Published in final edited form as:

Br J Haematol. 2008 November ; 143(3): 446–450. doi:10.1111/j.1365-2141.2008.07358.x.

Purification and Characterization of Sideroblasts from Patients with Acquired and Hereditary Sideroblastic Anemia

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Keywords

sideroblastic anemia; iron overload; mitochondria; oxidative stress; MDS

The sideroblastic anemias (SAs) are a heterogeneous group of inherited (rare) and acquired (relatively common) disorders of erythroid development characterized by iron accumulation within the mitochondria of developing erythroid cells, *i.e.* ringed sideroblasts (RS). Mutations in a few genes cause types of hereditary SA—including the gene that encodes the haem biosynthetic enzyme 5-aminolevulinic synthase (Cotter, *et al* 1994), a putative mitochondrial transporter ATP binding cassette b7 (Allikmets, *et al* 1999) an RNA modifying enzyme, pseudouridine synthase (Bykhovskaya, *et al* 2004), and a mitochondrial localized protein involved in iron-sulfur cluster biogenesis, glutaredoxin 5 (Camaschella, *et al* 2007). A large deletion of mitochondrial DNA in Pearson marrow pancreas syndrome causes SA in the context of a multi-system mitochondrial disorder (Rotig, *et al* 1990). Acquired SA is most commonly seen in myelodysplastic syndromes (MDS), where standard karyotypic analyses have not defined cytogenetic abnormalities that predict the presence of ringed sideroblasts. The significance of mitochondrial DNA mutations in the pathogenesis of SA and MDS remains controversial, with conflicting data as to whether mutation frequency is increased in marrow cells from patients with this group of disorders (Gattermann 2000, Reddy, *et al* 2002, Shin, *et al* 2003), and little direct evidence to link specific mutations with disease (Wulfert, *et al* 2008). Herein, we describe purification of

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sideroblasts from human marrow samples employing a simple, magnetic column-based method that was initially developed using a mouse model system (Martin *et al* 2005), and present characterization of the purified human cells.

This study included four males and two females, aged 70–85 years, with MDS and RS (3 refractory anemia with ringed sideroblasts [RARS], 3 multilineage dysplasia or excess blasts). One RARS patient provided two specimens for this study 10 months apart. One of the MDS patients progressed to acute myeloid leukemia ~4 months after a sample was obtained. The seventh patient in this study was a 26-year-old male with X-linked sideroblastic anemia. All marrow samples were collected with the approval of the Scripps Research Institute human subjects committee. The control marrow specimens lacked RS or evidence of dysplasia. Sideroblasts were purified by adaptation of a simple magnetic column-based method as described previously for purification of murine siderocytes (Martin *et al* 2005). Reactive oxygen species (ROS) production and mitochondrial membrane potential ($\Delta\Psi_m$) flow cytometry (fluorescent-activated cell sorting) assays, and protein carbonyl determination (oxyblot) were performed as described previously (Martin *et al* 2005).

Using a murine model of SA, we have previously demonstrated that the increased intracellular iron characteristic of sideroblasts or siderocytes (enucleated red cells containing iron-loaded mitochondria) can be exploited to obtain a highly purified population of these cells (Martin *et al* 2005). Here, we utilized the same method (passage of a cell suspension over a magnetic column in absence of any magnetic bead affinity reagent) for purification of sideroblasts and siderocytes from 8 fresh human marrow specimens. $0.29 \pm 0.07\%$ vs. $0.08 \pm 0.01\%$ of cells were recovered in the column-bound fraction (CBF) relative to starting material (SM) in diseased (N = 8) vs. normal (N = 2) whole marrows, respectively. This represents a 3.62-fold greater proportion of magnet⁺ cells in diseased than in normal samples (Fig. 1A). Cell counts included both nucleated cells and erythrocytes in the starting marrow specimens and purified samples.

Magnetic purification of bone marrow suspensions showed a significant enrichment in glycophorin A (GPA)⁺ and transferrin receptor (CD71)⁺ iron-overloaded erythroblasts, *i.e.* sideroblasts ($35.07 \pm 6.06\%$ of gated cells in CBF vs. 2.27 ± 0.67 and $2.40 \pm 1.35\%$ of gated cells in SM and column flow-through (FT), respectively; N=8; ***, P<0.0001; not shown). Morphology by light microscopy showed that >90% of purified cells were erythroid (erythroblasts to siderocytes) (Fig. 1B). Perl's iron stain demonstrated significant iron accumulation within cells in the magnet-purified fraction - including nucleated RS and siderocytes.

Purified sideroblasts showed a significantly increased mitochondrial membrane potential, $\Delta\Psi_m$, with geometric mean fluorescence intensity (GeoMFI) of 57.05 ± 11.79 relative to SM and FT fractions (5.60 ± 0.87 and 5.54 ± 0.91 GeoMFI, respectively; N=8; ***, P<0.0001; Fig. 2B). These results are similar to those observed when purifying murine sideroblasts/siderocytes in a mouse model of SA secondary to loss of the intramitochondrial antioxidant protein superoxide dismutase 2 (Martin *et al* 2005). Elevation of the mitochondrial membrane potential implies an increase in the H⁺ gradient within the mitochondria, and may reflect a defect in the distal portions of the electron transport chain or a defect in mitochondrial ATP synthesis.

