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Congenital Disorders of Ribosome Biogenesis and Bone Marrow Failure

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Diamond Blackfan anemia (DBA) is a congenital bone marrow (BM) failure syndrome that typically results in macrocytic anemia within the first year of life. DBA is also associated with birth defects, increased incidence of cancer, and other cytopenias. Shwachman-Diamond syndrome (SDS) is a multisystem disease characterized by exocrine pancreatic dysfunction, impaired hematopoiesis, and leukemia predisposition. Other clinical features include skeletal, immunologic, hepatic, and cardiac disorders. Treatment for these BM failure syndromes, including stem cell transplantation (SCT), will be discussed in this review.

Biol Blood Marrow Transplant 16: S12-S17 (2010) © *2010 American Society for Blood and Marrow Transplantation*

KEY WORDS: Ribosome, Bone marrow failure, Stem cell transplantation

CLINICAL ASPECTS AND MOLECULAR PATHOGENESIS OF DIAMOND BLACKFAN ANEMIA (DBA): KATHLEEN SAKAMOTO

Diamond Black Anemia (DBA) is an inherited hypoplastic anemia that usually presents in early infancy and was first described by Josephs, Diamond and Blackfan in the 1930s [1,2]. DBA is 1 of a group of genetic disorders referred to as "inherited bone marrow failure syndromes" [3]. The incidence of DBA is approximately 5 per million live births. DBA consists of bone marrow (BM) failure, birth defects, and a predisposition to develop malignancies [3]. The hematopoietic defect is characterized by progressive apoptosis of erythroid precursors, resulting in normochromic or macrocytic anemia. DBA is also associated with craniofacial, thumb, cardiac, and urogenital malformations [1]. Other hallmarks of the disease include reticulocytopenia, normocellular BM with decreased erythroid precursors, decreased neutrophil count, and variable platelet count with a normal BM cellularity [3]. The presence of macrocytosis, elevated adenosine deaminase, and increased fetal hemoglobin levels suggests that the phenotype of DBA is secondary to "stress erythropoiesis" (Table 1) [3].

The reasons for the clinical presentation of DBA soon after birth may because of decreased erythropoietin production, elevated hemoglobin level, and the switch to decreased O_2-affinity adult hemoglobin allowing for more oxygen delivery to tissues [3]. There are also data suggesting that erythroid progenitors are less sensitive to erythropoietin in the transition from fetal state to the adult state [3]. Furthermore, anemia results from a defect in erythropoiesis, rather than immune-mediated destruction [3]. BM from DBA patients demonstrated decreased formation of either early or late erythroid colony forming units and defective erythroid differentiation [3]. More recent data suggest that there is premature apoptosis of erythroid progenitors [3,4] in mammalian and zebrafish models [4,5].

In 1999, the first DBA gene was identified as being ribosomal protein subunit 19 (RPS19) (Table 1) [6,7]. Studies have demonstrated heterozygosity for mutations in RPS19 in 24% of patients [8]. In 2006, another DBA gene was recently identified as ribosomal protein 24 (RPS24) [7,9]. Since then, mutations in several other ribosomal proteins of the 40S ribosomal subunit (RPS17, RPS19, and RPS24) and the 60S ribosomal subunit (RPS5, RPL11, and RPL35A) [3,10,11]. More recently, Ebert et al. [12,13] reported that patients with myelodysplastic syndrome (MDS) and 5q- have haploinsufficiency of RPS14. This further supports the notion that insufficiency of ribosomal subunits contributes to erythroid failure. The consequence of RPS mutations is abnormal ribosomal biosynthesis.

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Financial disclosure: See Acknowledgments on page S16.

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1083-8791/10/161S-0004$36.00/0
doi:10.1016/j.bbmt.2009.09.012

Table 1. Genes involved in DBA and SDS

	Genes	Useful Laboratory Tests
DBA	*RPS19*	Erythrocyte adenosine deaminase
	RPS17	Hemoglobin F
	RPS24	
	RPL35A	
	RPL5	
	RPL11	
SDS	*SBDS*	Trypsinogen*
		Pancreatic isoamylase*
		Fecal elastase

DBA indicates Diamond Blackfan anemia; SDS, Shwachman-Diamond Syndrome.
*Currently, ranges for SDS have not been established in commercial labs.

The question remains as to why erythropoiesis is particularly vulnerable to ribosome deficiency.

