Myelodysplastic Syndromes –
Unexpected Insights Stimulate the Biological Landscape

Azra Raza[1] and Naomi Galili[2]

1. Professor of Medicine, and Director, Myelodysplastic Syndrome Center, St Vincent's Comprehensive Cancer Center;
2. Associate Professor of Medicine, New York Medical College, and Director, Bone Marrow Translational Laboratory, St Vincent's Comprehensive Cancer Center

Abstract

The biology of myelodysplastic syndromes (MDS) is characterised by excessive proliferation followed by intramedullary apoptosis of haematopoietic cells. Clonal expansion would at first seem to be incompatible with excessive apoptosis; however, a dual action of pro-inflammatory cytokines that stimulate the progenitor cells while inducing apoptosis in their maturing progeny could explain this paradox. Until recently, these were the only biological characteristics common to all subtypes of MDS. Novel insights emerging from the studies of micro RNAs (miRNAs), gene expression profiling and single nucleotide polymorphism (SNP) arrays are providing additional molecular and genetic features linking the heterogeneous phenotypes. Expression arrays have indicated that genes involved with ribosomal biogenesis and the translational processes controlled by the ribosomal proteins are deregulated in multiple MDS subtypes. Specifically, RPS14, which lies in the commonly deleted region (CDR) of patients with 5q- syndrome, was shown to be causative for that specific phenotype. Certain congenital anaemias also have abnormalities in ribosomal proteins, thus linking acquired and inherited diseases. In addition to excessive apoptosis and proliferation, deregulated ribosomal biogenesis appears to be another unifying mechanism between the syndromes of myelodysplasia. Finally, DNA microarray studies and SNP arrays are yielding novel data in terms of prediction of response to drugs and identification of patients at high risk of developing MDS. These biological insights, some quite unexpected, are opening the doors to entirely novel areas of research and will hopefully lead to better outcomes for patients through translational research.

Myelodysplastic syndromes (MDS) are clearly a disease of the elderly, the median age of patients being 70 years. Almost one-third of patients will progress to acute myeloid leukaemia (AML). These clonal heterogeneous stem cell disorders are characterised by the clinical presentation of variable cytopenias despite a generally cellular and dysplastic marrow. Cytogenetic abnormalities most commonly affecting chromosomes 5, 7, 8 and 20 are present in approximately 40–50% of MDS patients at diagnosis and greatly affect prognosis and survival. Progress in identifying biological cohesion between the various subtypes of MDS was achieved with the demonstration that the cytopenias in a significant proportion of patients may be due to an excessive pro-inflammatory cytokine-mediated apoptosis of maturing haematopoietic clonal cells. At a biological level, MDS is a disease of both the seed (cell) and the soil (bone marrow microenvironment). Therefore, it follows that treatment must be designed to target both.

This article will summarise some of the recent advances that, for the first time, shed light on how such an incongruent group of syndromes is linked through certain recurring and unifying themes.

Clonal Expansion

An emerging appreciation about malignant diseases in general, and MDS in particular, is the role that the microenvironment of the diseased cells plays in their pathology. MDS starts when a single pluripotential haematopoietic stem cell in the bone marrow acquires a growth advantage over its neighbours for an as yet unexplained reason. This leads to an expansion of the abnormal clone. Obviously, the critical question here is: what gives this particular stem cell a growth advantage? The answer would define the aetiology of cancers in general. The prevailing and widely accepted view is that the activation of oncogenes through elimination of tumour suppressor genes through mutations, deletions or hypermethylation is responsible. Other theories include accumulation of random mutations throughout the genomes of pre-cancerous cells or extensive aneuploidy in the chromosomes themselves. Such aberrations would lead to an unstable or critical state in the bone marrow through a peculiar self-organisation of the abnormal cells that pushes the system away from equilibrium and towards a 'tipping point'. Such a self-organised criticality could make individual stem cells prone to damage.

It is possible that MDS begins not in the stem cell but through a process of self-organisation of bone marrow cells towards an unstable state, which makes them sensitive to intracellular genetic or chromosomal aberrations. With age, the marrow cellularity diminishes considerably so that the physiological gradient of stromal stem cell signals could be perturbed, allowing a stem cell to escape the inhibitory signals and gain

a proliferative advantage over its neighbours. The proliferative advantage, although slight, could eventually lead to clonal expansion and, ultimately, a state of monoclonal haematopoiesis that is intuitively more susceptible to a malignant transformation than a system with polyclonal haematopoiesis. In fact, up to 40% women over 65 years of age have been found to have monoclonal haematopoiesis without manifesting any evidence of disease, and it is well known that MDS is more common in the elderly. In summary, therefore, clonal expansion of a haematopoietic stem cell could begin as a perturbation in its micro-environment, progressing towards mutations in the cell itself and eventual transformation to a malignant state.

