Translational efficiency in patients with Diamond-Blackfan anemia

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Background and Objectives. Diamond-Blackfan anemia (DBA) is a rare congenital pure red cell aplasia characterized by normochromic macrocytic anemia, reticulocytopenia, and normocellular bone marrow with a selective deficiency of erythroid precursors. Ribosomal protein S19 (*RPS19*), currently the only gene associated with DBA, is mutated in 25% of DBA patients, but its role in erythropoiesis is unknown. We attempted to elucidate the importance of RPS19 in translation in relation to the pathogenesis of DBA.

Design and Methods. We measured translation and proliferation rates in unstimulated and phytohemagglutinin (PHA)-stimulated lymphocytes isolated from DBA patients, as well as in K562 cells expressing several RPS19 mutants to directly test the effect of RPS19 mutations on translation. The effect of leucine on overall translation was also studied.

Results. We found that the level of translation was on average 48-73% of controls in both unstimulated and PHA-activated DBA lymphocytes irrespective of mutations in RPS19. The addition of leucine increased the translational level in RPS19-non-mutated DBA cells, but not in cells with an RPS19 mutation. In unstimulated DBA cells, proliferation was significantly impaired in both RPS19-mutated and non-mutated cells, but in both groups could be efficiently activated by PHA. Studies on K562 cells showed that RPS19 mutations affecting RPS19 conserved arginines R56Q and R62Q could significantly inhibit the rate of protein synthesis, indicating the importance of RPS19 in translation.

Interpretation and Conclusions. Our results indicate that inefficient translation may be the main cause of DBA, and administration of leucine may be beneficial for at least some DBA patients.

Key words: ribosomal protein S19, Diamond-Blackfan anemia, translation, proliferation, leucine.

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Diamond-Blackfan anemia (DBA; OMIM: 205900) is a congenital disorder affecting 5-7 children per million live births.[1,2] The diagnosis of DBA is based on the following criteria:[3] (i) normochromic macrocytic anemia presenting usually in the first year of life; (ii) normocellular bone marrow with a selective deficiency in red cell precursors; (iii) profound reticulocytopenia; (iv) normal or slightly increased platelet count, and normal or slightly decreased white blood cell count. The level of serum erythropoietin is usually increased. Erythrocytes often express several fetal markers (for example hemoglobin F, i antigen) and have an increased level of adenosine deaminase.[4,5] In about 40% of patients various congenital anomalies are present, including craniofacial dysmorphism, thumb and neck anomalies, congenital heart defects (mainly ventricular and septal defects), urogenital malformations and mental retardation.[2] Prenatal or postnatal growth retardation independent of steroid therapy is also often present. Most cases (80%) are sporadic with both sexes affected equally. The defect in erythropoiesis seems to be intrinsic to hematopoietic progenitors since the patient's bone marrow derived stromal adherent layer can support normal growth and differentiation of control progenitors,[6] and allogeneic bone marrow transplantation has been shown to be a curative option for the disease.[7] *In vitro* culture studies showed a poor response of early erythroid progenitors from DBA patients to various hematopoietic growth factors, including erythropoietin, interleukin-6 and granulocyte-monocyte colony stimulating factor used either alone or in combination.[8] Data from *in vitro* long-term cultures have brought evidence that besides the defect in erythropoiesis, granulocyte-macrophage and megakaryocytic lineages are also affected, suggesting a defect of more primitive hematopoietic progenitors.[6,9] Fifty to seventy percent of patients respond to standard steroid therapy, one third of them achieving remission, but many become transfusion dependent.[2,7] Steroid-resistant patients require a regular

program of red cell transfusion, which leads to iron overload, and an iron chelator must be administered to overcome associated complications.[3]

As for the genetic background, the first DBA locus has been mapped to 19q13.[2,10] and the gene for ribosomal protein S19 (*RPS19*) has been found to be mutated in 25% of patients with DBA.[11-13] In spite of considerable research, *RPS19* still remains the only gene associated with the DBA phenotype, and though more than 60 mutations have been described so far, there is a lack of a consistent relationship between the nature of the mutations and clinical phenotype or long-term responses to therapy.[7]

RPS19 is a component of a small ribosomal subunit (40S subunit), and in addition to being a building block of ribosomes, seems to play a specific role during ribosome biogenesis[14] and in the initiation of translation.[15-17]

Two mechanisms of RPS19 involvement in DBA pathogenesis have been proposed: (i) the DBA phenotype stems from the defective function of RPS19 in translation and/or (ii) the DBA phenotype is caused by the disruption of an extraribosomal function of RPS19. To support or reject the first proposed mechanism of RPS19 involvement in translation, we studied translational efficiency in DBA cells with or without a mutation in RPS19.