The precise role of RPS19 haploinsufficiency in the pathogenesis of DBA is not completely understood. The fact that DBA presents early in life suggests that RPS19 affects erythropoiesis early during development and alterations in blood cell production at birth activates specific pathways that lead to the erythroid phenotype [14]. To test this possibility, a zebrafish model of RPS19 deficiency was generated by injecting RPS19 morpholinos in embryos [4]. The embryos had developmental abnormalities very similar to humans, including craniofacial abnormalities, short fins, and defective erythropoiesis. Furthermore, erythroid progenitors were macrocytic and underwent premature apoptosis (Figure 1) [4].

Knockout of RPS19 in mice is embryonic lethal and heterozygotes lack a DBA phenotype [14]. RPS19 knockdown in mouse erythroleukemia cells resulted in G0/G1 arrest and increased expression of p21 and p27 and decreased expression of the anti-apoptotic proteins, Bcl-2 and Bad [15]. $CD34^+$ cells from DBA patients undergo apoptosis in in vitro culture assays [15]. Recently, gene therapy in $CD34^+$ cells from DBA patients with RPS19 mutations was shown to rescue the erythroid phenotype in mouse transplantation models [16].

There is strong evidence that erythroid defects due to RPS19 haploinsufficiency may be regulated at least in part, through p53-dependent pathways. Previous studies demonstrated that disruption of the nucleolus or genotoxic stress results in p53 upregulation [17]. Haploinsufficiency of RPS6 and RPL22 have also been shown to lead to activation of p53 [4,18,19]. More recently, rps19 and rps20 genes were found to be mutated in mice with a dark skin phenotype [20]. The phenotype of the rps19 mutant in mice was similar to DBA, including apoptosis of erythroid progenitor cells and growth retardation [3,20]. One simple explanation for the erythroid phenotype is that RPS19 activates stress-related pathways, resulting in p53 upregulation and induction of apoptosis of erythroid progenitors. However, it is more likely that the mechanism is more complicated, including activation of p53-independent pathways, which might affect erythroid specific differentiation pathways. Thus, the precise regulation of p53 downstream of RPS deficiency is still unknown.

DBA is characterized by heterogeneity in both clinical presentation and response to treatment. At this time, phenotypic and genotypic correlation to predict response to treatment has not been identified. DBA is also considered a cancer predisposition syndrome. In a recent review by Lipton and Ellis [21], of the 30 cases of cancer in DBA patients, 15 were hematopoietic malignancies. Although cancer is rare, the incidence is higher than what would be expected for the specific age group [21]. The prognosis is poor in DBA patients with cancer, mostly because of significant myelosuppression following chemotherapy [21].

The treatment of DBA consists of corticosteroids and red cell transfusions [1,3]. Studies in the 1950s demonstrated that steroids could improve anemia in DBA patients, despite the fact that DBA is not an

