



Diamond Blackfan Anemia

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Mutations affecting genes encoding ribosomal proteins cause Diamond Blackfan anemia (DBA), a rare congenital syndrome associated with physical anomalies, short stature, red cell aplasia, and an increased risk of malignancy. p53 activation has been identified as a key component in the pathophysiology of DBA after cellular and molecular studies of knockdown cellular and animal models of DBA and other disorders affecting ribosomal assembly or function. Other potential mechanisms that warrant further investigation include impaired translation as the result of ribosomal insufficiency, which may be ameliorated by leucine supplementation, and alternative splicing leading to reduced expression of a cytoplasmic heme exporter, the human homolog of the receptor for feline leukemia virus C (FVLCR). However, the molecular basis for the characteristic steroid responsiveness of the erythroid failure in DBA remains unknown. This review explores the clinical and therapeutic implications of the current state of knowledge and delineates important but as-yet-unanswered questions.

Introduction

Diamond Blackfan anemia (DBA; OMIM 205900) is a rare congenital red cell aplasia that classically presents with severe anemia in early infancy, often in association with physical anomalies and short stature. DBA moved into the scientific limelight after the unexpected identification of *RPS19*, the gene encoding ribosomal protein S19 (rpS19), as the first DBA gene.¹ The finding of mutations affecting further ribosomal protein genes has confirmed DBA as a disorder of ribosomal biogenesis or function, endorsed by the demonstration that haploinsufficiency of *RPS14* accounted for the macrocytic anemia associated with the 5q⁻ syndrome.²

This review examines how recent scientific advances can help in the understanding of clinical aspects of DBA, in particular to develop much-needed new treatments. (For a consensus discussion of the clinical management of DBA, see Vlachos et al.³)

Clinical and genetic features of DBA

Clinical presentation

DBA classically presents at 2-3 months of age; only 25% of affected babies are anemic at birth and hydrops is rare. Physical anomalies are present in up to 50% with a wide range of severity. These are typically craniofacial, including hypertelorism, flat nasal bridge, and high arched or cleft palate. Thumb abnormalities are seen in 20%, including the classical triphalangeal thumb. The anemia responds to steroid therapy in up to 70% of patients, but eventually approximately 40% of affected individuals are dependent on a long-term transfusion program unless they have a suitable donor for hemopoietic stem cell transplantation (HSCT). Spontaneous remissions may occur, even after many years of transfusion dependence.⁴ Remissions, whether spontaneous or treatment induced, are associated with a residual erythropoietic defect shown by a persistent mild macrocytic anemia with increased erythrocyte adenosine deaminase (eADA) activity.⁵ The overall actuarial survival of patients in the North American DBA Registry is 75.1% ± 4.8% at 40 years,⁶ although steroid nonresponders have a lower life expectancy. A high proportion of deaths are treatment related, including death from complications of HSCT and from iron overload, underscoring the need for new, safe, and effective treatments. Improved survival in DBA has revealed an increased risk of malignancy, with a higher

than predicted incidence of acute myeloid leukemia (AML) and osteogenic sarcoma.⁷ The exact risk for these and other cancers associated with DBA is not yet known.

DBA genetics

Approximately 50% of patients have mutations affecting a single allele of the ribosomal protein genes S19, L5, L11, or S26. Mutations in genes encoding ribosomal protein S7, S24, S17, S10, or L35a account for another small percentage of cases (summarized in Vlachos et al⁸). To date, all reported mutations have been heterozygous, which is consistent with the autosomal dominant pattern of inheritance most commonly seen in familial DBA. The majority of mutations described to date predict haploinsufficiency of the relevant ribosomal protein, with a small proportion having missense mutations of a normally expressed allele.

Genotype-phenotype correlation

It is clear that the phenotypic spectrum of DBA encompasses a wide range of severity, even within the same family, ranging from the classical syndrome described above to individuals with an isolated increase in eADA. No correlation has yet been found between the identity of the DBA gene and hematological severity, including response to steroids. However, craniofacial abnormalities are associated with mutations affecting *RPL5* or *RPL11*.⁹

Other congenital syndromes associated with ribosomal pathophysiology

Ribosomal pathophysiology has been implicated in other congenital syndromes, which may overlap sufficiently with DBA to cause diagnostic problems.