Design and Methods

Cell isolation and activation

After informed consent, peripheral blood mononuclear cells from DBA patients and healthy controls were isolated using Histopaque-1077 (Sigma-Aldrich) gradient centrifugation. All patients had normal leukocyte counts. Cells were divided into two groups and maintained in RPMI-1640 (Invitrogen/Gibco) medium with 10% fetal calf serum (Invitrogen/Gibco). In one group, phytohemagglutinin (PHA; Sigma-Aldrich) was added at a final concentration of 2 μg/mL to activate lymphocyte proliferation. After 2 days of culture, cells were counted, and the rate of translation and proliferation was measured. At the time of the measurement, lymphocytes represented 86.01% (±4.0%) and 79.34% (±3.6%) of viable cells (DAPI negative) in the control and PHA-activated group, respectively, as determined by FACS using specific antibodies against CD3, CD16/56, CD19 and CD45 (4-KOMBI TEST, Exbio, Czech Republic). T-lymphocytes represented 92.04% (±0.6%) and 92.15% (±1.6%) of lymphocytes in the control and PHA-activated group, respectively.

Expression of the mutated RPS19 in K562 cells

To obtain high cellular expression, we used the RevTet-On System (Clontech) according to the manufacturer's instructions. The wild type *RPS19* cDNA or cDNA carrying the RPS19 mutation previously identified in our DBA patients (R56Q; R62Q; 11 bp deletion leading to the frameshift at codon 66 and stop at codon 149)[13] were PCR-cloned into the pCRII plasmid using the TA Cloning Kit (Invitrogen). *RPS19* cDNA were excised by BamHI and EcoRV restriction enzymes, and recloned into BamHI and HpaI restriction sites in the pRevTRE vector (Clontech). Resulting vectors were verified by sequencing.

Vectors were introduced into the established K562-Tet-On cell line via retroviral-mediated transfer (Pantropic Retroviral Expression System; Clontech), and transduced cells were selected by hygromycin (400 μg/mL; Sigma-Aldrich). The K562-Tet-On cell line had been previously prepared by transduction of the human erythroleukemia cell line K562 with the pRevTet-On vector (Clontech), and transduced cells were selected by G418 (1 mg/mL; Sigma-Aldrich). The expression of introduced RPS19 was induced by doxycycline (1 μg/mL; Sigma-Aldrich), and verified by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using vector-specific primers. All experiments were done in the presence of doxycycline in the culture medium for at least 2 days. Doxycycline had no effect on cell proliferation and cytotoxicity.

Translation analysis

Proteosynthetic activity of cells was measured by the incorporation of radioactively labeled L-leucine into newly synthesized proteins. Viable cells were counted, washed twice in leucine-free RPMI-1640 medium (ICN), and incubated for an additional 15 minutes in the same medium. After centrifugation, cells were resuspended in RPMI-1640 containing only [^{3}H]-L-leucine (10 or 100 μCi/mL; MP Biomedicals; Amersham Biosciences). Three aliquots were taken after 5 and 10 minutes of incubation at 37°C, and were immediately mixed with ice-cold phosphate-buffered saline. After centrifugation at 4°C, cells were resuspended in 100 μL of bovine serum albumin (1 mg/mL; Sigma-Aldrich), and 1 mL of 10% trichloroacetic acid was added. The solution was incubated for 30 minutes on ice. Proteins were recovered by vacuum filtration on cellulose filters that were allowed to dry out. Filters were then dissolved in a scintillation cocktail to measure radioactivity on a Beckman LS1801 counter. When indicated, cells were cultured for 2 days in the presence of various concentrations of L-leucine (100, 350, 600, and 1100 μg/mL) prior to the translation measurement. Student's paired t-test was used for statistical evaluations.

Proliferation analysis

DNA biosynthetic activity, measured by the utilization of radioactively labeled deoxythymidine, was used as a marker for cellular proliferation. K562 cells expressing various variants of RPS19 and control cells were

diluted to 50 000 cells/mL. Every 24 hours for 4 consecutive days, six 200 μL aliquots were taken, and cells were cultured overnight with [^{3}H]-deoxythymidine (5 μCi/mL; Lacomed, Czech Republic) in microwell plates. Cells were harvested onto glass fiber mats by the Skatron cell harvester (Norway). Filters were then dissolved in a scintillation cocktail to measure radioactivity on the Beckman LS1801 counter.

The proliferation of lymphocytes was evaluated in the same way after 2 days in culture with or without PHA. Student's t-test was used for statistical evaluations.

