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Aberrant mitochondrial iron distribution and maturation arrest characterize early erythroid precursors in low-risk myelodysplastic syndromes

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Early erythroblasts from patients with refractory anemia (RA) and RA with ringed sideroblasts (RARS) show constitutive mitochondrial release of cytochrome *c*. Moreover, mature erythroblasts in RARS, but not in RA, display aberrant accumulation of mitochondrial ferritin (MtF). We analyzed cytochrome *c* release, MtF expression, and gene expression during erythroid differentiation in bone marrow cells from myelodysplastic syndrome (MDS) patients and healthy controls. Whereas none or few cultured erythroid cells from healthy individuals and RA patients expressed MtF, those from RARS patients showed MtF expression at an early stage, when cells were CD34$^+$ and without morphologic signs of erythroid differentiation. The proportion of RARS erythroblasts that were MtF$^+$ increased further upon in vitro maturation. Moreover, a significant overexpression of mRNA encoding cytochrome *c*, and proapoptotic Bid and Bax, was seen in freshly isolated cells from MDS patients. Genes involved in erythroid differentiation were also dysregulated in MDS cells. Importantly, GATA-1 expression increased during normal erythroid maturation, but remained low in MDS cultures, indicating a block of erythroid maturation at the transcriptional level. In conclusion, aberrant MtF expression in RARS erythroblasts occurs at a very early stage of erythroid differentiation and is paralleled by an up-regulation of genes involved in this process. (Blood. 2005;106:247-253)

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Introduction

The low-risk myelodysplastic syndromes (MDSs) refractory anemia (RA) and RA with ringed sideroblasts (RARS) are characterized by profound anemia and transfusion dependency, and a relatively low risk of progression to acute myeloid leukemia.[1,2] In RARS, the anemia is mirrored by hyperplastic but severely ineffective erythropoiesis due to increased apoptosis of erythroid progenitors.[3] The erythropoiesis of RA patients is also inadequate with apoptotic features, but may range from hypo- to hyperplastic, and shows no or few ringed sideroblasts.[4-6] The pathogenesis of RA anemia seems to be more heterogeneous, including T-cell–mediated bone marrow failure in a subset of patients.[7,8] We have recently demonstrated that the erythroid apoptosis of low-risk MDS is initiated at a very early stage of stem cells and is associated with mitochondrial release of cytochrome *c* with subsequent activation of caspase-9 and effector caspase-3. Importantly, in RARS, granulocyte colony-stimulating factor (G-CSF) inhibits spontaneous release of cytochrome *c*, loss of mitochondrial membrane potential, and caspase activation, and restores erythroid proliferation.[9,10]

Iron is predominantly stored in ferritin within cells. The multiple forms, or isoferritins, that can be found in human tissues are composed of variable proportions of 2 subunits: L-ferritin (light) and H-ferritin (heavy), encoded by genes located on chromosomes 11 and 19, respectively. Since free iron is potentially harmful to the cell, it is sequestered and detoxified to the less soluble ferric form by ferroxidase activity. H-ferritin (HF) exerts most of its ferroxidase activity in the cytosol.[11,12] Recently, a novel mitochondrial ferritin gene (MtF) was reported. This intronless gene contains a mitochondrial localization signal and is expressed in the mitochondrial matrix. It exhibits more than 75% sequence identity to the *HF* gene.[13,14] Mature erythroblasts from patients with X-linked sideroblastic anemia and RARS, in contrast to normal erythroblasts, express MtF[14]; however, it is still unknown at which stage of erythroid differentiation this abnormal expression appears.

The main task for the mitochondrion is to produce energy (adenosine triphosphate [ATP]). This occurs in the respiratory chain, consisting of 5 multiprotein enzyme complexes (I-V) and 2

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electron carriers, coenzyme Q and cytochrome *c*.[15] Cytochrome *c* is closely associated with complex IV (cytochrome *c* oxidase) and has a major function to mediate the electron transport between complex III and IV. However, cytochrome *c* is also a key player in the regulation of apoptosis.[16,17]

