

Molecular pathology of myelodysplastic syndromes: Biology of medullary stromal and hematopoietic cells (Review)

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Abstract. Myelodysplastic syndromes (MDS) have been defined as a disease entity based on clinical features and morphological findings. Despite similarities in clinical manifestations, genetic abnormalities occurring in hematopoietic cells are heterogeneous among the syndromes. However, recent investigations have revealed that there are common biological events in the bone marrow of MDS cases. Most notably, excessive apoptosis of hematopoietic cells was observed to be induced by the bone marrow microenvironment. The apoptosis was mediated by paracrine as well as autocrine factors, suggesting that medullary stromal and hematopoietic cells play a role in the pathology of disease. Pro-inflammatory cytokines, such as $\text{TNF}\alpha$, in the bone marrow microenvironment are predominantly paracrine mediators of apoptosis. Regarding autocrine stimulation mechanisms, it has recently been shown that the deregulation of ribosomal protein is capable of initiating a stress response in the hematopoietic cell through a p53-mediated signaling pathway. Thus, both the stromal cells of the bone marrow microenvironment and hematopoietic cells themselves possess a common and characteristic biology in this heterogeneous disease entity.

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1. Introduction

Myelodysplastic syndromes (MDS) are characterized by the clinical manifestation of a variable cytopenia, despite normo- or hypercellular bone marrow, with a subset of cases progressing to overt leukemia (OL). MDS occur predominantly in the elderly and thus, the prevalence of patients with MDS is on the increase due to an aging population and increased awareness of these syndromes. In MDS bone marrow, it has been postulated that hematopoietic stem cells are initially exposed to genetic hits that eventually cause abnormality, and then develop a growth advantage over their normal counterparts, leading to clonal expansion and monoclonal hematopoiesis (1,2) (Fig. 1). Cell cycle kinetic studies demonstrated that MDS bone marrow is actively proliferative and cycling more rapidly than even normal bone marrow cells (3). However, despite such active proliferation of bone marrow cells, the presence of cytopenias (ineffective hematopoiesis) remains to be elucidated (Fig. 1). This review summarizes the mechanism of the underlying ineffective hematopoiesis in MDS, which appears to be caused by the apoptotic death of hematopoietic cells. In early MDS, stromal and hematopoietic cells produce high levels of pro-apoptotic cytokines, whereas in advanced MDS or OL, these cells produce anti-apoptotic molecules (Fig. 1). Therefore, apoptosis was the key finding inducing common and significant biologic characteristics in different sub-types of MDS patients. Regarding MDS bone marrow, a number of additional common abnormalities have recently been reported and are evaluated in this review.

2. Excessive apoptosis in the MDS bone marrow: cytokines and the bone marrow microenvironment

Cytokines and their receptors. The paradox of cytopenia, despite cellular marrow in MDS patients, was resolved by findings regarding programmed cell death in the 1990s. The excessive proliferation of bone marrow cells in MDS patients appeared to correspond to an equally excessive intramedullary apoptosis of hematopoietic cells (4-7). This apoptosis was observed in all FAB categories of MDS patients, although the frequency was higher in the early stage of disease and proportionately lower in patients with an increasing percentage of blasts. Furthermore, it was shown that excessive apoptosis was mediated by a number of pro-apoptotic factors, including pro-

inflammatory cytokines that were overexpressed in the MDS bone marrow. These factors include tumor necrosis factor α (TNF α), interferon γ (IFN γ), transforming growth factor β (TGF β), interleukin 1 β , FAS-ligand (FAS-L) and inducible nitric oxide synthase (5-11). Pro-apoptotic factors, such as FAS-L were mainly produced by stromal cells, including macrophages, or partly by hematopoietic cells, whereas receptors, such as FAS and TNF receptor (TNFR), were expressed in hematopoietic cells (6,12).