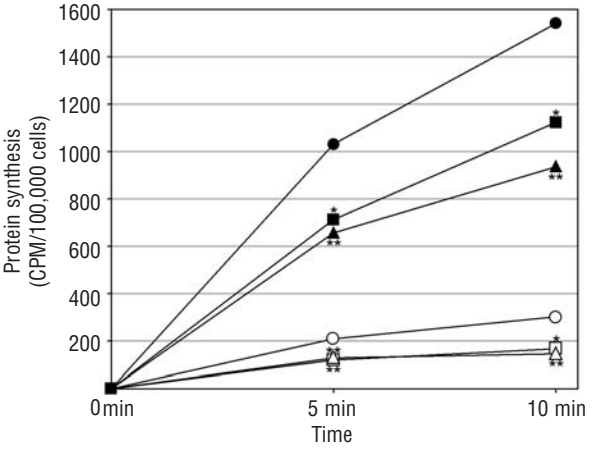
Sequencing analyses

Exon 54 of mammalian target of rapamycin (mTOR, also known as FK506 binding protein 12-rapamycin associated protein 1 [FRAP1]), comprising Thr2446 and Ser2448, was PCR-amplified by the primers 5'TCCCATGCTAATACCCACCC (forward) and 5'CACCCACCGACTGAAGCCC (reverse) in eight DBA patients. Exon 3 of Ras homolog enriched in brain (Rheb) comprising Asp60 was PCR-amplified by the primers 5'TAATGGAAGTAGGGGAGAATC (forward) and 5'GGTTTTCTTGGCATAGCTGG (reverse) in eight DBA patients. PCR products were cut out of the agarose gel, electroeluted, and sequenced from both ends on the ABI310 Genetic Analyzer (Applied Biosystems).

Results

Translation is reduced in DBA lymphocytes irrespective of mutations in RPS19

The main question we wanted to answer was whether mutations in RPS19 could alter translational efficiency in DBA patients. If not, this would imply that an RPS19 extraribosomal function, rather than its role in translation, is responsible for the DBA phenotype. Because of difficulties in obtaining large numbers of CD34$^+$ cells needed for our analyses, we used peripheral blood lymphocytes from DBA patients and healthy volunteers instead. Lymphocytes from healthy controls (n=9) and DBA patients with an RPS19 mutation (2x R62Q; R56Q; Del11bp) or without RPS19 mutations (n=8) were used to measure the rate of translation either at the basal state or after the addition of PHA, which is known to stimulate proliferation of human lymphocytes efficiently. Surprisingly, the average level of basal translation was significantly lower not only in patients with RPS19 mutations (p<0.007), but also in DBA patients without mutations in RPS19 (p<0.03). In both DBA groups the level of translation reached on average 48%-61% of control values, and no statistical differences could be found between the translational efficiency of DBA cells with RPS19 mutations and those without (Figure 1).



	Raw data (CPM/100,000 cells) Means		SD		Data normalized to controls	
Basal translation	*5 min*	*10 min*	*5 min*	*10 min*	*5 min*	*10 min*
Controls (n=9)	209.1	301.4	71.8	124.1	1	1
RPS19-NON-mutated (n=8)	118.5**	166.8*	46.4	68.0	0.5669**	0.5535*
RPS19-mutated (n=4)	127.2**	145.8**	8.5	14.4	0.6085**	0.4836**
Activated translation						
Controls (n=9)	1032.1	1540.9	186.1	209.4	1	1
RPS19-NON-mutated (n=8)	712.9*	1122.9*	296.2	471.7	0.6907*	0.7287*
RPS19-mutated (n=4)	656.7**	934.5**	141.1	287.7	0.6363**	0.6064**

Figure 1. Basal and activated translation in DBA cells. Basal translation and activated translation are defined as the amount of protein synthesized by lymphocytes at time 0, after 5 minutes and after 10 minutes, without or after the addition of PHA for 48 hours, respectively. The level of translation is expressed as the amount of radioactively labeled leucine that was incorporated into newly synthesized proteins in 100 000 cells. Raw data and values normalized to controls are shown in the table for greater clarity. *p<0.03; **p<0.007, with respect to controls.

The addition of PHA can increase translation by 5 to 7-fold (no statistical difference between DBA cells and controls); however, the level of translation was again significantly lower in both DBA groups irrespective of the mutations in RPS19 (on average 60%-63% [p<0.007] and 69-72% [p<0.03] of control values in the RPS19-mutated and RPS19-non-mutated DBA group, respectively). After PHA stimulation, RPS19-mutated DBA cells exhibited lower translational efficiency in comparison with RPS19-non-mutated DBA cells, but the difference was not significant (Figure 1).

The patients' characteristics together with the level of translation at the 5-minute time points are shown in Table 1. No consistent relationship was found between the level of translation and clinical phenotype or long-term response to therapy.

Mutations in RPS19 can significantly slow down cellular translation

Results from the DBA translation studies indicated that insufficient translation might be the cause of DBA.

Table 1. Patients' characteristics and the level of basal and activated translation normalized to controls at the 5 min time points.