Erythroid differentiation requires activation of the erythropoietin (Epo) receptor followed by activation of the *Jak-Stat* pathway. This process is modulated by a complex network of transcription factors via activation of a set of target genes. GATA-1 plays a crucial role in erythroid development, and can switch the common lymphoid progenitors and granulocyte/monocyte progenitors toward megakaryocyte/erythrocyte lineage.[18,19] Epo induces globin gene expression and can specifically promote late erythroid differentiation.[20,21] In addition, Epo cooperates with GATA-1 to induce $Bcl\text{-}x_L$ gene expression and to maintain erythroid cell survival during terminal maturation.[22,23]

The present study aimed at clarifying the pathophysiology underlying ineffective erythropoiesis in MDS with particular focus on mitochondrial abnormalities and iron distribution during erythroid maturation. We show that apoptosis, as evidenced by significant cytochrome *c* release in early RARS erythroblasts, is paralleled by an accumulation of aberrant mitochondrial ferritin. $CD34^+$ progenitors from MDS patients overexpress genes coding for erythroid maturation and hemoglobinization, but also proapoptotic genes. MDS erythroblasts show a defective GATA-1 expression pattern during erythroid maturation. Finally, we provide evidence that G-CSF inhibits erythroid apoptosis by targeting premitochondrial mechanisms rather than by affecting mitochondrial function, or defective iron distribution.

Patients, materials, and methods

Patients

The diagnostic procedure was performed according to the criteria put forth by the Nordic MDS Group.[24] Informed consent was obtained from patients and controls, and the study followed the guidelines of the ethical committee for research at Karolinska Institutet and the Declaration of Helsinki. Patients were classified according to the French-American-British (FAB) classification. We included 9 patients with RA and 10 with RARS with a median age of 68 years (range, 38-86 years) and 75 years (range, 52-82 years), respectively. At the time of sampling, 9 patients were transfusion dependent and 10 had a stable anemia. Normal bone marrow (NBM) samples were obtained from 19 healthy individuals.

CD34 separation and erythroblast cultivation

Bone marrow needle aspiration and mononuclear cell (MNC) isolation and cultivation were performed as previously described.[9] $CD34^+$ cells were separated from MNCs using the MiniMacs system (Miltenyi Biotec, Bergisch Gladbach, Germany). Following positive selection for CD34, the cells (0.1×10^6/mL) were cultivated for 14 days in Iscoves medium (Sigma, St Louis, MO) with standard supplements plus recombinant human interleukin-3 (rh-IL-3, 10 ng/mL; PeproTech House, London, United Kingdom), rh-IL-6 (10 ng/mL; PeproTech House), and rh–stem cell factor (rh-SCF, 25 ng/mL; Medical & Biological Laboratories, Nagoya, Japan). The medium was replenished every second day with the aforementioned cytokines to maintain the cultures at the same cell concentration. Epo (2 IU/mL; Roche, Basel, Switzerland) was added to the medium in the second week, beginning at day 7, and was replenished at days 9 and 11.

Cytoplasmic H-ferritin and mitochondrial ferritin

Cytocentrifuged specimens were fixed for 5 minutes in formalin vapors and then were analyzed for the distribution of cytoplasmic HF using a mouse monoclonal antibody (rH02) diluted 1:1000, and for MtF with a polyclonal antibody raised in mice against and specific for the recombinant mitochondrial protein, diluted 1:800. Bound antibody was detected by an immunoalkaline phosphatase method (streptavidin-biotin complex, LSAB2 Kit; Dakopatts, Glostrup, Denmark). The slides were counterstained with hematoxylin. Negative controls were performed by replacing the primary antibody with nonimmune mouse serum. On each specimen, the percentage of positive erythroblasts was determined.

Cytochrome *c* release

Translocation of cytochrome *c* from mitochondria to cytosol in hematopoietic stem cells undergoing erythroid differentiation was determined after 4-hour incubation with G-CSF.[10] Briefly, cells were stained with MitoTracker Red CMXRos (Molecular Probes, Eugene, OR), cytocentrifuged onto glass slides, and fixed and permeabilized in paraformaldehyde and Triton X-100, respectively. Cells were then stained with an anti–cytochrome *c* antibody (BD Biosciences), followed by a fluorescein isothiocyanate (FITC)–conjugated goat antimouse antibody (Sigma). Images were analyzed on a Leica DM RXA digital confocal microscope (Leica Microsystems, Wetzlar, Germany), and further processed using the Slide Book 4.0.0.6 analysis software (Intelligent Imaging Innovations, Goettingen, Germany).

