



REVIEW

Myelodysplasia and apoptosis: new insights into ineffective erythropoiesis

AA van de Loosdrecht^{1*} and E Vellenga¹

¹Department of Hematology, Academisch Ziekenhuis Groningen, Groningen, The Netherlands

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Myelodysplasia

The myelodysplastic syndrome (MDS) is a clonal hematopoietic disorder characterized by ineffective erythropoiesis associated with morphological evidence of marrow cell dysplasia, resulting in refractory pancytopenia despite normal or even hypercellular bone marrow. The impaired proliferation and differentiation program of myeloid, erythroid and megakaryocytic lineages, whereby the erythroid lineage is most pronouncedly affected, especially in refractory anemia with or without ringsideroblasts (RA/RARS), may become progressive during the course of MDS with eventually transformation to acute myeloid leukemia. Clinical heterogeneity has been demonstrated in MDS. The French–American–British (FAB) classification based on blood and bone marrow morphology has been useful in identifying prognostically subsets of MDS with respect to leukemic evolution and median survival. To improve the clinical and prognostic use of such classifications, the recent international prognostic

score system (IPSS) has been proposed which combines morphological, cytogenetic and clinical data.¹ Other features including morphology assessed by ultrastructural analysis and expression of certain oncogenes may contribute to predict prognosis but are not yet clearly defined.

The pathogenesis of MDS is poorly understood. Different pathogenic defects have been recognized. One of the main characteristics is the impaired *in vitro* erythroid colony formation in response to erythropoietin (Epo) and stem cell factor (SCF) stimulation.² Suspension culture assays have demonstrated that CD34⁺-sorted cells did have the capacity to differentiate to a certain degree, despite the strong impairment in the proliferative response.³ An impaired proliferation of erythroid progenitors in response to growth factor stimulation (SCF, Epo) was shown despite the fact that in some patients a differentiation along the erythroid lineage could be observed.³ The defects in the erythroid development in a subpopulation of patients with MDS is localized at an early stage of erythroid differentiation and is associated with persistent expression of CD34 antigen. The impaired *in vitro* growth may be in part due to an increased apoptosis despite the continuous presence of growth factors.

The *in vitro* data seems to be in paradox with *in vivo* data. Frequently, a hypercellular bone marrow is

*Correspondence: AA van de Loosdrecht, Department of Hematology, Academisch Ziekenhuis Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands Tel: (+31)50 3616161; fax: (+31)50 3614862; E-mail: a.van.de.loosdrecht@int.azg.nl
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observed whereby the erythroid lineage is prominently present. Furthermore, an uncoupling of proliferation and differentiation of erythroid progenitors in MDS could be shown.⁴ The proliferative response of erythroid progenitors in MDS is strongly impaired which might be related to the increased tendency to apoptosis, whereas cells retain the capacity to differentiate in some cases. To further emphasise ineffective erythropoiesis in MDS ferrokinetic studies may contribute to understanding its pathogenesis.⁵ Increased normal as well as decreased ETU (erythron transferrin uptake, indicating the proliferative status of erythroid progenitors *in vivo*) values were observed in MDS. A diminished ETU value correlated significantly with the presence of sTfR (soluble transferrin receptor) in cases with refractory anemia. A significant difference in ETU values was observed between RA and RARS (RA with ringsideroblasts). Most of the cases with an increased ETU value showed an augmented percentage of erythroblasts in the BM which was inversely related with serum Epo levels. Transfusion dependency was associated with normal to increased ETU levels and cytogenetic abnormalities. These observations demonstrate that different patterns of defects can be observed in the erythropoiesis in MDS whereby normal to increased ETU levels and the presence of cytogenetic abnormalities differentiate between cases of RA with ineffective erythropoiesis with regular transfusions and cases which are relatively transfusion independent.