Treacher Collins syndrome

Treacher Collins syndrome (TCS; OMIM 154500) is an inherited disorder of craniofacial dysmorphogenesis with hypoplasia of the facial bones and cleft palate. TCS shares clear phenotypic similarities with the craniofacial anomalies seen in DBA, especially in patients with mutations affecting *RPL5* or *RPL11*. However, TCS is not associated with anemia. Heterozygous mutations of *TCOF1*, which encodes treacle, a nuclear phosphoprotein, are seen in 90% of

affected families.¹⁰ Treacle is involved in the transcription of ribosome rDNA by interacting with upstream binding factor, a transcription factor for RNA pol I, the down-regulation of which inhibits the production of rRNA. Recently, mutations affecting POLRID and POLRIC, which encodes subunits of RNA polymerases I and III, have been identified in TCOF-ve patients,¹¹ confirming TCS as a disorder of ribosome biogenesis.

Cartilage hair hypoplasia

Cartilage hair hypoplasia (CHH; OMIM 250350) is an inherited disorder comprising skeletal abnormalities, abnormal hair, and impaired cell-mediated immunity. Whereas anemia is not a universal feature of CHH, there have been several case reports of severe macrocytic anemia in infancy with a hematological presentation very similar to that of DBA, including steroid-responsiveness in some cases, which persists to adulthood in 10% of patients.¹² Increased eADA has been variably reported. CHH is caused by mutations affecting *RMRP*, an untranslated gene that forms the RNA subunit of the RNase MRP complex¹³ that is involved in ribosomal assembly by cleavage of 5.8S rRNA, in cell-cycle control by cleavage of cyclin B2 mRNA at the end of mitosis, and in processing mitochondrial RNA.¹⁴

Shwachman-Diamond syndrome

Shwachman-Diamond syndrome (SDS; OMIM 260400) is an autosomal recessive disorder of exocrine pancreatic insufficiency, skeletal abnormalities, and BM dysfunction, with a high risk of myelodysplastic syndrome with progression to AML.¹⁵ *SBDS* loss-of-function mutations can be detected in approximately 90% of SDS patients.¹⁶ The *SBDS* protein has multiple interactions, and the precise function or functions responsible for the phenotypic features of SDS has not been established. With respect to ribosomal pathophysiology, the yeast ortholog of *SBDS* associates with the large ribosome subunit, a mutation that causes impaired subunit maturation.¹⁷

Myelodysplastic syndrome associated with isolated del(5q) chromosome abnormality

The 5q⁻ syndrome is an acquired clonal disorder with the distinct phenotype of macrocytic anemia with erythroid hypoplasia, and normal or raised platelet count with dysplastic hypolobulated megakaryocytes. The important overlap with DBA was established by the demonstration that haploinsufficiency of *RPS14* is responsible for the 5q⁻ erythroid phenotype.² *RPS14* haploinsufficiency does not account for the clonal dominance and megakaryocytic changes in the 5q⁻ syndrome, which have recently been attributed to miRNA-145 and miRNA-146a, which are located within the common deleted region.¹⁸ Knockdown of these miRNAs in mouse hemopoietic stem cells causes thrombocytosis, neutropenia, and megakaryocyte dysplasia. The therapeutic response to lenalidomide in the 5q⁻ syndrome is associated with cytogenetic response, suggesting that it acts by suppression of the clonal advantage caused by miRNA-145 and miRNA-146a haploinsufficiency, rather than by amelioration of the erythroid defect caused by *RPS14* allele loss.

Pathophysiology of DBA

Characterization of erythroid defect

The demonstration of impaired erythroid differentiation by purified CD34⁺ cells from patients with DBA provides strong evidence for the existence of a defect that is intrinsic to the erythroid progenitor cell. Commitment to the erythroid pathway is intact, with impaired erythropoiesis becoming apparent at the late erythrocyte burst-

forming unit stage, characterized by impaired proliferation and increased apoptosis. In vitro erythroid culture studies have confirmed a direct enhancing effect of glucocorticoids on both DBA and control erythroid progenitor cells,¹⁹ although additional indirect effects cannot be excluded.