Determination of mitochondrial ATP production rate (MAPR) in $CD34^-$ cells

Mitochondrial ATP production was determined as previously described.[25] $CD34^-$ cells (8×10^6 cells) were permeabilized for 10 minutes on ice in a solution containing digitonin 0.01%, KCl 150 mM, Tris (hydroxymethyl) amino methane 25 mM, KH_2PO_4 10 mM, EDTA (ethylenediaminetetraacetic acid, 2 mM), albumin 0.1% (wt/vol), pH 7.5. The permeabilization efficacy was more than 90% in all samples. After the treatment, the cells were centrifuged and resuspended in digitonin-free solution. Permeabilized cells (1.5×10^4 cells/mL) were added to 1-mL cuvettes containing firefly luciferase, D-luciferine 0.1 g/L, L-luciferine 4 mg/L, bovine serum albumin 1 g/L, $Na_2P_2O_7$ 1 μM, sucrose 150 mM, K_2HPO_4 15 mM, $MgAc_2$ 2 mM, EDTA 0.5 mM, adenosine diphosphate (ADP) 0.6 mM, adenosine monophosphate (AMP) 1 mM, and $p,^1p^5$-di(adenosine-5′) pentaphospate (DAPP) 2 μM, and one of the following substrate combinations: (a) glutamate 15 mM + succinate 15 mM (G + S) or (b) N, N, $N,^1N^1$-tetramethyl-1,4-phenyldiamine (TMPD) 2.25 mM + ascorbate 5 mM (T + A) and pH adjusted to 7. ATP production was recorded for 3 minutes at 25°C in a BioOrbit 1251 luminometer (Turku, Finland). Oligomycin 3 μg/mL was added and the residual ATP production was recorded for another 3 minutes. Finally, 10 μL of ATP 0.5 μM was added as internal standard; and the resulting increase in light emission was determined. All determinations were performed in duplicate. Oligomycin was found to quench the luminometric response with 14%, and was corrected for. An aliquot of the cells was sonicated and citrate synthase (CS) activity was determined. Mitochondrial ATP production was expressed as the difference in ATP production before and after the addition of oligomycin in relation to the cell's content of CS activity (units of ATP production/min per unit of CS).

Real-time quantitative polymerase chain reaction (PCR) analysis

Total RNA from $CD34^+$ cells extracted from MDS patients and NBM was isolated using the RNeasy minikit (Qiagen, Valencia, CA). cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) in a final volume of 50 μL, according to the manufacturer's protocol, with random hexamers as primers. The reaction was conducted at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes, on a 9600 GeneAmp PCR system (Applied Biosystems). Primers and probe combinations were designed to allow quantification of human cytochrome *c* using the Primer Express 1.0 software (Applied Biosystems). They were designed to amplify cytochrome *c* mRNA over the exon junctions to avoid DNA amplification. For human cytochrome *c,* 900 nM sense 5′-CAG TGC CAC ACC GTT GAA AA-3′, 900 nM antisense

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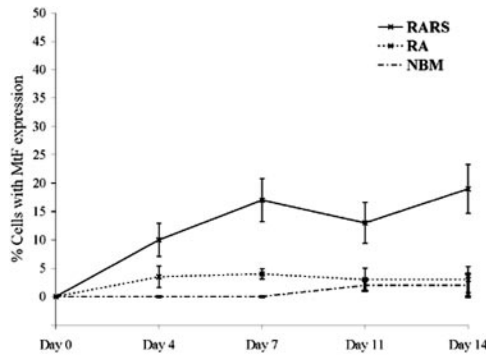

Figure 1. Higher expression of MtF in RARS. Expression of MtF was measured in 5 RARS, 4 RA, and 3 NBM samples. Data are shown as median ± SEM of cells with MtF expression. There were a few MtF$^+$ cells in NBM (0%-4%) and RA (median: 3% [0%-8%]) samples during 14-day cultivation. In RARS samples, there was a significant expression of MtF at day 4 (10%), with a continuous increase during the culture period (day 7: 17%; day 11: 13%; day 14: 19%).