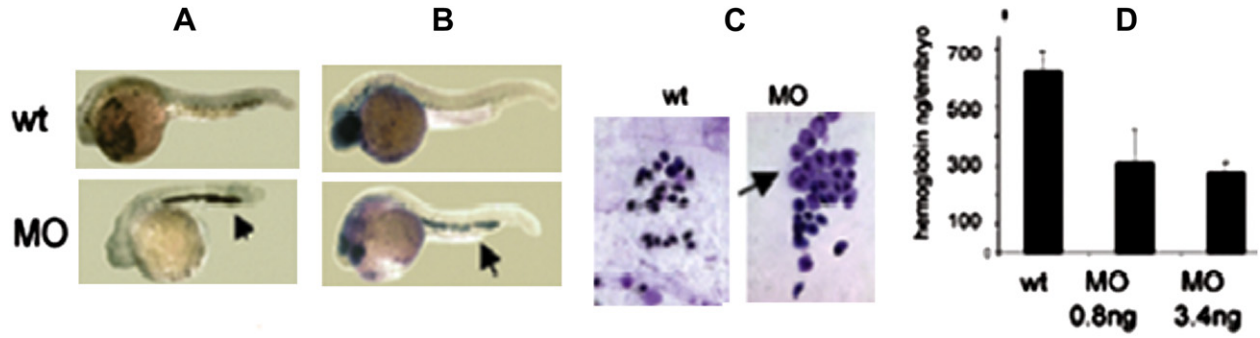

Figure 1. DBA phenotype in zebrafish embryos injected with RPS19 morpholinos. (A,B) At 72 high-power field, there are fewer blood cells in zebrafish embryos injected with RPS19 morpholinos and there are only few cells in the heart region: 2.5 ng morpholino per embryo, O-dianizidine staining, 30 embryos per group. (C) Erythroid cells from the blood of a morphant embryo are variable in size; some look like erythroblasts. Zebrafish erythrocytes differ from mammalian cells in that both primitive and definitive cells are nucleated. Blood smears were prepared from individual embryos; the results are representative of 6 to 10 embryos. (D) The level of hemoglobin is lower in morphants at day 5 in dependence with morpholino dose [4]. This figure and research was originally published as Danilova N, Sakamoto KM, Lin S. Ribosomal protein S19 deficiency in zebrafish leads to developmental abnormalities and defective erythropoiesis through activation of p53 protein family. *Blood.* 2008;112:5228-5237. © American Society of Hematology.

immune-mediated disease. Red cell transfusions are given to DBA patients with daily subcutaneous administration of the iron chelator, deferoxamine. Previous literature has reported that 79% of patients were responsive to steroids, 27% were nonresponsive, and 4% were never treated [1,3]. More recent data from the DBA registry demonstrated that 37% of patients receive corticosteroids and 31% receive red cell transfusions. Of the 35% of DBA patients who were not steroid responsive, 22% became steroid refractory after a period of time, and 33% could not be weaned to an acceptable dose [3]. Steroid treatment is often delayed until after 1 year of age to minimize effects on growth and development. Patients who do not respond within a month of starting steroid therapy are considered refractory and require red cell transfusions [3]. Remissions have been described with DBA patients; however, there are few data on the percentage of patients who were able to sustain red cell production for over 6 months without therapy [1,3]. With increased understanding of the molecular pathogenesis of DBA and the role of ribosomal biogenesis, there is potential for new therapeutic approaches to treat these patients in the future.

SHWACHMAN DIAMOND SYNDROME (SDS): CLINICAL AND MOLECULAR FEATURES: AKIKO SHIMAMURA

Shwachman Diamond Syndrome (SDS) is an autosomal recessively inherited syndrome characterized by BM failure, exocrine pancreatic dysfunction, and leukemia predisposition (reviewed in [22]). The most common manifestation of BM failure is neutropenia. Patients may also develop thrombocytopenia and anemia. A subset of patients progress to severe aplastic anemia (SAA). Erythroid macrocytosis and elevated fetal hemoglobin levels may be present. Clonal cytogenetic abnormalities frequently arise in the bone marrow [23]. Patients with SDS are at increased risk for myelodysplastic sydrome (MDS) and acute myelogenous leukemia (AML) [24,25].

Histologic data regarding the exocrine pancreas are limited. Much of the pancreatic tissue is replaced with fat amid a paucity of acini. The pancreatic ducts and islets appear intact. Pancreatic atresia with lipomatosis may be evident on imaging studies. If the pancreatic involvement is severe, patients may be symptomatic with steatorrhea, fat-soluble vitamin deficiency, and failure to thrive. Many patients lack clinical symptoms or their symptoms may resolve over time. Tests for exocrine pancreatic dysfunction include low pancreatic elastase, low serum trypsinogen, and low pancreatic isoamylase levels (Table 1) [26].

Additional clinical features have also been described for SDS. Skeletal abnormalities are commonly observed [27,28]. Skeletal findings include metaphyseal dysostosis, thoracic dystrophies, and low bone turnover osteopenia. Hepatomegaly with elevated transaminases may present in infancy. These hepatic abnormalities typically resolve spontaneously with age. An eczematous rash is also frequently reported in infancy. Patients may have immunologic compromise such as low immunoglobulin levels and reduced B and T cell number and function [29]. Cardiac, neurologic, and endocrine abnormalities have also been described.