As indicated by Raza *et al* (13), a second crucial paradox at this point was the presence of clonal expansion and monoclonality in cells that showed a tendency for apoptotic death. A model was proposed to explain this second paradox in which an unknown, poorly understood, initial lesion in a pluripotential hematopoietic stem cell leads to this cell developing a growth advantage (8). The cells are stimulated further to proliferate through the effects of pro-inflammatory cytokine TNF α . This proliferation eventually leads to a monoclonal hematopoiesis. Notably, as the daughter cells mature, TNF α driving the proliferation of the progenitors is capable of exerting a dual action, in that apoptosis is induced in the maturing progeny. This phenomenon was supported by demonstrating a shift in the expression of the cytokine receptors of hematopoietic cells in MDS. Pro-apoptotic receptor TNFRI and pro/anti-apoptotic receptor TNFRII were expressed differentially in response to the disease stages (12). The tendency to undergo apoptosis is not inherited uniformly by all the cells of the subsequent generations; instead, there is a spectrum of sensitivity to pro-inflammatory cytokines. The cells most sensitive to apoptosis are those undergoing apoptotic death in the bone marrow, whereas apoptosis-resistant cells actually enter the bloodstream. This phenomenon has been supported by the finding that granulocytes in MDS patients, although clonal in nature, are more resistant to apoptosis than granulocytes obtained from normal, healthy donors (14).

Early hematopoietic progenitor appears to develop a growth advantage and clonal expansion, leading to monoclonality. The subsequent generations of this transformed clone have an unequal tendency towards apoptotic death in the presence of increased amounts of a cascade of pro-inflammatory cytokines. One of the key systems for this switching appears to be TNF α and its receptors. Therefore, MDS should be considered as a disease of not only hematopoietic cells, but also of the bone marrow microenvironment, a concept that becomes critical when developing therapeutic strategies. Thus, cell proliferation followed by marked apoptosis was the most significant biological characteristic present in all subtypes of MDS.

Angiogenic factors. The bone marrow microenvironment of MDS is also affected by the production of angiogenic factors and matrix metalloproteinases (MMPs), which may regulate marrow microvascular density (MVD). Bellamy *et al* (15) have reported that in hematopoietic cells of MDS, the production of vascular endothelial growth factor (VEGF) and its receptor may promote leukemia progenitor self-renewal and cytokine elaboration and may also provide a biological condition for the generation of foci of abnormal localized immature precursors in advanced disease. Verstovsek *et al*

(16) reported that VEGF expression is the determining factor of the biological behavior of MDS and AML. Findings of a recent study revealed a significant increase in marrow MVD in *de novo* AML patients compared to controls (17). In MDS marrow, MVD was higher than that of controls, but lower than that of *de novo* AML patients. MDS patients exhibited a significantly lower MVD following transformation to OL, as demonstrated by a pairwise comparison of samples collected from the same patient on diagnosis and at leukemic evolution. Furthermore, AML secondary to MDS revealed a MVD lower than that of *de novo* AML patients, similar to that of healthy controls. Genes encoding for pro-angiogenic factors exhibited a similar expression pattern. Of note, TGF β , an anti-angiogenic mediator, exhibited an opposite expression pattern, since its expression was higher in AML secondary to MDS than in MDS and *de novo* AML.

Matrix metalloproteinases. Concerning the role of MMP in MDS bone marrow, Iwata *et al* (18) demonstrated that stromal cells induced the expression of MMP-9 in monocytes. MMP-9 expression was inversely correlated with bone marrow cellularity and percentage of cytogenetically abnormal cells. In their study, Travaglini *et al* (19) showed that MMP-2 and MMP-9 expression levels were higher in MDS patients than in healthy controls. The two MMPs localized in the cytoplasm of maturing myeloid cells. A positive correlation was observed between MMP-2 erythroblast expression and erythroid dysplasia, and an inverse correlation was observed between MMP-2/MMP-9 myeloid expression and marrow blast cell percentages. Particularly, in early MDS, the abnormal MMP expression profile correlated with an increased apoptotic rate, and high MMP levels were associated with longer overall and evolution-free survival.

