression of a *bcl-2* transgene renders these cells almost completely resistant to apoptosis and deletion of *bim* gene induces their partial resistance to death (164, 165). Importantly, the anti-apoptotic function of Bcl-2 in mast cells depends on its association with the heat shock protein HSP90β, which is enforced by kit-ligand, and the disruption of this interaction provokes caspase-dependent apoptosis (166).

Caspase-9 and Apaf-1 are required for mast cell apoptosis as both Apaf1$^{-/-}$ and caspase-9$^{-/-}$ mast cells fail to die by apoptosis upon growth factor starvation. Interestingly, these Apaf1$^{-/-}$ and caspase-9$^{-/-}$ mast cells were also functionally dead, e.g. did not degranulate in response to stimulation through the FcεR (167), but whether this observation indicates a non apoptotic function of caspase-9 and Apaf-1 in mast cells remains to be determined.

Nearly all systemic mastocytosis, which are characterized by clonal proliferation of mast cells, involve the D816V gain-of-function mutation of the receptor tyrosine kinase Kit that promotes receptor autophophorylation (in the absence of Kit-ligand or SCF). This mutation in the kinase domain interferes with binding of classical small molecule tyrosine kinase inhibitors such as imatinib but the novel

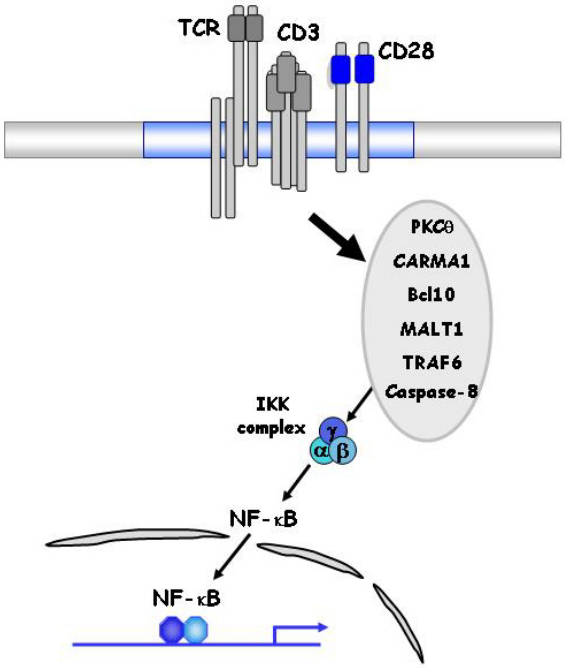

Figure 5. The role of caspase-8 in NF-κB activation upon stimulation of T cells. T cell stimulation induces the formation of a multi-molecular platform known as CBM (Carma 1 / Bcl-10 / MAL-1) complex in which caspase-8 is recruited and activated. In turn, caspase-8 activates NF-κB. The CBM complex forms in lipid rafts of the plasma membrane, which involves protein kinase-C τ and the ubiquitin ligase TRAF6.

molecules that specifically target this mutation such as EXEL-0862 reactivate caspase-dependent apoptosis in human mast cells (168).

14. CASPASES AND LYMPHOCYTE DEATH OR PROLIFERATION

Caspases are involved in the death of many lymphocyte populations, including negatively selected $CD4^+CD8^+$ double-positive T cells, activated B cells in the germinal centre and chronically restimulated peripheral T cells (for review, see (169)). However, apoptosis can occur in lymphocytes independently of caspases, e.g. in thymocytes that fail to express a correctly rearranged TCR (for review, see (170)), whereas these enzymes function at specific checkpoints during immune-cell development, differentiation and proliferation, independently of their role in apoptosis (18, 171).

Caspase-8 was linked to lymphocyte activation in response to specific stimulation such as TcR stimulation (172). Unlike caspase-8–deficient mice, which die *in utero*, caspase-8-deficient humans are immunodeficient and demonstrate hypogammaglobulinemia, recurrent viral infections and mild lymphadenopathy. These patients are homozygous for an inherited mutation in *caspase-8* gene: a single aminoacid change abrogates enzymatic activity and destabilizes the protein. Their B cells, T cells and NK cells show defective activation through antigen receptors (173). The difference between mice and human cannot be explained by the expression of caspase-10, a homologue of caspase-8 that is not expressed in mice, as caspase-10 deficiency generates an autoimmune rather than an immunodeficient disease (174). T-cell specific deletion of caspase-8 in mice provokes a lymphoproliferative rather than immunodeficient phenotype (175).

