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Apoptotic pathways to death in myelodysplastic syndromes

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Myelodysplastic syndromes (MDS) are heterogeneous hematopoietic stem cell diseases characterized by myeloid dysplasia and increased apoptosis. Pathways to death targeting mitochondria are activated downstream of death receptor, Fas and endoplasmic reticulum. Some molecular events, such as overexpression of oncogenes, ectopic expression of non-hematopoietic genes, and gene expression in conditions of haploinsufficiency, identified in these diseases have been used to generate mouse models, which recapitulate the features of MDS or MDS/acute myeloid leukemia (AML). These molecular defects altered protein-RNA transport, ribosome biogenesis, transcription and signaling, leading to a block of maturation, cellular stress and apoptosis. Mouse models are, therefore, useful for identifying mechanisms of cell death and testing new drugs.

Introduction

Apoptosis controls tissue homeostasis through mitochondria which are the central organelles targeted by all types of apoptotic stimuli. Excessive apoptosis and impaired differentiation are observed in the MDS, a heterogeneous group of clonal stem cell disorders. Chromosomal abnormalities including deletions, amplifications, and translocations have been identified in MDS cells. Mouse models of MDS, all characterized by hematopoietic cell dysplasia, apoptosis and long time to transformation have been generated. The molecular basis of apoptosis in these models does, however, remain unclear.

Apoptotic pathways to death

Physiological cell death usually occurs through apoptosis, involving the activation of cysteine proteases, known as caspases, or the activation of non-caspase molecules. The proteolytic events mediated by caspases lead to morphological and structural changes defining the apoptotic phenotype, such as cell shrinkage, blebbing of the plasma membrane, nuclear fragmentation, chromatin condensation and phosphatidylserine exposure which is an *eat me* signal for macrophages. The apoptotic pathways are controlled by the Bcl-2 family members both in mitochondria and in the endoplasmic reticulum (ER). Anti-apoptotic Bcl-2 and Bcl-x_L serve as guardians of the mitochondrial outer membrane integrity by inhibiting the oligomerization of pro-apoptotic Bax/Bak anchored into this membrane. Inhibition of Bcl-2/Bcl-x_L by pro-apoptotic BH3-only proteins (Bad, Bik, NOXA) or direct activation of Bax/Bak (tBid, Bim, PUMA) induces mitochondrial outer membrane permeabilization causing the release of the caspase-activating protein, cytochrome *c*, and other mediators of genome destruction, the apoptosis-inducing factor and endonuclease G. Inhibition of caspases blocks most of the phenotypic changes linked to apoptosis, but does not prevent cell death which can occur by autophagy or necrosis. Other mitochondrial functions are important for cell survival, such as the mitochondrial respiratory chain whose disruption generates reactive oxygen species causing lipid peroxidation, membrane damage, rupture of lysosomes and subsequent hydrolysis of proteins, nucleic acids and lipids. However, caspases may also contribute to the arrest of electron transport by inducing the cleavage of proteins of respiratory chain complexes including p75 NDUSF1. Thus, cell death might be determined by mitochondrial outer membrane permeabilization while not restricted to effector caspase activation.[1]

Bcl-2 family members control apoptosis even when caspases are neutralized, showing that Bcl-2 proteins control a cell death dependent checkpoint that is upstream of the caspases. At the level of the ER, Bcl-2 regulates the unfolded protein response which is an evolutionary conserved adaptative response to the

accumulation of misfolded proteins. The unfolded protein response stimulates the induction of chaperones and transporters for retrograde transport of proteins into the cytosol, ubiquitination and proteasome-mediated destruction. These pathways protect cells from death. In conditions of prolonged stress, ER could transmit the apoptotic signals to the mitochondria, through caspase-dependent cleavage of ER-resident proteins and Ca^{2+} release. For instance, the ER-resident Bcl-2 associated protein, BAP31 is cleaved in a caspase-dependent manner and the proteolytic fragment generated after cleavage can induce the release of Ca^{2+} and trigger mitochondrial cell death.[2] Bcl-2 or Bcl-xL prevents ER stress mediated by increased Ca^{2+} stores. Bcl-2 or Bcl-x_L induces a continuous depletion of ER Ca^{2+} stores through direct interaction with inositol trisphosphate receptors, which protect cells from death.[3] Conversely, Ca^{2+} leakage increases in double knockdown $Bax^{-/-}/Bak^{-/-}$ cells, which may contribute to cell death.[4] Evidence for a physical connection between ER and mitochondria has been provided and Ca^{2+} efflux from the ER regulates mitochondrial swelling and fission. Furthermore, ER stress induced the translocation of the BH3-only protein, Bim, to the ER and subsequent activation of caspase-12 (a paralog of human caspase-4) in mice leading to apoptosis independently of mitochondrial damage.[5]