Tissue specificity

Whereas impaired ribosomal biogenesis should affect all cell types, the extreme demand placed on ribosomal biogenesis in proerythroblasts by the combination of globin synthesis and rapid proliferation may underlie the erythroid specificity of the DBA phenotype. However, other tissue- or cell-specific mechanisms may also apply. Ribosomal protein haploinsufficiency may also have an indirect effect; abnormal pigmentation in the *RPS19*- or *RPS20*-mutated Dsk mouse is caused by melanocyte stimulation by kit ligand (stem cell factor) produced by keratinocytes.²⁰

Impaired ribosomal biogenesis

Haploinsufficiency affecting individual ribosomal proteins perturbs the precise stoichiometry of ribosomal biogenesis, which entails the coordinated assembly of proteins in association with processing of pre-rRNA.²¹ Knockdown of individual ribosomal proteins induces defective pre-rRNA processing, with a characteristic pattern of accumulation of intermediate forms depending on the identity of the deficient protein.²² Equivalent abnormalities are seen in fibroblasts or lymphoblastoid cells derived from patients with DBA. Imbalances of ribosomal proteins and other factors involved in ribosome assembly lead to the accumulation of p53, a process mediated through interactions between Mdm2/Hdm2 and free ribosomal proteins, including L5, L11, L23, or S7, and resulting in cell-cycle arrest or apoptosis.²³ Inactivation of p53 prevents the cell-cycle arrest but does not restore rRNA processing.

Central role for p53 activation in the pathophysiology of DBA

The role of p53 in the ribosomal surveillance checkpoint described above and the observation of apoptosis in DBA erythroid cultures makes p53 activation an attractive candidate mechanism in the pathogenesis of DBA.

Role of p53 in physical anomalies

There is convincing evidence in animal models for a role of p53 in mediating the physical anomalies associated with ribosomal pathology. The abnormal pigmentation in the *RPS19*- or *RPS20*-mutated Dsk mouse is p53 dependent,²⁰ and the phenotype of *RPL11* deficiency in the zebrafish, which normally results in embryonic lethality due to cranial malformations, can be rescued by simultaneous knockdown of p53.²⁴ Neural crest apoptosis in the *TCOF1*-knockdown mouse model of TCS can be rescued by p53 inhibition with pifithrin- α or on a p53-deficient genetic background.²⁵ The triphalangeal first digit and retinal abnormalities seen in the *RPL24* hypomorph Bst mouse²⁶ are also alleviated on a p53-null background.

Role of p53 in erythroid failure

Knockdown of *RPS19* in mouse fetal liver cells results in increased levels of p53 and p21, with reduced proliferation in association with G1/S phase delay, but no increase in apoptosis.²⁷ Suppression of p53 in the *rpS19*-deficient zebrafish alleviates impaired erythropoiesis,²⁴ whereas a p53-deficient background rescues the anemic phenotype of a mouse model of the 5q⁻ syndrome.²⁸ Evidence in human cells comes from a study by Dutt et al of primary BM-derived CD34 cells, in which reduced levels of *rpS19* or *rpS14* were shown to

cause selective induction of p53 in cells of the erythroid lineage. Nutlin, which blocks the association of Hdm2 with p53, had a similar effect, whereas inhibition of p53 by pifithrin- α rescued the erythroid knockdown phenotype.²⁹

Role of p53 in cancer predisposition

In the context of p53 activation, there should be a strong selective advantage to cells carrying loss-of-function mutations affecting p53. Parallels may be drawn between the increased incidence of pediatric osteogenic sarcoma in DBA⁶ and that in Li-Fraumeni syndrome, in which there is congenital p53 deficiency. Zebrafish with heterozygous mutations affecting ribosomal protein genes have an increased incidence of malignancy, notably with malignant peripheral nerve sheath tumor, an otherwise rare tumor usually associated with loss-of-function p53 mutations.³⁰

Polymorphisms affecting the p53 pathway: potential role in the variable phenotype of DBA

There are several functionally significant variations affecting the p53 pathway that provide a potential mechanism for phenotypic variation in DBA, even within the same family, and also individual cancer risk. For example, a polymorphism leading to increased expression of *HDM2* and thus to lower levels of p53 would be predicted to ameliorate the erythroid defect in DBA.

p53 and mechanism of action of steroids

In most cell types, dexamethasone and p53 have opposing actions. Erythroid-specific interactions between the activated GR and p53 could therefore provide a potential mechanism of action for steroids in DBA. For example, mouse fetal liver erythroblasts have a greater response to dexamethasone on a p53-deficient genetic background.³¹

Therapeutic implications of a central role for p53

Suppression of the p53 response to enhance erythropoiesis would be predicted to increase the risk of cancer. However, advances in understanding of the role of p53 in the pathophysiology of DBA may inform other treatment decisions, such as in the avoidance of radiotherapy in HSCT conditioning regimens. In addition, ablative conditioning therapy and its associated complications may be unnecessary in HSCT or gene therapy, because the transplanted normal or genetically corrected graft HSC should have a competitive advantage over host cells with impaired ribosomal biogenesis.