Niche-induced myelodysplasia: bone progenitor dysfunction. The majority of normal and malignant tissues appear to form 'stroma' with specific mesenchymal cell involvement in the regulatory niches of stem cells. By examining the manner in which mesenchymal osteolineage cells of the bone marrow modulate hematopoiesis, Raaijmakers *et al* (20) showed that deletion of *Dicer1* disrupts the integrity of hematopoiesis in mouse osteoprogenitors, but not in mature osteoblasts. These mice exhibited myelodysplasia followed by the development of AML that had acquired several genetic abnormalities, whereas *Dicer1* remained intact. As a result of *Dicer1* deletion, the expression of *Sbds* was reduced. *Sbds* is the gene mutated in Schwachman-Bodian-Diamond syndrome, a human bone marrow failure and leukemia pre-disposition condition. As expected, deletion of *Sbds* in mouse osteoprogenitors induced bone marrow dysfunction with myelodysplasia. Therefore, perturbation of specific mesenchymal subsets of stromal cells disorders differentiation, proliferation and apoptosis of heterologous cells, and disrupts tissue homeostasis. This study clearly indicated the essential role of niche as well as the microenvironment in the pathogenesis of MDS.

3. Recent findings for hematopoietic cell defects of MDS

The majority of MDS and AML patients harbor cytogenetic and molecular defects that identify entities with specific

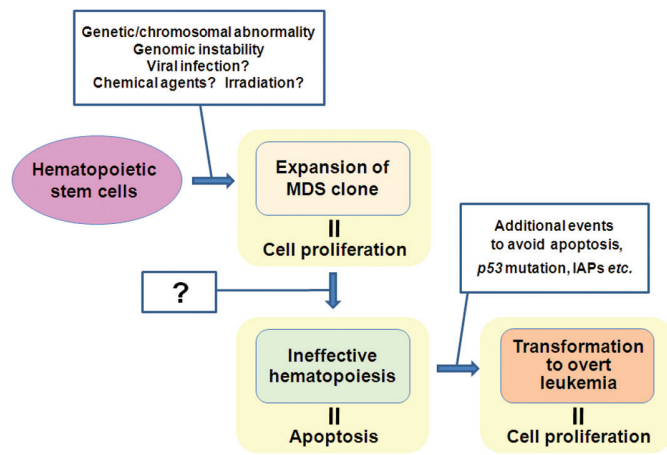


Figure 1. Schematic illustration of the development of myelodysplastic syndromes (MDS) and overt leukemia (OL) with characteristic biological events. The initial expansion of the MDS clone should be associated with cell proliferation, whereas ineffective hematopoiesis occurs with apoptosis. The evolution of OL is accompanied by cell proliferation by avoiding the apoptotic pathways.

biological/clinical features and distinct therapeutic responses (21-26). The incidence of chromosomal abnormalities is 40-60% in *de novo* MDS, 50-60% in AML and 70-90% in secondary MDS/AML (sMDS/sAML). A significant point that indicates a distinct pathogenesis for MDS and AML is that the genetic defects observed in *de novo* MDS are different from those of *de novo* AML. In *de novo* MDS, the incidences of chromosomal lesions with deletions (50%) and numerical defects (10%) dominate over the incidence of chromosomal translocations (<5%), although the most common cytogenetic abnormalities of AML are the balanced translocations (40%) (22). Thus, the hematopoietic cell (or myeloid blast) defects of MDS appear to be distinct from those of *de novo* AML.

Genetic bases of 5q syndrome. A special type of MDS involving an isolated del(5q) is called 5q⁻ syndrome (27). The syndrome is characterized by macrocytic anemia, normal or slightly elevated platelet counts, normal blast count, hypolobated megakaryocytes and a long-term risk of OL evolution of approximately 10% (28).

Bone marrow in 5q⁻ syndrome is essentially normo- or hypercellular with >90% of the hematopoietic stem cells being clonal (29). Despite the expansion of immature hematopoietic cells, the clinical manifestation is anemia, designated as ineffective hematopoiesis. By knocking down all 40 genes located within the common deleted region (CDR) at 5q32-33, Ebert *et al* (30) demonstrated that a decreased expression of RPS14 results in poor erythroid development and increased erythroid apoptosis. Forced expression of RPS14 in del(5q) marrow progenitors rescued the disease phenotypes. RPS14 is a ribosomal gene, coding for a component of the ribosomal subunit 40S. Other ribosomal genes, in particular RPS19, have been shown to cause Diamond-Blackfan anemia (31), also characterized by a macrocytic anemia. In addition, animal models have demonstrated that defects in ribosomal genes may increase the risk of cancer (32).