Excessive Proliferation–Apoptosis Mediated Through Cytokines

In the early 1990s, excessive proliferation and excessive apoptosis were recognised as biological characteristics that were common to all types of MDS and explained the inconsistency of finding cytopenias in the presence of a cellular bone marrow.[1] Furthermore, it was shown that the apoptosis may be largely mediated by a set of pro-inflammatory cytokines, the prime initiator of the cascade being tumour necrosis factor alpha (TNF-α). The paradox here was the existence of clonal expansion if the MDS cells are prone to die prematurely by programmed cell death. We proposed a model to explain this paradox that could result from the dual actions of a cytokine such as TNF-α that stimulates the proliferation of and hence clonal expansion in early progenitor cells, but induces apoptosis in their maturing progeny.[2,3] Furthermore, it is clear that the propensity to undergo apoptosis is not something that is parcelled out equally to all daughter cells in the clone. In fact, the spectrum of sensitivity to apoptose ranges from cells that are highly sensitive to a subset of mature peripheral blood granulocytes whose resistance to undergoing apoptosis is more than even that of granulocytes from normal individuals.

In summary, therefore, a haematopoietic stem cell that has developed a proliferative advantage over its neighbours, for an as yet unexplained reason, undergoes clonal expansion. The progeny of this clone has a spectrum of sensitivity for premature apoptosis, with some daughters dying early in the marrow while others with resistance reach the peripheral blood as mature granulocytes. A major mediator of the excessive apoptosis appears to be the cascade of pro-inflammatory cytokines.

Finally, excessive apoptosis, which was the first unifying biological link between the different types of MDS, and proliferation are tightly controlled by the p53 tumour-suppressor proteins. Previous work had failed to pinpoint the precise mechanistic basis of this association between p53 and apoptosis in MDS; however, thanks to recent dramatic technological improvements, entirely unexpected new areas of research are emerging that both explain the molecular basis of some of these biological insights and provide further proof of thematic cohesion between the syndromes of myelodysplasia.

Micro RNAs/RNA Interference, DNA Microarrays and Single Nucleotide Polymorphism Arrays

Rapidly developing technical advances in areas such as molecular arrays and RNA inhibition have resulted in the accumulation of enormous amounts of data that have led to fundamental changes in the way that genetics and basic biological mechanisms can be examined. The goal of personalised medicine is no longer just a pipe dream, but rather a process that is now feasible. Three of these

emerging technologies are now being used to help develop improved outcomes for patients with MDS.

Micro RNA Interference

Until recently, RNAs that do not encode proteins have been associated with the mechanism of transferring information from the DNA 'gene' to the production of the functional protein. Therefore, ribosomal RNA and transfer RNA (tRNA) were the most abundant and well-known examples of non-coding RNA. In 1993, a gene *lin-4* in *Caenorhabditis elegans* was identified that encoded a very small 22 nucleotide RNA molecule that was complementary to a second gene *lin-14*.[4] As *lin-4* negatively regulates the LIN-14 protein, it was suggested that it does so by antisense interaction with the *lin-14* message. This report, while interesting, did not attract much attention. That changed dramatically with the discovery by Fire and Mello in 1998 that small double-stranded RNA molecules could specifically suppress gene function[5] (RNAi, for which they received the Nobel Prize for Medicine in 2006). Multiple papers appeared describing the use of RNAi in several organisms to dissect gene function. Most importantly, it was realised that the original discovery of *lin-4* was not just an isolated curiosity, but was in fact a negative regulatory mechanism found in all species from bacteria to mammals. In addition, the pathways that are involved in both RNAi and the small regulatory RNA (termed micro RNAs [miRNA] in the human genome) were found to be remarkably similar.[6] The 20–22 nucleotide miRNA binds to the 3' untranslated end of the mRNA, thereby inhibiting translation by suppression or cleavage of the messenger RNA (mRNA) molecule. As perfect complementation of sequence is not necessary for binding, multiple miRNAs can inhibit a single mRNA and expression of a single mRNA can be suppressed by more than one miRNA. Sequencing analysis predicts that hundreds, if not thousands, of miRNAs exist in the human genome. Work is now in progress to identify specific gene targets of miRNAs and their role in regulating multiple biological pathways, including that of haematopoiesis.