5′-CCC CAG ATG ATG CCT TTG TT-3′, and 250 nM probe 6-FAM-CCC TGG ATA CTC TTA CAC AGC CGC CAA-TAMRA were used. The various primer and probe combinations allowing amplification of β-globin (HBB), GATA-1, DLK-1, Bax, Bid, and 18S ribosomal RNA cDNAs were purchased as Assays on Demand (Applied Biosystems). The 18S rRNA served as an endogenous control to standardize the amount of sample cDNA added to a reaction. The TaqMan Universal PCR Master Mix (Applied Biosystems) was used for the reaction mixtures of 25 μL, as recommended by the manufacturer. Real-time PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using the Sequence Detector Systems software. The conditions for the PCR amplification were as follows: 1 cycle at 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative expression of mRNA was calculated with the ΔΔCt method.

Statistical analysis

Statview 5.0 analysis software (SAS Institute, Cary, NC) was used for analysis of quantitative data. Results are presented as mean values ± SD, or as median values plus range, when appropriate. Paired Student *t* tests were used for comparison of related samples. The calculation was made using the nonparametric Mann-Whitney *U* test (2-tailed) for mRNA expression. *P* values less than .05 were considered statistically significant.

Results

Mitochondrial ferritin expression in RARS cultures during erythroblast maturation

Immunostaining was performed for MtF and cytoplasmic HF at days 0, 4, 7, 11, and 14 in 4 RA, 5 RARS, and 3 NBM. Freshly isolated CD34$^+$ cells from all individuals stained negative for MtF. NBM showed almost no MtF$^+$ cells (0%-4%), and only a small proportion of RA erythroblasts were positive (median: 3% [0%-8%]) during culture. By contrast, a distinct staining was detected in RARS erythroblast cultures, which showed an early increase in MtF$^+$ cells and a continuous increase of these cells during the culture period (Figure 1). HF showed a variable expression (Table 1). A moderate increase was observed during normal erythroid maturation. MDS samples showed more HF during the first week of maturation, indicating that more excess iron has to be handled during early erythroid differentiation in MDS. In contrast to the MtF data, there was no difference between RARS and RA samples regarding HF expression.

Correlation between spontaneous cytochrome *c* release and MtF expression

We have recently shown that spontaneous cytochrome *c* release from mitochondria occurs at an early stage of erythroid maturation in MDS-derived progenitor cells.[10] The relation between spontaneous release of cytochrome *c* and MtF expression was therefore investigated in RARS and RA during erythroblast culture. Cytochrome *c* release was more pronounced in RARS, in line with our previous findings.[10] A positive correlation was observed between MtF expression and cytochrome *c* release in RARS at day 14 ($r^2 = 0.8$) (data not shown).

No significant impairment of mitochondrial ATP production in MDS marrow cells

Modification of a method developed for analysis of ATP production of intact fibroblasts[25] allowed us to reduce the number of cells required for analysis from 50×10^6 to 8×10^6. This was sufficient for analyzing mononuclear cells, but not for erythroblasts. Activity was determined in 3 RA, 5 RARS, and 2 NBM, in the absence and presence of G-CSF (Figure 2). In 2 cases, MNCs and CD34-depleted MNCs were investigated in parallel, with no difference in the results (data not shown). ATP production was determined in the presence of 2 substrate combinations. The endogenous substrates glutamate + succinate (G + S) are normally metabolized in mitochondria, and use all the respiratory chain complexes for ATP production. On the contrary, the artificial substrate combination TMPD + ascorbate (T + A) selectively delivers electrons to cytochrome *c,* and is thus dependent only on complex IV for the energy transduction to ATP. There was no significant difference between patients and controls, either between RA and RARS, in energy transduction from the substrate combinations G + S and T + A; although some MDS cases showed reduced levels of MAPR (Figure 2A-B).

MDS progenitors show aberrant expression patterns of HBB, GATA-1, and DLK-1

RARS reticulocytes may show reduced hemoglobin concentration.[26] To follow up on our functional assessment of MDS erythroblasts, we therefore assessed one of the genes encoding for hemoglobinization by studying the profile for HBB during erythroid maturation by real-time quantitative PCR. NBM showed low

Table 1. H-ferritin expression during erythroblast maturation in MDS and NBM

	% HF, d 0	% HF, d 4	% HF, d 7	% HF, d 11	% HF, d 14
RARS	6 (0-38)	28.5 (3-53)	36 (10-46)	37 (12-61)	39 (23-65)
RA	18 (16-66)	44 (15-53)	39 (24-46)	49 (33-58)	65
NBM	20 (12-24)	12 (3-15)	16 (11-31)	41 (22-49)	34 and 41

Immunostaining for H-ferritin (HF) was performed at day 0, 4, 7, 11, and 14 of erythroblast culture, as detailed in "Patients, materials, and methods." Results are shown as median values plus range (RA, n = 4; RARS n = 5; NBM, n = 3).