Other cell-cycle checkpoints

p53 activation is a feature of many facets of cell-cycle regulation, and the ribosomal biogenesis surveillance checkpoint may not be the sole mechanism in DBA. In CHH, the occurrence of anemia and increased cancer risk is predicted by *RMRP* mutations resulting in reduced mRNA cleavage activity, which are involved in cell-cycle control, whereas those resulting in reduced rRNA cleavage, which are predicted to trigger the ribosomal biogenesis surveillance checkpoint, are associated with skeletal dysplasia.¹⁴ siRNA knockdown of *RPS19* in mouse fetal liver cells results in G1/S cell-cycle delay.²⁷ By contrast, expression of the *RPS19* R62W missense mutation in the mouse induces cell-cycle arrest in G2/M.³² Primary fibroblasts from a DBA patient with a truncating *RPS19* mutation showed arrest at G1, whereas those with *RPS24* deficiency showed perturbed S-phase progression, suggesting the occurrence of ribosomal protein gene- or mutation-specific effects.³³

Other potential mechanisms in the pathophysiology of DBA

The evidence presented above strongly supports a central role of p53 underlying the phenotype of DBA. However, there are other potential mechanisms for the erythroid failure observed in DBA, including reduced translational efficiency and abnormalities in heme metabolism, which warrant further consideration.

Reduction in rate of protein synthesis

A reduction in the overall rate of polypeptide chain initiation will disproportionately and specifically affect mRNAs that are least efficiently translated.³⁴ Therefore, ribosomal insufficiency might affect erythropoiesis preferentially by affecting the level of expression of critical erythroid proteins that are subject to translational control. The pathogenesis of the erythroid defect in DBA could therefore be due to the failure of a specific protein to reach a threshold level at a critical stage, for example, by perturbing the stoichiometry of multiprotein erythroid-specific complexes or by a more selective influence on the translation of a critical protein.

A global reduction in translation might also be relevant to the pathogenesis of DBA. PHA-stimulated DBA lymphocytes have been shown to have reduced levels of translation that can be increased by the addition of leucine.³⁵ Leucine and the other branched-chain amino acids influence protein synthesis by signaling through the mammalian target of rapamycin (mTOR) pathway. mTOR is central to the control of translation in response to cytokine signaling and nutrient availability. There is evidence of an interaction between glucocorticoids and the mTOR pathway; the anti-inflammatory action of glucocorticoids in human monocytes and myeloid dendritic cells is inhibited by rapamycin. In addition, glucocorticoids can block the expression of TSC2, a negative regulator of mTOR, and enhance signaling through mTOR.

A successful trial of oral leucine supplements has been reported in a child with steroid-nonresponsive DBA, whose cells had shown a good in vitro response to leucine.³⁶ The patient increased in weight and in general well-being and became transfusion independent over the course of 6 months. The use of nutritional supplementation with branched-chain amino acids in the management of DBA is potentially very interesting, warranting the establishment of controlled clinical trials.

Alternative splicing of FLVCR1

DBA erythroid cells have been shown to express alternatively spliced *FLVCR1* isoforms, with reduced FLVCR protein expression and reduced functional activity.³⁷ *RPS19*-down-regulated K562 cells show similar alternative splicing and down-regulation. *FLVCR1*-null mice have craniofacial and limb deformities, lack definitive erythropoiesis, and die mid-gestation. However, neonatal deletion of *FLVCR1* causes a severe macrocytic anemia with maturation arrest at the proerythroblast stage. *FLVCR1* encodes a cytoplasmic heme exporter that is associated with feline red cell aplasia. FLVCR mediates heme export from macrophages and plays a role in the regulation of hepatic iron. Free heme toxicity is believed to be the mechanism for the erythroid failure. Impaired iron homeostasis secondary to FLVCR dysfunction might contribute to the severity of transfusion-related iron overload in DBA.