Patient (sex)	*Level of basal translation*	*Level of activated translation*	*Mutation in RPS19*	*Age at diagnosis (months)*	*Malformations*	*Status at the time of experiments*
1:CZ23 (F)	0.2122	0.3936		1	Vesicorenal reflux	Transfusion-dependent
2:CZ2 (M)	0.3881	0.6615		1	Flat thenar	Steroid-dependent
3:CZ14 (M)	0.4221	0.2357		1	Flat thenar	Steroid-dependent
4:CZ21 (M)	0.4737	0.4611		1	Vesicorenal reflux	Transfusion-dependent
5:CZ6 (F)	0.5541	0.5565	G167A; Arg56Gln	2		Remission, free of treatment
6:CZ10 (M)	0.5685	0.8646		10	Atrioseptal defect	Steroid-dependent
7:CZ9 (F)	0.5902	0.5052	Del196-206; Frameshift at codon 66, stop at codon 149	2		Steroid-dependent
8:CZ7 (F)	0.6262	0.8621	G167A; Arg56Gln	1		Transfusion-dependent
9:CZ17 (M)	0.6634	0.6215	G185A; Arg62Gln	2	Epicanthus, hypertelorism	Remission, free of treatment
10:CZ19 (M)	0.7746	1.1125		2	Flat thenar	Steroid-dependent
11:CZ12 (M)	0.7942	0.8088		3	Flat thenar	Steroid discontinued, transfusions
12:CZ25 (F)	0.9022	0.9876		4	Thumb anomaly	Steroid-dependent

Patients are arranged in order of basal translation. Patients CZ6 and CZ7 are sisters. Adenine in start codon is taken as +1.

We were, therefore, interested to know whether the lowered translation, at least in DBA cells with an RPS19 mutation, could be attributed solely to mutations in RPS19. We, therefore, cloned wild type and mutated *RPS19* cDNA previously found in our DBA patients[13] (R56Q; R62Q; Del11bp) into an expression vector. The human erythroleukemia cell line K562 was stably transduced with the retroviral vectors to overproduce the mutated RPS19 protein. Expression of mutated *RPS19* mRNA exceeded expression of endogenous *RPS19* by at least a factor 20, as determined by real-time RT-PCR. As shown in Figure 2, point mutations in RPS19 hot spots (R56Q; R62Q) can significantly lower the level of translation to 56%-63% that of controls ($p<0.005$). The forced expression of the wild type and Del11bp-RPS19 mutant proteins did not affect cellular translation in K562 cells.

Proliferative capacity is significantly reduced in DBA lymphocytes irrespective of mutations in RPS19, but can be increased by PHA

Proliferation activity was measured in unstimulated and PHA-stimulated lymphocytes from DBA patients and healthy controls, by the incorporation of [^{3}H]-deoxythymidine into newly synthesized DNA. Figure 3 shows that DBA cells had a significantly lower proliferative capacity irrespective of RPS19 mutations ($p<0.02$), reaching on average 49% and 43% of control values in the RPS19-mutated and RPS19-non-mutated DBA group, respectively.

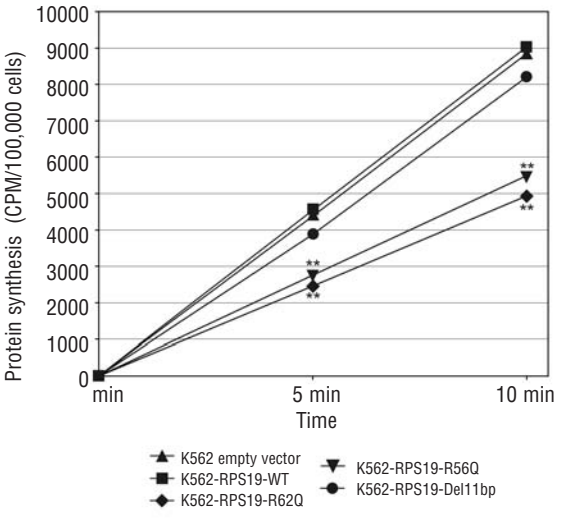
The addition of PHA dramatically increased proliferation in both DBA groups up to 88% and 77% of control values in the RPS19-mutated and RPS19-non-mutated DBA group, respectively. Again, no statistically significant differences was observed between RPS19-mutated and RPS19-non-mutated DBA cells. Interestingly, PHA activated proliferation more readily in both DBA groups than in controls (508-fold, 462-fold and 277-fold in the DBA RPS19-non-mutated group, RPS19-mutated group and controls, respectively; $p<0.05$).

Proliferation is not impaired in K562-RPS19 cells

Proliferation was also measured in the K562 cells producing various RPS19 variants. As shown in Figure 4, no statistically significant differences were noted between K562-RPS19 variants and control cells.