Using large parallel sequencing of small RNA libraries, Starczynowski and Karsan (33) identified two down-regulated

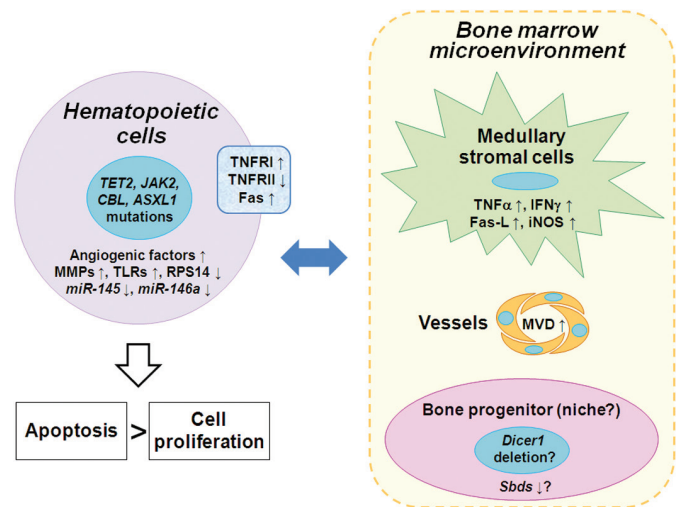


Figure 2. Abnormalities in hematopoietic cells and bone marrow microenvironment such as stromal cells, vessels and bone progenitors identified in MDS. Genetic mutations described in the nucleus of the hematopoietic cells do not include conventional mutations/abnormalities such as abnormalities of NRAS, FLT3, MLL, NF1, CSF1R, KIT, CDKN2B, TP53, RB1, EVI1, IRF1, AML1, WT1 and NPM1.

micro-RNAs, *miR-145* and *miR-146a*, located close to the CDR on 5q and with an abundant expression in marrow progenitors. Knock-down of *miR-145* and *miR-146a* leads to a phenotype with key features of the 5q⁻ syndrome; i.e., hypolobated megakaryocytes and peripheral thrombocytosis. Moreover, predicted targets for *miR-145* and *miR-146a* were associated with the innate immune response pathway. Two genes in the Toll-like receptor signaling pathway were verified as true targets: TIRAP for *miR-145* and TRAF6 for *miR-146a*. TIRAP interacts with TRAF6 and subsequently results in the activation of NF-κB. A proportion of mice transplanted with TRAF6 overexpressing marrow developed bone marrow failure or AML in due course.

A mouse model of 5q⁻ syndrome has been generated (34). Since the CDR of 5q⁻ syndromes is split into two regions in mice, on chromosomes 11 and 18, chromosomal engineering was used to delete small segments within these regions. The only deletion that exhibited a clear phenotype was the *Rps14* gene, leading to macrocytic anemia. *Rps14*-deficient mice were then bred with *p53* knockout mice resulting in a reversal of the phenotype. This reversal indicates that *p53*-mediated cell death or growth inhibition may be crucial for RPS14-induced anemia in 5q⁻ syndrome.

As summarized by Jädersten and Eva Hellström-Lindberg (35), various genetic alterations cooperate to induce the characteristic phenotype of 5q⁻ syndrome. The anemia is likely to be caused by RPS14 deficiency, while hypolobated megakaryocytes and thrombocytosis may be the result of a deficiency of *miR-145* and *miR-146a*. However, the CDR of 5q⁻ syndromes contains a number of other genes that may be of importance for clonal expansion, such as the tumor suppressor gene, SPARC (36). SPARC is anti-adhesive and modulates the cell matrix, induces apoptosis and may inhibit angiogenesis (37). Haploinsufficiency of SPARC may increase the adhesion of the hematopoietic stem cells to the supporting stromal cells and provide a clonal advantage for proliferation.