Myelopoiesis in particular has been shown to be regulated by miRNA, but studies are rare. Initial studies in aberrant erythropoiesis and erythroleukaemias implicated increased expression of miRNA-221 and -222 resulting in inhibition of the c-kit receptor.[7] Normal erythropoiesis was later shown to be associated with decreased expression of miRNA-150.[8] miRNA-150 was also shown to direct differentiation of megakaryocytic–erythrocyte precursor cells towards megakaryocyte differentiation rather than erythrocyte differentiation through its interaction with the transcription factor MYB.[9] Recent studies have shown that miRNA-150 and -155 play a role in the myeloproliferative diseases polycythaemia vera and primary myelofibrosis.[10–12] While the heterogeneity inherent to MDS has hampered studies in this disease, initial findings suggest that aberrant miRNA-controlled expression may contribute to disease pathology. The haploinsufficiency of the commonly deleted region (CDR) on the long arm of chromosome 5 in 5q- syndrome MDS contains three miRNAs. Boultwood et al.[13] examined their expression levels in 5q- syndrome MDS compared with refractory anaemia (RA) with normal karyotype and controls by quantitative polymerase chain reaction (PCR). They found that while miRNA-145 expression was slightly increased in 5q patients, there was no significant difference between the other two miRNAs in the MDS patients versus controls. Additionally, a strong increase in miRNA-125b was found in bone marrow cells from patients diagnosed with AML and MDS containing a rare but consistent t(2;11)(p21;q23) karyotype.[14] *In vitro* studies showed that overexpression of miRNA-125b disrupted

CD34+ cell differentiation and also inhibited terminal monocyte and granulocyte differentiation in cell lines. Most recently, Hussein et al.[15] looked at expression levels of these haematopoiesis-associated miRNAs (-150, -155, -221 and -222) in marrow cells of 52 MDS patients of all subtypes (eight with isolated del(5q)) compared with controls. Surprisingly, miRNA-150 increased expression was the only perturbation detected and this was confined to patients with del(5q). The expression of MYB, one of the targets of miRNA-150, was found to be inversely correlated with miRNA-150 expression, suggesting that decreased levels of MYB, a main component of erythropoiesis,[16] may contribute to the anaemia found in 5q- syndrome patients.

DNA Microarrays

The ability to simultaneously measure the expression of virtually all known genes in a limited number of cells has dramatically changed basic biology and medical research. In the field of oncology and haematology, numerous studies have established expression profiles that aid in the diagnosis and classification of tumour types. However, for MDS the heterogeneity of the tumour cells together with the confounding influence of an abnormal bone marrow microenvironment has somewhat limited the use of this technology. Initial studies focused on limiting heterogeneity by use of CD34+ cells combined with selection of specific French–American–British (FAB) subtypes. While these studies gave clues in terms of the biological pathways deregulated in MDS and therefore most likely involved with the pathology of the disease, they were of limited clinical benefit. Our group sought to approach the problem in a different manner. The del(5q) MDS patients were shown in a phase II clinical trial to be highly responsive to therapy with lenalidomide, a derivative of thalidomide.[17] Interestingly, in a second phase II trial approximately 25% of patients without del(5q) were also responsive to this agent.[18] No apparent clinical parameters could predict which non-del(5q) patients were likely to respond. We postulated that perhaps there was an expression profile that could identify these responding patients. Using pre-therapy marrow mononuclear cells and the Affymetrix 2.0 DNA array, a unique 32-gene expression profile was generated that separated responders, irrespective of karyotype, from non-responders.[19] This profile comprised many erythroid genes that were underexpressed in responders compared with the non-responders. Further in vitro studies demonstrated that these genes are initially expressed at very low levels in CD34+ cells, but increase expression when differentiated along the erythroid lineage. Thus, patients presenting primarily with a defect in erythroid differentiation, whether due to selective apoptosis or other mechanisms, responded to lenalidomide. This profile was also used to predict response with 80% accuracy. The ability to predict lenalidomide response in non-del(5q) patients would be of significant clinical value, as such patients are not currently US Food and Drug Administration (FDA)-approved to receive this drug, which could alleviate their severe anaemia. A clinical confirmatory trial is currently being conducted.