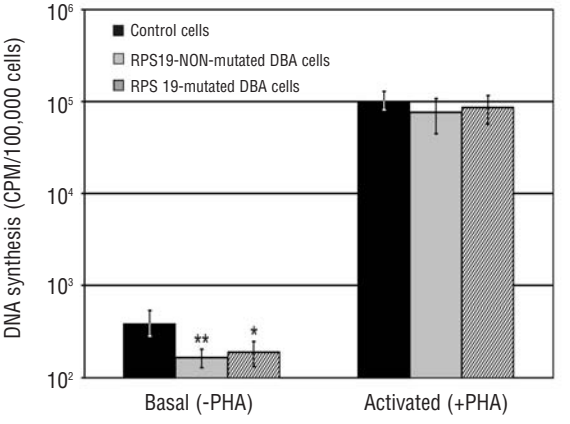
Leucine improves translational efficiency in RPS19-non-mutated DBA lymphocytes, but not in cells with an RPS19 mutation

If insufficient translation is the cause of the DBA phenotype, then stimulation of translation might be beneficial for patients. There are several studies suggesting that leucine, in addition to being a substrate for protein synthe-



	Basic data (CPM/100 000 cells)				Normalized data to controls	
	Means		SD			
	5 min	*10 min*	*5 min*	*10 min*	*5 min*	*10 min*
K562-empty vector	4380.4	8842.3	870.2	1619.9	1	1
-RPS19-WT	4539.7	9025.2	1891.1	5056.5	1.0364	1.0207
-RPS19-R62Q	2455.9**	4933.8**	565.0	918.3	0.5607**	0.5580**
-RPS19-R56Q	2756.1**	5496.6**	228.1	875.5	0.6292**	0.6216**
-RPS19-Del11bp	3875.0	8209.9	574.8	1291.5	0.8846	0.9285

Figure 2. Translation rates in K562 cells expressing a mutated RPS19 protein. Translation rate of the transduced K562 cells is defined as the amount of protein synthesized by cells within 5 and 10 minutes. The level of translation is expressed as the amount of radioactively labeled leucine that was incorporated into newly synthesized proteins in 100 000 cells. Raw data and values normalized to controls are shown in the table for greater clarity. ***p*<0.005, with respect to controls. Five independent experiments were done in each group.



	Normalized data to controls	
	-PHA	*+PHA*
Controls (n=16)	1	1
RPS19-NON-mutated (n=8)	0.4351**	0.7755
RPS19-mutated (n=4)	0.4980*	0.8837

Figure 3. Basal and activated proliferation in DBA cells. Basal proliferation (-PHA) and activated proliferation (+PHA) are defined as the level of radioactively labeled deoxythymidine incorporated by 100,000 cells, without or after the addition of PHA for 48 hours, respectively. Means and standard deviations are shown. Values normalized to controls are shown in the table for greater clarity. **p*<0.02; ***p*<0.0001, with respect to controls.

sis, also plays an important role in regulating protein synthesis by acting as a nutrient signal to stimulate translation initiation.[18,19]

We, therefore, added leucine into the culture medium to final concentrations of 100, 350, 600, and 1100 μg/mL, and measured the level of translation in cells from healthy controls and DBA patients with or without a mutation in RPS19 (Figure 5). In controls, leucine significantly increased translation at all concentrations, having its greatest effect at 600 μg/mL (+51%). In contrast, a slight decrease was observed in the RPS19-mutated DBA group, and only a modest increase was documented in the DBA RPS19-non-mutated group at 350 and 600 μg/mL. However, the highest leucine concentration had the best stimulatory effect, increasing translation in DBA RPS19-non-mutated cells by 55%. No correlation was found between the level of basal or activated translation and the response to leucine.

Domains of mTOR and Rheb that integrate signals from nutrient status are not mutated in DBA patients

Results from leucine cultures indicated that the

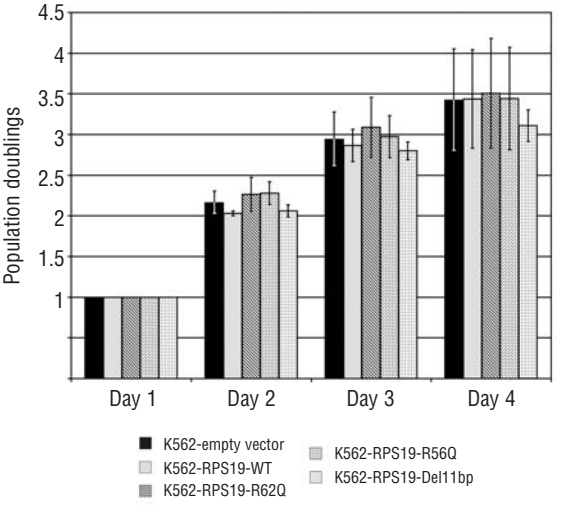
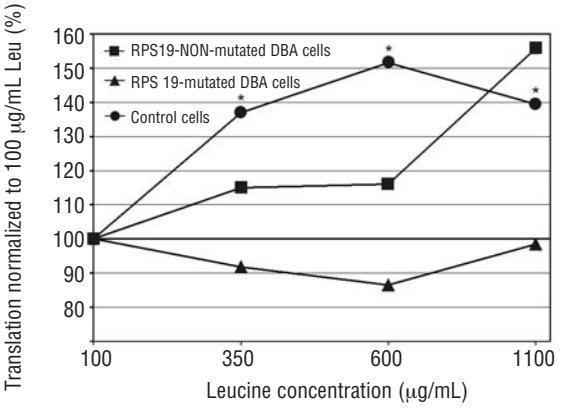


Figure 4. Proliferation of K562 cells expressing a mutated RPS19 protein. Population doubling normalized to day 1 was measured by overnight incorporation of radioactively labeled deoxythymidine in 200 μL cell aliquots taken from respective cultures over the course of 4 consecutive days. Means and standard deviations are shown. Five independent experiments were done in each group.