Novel gene mutations identified in MDS. Although the frequency is not usually high, various novel mutations have been identified in patients with MDS. JAK2 mutations (the most common one being V617F) were found in a significant proportion of patients with myeloproliferative neoplasms (MPN) (38), leading to the constitutive activation of proliferative and survival signaling in hematopoietic cells. In addition, an activating mutation in the thrombopoietin receptor (MPL) has been found in a small proportion of essential thrombocythemia and primary myelofibrosis (39). Multipotent hematopoietic stem cells with these mutations generate a myeloid clone that expands to replace hematopoietic cells without the mutation (40). These types of gene mutations have also been reported in MDS and MDS/MPN. JAK2 mutations occur in approximately 50% of patients with RARS with marked thrombocytosis (RARS-T), although the mutation is extremely rare in RARS with normal platelet count (41,42).

In 2009, mutations in the *Ten-Eleven Translocation-2* (*TET2*) gene located on 4q were found in MDS and MPN (43-47). Among 320 patients with myeloid malignancies, *TET2* mutations were found in 12% of patients with MPN, 19% of MDS and 22% of patients with CMML (44). Moreover, hematopoietic stem cells (HSC) harboring the mutation had a growth advantage compared to normal HSC. Another study revealed that 26% of 102 MDS patients carried *TET2* mutations (46). These *TET2* mutations were found to be more frequent in IPSS low (41%) and intermediate-1 risk (27%) MDS patients than in those with intermediate-2 (13%) and high-risk (14%) MDS. However, the prognostic significance of *TET2* remains controversial (48). Although the cellular function of *TET2* is unknown, *TET1* was revealed to mediate the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, and was involved in the epigenetic control of the gene expression (49). Therefore, *TET2* appears to be involved in DNA methylation.

Recently, mutation of the *C-b1 E3 ubiquitin ligase gene* (*CBL*) that is located on chromosome 11q was found in MDS and MDS/MPN. This ligase is involved in the degradation of receptor tyrosine kinases, and its inactive mutation may cause a growth advantage in the hematopoietic cells. The *CBL* mutation was identified in 2 of 38 (5%) patients with CMML and 10 of 110 (9%) with secondary AML (50). However, only 1 of 115 (1%) of the patients with MDS carried mutant *CBL*. Mutations were revealed to associate with poor survival on multivariate analysis.

Another recently identified gene mutation in MDS is the polycomb-associated gene *ASXL1*, which is involved in the regulation of chromatin remodeling. *ASXL1* mutations were found in 11% of 35 MDS patients and 43% of the 39 CMML cases (51). Boulton *et al* studied 300 patients with MDS and AML and found that 62 (21%) patients carried an *ASXL1* mutation (52). The mutation was observed in 5 of 79 (6%) RA patients, 17 of 55 (31%) RAEB-1 or RAEB-2 patients and 17 of 67 (25%) AML patients. Thus, the mutation was more frequent in high-risk MDS and AML patients compared to lower-risk MDS patients.

Uniparental disomy. Small genetic lesions, such as uniparental disomy (UPD), have been identified by high-density single nucleotide polymorphism (SNP) arrays. In MDS patients,

10-15% of those with normal karyotypes had regions of DNA that were derived from only one parent. This phenomenon has been termed UPD (53-55). In addition, small genetic lesions such as amplifications and deletions, which had remained undetected on conventional cytogenetics or FISH analyses, were also identified. UPDs were constitutional and not limited to the clonal cells, whereas the amplifications and deletions proved to be acquired. These data suggest that individuals who are born with constitutional UPDs are predisposed toward genetic instability and are at an increased risk of developing MDS. For example, the *TET2* gene mutation is known to be frequently affected by UPD (47).