Expression microarray studies in MDS have sought to identify molecular pathways that could explain the diverse clinical phenotypes evolving from marrows with common features of dysplasia and increased proliferation with excessive apoptosis. Initial studies by Boultwood et al.[13] showed that Wnt/B-catenin signalling and protein ubiquitination pathways were deregulated in 5q-syndrome patients compared with RA with normal karyotype or healthy controls. Wnt/B-catenin signalling regulates stem cell fate and transformation events and both pathways have been implicated in other myeloid malignancies.[20–22] Additionally, this group hypothesised

that the observed decreased expression in 5q- patients of the two ribosomal genes in the CDR – RBM22 and RPS14 – may be important in the development of 5q- syndrome, analogous to the causative role played by the ribosomal gene RPS19 in Blackfan-Diamond syndrome.[23] Indeed, Ebert et al.[24] shortly thereafter used RNAi to inhibit each gene of the CDR individually in CD34+ cells cultured to produce mature erythrocytes or mature megakaryocytes as the functional readout. Only inhibition of RPS14 resulted in blocked erythropoiesis in the presence of normal megakaryocyte differentiation – a recapitulation of the 5q- phenotype. Forced expression of RPS14 in CD34+ marrow cells from 5q- patients re-established erythrpoietic differentiation, showing that RPS14 loss on one allele was the causative event for the 5q- syndrome. Further studies[25] showed that 55 genes associated with ribosome and protein synthesis were differentially expressed in RA with normal karyotype and 5q- syndrome patients compared with controls. Confirming the importance of deregulated ribosomal genes, Sohal et al.[26] used meta-analysis to compare the CD34+ expression profiles of 60 cases of non-del(5q) MDS patients with 52 normal controls. This study showed that ribosomal protein genes were the most significantly deregulated class of genes in the MDS group, with decreased expression in many of those genes. Therefore, results of expression arrays point to deregulated ribosomal biogenesis, in addition to excessive apoptosis and proliferation, as a unifying mechanism in the evolution of MDS.

Single Nucleotide Polymorphism Arrays

Sequencing results of the human genome show that there is great variation within individuals at the single nucleotide level. This variation, known as SNPs, most typically has no deleterious effect and therefore is highly conserved in different populations. Therefore, it is possible to use SNPs as genetic markers that may be associated with specific diseases, clinical presentation and response to therapy or prognosis. SNP arrays are essentially DNA arrays that detect specific SNPs resulting in a 'SNP map' of individuals. Currently, >50 common SNP variations have been found to be associated with a number of diverse diseases including type 2 diabetes, immune disorders and cardiac disease.[27] More recently, copy number variation (CNV, a short or long sequence of nucleotides) has been found to account for much of the variation seen in the genome and to be associated with disease phenotype.[28] CNV is now incorporated into the newer high-density versions of SNP arrays. An additional advantage of these arrays is that it is possible to detect copy-neutral loss of heterozygosity (LOH). LOH reflects the loss or gain of an allele. However, in some instances while two alleles are present, they are not from both parents; instead, one is inherited from a single parent and duplicated. This is referred to as uniparental disomy (UPD) or copy-neutral LOH. When this allele is abnormal, it may result in a disease phenotype. Several recent studies of MDS have found that some patients with marrow cells that have cytogenetically normal karyotypes actually contain numerous CNV and UPD.[29,30] These changes are clonal and are not found in the normal tissues of the patients. While the percentage of patients with these abnormalities varies between studies, it appears to be a significant subgroup within cytogenetically normal MDS patients. The clinical utility of such studies is clearly demonstrated by Heinrichs et al.,[31] who examined the clinical course of two patients with low-risk International Prognostic Scoring System (IPSS) scores and normal karyotype who were found to have UPDs on chromosome 7q. These patients had unexpectedly rapid disease progression, more suggestive of that normally seen in patients with karyotypic deletions of chromosome 7, for whom therapy is usually aggressive. Regions of UPD/CNV may

contain mutations that can result in deregulation of molecular pathways essential to normal proliferation and differentiation. As such UPD/CNV detection can identify regions that should be sequenced in detail in other MDS patients in an attempt to find new therapeutic targets. Use of high-density SNP arrays thus has the potential to improve the diagnosis, prognosis and clinical management of MDS.