	Leu100	Leu350		Leu600		Leu1100	
	Means	Means	SD	Means	SD	Means	SD
Controls (n=7)	100	137.0*	31.6	151.5*	43.3	139.5*	45.4
RPS19-NON-mutated (n=3)	100	115.0	18.5	116.0	21.0	155.8	34.1
RPS19-mutated (n=3)	100	91.8	5.9	86.6	12.4	98.4	19.4

Figure 5. Translation in DBA cells after the addition of leucine. Translation was measured after the addition of various concentrations of leucine to the culture medium for 48 hours. The level of translation is normalized to the leucine concentration of 100 μg/mL [a standard concentration in various culture media]. Basic data are shown in the table for greater clarity. SD, standard deviation; *$p<0.05$, with respect to leucine 100 μg/mL.

leucine regulatory pathway could be impaired in DBA patients. Since the stimulation of translation initiation by leucine administration involves the mTOR pathway,[20] we focused on its two key components – Rheb (Ras homologue enriched in brain) and mTOR (mammalian target of rapamycin). Because the structure of both genes is rather complex (*mTOR* consists of 75 exons including alternative exons; *Rheb* consists of 35 exons including alternative exons), we decided, as the first step, to perform a mutational analysis of currently known *hot spots* in Rheb and mTOR that have been identified to be critical for nutrient activation and regulation of the mTOR pathway. Dominant negative mutants of Rheb (Asp60Lys; Asp60Val) that blocked nutrient activation of the mTOR pathway have been described.[21] We therefore screened the Rheb exon 3 comprising codon 60 in eight DBA patients, but we found no alterations. In mTOR, Thr2446 and Ser2448 were identified as nutrient-regulated phosphorylation sites that integrate signals from nutrient status and growth factors to control the regulation of protein translation.[22] Screening of mTOR exon 54, comprising both codons, revealed mutations in eight DBA patients.

Discussion

DBA patients suffer from profound aregenerative anemia that is often associated with various physical anomalies. A heterozygous mutation in RPS19 has been found in 25% of patients, but no causative genetic alterations have been identified so far in the remaining patients. Understanding the role of RPS19 could provide a clue toward discovering the molecular principles of DBA also in patients without RPS19 mutations, which could lead to more specific and efficient treatment for DBA.

In theory, RPS19 could have an extraribosomal function indispensable for erythropoiesis, and other members of the RPS19 hypothetical pathway would be the most probable candidates as DBA-associated genes. Alternatively, since RPS19 is a structural and functional component of a small ribosomal subunit, its primary role could be expected to be in the process of translation. We, therefore, attempted to discriminate between the two possibilities. We assumed that if translation was not affected by mutations in RPS19, then an extraribosomal function of RPS19 would be responsible for the pathogenesis of DBA.

Since translation is an omnipresent process, we reasoned that if mutations in RPS19 could affect translational efficiency, then lowered translational capacity should be detectable in all cells. Because lymphocytes can be easily isolated and activated to proliferate by PHA, we used these cells for our analyses. Indeed, as would be expected for DBA cells carrying a heterozygous mutation in *RPS19*, their rate of translation was on average slightly more than half of control values. Surprisingly, however, also the majority of DBA patients without mutations in RPS19 showed a defect in protein synthesis. In fact, patients with and without a mutation in RPS19 could not be distinguished by the level of translation. While the translation in patients with mutations in RPS19 showed less overall variation in the unstimulated state and ranged from 55-66% and 44-55% that of controls at the 5 and 10 min time points, respectively (61% and 48% on average), the protein translation in samples with normal RPS19 showed more variation, from 21-90% and 25-88% of control values at the 5 and 10 min time points, respectively (57% and 55% on average). Upon stimulation, average translation in both RPS19-mutated- and RPS19-non-mutated groups also remained significantly lower than in controls. These findings suggest that impaired translation could generally underlie the pathogenesis of DBA. The greater variability in patients without an RPS19 mutation may reflect the genetic diversity underlying DBA in these samples, with different genes likely affecting protein synthesis to different extents. It does, however, need to be said that in some patients (for example CZ25) only a mild decrease in translation was detected, indicating that a non-protein-synthesis-related mechanism could still be an underlying cause in some DBA patients.