Apoptosis signaling

So far, two different pathways can be recognized which trigger the apoptotic process. The caspase-8/3 pathway is activated by different death receptors while the caspase-9/3 pathway is triggered by mitochondrial disruption which will be highlighted in the following part especially in conjunction with MDS.⁶

Death receptors

Apoptosis plays a central role in the development and homeostasis of eukaryotic cell processes.⁷ Aberration of normal physiological apoptosis may be detrimental; failure of cells to initiate apoptosis after DNA damage may contribute to malignant transformation. Death receptors, cell surface receptors that transmit apoptosis

signals by specific ligands, play a central role in initiating apoptosis. These receptors belong to the tumor-necrosis-factor (TNF) receptor gene family among which are CD95 (Fas/APO1), TNFR1 (p55/CD120A), Death Receptor (DR)-3 (APO3), DR4 and DR5 (APO2).^{7,8} The ligands of the death receptors are structurally related molecules that belong to the TNF gene superfamily (CD95L, TNF, APO3L, APO2L).^{9,10} CD95 ligation leads to clustering of receptor death domain FADD (Fas-associated death domain) which activates procaspase-8, a cysteine protease. Caspase-8 activates downstream effector caspases committing the cell to undergo apoptosis. TNF trimerizes TNFR1 upon binding after which TRADD (TNFR-associated death domain) complexed to FADD-TNFR1-TRADD complex activates procaspase-8, thereby initiating apoptosis. Furthermore, TRADD may bind with TNFR-associated factor-2 (TRAF2) and receptor interacting protein (RIP) to stimulate nuclear transcription factors such as nuclear factor- κ -B (NF- κ B) and AP-1. DR3 triggers responses that resemble those of TNFR1. Signalling through DR4 and DR5 to caspases appears distinct and has not yet been fully identified.

In normal hematopoiesis, the development of the erythroid lineage is dependent on Epo and the presence of the Epo receptor (EpoR). The expression of the EpoR correlates with the biological responsiveness being low on primitive erythroid precursors, high on proerythroblasts and is down-regulated in the late basophilic erythroblast stage.¹¹ With Epo deprivation erythroid progenitors undergo apoptosis which might be regulated by the expression of (proto)oncogenes such as *bcl-X_L* and *bcl-2* (see in detail below). High levels of *bcl-X_L* and *bcl-2* during later phases of erythroid differentiation may prevent erythroblasts from apoptosis. In addition, in normal hematopoiesis, Fas is rapidly up-regulated in early erythroblasts and expressed at high levels through terminal differentiation.¹² In contrast, Fas-L is selectively induced in early differentiating Fas-insensitive erythroblasts. Fas-L bearing mature erythroblasts displayed a Fas-based cytotoxicity against immature erythroblasts which is abrogated by high levels of Epo.¹² These findings suggest a negative feedback apoptotic control mechanism between mature and immature erythroblasts dependent on Epo and thereby regulating erythrocyte production in physiologic erythropoiesis. Fas cross-

linking in mature erythroblasts is not able to transduce a death signal even in the absence of Epo, suggesting that Fas in mature erythroblasts is not coupled to the apoptotic machinery. In approximately 50% of patients with MDS, increased Fas and Fas-L expression in particular on erythroblasts were found as compared to erythroblasts in normal individuals.^{13–16} These findings of Fas/Fas-L expression in MDS might be related to the differentiation defect and result in a dysfunctional negative feedback control mechanism and an increased tendency to apoptosis. A second possibility regarding the involvement of Fas/Fas-L in the apoptotic signaling in MDS might be due to an altered function of cells in the microenvironment. Fas-L expression has been demonstrated on CD68⁺-cells (reflecting bone marrow macrophages), suggesting that the bone marrow microenvironment may play a role in and/or initiating apoptosis of Fas⁺-erythroblasts.¹⁵ A role for macrophages as one of the major cellular components in the bone marrow microenvironment was further supported by studies demonstrating increased erythroid proliferation and terminal differentiation leading to enucleation that was mediated through macrophages.^{17,18} In the absence of macrophages erythroid cells mature to late erythroblasts but fail to enucleate. A novel protein Emp (erythroblast macrophage protein) has now been fully characterized which mediates attachment of erythroblasts to macrophages, preventing apoptosis and potentiating further maturation.¹⁸

These pathways are of importance in MDS since it was shown that TNF α levels were significantly elevated in patients with RA compared to patients with RAEB/RAEB-t. The presence of TNF α inducible apoptotic DNA-fragmentation in MDS and a reduced number of apoptotic cells in the presence of anti-TNF α -receptor antibodies supports its involvement.¹⁹