RA indicates refractory anemia; RARS, RA with ringed sideroblasts; and NBM, normal bone marrow.

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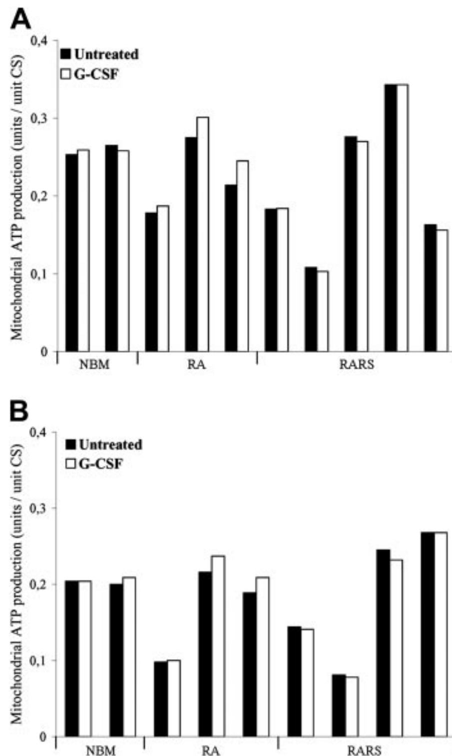

Figure 2. No difference in mitochondrial ATP production between MDS and NBM. (A) Mitochondrial ATP production rate (MAPR) was measured in $CD34^-$ cells from 2 NBM, 3 RA, and 5 RARS samples. There was no significant difference between MDS and NBM. Moreover, G-CSF did not have any significant effect on the MAPR determined with the substrate combination glutamate + succinate. (B) MAPR was measured with the substrate combination TMPD + ascorbate. There was no significant difference in ATP production between MDS and NBM. In addition, G-CSF did not influence the results in this system.

HBB levels in $CD34^+$ cells, but a 458-fold increase during the 14-day culture period. MDS $CD34^+$ cells showed significantly higher HBB levels (23-fold, $P = .001$), less increment during maturation (77-fold), but day-14 levels in parity with NBM (Figure 3A). RARS cells had higher expression than RA cells; however, the number of RA patients was too small to allow a statistical comparison (data not shown). GATA-1, a transcription factor with a wide range of activities, is essential for erythroid maturation and stimulates HBB expression, for example.[27] GATA-1 may also be cleaved by caspase-3 in a negative feedback loop regulating erythroid proliferation and maturation.[28] We found that GATA-1 was overexpressed in MDS $CD34^+$ cells (6-fold, $P = .001$). However, while GATA-1 increased 50-fold in NBM, the increment was almost absent in differentiating MDS erythroblasts (Figure 3B). In this aspect, there was no difference between RA and RARS. To confirm these findings, we examined the protein expression of GATA-1 in RARS and NBM at the aforementioned time points. GATA-1 expression was not seen during the first week of cultivation, but was readily detected during the second week after Epo addition, however, lower in RARS compared with NBM (data not shown). Microarray studies on early MDS progenitors have revealed up-regulation of DLK-1, a gene thought to be involved in differentiation.[29] We found significantly increased DLK-1 expression in MDS $CD34^+$ cells, compared with NBM, thus confirming previous gene profiling studies (26-fold, $P = .001$). DLK-1 expression decreased in MDS progenitors during erythroid differentiation, with no difference between normal and MDS progenitors at day 14, and with no obvious differences between RA and RARS (Figure 3C).

MDS progenitors show an overexpression of cytochrome *c*, Bax, and Bid

To further elucidate the mechanisms contributing to constitutive apoptosis in MDS, we investigated mRNA levels of apoptosis-related genes in freshly isolated $CD34^+$ in 6 RARS, 2 RA, and 13 NBM. MDS patients showed a nearly 4-fold (5.66 vs 1.57; $P = .006$) increase in the relative expression of cytochrome *c* mRNA level compared with NBM (Figure 4).