To test whether mutations in RPS19 can affect translation directly, we overexpressed several types of mutant RPS19 proteins in K562 cells. Overexpression of

RPS19 variants mutated in conserved arginines 56 and 62 significantly lowered the rate of translation in K562 cells, indicating that RPS19 is important for efficient translation, and that the R56Q- and R62Q-mutated RPS19 proteins may directly compete with wild type RPS19 during ribosome biogenesis or initiation of translation. In the K562-Del11bp cells the deletion did not influence translation, while the level of translation in the DBA patient with the Del11bp mutation (CZ9) was 43%-59% that of controls. This discrepancy can be simply explained by the fact that the deletion results in a functionless RPS19 protein (it is highly unstable compared to the wild type protein and not capable of localizing to nucleoli; *manuscript in preparation*), leaving the patient's cells RPS19 haploinsufficient, while in the K562-Del11bp cells all endogenous *RPS19* alleles remained intact, and translation was not, therefore, affected. On the other hand, overexpression of wild type RPS19 did not increase the level of cellular translation, suggesting the need for precise stoichiometry during the assembly of ribosomes.

A primary role for RPS19 in ribosome biogenesis and translation is also supported by the results of Ebert and colleagues.[23] To determine the specific role of RPS19 in erythroid differentiation, the authors used DNA microarrays to track changes in mRNA levels of several thousands genes during this process. It was demonstrated that during erythroid differentiation the expression of *RPS19* is tightly coordinated with that of other ribosomal proteins, and its overall expression is not erythroid specific relative to other ribosomal proteins.

Two specific roles for RPS19 in translation have been proposed – it takes part in the initiation of translation,[15-17] and it seems to be important for ribosome biogenesis, at least in yeast, because the yeast RPS19 homolog is strictly required for maturation of the 3'end of 18S rRNA.[14] These data collectively suggest that inefficient translation due to mutations in RPS19 and other yet-to-be identified translation-related proteins could be the molecular cause of DBA in the majority of patients.

Could insufficient translation lead to the symptoms of DBA? It has been demonstrated that the expression of RPS19 and other ribosomal proteins is relatively high in $CD34^+$ cells,[23-25] indicating the need for highly effective translation in primitive hematopoietic progenitors. Lowered protein synthesis has already been reported to affect erythropoiesis in particular. The immunosuppressive drug rapamycin has been shown to specifically inhibit translation of ribosomal protein mRNA,[26] leading to a general decrease in protein synthesis and to a prolongation of the G_1-phase.[27] The addition of rapamycin into semisolid cultures of murine bone marrow cells inhibited the formation of BFU-E-derived colonies by nearly 60%, whereas the formation of CFU-GM colonies was reduced by only 30%,[28] indicating that erythropoiesis is more sensitive than granulocyte-macrophage lineage differentiation to reduced protein synthesis. Rapamycin also blocks the proliferative response of primary bone marrow cells to hematopoietic cytokines (interleukins-3, -4, and -6, granulocyte and granulocyte-monocyte colony-stimulating factors, stem cell factor), and it inhibits the multiplication of hematopoietic progenitors *in vitro* and hematopoietic recovery after myeloablation *in vivo*.[29] Inefficient translation could thus result in reduced proliferation.

This conclusion is also supported by our results. We measured DNA biosynthetic activity as a marker of proliferation, and found that the average basal proliferation of DBA lymphocytes reached only 43-49% of control values irrespective of mutations in RPS19. This is in agreement with numerous reports that DBA cells have been found to be hypoproliferative, and it has been suggested that inefficient proliferation is the cause of DBA.[9,24,30,31,32] Based on these results we suggest that insufficient translation may be the primary cause of the hypoproliferation observed in DBA cells.

Cell proliferation and differentiation are also key processes in embryonic development, and decreased efficiency of translation would be expected to result in various physical malformations, as documented in about 40% of DBA patients. This theory is supported by the so-called *Minute* phenotype in *Drosophila melanogaster*, which is associated with mutations in about 50 loci.[33] Affected flies have a small body, thin bristles and delayed larval development. Some loci have been shown to encode ribosomal proteins, and at least 13 ribosomal proteins have been unambiguously correlated to the *Minute* phenotype.

Moreover, there is a recent report that DBA patients without RPS19 mutations have reduced gene expression of all ribosomal proteins in comparison with patients with aplastic anemia.[34] Using a microarray technique and K-mean cluster analysis, it was demonstrated that 28 of all 79 ribosomal proteins also belonged to the group of genes whose mRNA expression levels were invariably lower in DBA patients without RPS19 mutations than in controls with aplastic anemia.