A main function of caspase-8 in T cells appears to be NF-κB activation. Upon TcR stimulation, a multimolecular complex called CBM forms at the level of plasma membrane lipid rafts. This CBM complex includes Carma1 (CARD-membrane-associated guanylate kinase protein 1), Bcl-10 (B cell lymphoma-10) and the paracaspase MALT1 (mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1) (Figure 5). Caspase-8 is recruited in this complex and enzymatically activated in the absence of any processing. Active caspase-8 is required for the recruitment of IKK (Inhibitors of NF-κB (I-κB) kinase) complex that activates NF-κB (172, 176). CARMA1 recruitment to plasma membrane lipid rafts and Bcl-10-dependent caspase-8 activation involve phosphorylation events mediated by PKCθ (protein kinase C-θ) and PDK1 (phosphoinositide-dependent kinase 1) (177, 178). The ubiquitin ligase TRAF6, which binds to active caspase-8 upon TCR stimulation, also facilitates its movement into lipid rafts (177). Interestingly, in T cells, caspase-8 is required for NF-κB activation in response to TcR stimulation but is dispensable for NF-κB activation in response to TNF (172).

If caspase-8 is required for TcR-induced NF-κB activation in T-cells, short prodomain caspases such as caspase-3 and caspase-7 could play a role in the propagation of NF-κB activation through the cleavage of PARP1 in inflammatory cells, (179). Caspase-generated PARP1 fragments could interact with both the p50 and the p65 subunits of NF-κB and with the co-activator p300 (48). Whether this mechanism operates in lymphocytes remains unknown.

The death domain adaptor FADD and the caspase-8-like cFLIP could also play a role in lymphocyte expansion in response to stimulation (172, 180, 181). FADD was shown to localize in the cell nucleus where it could be phosphorylated by casein kinase 1α and FADD dephosphorylation could be a signal for progression through G_2/M cell-cycle checkpoint (182-186). The role of cFLIP remains controversial. It could affect *ex vivo* T cell expansion after activation as a T-cell specific deletion of *cflip* gene generates T cells with impaired proliferation and survival (186-188). Analyses performed *in vivo* rather than *ex vivo* suggest that FADD and caspase-8 functions are more important in $CD8^+$ than in $CD4^+$ T cells but their precise effects remain elusive as they vary from one animal model to another (for review, see (169)). In B cells, caspase-8 could also mediate the response to toll-like receptors ligands but this effect appears to be independent of NF-κB (189).

Caspase-3 has a conventional apoptotic function in T cells and a less conventional cell-cycle regulatory effect in B cells (190). This later effect involves caspase-3-mediated cleavage of the CDK inhibitor $p21^{WAF1/CIP1}$, which disrupts its ability to associate with proliferating cell nuclear antigen (PCNA) (191).

15. CONCLUSIONS

Caspases control the life span of several blood cell types through their well known function in apoptosis. These enzymes are signaling molecules in the differentiation pathway of specific hematopoietic cell types and the proliferation of some immune cells in response to antigen stimulation. The non-apoptotic functions of caspases implicate a subtle regulation. Pathways of caspase activation that do not lead to cell death have been partly identified. These pathways appear to differ from one cell type to another in the hematopoietic system. A first consequence of these observations is that the presence of activated caspases cannot be sufficient evidence for cell death identification. Another consequence is that complete identification of the pathways leading to caspase activation in the absence of cell death could provide insights in the pathophysiology of several hematological diseases. For example, low grade myelodysplastic syndromes are characterized by an excess in blood cell precursor, caspase-dependent apoptosis. Conversely, monocytes from patients with chronic myelomonocytic leukemia fail to activate caspases in response to M-CSF and to differentiate correctly into macrophages. These observations suggest that further understanding of caspase functions in hematopoiesis should help to design novel pharmaceutical strategies in the future.

16. ACKNOWLEDGEMENTS

Our group is supported by the Ligue Nationale Contre le Cancer (Equipe labellisée). The Ligue Nationale contre le cancer also supports AJ and LG. We apologize to the authors who made important contributions to the field, but have not been cited due to space limitation.

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Abbreviations: Epo: erythropoietine, Jak2: Janus kinase 2, Nix: Nip-like protein, Mcl-1: Myeloid leukemia-1, CAD: Caspase-activated DNase, ICE: IL-1β converting enzyme, DISC: Death inducing signaling complex, HSC; Hematopoietic stem cells, PARP: Poly (ADP-ribose) polymerase, NF-E2: Nuclear factor-erythroid 2, SCF: Stem cell factor, VEGFR: Vascular endothelial growth factor receptor, M-CSF: Macrophage colony stimulating factor, GM-CSF: Granulo-monocyte colony stimulating factor, G-CSF: granulocyte-colony stimulating factor, NPM; nucleophosmin, PAK-2: p21 activated kinase 2, PAI-2: Plasminogen Activator Inhibitor 2, HPK1: hematopoietic progenitor kinase 1, FADD: Fas associated death domain,

Key Words: Caspase, Differentiation, Hematopoiesis, Review

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