Over 90% of patients with SDS harbor biallelic mutations in the *SBDS* (Shwachman-Bodian-Diamond Syndrome) gene, which encodes a highly conserved, ubiquitously expressed protein [30]. *Sbds−/−* mice exhibit early embryonic lethality [31]. The SBDS protein is localized throughout the cell and shuttles in and out of the nucleolus, the major cellular site of ribosome biosynthesis [32]. Mammalian SBDS binds to the 60S large ribosomal subunit [33]. Several screens for SBDS-associated proteins have identified ribosomal proteins and assembly factors [33-36]. Knockdown of the yeast orthologue, *Sdo1*, results in a slow growth phenotype, which is suppressed by mutations in *Tif6* [37]. Tif6 is a ribosome assembly factor that must be released from the 60S subunit to allow the joining of the 40S and 60S ribosomal subunits into the mature 80S ribosome. Tif6 suppressor mutations result in diminished binding of Tif6 to the 60S ribosomes. Based on these data, a model has been proposed, whereby Sdo1 promotes the dissociation of Tif6 to allow the joining of the 40S and 60S ribosomal subunits [37]. Thus, in contrast to the situation in DBA where loss of ribosomal subunit proteins impairs the formation of the 40S or 60S precursor subunit, SBDS functions at a later stage of ribosome biogenesis after the 40S and 60S subunits have been assembled.

SBDS has also been implicated in additional cellular functions that likely also contribute to the clinical phenotype. SBDS functions in mitotic spindle dynamics by promoting spindle stabilization [38]. SBDS loss also affects actin polymerization [39] and neutrophil chemotaxis [40]. SBDS has also been implicated in cellular stress responses [41].

The diagnosis of SDS is easily missed because cytopenias may be mild or intermittent and exocrine pancreatic dysfunction may be asymptomatic. The full clinical phenotypic spectrum for SDS is poorly understood. Most studies describing the SDS phenotype were conducted prior to the advent of genetic testing. Diagnosis is further complicated by the finding that around 10% of SDS patients lack identifiable *SBDS* mutations [30]. Because SDS is an autosomal recessively inherited disorder, all siblings of an SDS proband should also be tested.

The only curative treatment for BM failure in SDS is a hematopoietic stem cell transplant (HSCT); however, patients with SDS are at increased risk for

transplant regimen-related toxicity as discussed later. Neutropenia often responds to granulocyte-colony stimulating factor (G-CSF). Coagulopathy may result from thrombocytopenia or from vitamin K deficiency secondary to pancreatic insufficiency. Treatment of coagulopathy includes platelet transfusions, and vitamin K supplementation. Treatment of active bleeding with antifibrinolytic agents such as aminocaproic acid may also be considered as clinically indicated. Symptomatic or severe anemia is treated with red cell transfusions. The clinical management of cytogenetic clones arising in the BM must be considered within the context of the BM morphology, the peripheral blood counts, and the specific cytogenetic findings. HSCT should be considered for MDS or AML arising in patients with SDS, although data are lacking regarding optimal treatment regimens. Because patients with SDS are at increased risk for hematologic malignancies, regular monitoring of the blood counts and BM are recommended [42].

STEM CELL TRANSPLANTATION FOR DISEASE WITH RIBOSOMAL DEFECTS: STELLA DAVIES

Transplant For DBA

Experience with transplantation for DBA is limited, as the majority of children can be managed with a combination of steroids and transfusion with or without chelation. Steroid nonresponsiveness and high transfusion needs with associated iron overload is the most frequent indication for the procedure in those children who have been transplanted. The careful identification of children most likely to benefit from transplantation is 1 of the most important challenges that continually needs to be addressed, as outcomes of transplant improve, a change that must be balanced against parallel advances in chelation that can improve supportive therapy.

Several registry series published in the last 5 years have indicated that reasonable outcomes can be obtained for children with DBA receiving transplant from an HLA-matched sibling donor, Roy et al. [43] described outcomes of 61 transplants for DBA reported to the Center for International Blood and Marrow Transplant Research (CIBMTR). Sixty-seven percent of cases (n = 41) received stem cells from a matched related donor, median age was 7 years (range: 1-32 years), and the majority of cases (>64%) had received >20 prior transfusions. Day 100 mortality was significant at 18%, and survival at 3 years was 64%. Survival was significantly reduced in recipients of alternative donor grafts (unrelated [n = 12] or mismatched family member [n = 8]) compared with sibling donor grafts (76% versus 39%; $P = .005$). In addition, 3-year survival was lower in recipients with a reduced Karnofsky performance score (KPS) (42% KPS <90% versus 75% KPS >90%; $P = .011$), suggesting that outcomes are improved with earlier transplant as might be expected. This study offers the largest series of transplanted DBA cases yet, but as a registry series is necessarily limited by few details regarding the rationale in selecting transplantation for individual patients.