The so-called intrinsic pathway of apoptosis is connected to the death domain receptors of the tumor necrosis factor-α family. Upon stimulation, these receptors recruit the adapter protein Fas-associated death domain (FADD) that, in turn, recruits and activates the initiator caspase-8 in the death-inducing signaling complex (DISC). Depending on the cell type, caspase-8 activated at the DISC level either directly activates effector caspases and/or cleaves the BH3-only protein that connects the extrinsic to the intrinsic, mitochondrial-dependent pathway. Caspase-8 also turns off the nuclear factor-kappa B (NF-κB) survival signal by cleavage of RIP. Recently, a caspase-8L has been shown to cleave BAP31 downstream of Fas activation indicating that the ER could participate as an intermediate organelle in death receptor-induced mitochondrial apoptosis in some types of cells.[6] In the context of prolonged ER stress, both ER-resident caspase-dependent and independent cell death programs are initiated (Figure 1).

Deregulation of apoptotic pathways to death in myelodysplastic syndromes

Normal hematopoiesis, or at least erythropoiesis, is regulated in the bone marrow by death receptor Fas-mediated apoptosis through interaction with the Fas ligand.[7,8] Furthermore, the non-lethal functions of caspases are required for normal erythroid, megakaryocytic and monocytic cell differentiation without features of apoptosis in cytokine-dependent conditions.[9,10,11] For instance, caspase-3-like activity is necessary for the maturation of basophilic erythroblasts into polychromatophilic erythroblasts[9] and for the maturation of megakaryocytes into platelets.[10]

MDS are characterized by ineffective hematopoiesis resulting in peripheral blood cytopenias despite the hypercellular dysplasia of bone marrow. The ineffective hematopoiesis is partly due to increased apoptosis of the bone marrow myeloid precursors, as demonstrated in the 1990s.[12] These data were confirmed later by evidence of increased apoptosis in erythroid cells derived from liquid culture of MDS $CD34^+$ progenitors.[13,14] These cells activate the extrinsic pathway to death, starting at the level of the death receptors, Fas and FasL, both overexpressed at the surface of mature erythroid precursors. An excess of soluble Fas or the ectopic expression of a dominant negative mutant of FADD prevents caspase activation and apoptosis. Furthermore, expression of mutant Bcl-2 targeted to the ER inhibits mitochondrial outer membrane permeabilization and apoptosis through inhibition of Fas-dependent cleavage of BAP31, confirming a Fas-ER-mitochondrial pathway to death in MDS erythroid cells.[15]

Increased caspase activation could be related to impaired differentiation. This was suggested, for instance, by a reported case of familial mild thrombocytopenia due to a point mutation of the gene encoding cytochrome *c*. In this case, localized cytochrome *c* release led to altered megakaryocytic differentiation and premature platelet formation.[16] The dysplastic and apoptotic phenotype of MDS could not be explained by a unique chromosomal abnormality. Several mutations (*RAS*, *AML1*, *FLT3-ITD*, *NPM1*), overexpression of oncogenes (*EVI-1*) or balanced translocations (e.g. *NUP98-HOXD13*) have been reported in MDS or MDS/AML. However, the link between these molecular defects and the phenotype is unclear in most cases. Interestingly, a recent publication demonstrated a genotype to phenotype relationship in the 5q- syndrome. T. Golub's group showed that, in conditions of haploinsufficiency, the expression of *RPS14*, a gene of the 5q common deleted region, inhibits erythroid cell growth, induces apoptosis and erythroid cell dysplasia and promotes megakaryocytic cell growth.[17] Studies of animal models expressing molecular abnormalities identified in MDS patients should help to understand the contribution to phenotype.

Animal models

Apoptosis is a major criterion to validate MDS animal models, together with dysplasia and long time to transformation. Among the published mouse models, three different mechanisms of oncogenesis have been identified: (i) overexpression of oncogenes (*MDS1/EVI-1*, *AML1*, *RAS**), (ii) gene expression in conditions of haploinsufficiency (*NPM1*) and (iii) ectopic expression of a gene normally absent or expressed at a low level in hematopoietic tissues (*HOXD13*, *MLF1*).