Modulation of gene expression by alternative splicing is prominent in erythroid differentiation.³⁸ Orru et al have shown coimmunoprecipitation of splicing factors with rpS19,³⁹ suggesting that the

regulation of splicing may be a more generalized phenomenon in DBA. Interestingly, there are functional differences between kit isoforms, providing another possible link with mouse models of anemia. The anemia seen in Steel and White Spotted mice, which are deficient in stem cell factor and its receptor (kit), respectively, closely resembles that of DBA, being macrocytic and associated with high eADA activity.

Alternative splicing is influenced by histone and chromatin modification, making it a potential target for the therapeutic effect of steroids, which are known to induce epigenetic effects through modulation of histone acetylation and methylation.

Aspects of DBA for further studies

Age at presentation

The age at which the erythroid failure in DBA becomes manifest coincides with the fetal adult switch; one possible explanation is that fetal erythropoiesis is less vulnerable to ribosomal insufficiency. This may be related to differences in cell-cycle kinetics or to protection by a maternal factor and warrants further investigation.

Cellular and molecular basis for spontaneous remission

Spontaneous remission in DBA may be analogous to a recently described new clonal phenotype in Fanconi anemia,⁴⁰ in which the underlying genetic disorder is expressed, but with a paradoxical lack of the predicted cell-cycle arrest in G2. The mechanism of this checkpoint attenuation is not clear, but the affected peripheral blood leukocytes show low expression of p53 and CHK1. The phenomenon is associated with a milder hematological phenotype, but offers no protection against—and may even promote—malignant transformation. In the series described by Janov,⁴ 2 DBA patients who experienced spontaneous remissions after 13 and 15 years of transfusion dependence subsequently developed AML, which would be consistent with the emergence of a clone with attenuation of a p53-mediated checkpoint.

The cellular and molecular analysis of hemopoiesis during spontaneous remission provides a potential tool for elucidation of the molecular pathogenesis of DBA, as well as the basis for its attenuation. It is therefore important to determine whether spontaneous remission in DBA is characterized by clonal hemopoiesis, to assess the integrity of p53 pathways, and to analyze pre-rRNA processing.

Steroid nonresponsiveness

In 30% of patients with DBA, the anemia does not respond to steroids and in steroid responders, the required maintenance dose varies widely between individuals. Correlation between clinical steroid responsiveness and steroid effects in different cellular models provides a tool with which to assess the biological significance of in vitro observations, whereas elucidation of the molecular mechanisms of glucocorticoid resistance may offer new therapeutic options.

Identity of outstanding DBA genes

In approximately 40% of DBA cases, the gene or genes responsible remain to be identified. The identity of other genes will be of clinical benefit, facilitating accurate diagnosis and reproductive choices in affected families, and excluding clinically silent DBA in potential family donors for HSCT. It may also help to elucidate the pathophysiology of DBA. Sequencing of all ribosomal protein genes is clearly warranted, including screening for large deletions,⁸

but other candidate genes may be indicated by the potential molecular mechanisms for DBA discussed above; broadening the search may identify the exception that proves the rule. Studying the genotype-phenotype correlation in cells with missense dominant-negative mutations affecting ribosomal protein genes will also be of value in identifying critical interactions in the pathophysiology of DBA.

Conclusions

The discovery of RPS19 as the first DBA gene has led to exciting scientific research in DBA and other ribosomal disorders. Whereas there is compelling evidence for the involvement of p53 in the pathogenesis of DBA, no single proposed mechanism so far accounts for all facets of DBA and yet none is mutually exclusive. The relative importance of the different mechanisms in in vitro studies is likely to be influenced by the culture system used, providing an explanation for the lack of consistent correlation between the in vitro erythroid defect and clinical severity.¹⁹ It may transpire that, in vivo, each may play a part to a various degree, in a portfolio of pathophysiological processes, the differing contributions of each mechanism explaining phenotypic variability, including steroid responsiveness and risk of cancer.

There remains no real alternative to steroids in the treatment of DBA, and treatment-related complications remain an important cause of morbidity and mortality. It is important that continuing interest in the fascinating science of DBA does not eclipse the human aspects of the disorder, and that new treatments can develop from scientific advances.

Disclosures

Conflict-of-interest disclosure: The author declares no competing financial interests. Off-label drug use: None disclosed.

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