The DBA registry, that now includes more than 420 patients, updated their results in 2006, describing transplant outcomes in 15 alternative donor graft recipients (2 mismatched related, 4 unrelated cord blood [CB], 8 unrelated BM, and 1 unrelated peripheral blood stem cell [PBSC]) and 21 sibling donor recipients [44]. Outcomes in this report were similar to those reported by CIBMTR (survival 72% for sibling graft recipients versus 19% for alternative donor recipients), and it is likely that there may be significant overlap of cases between the 2 reports. The majority of patients were selected for transplant because of transfusion dependence, although 2 cases had SAA, and 1 significant thrombocytopenia.

The AA committee of the Japanese Society of Pediatric Hematology has reported outcomes of 19 transplants (13 matched siblings, 6 alternative donors) for DBA in Japan [45]. All the patients in this series had been extensively transfused, and many reported significant toxicities from steroids. In this series, all the children who received BM as a stem cell source (either from a sibling or an unrelated donor) were surviving. In contrast, none of the 3 unrelated CB recipients survived, although 2 sibling CB recipients were alive and free of disease. The authors of this study suggest that BM might be preferred over CB for children with DBA.

The disappointing results of transplantation from alternative donors in these reports may reflect advanced disease at time of transplant, or may be a consequence of the transplants strategies applied. The majority of recipients of alternative donor grafts received a total body irradiation (TBI)-based preparative, regimen, whereas the majority of sibling donors did not. Ostronoff et al. [46] have described a successful sibling donor transplant in an infant with DBA using a nonmyeloablative (NMA) preparative regimen (200 cGy TBI and 90 mg/m^2 fludarabine [Flu]), and is possible that reduced intensity conditioning (RIC) or NMA strategies could improve outcomes of alternative donor grafts. Preimplantation genetic diagnosis has been used to identify embryos that are unaffected with DBA and are HLA-matched with an affected child, to make available a matched donor for a transfusion-dependent child [47]. The need for this complicated and expensive approach will be obviated if outcomes using unrelated stem cell sources are improved. Investigators are also pursuing potential gene therapy approaches, and have shown that RPS19 gene transduction can correct the defect in erythroid

development in a murine model [16]. Additional work to develop safe vectors for clinical use will be needed before clinical trials can start in children with DBA.

Transplant For SDS

Children with SDS may be candidates for transplantation because of progressive cytopenias, or evolution to myeloid malignancy (MDS or AML). Development of clonal hematopoiesis necessarily causes anxiety, although caution should be used in proceeding to transplant for this indication alone. Cunningham et al. [48] reported isochromosome 7q in 8 children with SDS. Three children were transplanted and only 1 survives, whereas the untransplanted children are all surviving without evidence of progression to MDS or AML. These authors suggest that isochromosome 7q has a specific and benign clinical history in persons with SDS and alone should not be considered an indication for transplant.

Early reports of transplantation for SDS have identified significant toxicities, including an excess of cardiac toxicity, likely related to the underlying disease [49]. The largest reported series of transplants comes from the European Group for Blood and Marrow Transplantation, who described 26 transplants, 19 using unrelated donor stem cells, 6 sibling donor stem cells, and 1 other graft [50]. Treatment-related mortality (TRM) was high at 35%, with a follow-up of 1.1 years, whereas overall survival (OS) was 64.5%. Most of the fatal toxicity was because of organ failure and infections secondary to the preparative regimen. These data are similar to those reported by the French neutropenia registry describing 60% event-free survival (EFS) in 10 transplant recipients (6 sibling donors, 4 unrelated donors) [51].

RIC has been investigated in SDS with good outcomes, at least in early reports. Bhatla et al. [52] described 7 patients treated with RIC chemotherapy (Campath, Flu, and melphalan [Mel]; 4 sibling donors, 3 unrelated donors); all were surviving at the time of report. Similarly, Sauer et al. [53] report 3 children with SDS conditioned with Flu, treosulfan, and Mel prior to transplant, 1 receiving cells from a sibling donor, 1 unrelated donor, and 1 unrelated CB graft. Two of the 3 children were alive at time of report. Taken together, these data provide encouraging evidence that survival after transplant for SDS can be improved by the use of RIC preparative regimens.

ACKNOWLEDGMENTS

Financial disclosure: Abbott Laboratories, Inc. and Genentech, Inc. (K.M.S.). *Grants*: K.M.S. is supported by the St. Baldrick's Foundation and the NIH/NHLBI. S.M.D. is supported by the National Cancer Institute.

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