MDS1-EVI-1, *AML1* and *AML1-MDS1-EVI1* oncogenes

The *MDS1-EVI1* gene located on 3q26 encodes a transcription repressor and generates two protein isoforms, MDS1-EVI-1 and EVI1. Overexpression of EVI-1 has transforming activity, while the MDS1-EVI1 protein does not have such activity. Buonamici *et al.* used a retrovirus to overexpress EVI-1 in murine hematopoietic stem cells and transplant these cells into irradiated

recipients.[18] Ten months after transplantion, mice died from pancytopenia and their bone marrow showed erythroid and megakaryocytic hyperplasia and dyserythropoiesis. Bone marrow cells showed an impaired response to erythropoietin which could reflect inhibition of erythroid differentiation by EVI-1. Furthermore, caspase-3 activity was enhanced, suggesting that apoptosis was increased. However, the time to disease development was long and retroviral insertional activation of genes may have co-operated with EVI-1 to cause the MDS.

Molecular defects of the *AML1/RUNX1* fusion gene are detected in more than 20% of MDS, and are mostly mutations of one copy. However, AML1$^{+/-}$ mice develop a myeloproliferative disorder rather than a MDS. In contrast, mice receiving a bone marrow transplant using cells infected with retrovirus vectors harboring AML1/RUNX1 mutations die of MDS-refractory anemia with excess blasts (RAEB) or MDS-AML. All mice had multilineage dysplasia. AML1 mutations of the C-terminal domain led to leukocytopenia and erythroid dysplasia (MDS-RAEB phenotype), while AML1 mutations of the N-terminal Runt binding domain induced an acute disease with leukocytosis and severe hepatosplenomegaly (MDS-AML phenotype).[19] The AML1-MDS1-EVI1 fusion protein also has a transforming activity. However, both mutated AML1 and AML1-MDS1-EVI1 protect cells from death rather than induce apoptosis, possibly by activation of the Ras-mitogen-activating protein kinase pathway downstream of AML1 mutations. Thus, while EVI1 mice models have a MDS phenotype, AML1 mice models do not fit the definition of MDS, because they lack the apoptotic phenotype and their disease rapidly transforms into AML.

N-RAS/BCL-2*

N-, K- or H-ras activating mutations are identified in 10% of MDS/AML. Irradiated mice transplanted with bone marrow cells retrovirally expressing an active mutant of N-*RAS* develop a myeloproliferative disease in 60% cases and a MDS in 30%. Omidvar *et al.* showed that mice transgenic for N-*RAS*D12 developed a disease similar to MDS with myeloid dysplasia and apoptosis, while the co-expression of N-RasD12 with Bcl-2 induced AML.[20] This model demonstrated considerable genetic instability with an increased frequency of double-stranded DNA breaks and an increased error-prone repair of DNA by non-homologous end-joining that may predispose to myelodysplasia.[21] This model is, therefore, suitable for studying the effects of new drugs.

Nucleophosmin 1 (NPM1)

NPM1 is a highly conserved phosphoprotein that shuttles rapidly between the nucleus and cytoplasm, regulating the transport of pre-ribosomal particles. NPM1 is implicated in ribosome biogenesis, response to stress stimuli and maintenance of genome stability by inhibition of p53 and the DNA fragmentation activity of caspase-activated DNase (CAD/DFF40). A mutated form of NPM1, which is aberrantly localized in the cytoplasm, has been identified in *de novo* AML with a