Toll-like receptor. Toll-like receptors (TLRs) play a crucial role in host defense against invading microorganisms by identifying pathogen-associated molecular patterns (56,57). A number of endogenous molecules have been reported to be ligands of TLRs (58). Some of these molecules are known to be expressed in cancer tissue and to activate intracellular signal pathways via TLRs during cancer progression. At the time of initial diagnosis, MDS bone marrow hematopoietic cells tended to express higher levels of TLR2, TLR4 and TLR9 than control bone marrow cells (59). Among these TLRs, TLR9 exhibited a significant decrease of expression at the time of transformation to OL. The expression of TLR9 and TNF α showed a significant correlation in bone marrow cells from patients with MDS and OL. Bone marrow cells in MDS exhibit frequent apoptosis, whereas OL cells are prone to immortality. Thus, TLR9 may be associated with the regulation of apoptotic/proliferative signals via TNF α in the MDS bone marrow.

4. Animal models of MDS

Although MDS is characterized by numerous complex phenotypes, such as ineffective hematopoiesis, peripheral blood cytopenias, morphological dysplasia of hematopoietic cells and susceptibility to OL evolution, attempts have been made to generate effective animal models mimicking MDS pathology. These models include *Evi-1* overexpression in a mouse bone marrow transplantation (BMT) system, *Npm1* hetero mice, *Nup98/HoxD3* transgenic mice, mutated *Aml1*-bearing BMT system and *Dicer1* deletion mouse system. In mouse hematopoietic organs, a forced expression of *Evi-1* initially determines an excessive proliferation of bone marrow cells with the down-regulation of genes involved in erythroid differentiation and platelet formation followed by a profound and fatal peripheral cytopenia resembling human MDS (60). However, the condition does not progress to leukemia unexpectedly. *Npm1*^{+/+} mice exhibited features resembling MDS, although *Npm1*^{-/-} mice showed embryonic lethality (61). In contrast to the *Evi-1* model, these mice developed hematological malignancies of myeloid as well as lymphoid origin after a 2-year follow-up.

Similarly, *Nup98/HoxD13* transgenic mice develop a disease that faithfully exemplifies all the key features of MDS, including peripheral blood cytopenias, bone marrow dysplasia, apoptosis and transformation to OL (62). These mice exhibit a uniformly fatal MDS, dying of either severe anemia/leucopenia or leukemic evolution. However, mice with

BMT of *Nup98/HoxD13* cells did not develop OL. A recent study demonstrated that the BMT of *Aml1* mutants exhibited MDS-like features and evolution to OL (63). In this model, different mutations of *Aml1* resulted in different types of MDS. Finally, results from the *Dicer1* deletion mouse system were highly significant (20). As described above in the section on microenvironment-niche-induced myelodysplasia, these results clearly revealed the significance of niche-like bone progenitor cells in the pathogenesis of MDS.

5. Conclusions

As shown in Fig. 1, a heterogeneous disease entity, MDS, is a complex regulation of cell dynamics, resulting in cellular proliferation and/or apoptosis. Under the various phenotypic manifestations, common biology may exist as regulatory mechanisms. One of the most characteristic of these mechanisms, distinct from AML and MPN, is the induction of apoptosis in hematopoietic cells. Fig. 2 summarizes the genetic/molecular modifications recently identified in the bone marrow of MDS. Interaction between hematopoietic cells and the bone marrow microenvironment is likely to define the balance between apoptosis/cell proliferation of MDS bone marrow cells.

Apoptosis is induced in hematopoietic cells only when the subsequent generations of the initial MDS progenitor cell mature and begin to express the appropriate cytokine receptors. These insights led to the novel translational approach of using anti-cytokine therapy to improve the cytopenias by protecting the maturing cells from dying. This strategy was attempted without a serious concern for causing leukemic transformation as the therapeutic procedure did not affect earlier progenitors. Since the rate of apoptosis is inversely related to the risk of transformation, this strategy was ideal for patients with lower-risk MDS where anti-TNF agents such as thalidomide, lenalidomide, infliximab and etanercept have been found to be effective in improving cytopenias in a subset of patients. Notably, even high-risk patients have the same incidence of apoptosis in the maturing cells as lower-risk patients. The fact that the immature blasts are not dying, accounts for the lower overall incidence of apoptosis in this group.

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