G-CSF has no effect on MtF expression, ATP production, or cytochrome *c* mRNA expression

Finally, we studied the effect of G-CSF on MtF, HF expression, and ATP production. We found that addition of G-CSF (4-24 hours) did not significantly reduce the MtF^+ and/or HF^+ in any individuals. On the contrary, MtF^+ cells increased in some RARS cultures after prolonged G-CSF incubation (data not shown). Similarly, no effect of G-CSF on MAPR was detected in MDS marrow cells (total activity, $P = .50$; complex IV, $P = .28$) (Figure 2A-B). Short-term incubation with G-CSF diminishes apoptosis in MDS cells by

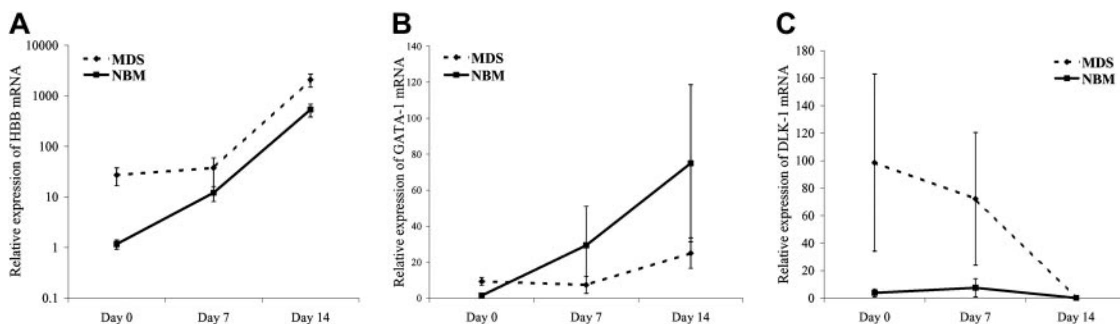

Figure 3. The relative expression of HBB, GATA-1, and DLK-1 is significantly higher in MDS $CD34^+$ cells. The relative expression of HBB, GATA-1, and DLK-1 mRNA was measured in freshly isolated $CD34^+$ cells (day 0), intermediate phase (day 7, $CD36^+$ cells), and late phase (GpA^+ cells) obtained from 6 RARS, 2 RA, and 13 NBM samples and normalized against 18S rRNA, with NBM as calibrator. Data shown are mean ± SEM. (A) At day 0, the relative expression of HBB was approximately 23-fold higher ($P = .001$) in low-risk MDS compared with NBM. At days 7 and 14, the relative expression of HBB was higher in MDS compared with NBM (37.6 vs 12.1 and 2089.6 vs 535.5, respectively). However, the fold index was higher in NBM compared with MDS at day 0 (day 7: MDS = 1.4-fold, NBM = 10.4-fold; day 14: MDS = 77-fold, NBM 458-fold). (B) At day 0, the relative expression of GATA-1 was approximately 6-fold higher ($P = .001$) in low-risk MDS compared with NBM. The relative expression of GATA-1 was lower in MDS compared with NBM at days 7 and 14. (C) The relative expression of DLK-1 was 26-fold and 9.7-fold higher in MDS compared with NBM at days 0 and 7, respectively. There was almost no expression of DLK-1 in GpA^+ cells in MDS and NBM at day 14.

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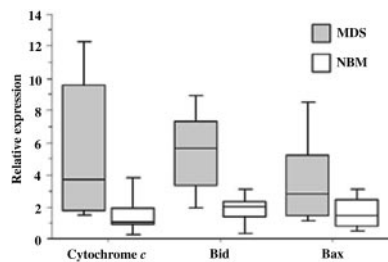

Figure 4. Relative expression of proapoptotic Bax, Bid, and cytochrome *c* mRNA is higher in low-risk MDS. Relative expression of Bax, Bid, and cytochrome *c* mRNA was measured in freshly isolated CD34$^+$ cells obtained from 6 RARS, 2 RA, and 13 NBM samples and normalized against 18S rRNA and using NBM as calibrator. The relative expression of Bax and Bid was higher in MDS compared with NBM (Bax: 3.73 [1.15-9.75] vs 1.69 [0.31-3.84]; Bid: 5.44 [1.78-9.45] vs 1.85 [0.21-3.17]). The relative expression of cytochrome *c* was nearly 4-fold higher in MDS (5.66 [1.49-13.41] vs 1.57 [0.2-4.19]). We also investigated the genes encoding Bax and Bid, 2 proapoptotic proteins implicated in mitochondrial apoptosis signaling, in MDS and NBM in freshly isolated stem cells (CD34$^+$) and at day 7 (intermediate phase, CD36$^+$ cells) and day 14 (GpA$^+$ cells). Higher expression of Bax and Bid was recorded in MDS at day 0 ($P = .07$ and $P = .006$, respectively), and a similar pattern was seen at days 7 and 14 (data not shown).