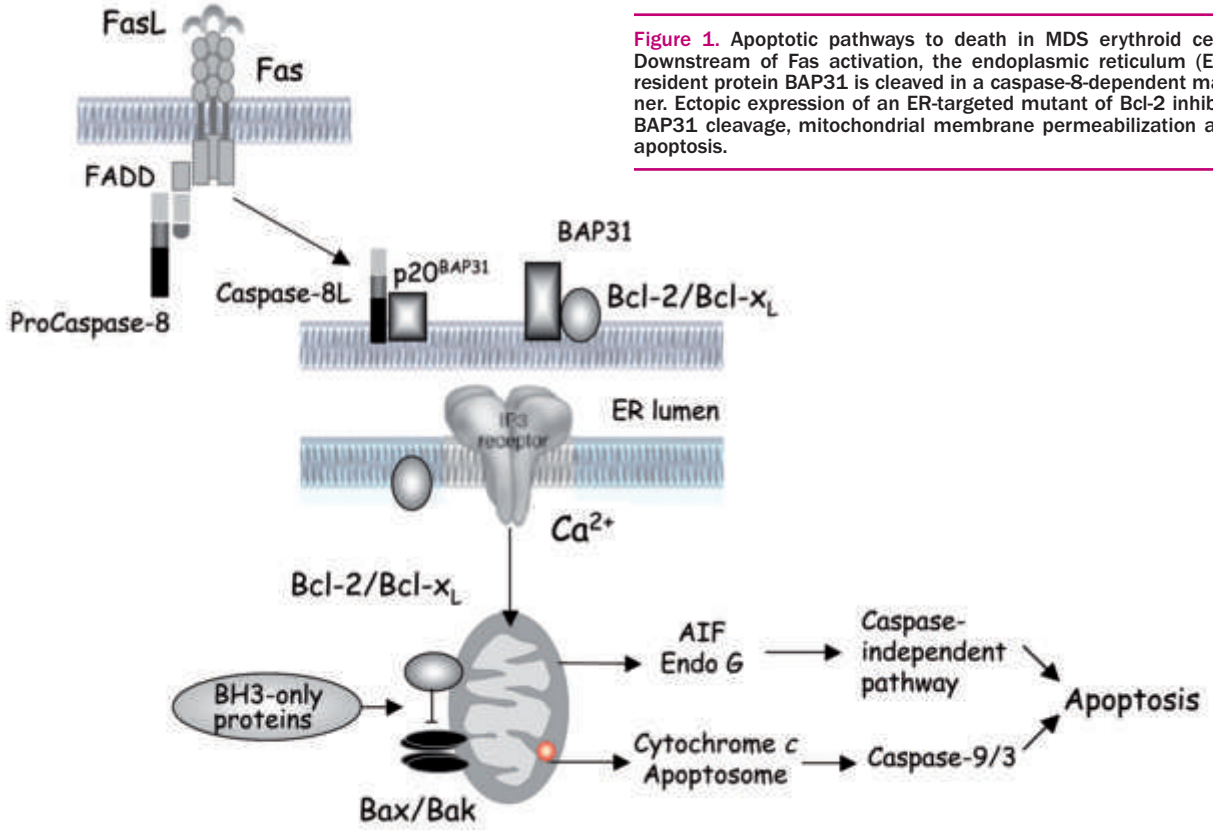

Figure 1. Apoptotic pathways to death in MDS erythroid cells. Downstream of Fas activation, the endoplasmic reticulum (ER)-resident protein BAP31 is cleaved in a caspase-8-dependent manner. Ectopic expression of an ER-targeted mutant of Bcl-2 inhibits BAP31 cleavage, mitochondrial membrane permeabilization and apoptosis.

normal karyotype. This mutant may have a dominant negative effect on the remaining wild-type protein and promotes cell survival. Grisendi *et al.* reported that *NMP1*$^{+/-}$ mice have erythroid cell dysplasia without anemia, megakaryocyte dysplasia with elevated platelet counts and increased apoptosis.[22] *NPM1* is also implicated in a translocation t(3;5) (q25.1;q34) with *MLF1*. The *NPM1-MLF1* fusion gene induces cell apoptosis when ectopically expressed in the K562 cell line. Both the N-terminal domain of MLF1 and the nuclear localization sequence of *NPM1* are required for apoptosis induction. Finally, these two latter models suggest that the reduction of *NPM1* gene dosage to heterozygosity predisposes to cell death and that NPM1 is required to prevent apoptosis in myeloid cells.

NUP98-HOXD13

The nucleoporin *NUP98* gene on chromosome 11p15 is implicated in several balanced translocations in MDS-AML diseases, involving fusion partners that belong to the clustered homeobox gene family (*HOXA11*, *HOXA13*, *HOXC11*, *HOXD11* and *HOXD13*). In contrast to the members of the *HOXA* cluster genes, whose overexpression leads to cell transformation, the *HOXD* cluster of genes are not normally expressed in hematopoietic cells. *NUP98-HOXD13* translocation results in aberrant expression of HOXD13 in hematopoietic cells, which is thought to deregulate the unidirectional and bidirectional transport of proteins and RNA-protein between the cytoplasm and the nucleus. The *NUP98-HOXD13* fusion gene is generated by t(2;11)(q31;p15), a translocation that has been described in MDS and AML patients. After 4 to 7 months, mice developed a typical MDS with peripheral cytopenias, bone marrow dysplasia and apoptosis. After 10 months, some of them developed AML or died of severe anemia and leukopenia. Bone marrows were hypercellular with impaired megakaryocytic differentiation.[23] In the present issue of Haematologica, Aplan's group reports an *in vitro* analysis of undifferentiated lineage-negative hematopoietic progenitors from mice expressing the *NUP98-HOXD13* fusion gene.[24] The progenitors had decreased expansion capacities and impaired differentiation and the more mature precursors were apoptotic. The same group also reported this year that half of the NUP98-HOXD13 mice with MDS develop AML with a latency that suggests a secondary event. N-*RAS* and K-*RAS* mutations, but not *FLT-3*, *TP53*, *AML1*, or *NPM1* mutations, have been demonstrated to complement the fusion gene with regards to inhibition of apoptosis and induction of proliferation, both favoring leukemic transformation.[25]