Based on our results and published literature, we therefore propose that the primary cause of DBA in the majority of patients may be inefficient translation. This is due to mutations in RPS19 in 25% of them and as yet unidentified alterations in other translation-related proteins in most of the rest. According to our hypothesis, during a critical stage of erythroid differentiation, a relatively high level of translation would be required to translate specific transcript(s) that upon reaching the threshold level trigger differentiation along the erythroid pathway. If the threshold level is not reached, the cells will not differentiate, and will be hypoproliferative. Hypoproliferation, at a specific time during ontogenesis, caused by suboptimal amounts of specific factor(s) due to insufficient protein synthesis, could thus lead not

only to erythroid defects, but also to the physical anomalies observed in DBA patients. As for the patients with only a mild decrease in translation, a specific factor may just represent a DBA candidate gene. If our model of DBA is correct, then hypoproliferation/erythroid defects could be corrected by at least three mechanisms – (i) an overall enhancement of translation; (ii) an increase in transcription of a specific mRNA; and (iii) an increase in the stability of a specific mRNA or protein. All three mechanisms should result in an increased amount of a specific key protein, but the second and third mechanisms act independently on the level of translation. We, therefore, suggest that using a proper stimulus that can activate either mechanism could revert hypoproliferation of DBA cells. Our suggestion is based on the following examples.

First, following stimulation by PHA the proliferation rate of DBA lymphocytes reached up to 77% and 88% of control values in the RPS19-non-mutated and RPS19-mutated DBA groups, respectively. In fact, DBA lymphocytes are activated by PHA significantly better than are control lymphocytes ($p<0.05$), implying that DBA cells can be activated to proliferate using the proper stimulus irrespective of their lowered translation. Second, we found no alterations in the proliferation rates of the K562-RPS19-R56Q and K562-RPS19-R62Q cell lines in spite of their halved translation, which was apparently efficient enough to cover the demand for all proteins required for their proliferation. The K562 cell line is a highly proliferative human erythroleukemia cell line[35] that abundantly expresses the BCR/ABL fusion protein,[36] which is likely the stimulus that drives the K562 cells to proliferate. Third, a majority of DBA patients respond to glucocorticoids, and many of them can become free of treatment. Indeed, two of our patients with a mutation in RPS19 (CZ6; CZ17) initially treated with prednisone were in remission and free of treatment at the time of our experiments, yet the level of their translation remained only 44-66% that of control values. Fourth, Ebert *et al.*[23] have shown that the dexamethasone enhancement of erythropoiesis in DBA patients is independent of RPS19. Dexamethasone does not increase transcription of ribosomal proteins (RPS19 inclusive), indicating that the number of ribosomes and hence the level of translation should not be altered by dexamethasone treatment. Glucocorticoids are, therefore, a stimulus that can induce proliferation of DBA cells in an RPS19-independent manner, likely through the direct activation of transcription of a differentiation-inducing factor mRNA.[23]

Since steroid therapy does not induce remission in all patients, and no specific dose-dependent factor required for erythropoiesis in DBA has been identified so far, we searched for a way to increase the overall translational rate in DBA patients. There are several reports describing the important role of the amino acid leucine in regulating protein synthesis by acting as a nutrient signal. Oral administration of large doses of leucine in rats stimulates protein synthesis in skeletal muscle and adipose tissue.[18,19] We showed that leucine was also capable of stimulating translation by more than 50% in control lymphocytes. More importantly, high doses of leucine also stimulated translational efficiency in DBA cells, but only in those without RPS19 mutations. This is consistent with the supposed mechanism of leucine's action on translation. Although this mechanism is not fully understood, it involves the mTOR pathway.[20,37] Via this pathway, leucine boosts translation by enhancing the activation of translation initiation factors that regulate mRNA binding to the ribosomal complex, and by specific up-regulation of ribosome biosynthesis through the ribosomal protein S6 kinase.[37,38] Since RPS19-mutated DBA cells are haploinsufficient in wild type *RPS19* mRNA production, even the increased translation initiation cannot reverse the lack of functional ribosomes. Our results suggest that giving leucine to DBA patients, especially to those without mutations in RPS19, might be of some benefit.

The different kinetics of leucine activity in control cells and DBA cells suggested that DBA cells might have a higher threshold for the leucine-mediated stimulation of translation, indicating a possible impairment of the mTOR pathway. We therefore performed mutational analysis of currently known *hot spots* in Rheb and mTOR, the two key proteins of the pathway, which have been shown to regulate the mTOR pathway in response to nutritional status and growth factors.[21,22] However, we did not detect any alterations.

In summary we conclude that reduced translational efficiency could be the cause of the majority of cases of DBA, and new therapeutic strategies for this congenital anemia could be targeted to enhance the level of translation and/or be based on the identification of a dose-dependent factor that is necessary for efficient erythropoiesis.

JC and RC developed the project, designed the experimental procedures and performed most of the experiments; RC wrote the manuscript; DP and KP diagnosed DBA in patients and provided their clinical data; LD performed proliferation tests; JP helped with results, analyses and interpretations. The authors declare that they have no potential conflict of interest.

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