inhibition of cytochrome *c* release and subsequent caspase activation.[10] We therefore investigated the effect of G-CSF on cytochrome *c* mRNA levels in MDS progenitors. Addition of G-CSF (4 hours) to freshly isolated CD34$^+$ cells showed no significant effect on cytochrome *c* mRNA levels in MDS or NBM (data not shown).

Discussion

Iron deposited in perinuclear mitochondria of ring sideroblasts consists of aberrant mitochondrial ferritin, MtF, and this seems to be a typical finding both of X-linked and acquired sideroblastic anemia, but is not seen in other types of low-risk MDS.[14] We demonstrate herein that MtF in RARS is expressed early (day 4) during erythroblast culture, when cells remain CD34$^+$ and without morphologically visible signs of erythroid maturation and iron accumulation, and that MtF expression continues to increase during differentiation. We also confirm the previous negative finding for RA without ringed sideroblasts, which shows a very low expression of MtF without variation over time. As previously demonstrated, both RARS and RA erythroblasts showed significant cytochrome *c* release from the intermembrane space of mitochondria into the cytosol during all stages of differentiation. This event was, however, more marked and progressive in RARS.[10] Importantly, a significant correlation between cytochrome *c* release and MtF expression at day 14 was observed, suggesting that these 2 events are closely linked. However, the causal relationship remains to be clarified. A very recent study has shown that overexpression of MtF in a cell line limits the availability of iron for heme synthesis.[30] Taken together with the current findings, the latter results may thus help to explain the defective erythropoiesis in low-risk MDS, in particular in RARS.

Excess iron can induce apoptosis via reactive oxygen species production and subsequent damage of mitochondrial function. Iron accumulation is much more prominent in RARS than in RA, which may contribute to the higher rate of cytochrome *c* release in RARS. However, it is also conceivable that the elevated number of cytochrome *c*–releasing erythroblasts merely reflects a higher cell survival rate in the RARS cultures. The intact HBB expression in the RARS cultures lends support to this interpretation. Since aberrant MtF accumulation and cytochrome *c* release are present in very early erythroblasts, iron is likely to be an important player in the ineffective erythropoiesis of these patients, as well as in the biology of the disease. Cells derived from RA patients also show release of cytochrome *c,* but only minimal MtF expression. It is conceivable that RA erythroblasts survive less well in the cultures, resulting in fewer cytochrome *c*–releasing cells. However, it seems clear that aberrant iron accumulation, evidenced by MtF accumulation, is not a typical finding of RA, and that alternative pathways to erythroid apoptosis are activated in these cells. We found a high proportion of HF$^+$ cells both in MDS and NBM. HF is an intermediate step to introduce the labile iron to the mitochondria, and it has been shown that HF has an antiapoptotic activity unrelated to its ferroxidase activity and to its capacity to modify cellular iron metabolism.[31] However, although early MDS erythroblasts showed more HF expression compared with NBM, no significant difference was found between the groups.

We and others have documented a dissipation of the mitochondrial membrane potential in RARS,[9,32] and have shown that this is a rather late phenomenon that is not involved in the initiation of apoptosis. We hypothesized that impaired mitochondrial function in MDS bone marrow cells might be related to an impaired ATP production of the mitochondrial respiratory chain. Therefore, we developed a method to measure the ATP-producing activity of all complexes, as well as of complex IV in separated bone marrow cells. After refinement of this technique, it was possible to use as few as 8×10^6 cells, which allowed for the analysis of MNCs, but not of cultured erythroblasts. In CD34$^-$ cells obtained from a limited number of patients and controls, there was no clear difference in terms of mitochondrial ATP production, even though a few MDS cases presented with low levels. ATP production is fundamental for cell survival, and it is possible also that cell suspensions with substantial mitochondrial damage in terms of cytochrome *c* release and MtF accumulation are able to compensate for this defect.