Conclusion

Mouse models of MDS characterized by both dysplasia and apoptosis are based on alterations of genes involved in ribosome biogenesis, protein and RNA-protein transport, transcription, and signaling. These genes control cellular responses to stress stimuli, genomic stability and differentiation. The molecular basis of apoptosis is largely unknown, but may involve ER or mitochondrial dysfunction either by enhancement of pro-apoptotic signals (disturbances of Ca^{2+} homeostasis, production of reactive oxygen species, mitochondrial outer membrane permeabilization) or default of anti-apoptotic or survival signals (adaptative responses, cytokine signaling, DNA repair). These animal models are useful for understanding the mechanisms leading to the priming of apoptosis and for the identification of targets for therapeutic strategies.

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The pathogenesis of classical Hodgkin's lymphoma: what can we learn from analyses of genomic alterations in Hodgkin and Reed-Sternberg cells?

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Classical Hodgkin's lymphoma (HL) is one of the most common human malignant lymphomas. Due to its characteristic histopathological, molecular and clinical features HL can be distinguished from all other lymphomas, the so-called non-Hodgkin's lymphomas. Two findings, in particular, make HL a unique malignant hematopoietic disease: first, the malignant mononucleated Hodgkin- and the multinucleated Reed-Sternberg cells (in the following termed Hodgkin/Reed-Sternberg [HRS] cells) represent only a small fraction of cells in the affected lymph nodes and are embedded in an infiltrate of reactive hematopoietic cells. Among others, these reactive cells are composed of B- and T-lymphocytes, eosinophils and plasma cells, and they are usually considered to be non-malignant. Second, the malignant HRS cells do not display a phenotype that can be assigned to a defined hematopoietic cell type.[1] The origin of HRS cells has, therefore, long been a matter of debate. With the description of clonally rearranged immunoglobulin (Ig) genes with a high frequency of somatic mutations it became clear that HRS cells are in most cases derived from germinal center (GC) or post-GC B cells.[2] It is of particular interest to note that, despite their B-cell origin, HRS cells have, in most cases, lost the B-cell-specific gene expression program. This is in contrast to most other B-cell-derived lymphomas, the gene expression pattern of which usually reflects the differentiation stage of their respective cell of origin. Furthermore, it is unclear how such cells survive, as GC B cells with defective Ig production usually undergo apoptotic cell death.

Clinically, HL is curable at the early stages in the vast majority of cases. However, the treatment of patients with advanced disease stages or insufficient response to the initial standard treatment options is challenging.[3] Furthermore, treatment succes is linked to considerable long-term toxicity, including the risk of treatment-related secondary malignancies. It is, therefore, imperative to develop less toxic therapeutic strategies, in particular non-genotoxic treatments based on, for example, inhibition of signaling pathways required for growth and survival of the tumor cells or enforcement of a cellular differentiation program leading to growth arrest and apoptosis. Knowledge of the key genomic and molecular defects of HRS cells is fundamental for the development of such treatment strategies.

The rareness of the tumor cells in the affected lymph nodes has been and is still a major obstacle to the identification of molecular and genomic defects in HRS cells and to the possibility of performing functional analyses with primary HRS cells. Although the purification of viable primary HRS cells from patients' lymph nodes has been described,[4] functional studies with these cells turned out to be nearly impossible because of the lack of suitable sample material and the insufficient number of cells. The current knowledge of molecular defects in HL is, therefore, based almost entirely on work with cell lines, albeit well-characterized, the analysis of primary HRS cells by immunohistochemistry and the analysis of micromanipulated HRS cells, as performed in the study by Hartmann *et al.* published in this issue of the journal.[5]

Deregulated signaling pathways in classical Hodgkin's lymphoma

Despite great efforts and advances in the last years, the pathogenesis of HL has not been clarified. In particular, although at the genomic level several recurrent alterations have been described, no unifying genomic defect specific to the malignant HRS cells has yet been identified. However, at the molecular level, a number of characteristic molecular defects have been demonstrated. These include activation of the transcription factors nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), members of the STAT signaling pathway and dereg-