Implication of mitochondria in apoptosis

It has long been assumed that mitochondria are not part of the apoptotic process since mitochondria remained morphologically intact during the apoptotic process. Furthermore, cells lacking mitochondrial DNA were fully competent to undergo apoptosis. Only recently, it became evident that mitochondria are the main regulators of apoptosis.^{20,21} By using different fluoro-

chromes (Rhodamine 123, DiOC6, JC1) measuring mitochondrial transmembrane potential ($\Delta\Psi_m$), it became evident that a decrease in $\Delta\Psi_m$ is a distinct (early) feature of apoptosis in different cell types using different apoptosis inducers.²² The change in $\Delta\Psi_m$ resembles the opening of mitochondrial permeability transition pores (MPT) or mitochondrial megachannel.^{23,24} At high levels of conductance the MPT is permeable to molecules up to 1.5 kDa culminating in large amplitude swelling of the mitochondria matrix. After large amplitude swelling, rupture in the outer membrane permits the release of cytochrome c (apoptotic protease factor-2; Apaf-2), a 12 kDa protein, as well as other apoptotic inducing factors.^{25–27} Cyclosporine-A (CsA) blocks the onset of MPT, cytochrome c release, and apoptotic cell death, showing that MPT is required for cytochrome c release and apoptose. Subsequently, cytochrome c coupled to Apaf-1 activates Apaf-3/caspase-9 initiating the proteolytic cascade leading to apoptosis. Recently, it has been demonstrated that crosstalk exists between the activation of the caspase-8/3 pathway and the cytochrome c release.^{28,29} A cytosolic protein, caspase activating factor (CAF), which is activated by caspase-8, modulate the $\Delta\Psi_m$ resulting in the release of cytochrome c.²⁹ Cytochrome c release could be demonstrated after mechanical disruption of the outer mitochondrial membrane.²⁴ So far no studies with regard to the mitochondrial disruption have been performed with erythroblasts of patients with MDS. It is conceivable that iron accumulation in mitochondria of MDS patients damages the mitochondrial membrane, resulting in the release of cytochrome c and activation of the caspase-9/3 signaling route.

The function of caspase-3 is not only the cleavage of proteases but can also affect the cytoskeleton formation. Recently, it has been demonstrated that caspase-3 binds to F-actin during apoptosis, leading to the cleavage of actin into 15 kDa and 31 kDa fragments.³⁰ In MDS, a defective F-actin polymerization in neutrophils with aberrant kinetics of actin reorganization was demonstrated.³¹ An abnormal pattern of F-actin polymerization may impair β_2 -mediated neutrophil functions and—in general—may influence dynamic activities of cellular surfaces such as endo- and exocytosis. These findings are of interest since impaired or altered signaling through caspase-9/3 may be involved in defective enucleation of erythroblasts in MDS.

Regulation of cytochrome c release

It has been shown that Bcl-2 enhances cell survival by modulating the membrane potential.^{32,33} At present 15 bcl-2 family members have been identified in mammalian cells. Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum (ER) and nuclear envelope. Overexpression of bcl-2 and bcl-X_L prevents the release of cytochrome c.^{34–36} Only recently, it was shown that bcl-2 may inhibit the generation of Apaf-1 and thereby interfering the downstream signaling pathway.³⁷ The bcl-2 family is regulated by cytokines and death survival signals at different levels both transcriptionally and by posttranslational regulation.^{32,33} Competing dimer interactions of apoptosis-preventing (bcl-2; bcl-X_L) and apoptosis-inducing members (Bax; Bad; Bak; Bid; Bcl-X_S) may be involved in the regulation of apoptosis.³⁸ No binding of cytochrome c with Bcl-X_S could be demonstrated so far. As mentioned, the expression of bcl-X_L and bcl-2 during later phases of erythroid differentiation may prevent erythroblasts from apoptosis in normal hematopoiesis.