An overexpression of proapoptotic Bax, Bid, and cytochrome *c* mRNA in freshly isolated CD34$^+$ progenitors from MDS patients was revealed in the current study. Overexpression of cytochrome *c* may be the result of a compensatory reaction to the leakage from the mitochondrial membrane space. However, the possibility of the overexpression as a primary phenomenon cannot be ruled out at present. Transcriptional up-regulation of cytochrome *c* was previously shown in response to a variety of apoptosis stimuli; however, simple overexpression of cytochrome *c* was, by itself, insufficient to induce apoptosis.[33] Microinjection of cytochrome *c* into the cytoplasm of intact cells is, on the other hand, a potent stimulus for cell death.[34] The higher expression of Bax and Bid in CD34$^+$ cells from MDS patients confirms previous findings using flow cytometry,[35] but here we show that elevated levels of these genes persist throughout in vitro erythroid differentiation. We previously showed that G-CSF reduces mitochondria-dependent apoptosis in MDS progenitors through inhibition of cytochrome *c* release and caspase activation. In the present study we investigated whether this effect is mediated through a direct effect on the mitochondria of MDS erythroblasts, or through activation of compensatory mechanisms for cell survival. We demonstrated that G-CSF had no effect on mitochondrial respiratory chain activity or aberrant MtF expression, but did exert a highly significant inhibitory effect on cytochrome *c* release, in line with our previous studies. As a matter of fact, in a few samples the MtF expression increased after 24-hour incubation with G-CSF. The few samples where we could assess the effects of G-CSF on mRNA expression in CD34$^+$ cells showed no significant changes (data not shown). It is likely that

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G-CSF up-regulates compensatory mechanisms for cell survival, allowing moderately damaged erythroblasts, including MtF+ cells, to survive in culture. The antiapoptotic effects of G-CSF in mature neutrophils have been extensively studied. Hence, it has been shown that G-CSF causes down-regulation of Bax expression[36] or, alternatively, prevents Bax translocation from the cytosol to mitochondria,[37] and subsequently inhibits caspase activation. G-CSF may also inhibit Bid truncation and translocation to the mitochondrial outer membrane.[38] Additional studies are warranted to further elucidate the mechanism underlying the antiapoptotic activities of G-CSF in bone marrow stem cells.

Ineffective hematopoiesis is a hallmark of MDS, and anemia is the predominant symptom in these patients. However, we found an overexpression at the stem cell level of genes involved in erythroid differentiation (HBB, GATA-1, and DLK-1). It is documented that the expression and function of the Epo receptor are intact in MDS,[39,40] and activation of this receptor enhances GATA-1 expression.[18,41,42] While the expression of HBB during erythroid differentiation was relatively normal in the MDS cultures, GATA-1 expression was clearly defective and failed to increase during cultivation. This may indicate that erythroid maturation is inhibited at the transcriptional level. It is also possible that the caspase-3 activation previously demonstrated in these cultures results in cleavage of GATA-1, thus leading to a defective signaling for erythroid differentiation.[28] Indeed, one may speculate that the defective erythroid maturation and/or increased apoptosis may lead to a compensatory overexpression of genes and subsequent hyperplastic bone marrow in an attempt to overcome the erythroid arrest. This could explain the expansion of erythroid progenitors seen in the bone marrow of MDS patients, and in particular in RARS. We also found a higher level of DLK-1 mRNA in the hematopoietic stem cell fraction of MDS patients, which is in agreement with studies by Miyazato et al,[29] who have identified an elevated expression of DLK-1 by global gene profiling analysis. Our findings not only support this publication, but also validate our in vitro culture assay in terms of differentiation of MDS stem cells toward the erythroid lineage.

In conclusion, our data provide evidence for impaired erythroid maturation at the stem cell level in MDS with subsequent overexpression of genes coding for erythroid maturation and hemoglobinization. Whether the primary cellular defect during erythroblast development is a block in differentiation due to defective gene expression or enhanced apoptotic signaling remains to be elucidated. We also demonstrate that the mechanism of erythroid failure differs between RA and RARS patients. In RARS, mitochondrial release of cytochrome *c* is closely linked to aberrant iron distribution at an early stage of differentiation, while alternative and probably more heterogeneous mechanisms are involved in RA. The current findings thus shed some light on the molecular pathogenesis of ineffective erythropoiesis in MDS.

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