Summary

During the last three decades, research in MDS has included both semantic debates related to classification of the heterogeneous syndromes into more uniform groups and efforts to understand the biology of the disease. This article has focused on characteristics that explain the underlying clinical manifestations of the disease such as excessive apoptosis being the cause of cytopenias, and also reviewed the distinctive themes that unify this seemingly unrelated group of syndromes. The paradox here is that if most of the clonal population is destined for a premature programmed cell death, what accounts for the continued expansion of the clone? At least one possible explanation involves the following steps. An as yet obscure initiating event imparts a growth advantage on an early haematopoietic stem cell that is able to continue proliferation despite the presence of pro-inflammatory cytokines. This clonal expansion accounts for eventual monoclonal haematopoiesis in MDS. However, the maturing daughters of this clone appear to inherit a spectrum of sensitivity to undergo apoptosis in the presence of the pro-inflammatory cytokines, ranging from exquisitely sensitive in the vast majority to highly resistant in a minority that eventually reach terminal maturation and make it to the peripheral blood. In short, the cytokines serve a dual purpose of causing expansion of the abnormal clone at the progenitor level while inducing apoptosis in their maturing progeny.

For almost a decade, excessive apoptosis in varying degrees was the only biological theme common to all the syndromes of MDS. All this began to change with the observation that haploinsufficiency of two ribosomal genes, RBM22 and RPS14, deleted because of their location in the CDR of 5q- patients, may be the cause of 5q-syndrome. Ebert used RNAi to inhibit one of the 40 genes of the CDR at a time and showed that it was the knockdown of RPS14 alone that recapitulated the anaemia and high platelet count of the 5q-syndrome by causing apoptosis in erythroid cells while preserving differentiation of the megakaryocytes. This RPS14 insufficiency biologically links the acquired anaemia of 5q- syndrome with some congenital disorders where other ribosomal protein genes such as RPS19 and RPS24 are involved (Blackfan-Diamond anaemia, etc.). It has now been shown that defective ribogenesis may be the second common theme underlying all of the MDS syndromes as ribosomal protein genes appear to be the most significantly deregulated class of genes in even the non-del(5q) type of MDS, with decreased expression in many of those genes. While lenalidomide produces complete transfusion independence in almost 70% of the del(5q) patients with low or intermediate-1 MDS, only ~25% of the non-del(5q) patients have this type of response. Using DNA microarrays, a unique signature was identified comprising 30 erythroid genes whose decreased expression is associated with a response to the drug irrespective of karyotype.

Concluding Remarks

As shown in this article, it is the dual action of pro-inflammatory cytokines that account for both a clonal expansion and variable cytopenias of MDS. It logically follows that targeting these cytokines, or the prime initiator of the cascade, TNF-α, would produce a dual benefit: on the one hand arrest of clonal expansion, and on the other hand improvement in the cytopenias. Thalidomide, an anti-TNF agent, did in fact accomplish just this in a subset of patients. Lenalidomide, its analogue, was more potent and less toxic, and specifically effective in lower-risk MDS patients with del(5q) while also benefiting a smaller group of patients without del(5q) abnormality. Patients with higher-risk disease, especially those in transformation, respond better to hypomethylating agents, which are assumed to reactivate tumour suppressor genes. These constitute the sum of FDA-approved drugs for the present, a paltry number that barely benefits half of all patients. The next wave of treatment strategies is likely to be developed as a result of the biological information generated through the elaborate technologies described in this review. For example, once the erythroid signature that identifies cells exquisitely sensitive to the actions of lenalidomide is confirmed, a diagnostic test to pre-select patients for therapy can easily be developed. Targeting defective ribogenesis would be another obvious strategy to pursue. Thus, new technologies have already unravelled surprising areas of research in MDS, as summarised in this article, and the next decade will witness the translation of these into novel therapies for an increasing number of our patients. ■

Azra Raza is a Professor of Medicine and Director of the Myelodysplastic Syndrome (MDS) Center at Saint Vincent's Comprehensive Cancer Center in New York. The author of more than 250 manuscripts, Professor Raza's interests centre on the aetiology, biological understanding and treatment of MDS and the leukaemias that arise from these syndromes.

Naomi Galili is Director of the Bone Marrow Translational Laboratory at Saint Vincent's Comprehensive Cancer Center in New York. She has collaborated with Professor Raza on multiple studies on the biology of myeloid disease, with special emphasis on myelodysplastic syndromes and the response of patients to novel therapeutic agents.

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