(Proto)oncogenes and apoptosis in MDS

By *in vivo* labelling studies with thymidine analogues and by morphological examination of bone marrow cellularity in MDS it is hypothesized that MDS is a highly proliferative disorder, in contrast to the *in vitro* growth characteristics of colony forming units.^{39,40} There is increasing evidence that increased intramedullary apoptose in MDS is involved in the observed pancytopenia.^{41–46} However, the degree of apoptosis observed in the bone marrow samples varies widely. Some authors report on high estimates of apoptosis of over 75% of the cells while others observe less than 10% of apoptotic cells.^{41–48} It is unlikely that these differences can be ascribed to a variety in patient samples. It is more likely that the techniques used, as well as the preparation of the bone marrow material, might be of importance. Augmented apoptotic cell death of the erythroid lineage was restricted to early MDS subtypes (RA/RARS) in contrast to more advanced stages of MDS (RAEB/RAEB-t).^{47,49} Apoptosis was shown to be largely restricted to the CD34⁺-compartment reflecting the more primitive progenitor cells.^{49,50} The ratio of expression of c-myc (inducer of apoptosis) to bcl-2 oncoproteins among CD34⁺-cells

was significantly increased for MDS (RA) as compared to CD34⁺-cells from normal and AML individuals.⁵¹ CD34⁺-cells in early MDS were also associated with a significant higher ratio of pro- and anti-apoptotic bcl-2 family proteins than in advanced disease.^{49–51} However, the degree of apoptosis could not be correlated to these ratios. It is hypothesized that the decrease in apoptosis in evolving acute myeloid leukemia results from clones that escape apoptotic control.

Apoptosis in MDS: implications for intervention?

The data reviewed on the pathogenesis of ineffective hematopoiesis in MDS support the hypothesis that apoptosis might play an essential role in (pan)cytopenias. Anti-cytokine based therapies, e.g. anti-TNF α , may inhibit apoptosis by binding TNF α and/or by down-regulating Fas receptors (CD95). More downstream in the signalling cascade of apoptosis, caspase inhibitors as well as blocking other pro-apoptotic proteins by anti-sense strategies, may interfere with ineffective erythropoiesis in early stages of MDS. Treatment of patients with MDS with G-CSF and Epo was associated with decreased numbers of apoptotic CD34⁺-bone marrow cells and sustain differentiation of erythroid precursors.⁵² A synergy of Epo and G-CSF could be demonstrated. The role of GM-CSF in MDS may particularly be of relevance to abrogate the neutropenia and, to a lesser extent, influencing erythropoiesis.⁵³ Since high levels of TNF α may suppress endogenous Epo production, combination therapies with anti-cytokine-based strategies with Epo(\pm G-CSF) may further circumvent ineffective erythropoiesis.

Since cyclosporine may interfere with the release of cytochrome-c by blocking MPT, cyclosporine may be useful in MDS to prevent apoptosis. This approach may be particularly of relevance in hypoplastic MDS resembling forms of severe aplastic anemia (AA). In addition, immunosuppressive treatment with ATG (anti-thymocyte globulin) have shown improvement of pancytopenias in MDS, suggesting altered cellular immunity by interfering with autologous reactive T-cells clones against erythroid precursors in MDS.⁵⁴ Finally, the thiol derivative amifostine as a scavenger of oxygen-reactive species may restore hematopoiesis in MDS by inhibition of apoptosis.^{55,56}

These approaches might be of interest in (sub)groups of patients with MDS in which apoptosis plays a prominent role in ineffective hematopoiesis. It should be emphasized that patients with high risk MDS can be cured by intensive chemotherapy followed by an autologous or allogeneic stem cell transplantation.^{57,58} In addition, immuno-modulatory strategies, as discussed above, might further restore ineffective hematopoiesis, or hence play a role in minimal residual disease.

Summary

Recent evidence suggests that intramedullary apoptosis in MDS contributes to the ineffective erythropoiesis with anemia. Although conflicting data exist about the level of apoptosis in bone marrow erythroblasts, apoptosis might play an important role in MDS but cannot explain in all cases the observed refractory anemia. To further unravel the precise mechanisms, more insight into the signaling pathway of apoptosis in MDS is warranted. In addition, the role of ineffective terminal differentiation of the erythropoiesis related to the apoptotic signaling pathways may further help understanding the pathogenesis of MDS.

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