ROS sensitive dyes dihydroethidium (DHE—sensitive to superoxide) and 5,6 chloromethyl 2',7' dichlorodihydro-fluorescein diacetate (CM-H₂DCFDA—a fluorescein derivative sensitive to peroxide and mixed ROS) were used to measure real-time peroxide and superoxide production in unfractionated marrow, magnet purified and flow through

fractions. Most cells in the magnet-purified fraction (CBF) produced ROS, whereas few cells from the SM and FT fractions showed significant dye oxidation ($71.27 \pm 5.05\%$ of gated cells vs. 1.26 ± 0.22 and $0.89 \pm 0.16\%$ of gated cells, respectively; $N=8$; ***, $P<0.0001$; Fig. 2 A). These results were consistent with comparisons of magnet purified siderocytes from the murine model system (Martin *et al* 2005).

Sideroblasts purified from 3 patients (CBF fractions) showed a significant increase in the amount and complexity of protein oxidative modification, measured as carbonyls (Fig. 2 C), when compared with an equivalent amount of protein from starting material, or from cells that passed through the magnetic column. This is similar to the enrichment for oxidized proteins observed when purifying sideroblasts/siderocytes from murine marrow or peripheral blood (Martin *et al* 2005). This association between excess iron and protein oxidation raises the possibility that redox active iron is a source of protein-damaging ROS via Fenton chemistry in developing erythrocytes.

We have demonstrated a simple and effective method for the enrichment of sideroblasts/siderocytes from marrow specimens of patients with SA or with myelodysplasia with ringed sideroblasts. These cells, purified on a magnet to take advantage of their high iron content, show evidence of increased oxidant production, altered mitochondrial function and oxidative damage to protein. We anticipate that this purified cell population will be useful for additional analyses to delineate other molecular and biochemical lesions characteristic of acquired SA.

Acknowledgments

We thank Dr. Mathieu Marella and Pr. Takao Yagi, TSRI, for assistance and use of their microscope. This work was supported by grant NIDDK RO1 DK62473 from the National Institutes of Health awarded to J.S.F. and The Stein endowment fund. This is TSRI MS #19210. F.M. Martin and J.S. Friedman designed the experiments. J. Prchal, J. Nieva, A. Saven, J. Andrei, G. Aripally, S. Bottomley, J. C. Barton and K. Bethel provided the biospecimens and comments on the manuscript. F.M. Martin performed the experiments. F.M. Martin and J.S. Friedman analyzed and interpreted the data and wrote the manuscript.

References

- Allikmets R, Raskind WH, Hutchinson A, Schueck ND, Dean M, Koeller DM. Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). *Human Molecular Genetics*. 1999; 8:743–749. [PubMed: 10196363]
- Bykhovskaya Y, Casas K, Mengesha E, Inbal A, Fischel-Ghodsian N. Missense mutation in pseudouridine synthase 1 (PUS1) causes mitochondrial myopathy and sideroblastic anemia (MLASA). *American Journal of Human Genetics*. 2004; 74:1303–1308. [PubMed: 15108122]
- Camaschella C, Campanella A, De Falco L, Boschetto L, Merlini R, Silvestri L, Levi S, Iolascon A. The human counterpart of zebrafish shiraz shows sideroblastic-like microcytic anemia and iron overload. *Blood*. 2007; 110:1353–1358. [PubMed: 17485548]
- Cotter PD, Rucknagel DL, Bishop DF. X-linked sideroblastic anemia: identification of the mutation in the erythroid-specific delta-aminolevulinate synthase gene (ALAS2) in the original family described by Cooley. *Blood*. 1994; 84:3915–3924. [PubMed: 7949148]
- Gattermann N. From sideroblastic anemia to the role of mitochondrial DNA mutations in myelodysplastic syndromes. *Leukemia Research*. 2000; 24:141–151. [PubMed: 10654450]
- Martin FM, Bydlon G, Welsh ML, Friedman JS. A Method for Rapid Mouse Siderocyte Enrichment. *Experimental Hematology*. 2005; 33:1493–1499. [PubMed: 16338492]
- Reddy PL, Shetty VT, Dutt D, York A, Dar S, Mundle SD, Allampallam K, Alvi S, Galili N, Saberwal GS, Anthwal S, Shaikh M, Suleman S, Kamal SY, Raza A. Increased incidence of mitochondrial cytochrome c-oxidase gene mutations in patients with myelodysplastic syndromes. *British Journal of Haematology*. 2002; 116:564–575. [PubMed: 11849212]

- Rotig A, Cormier V, Blanche S, Bonnefont JP, Ledeist F, Romero N, Schmitz J, Rustin P, Fischer A, Saudubray JM, Munnich A. Pearson's marrow-pancreas syndrome. A multisystem mitochondrial disorder in infancy. *Journal of Clinical Investigation*. 1990; 86:1601–1608. [PubMed: 2243133]
- Shin MG, Kajigaya S, Levin BC, Young NS. Mitochondrial DNA mutations in patients with myelodysplastic syndromes. *Blood*. 2003; 101:3118–3125. [PubMed: 12446454]
- Wulfert M, Kupper AC, Tapprich C, Bottomley SS, Bowen D, Germing U, Haas R, Gattermann N. Analysis of mitochondrial DNA in 104 patients with myelodysplastic syndromes. *Experimental Hematology*. 2008; 36:577–586. [PubMed: 18439489]

Fig.1. Magnetic purification of marrow aspirates from patients with RS allowed purification of iron-laden erythroid precursors

A. Magnetic columns enabled the recovery of a significant fraction of the marrow starting material. Bar graph shows the % of magnetically-purified cells in normal ($0.08 \pm 0.01\%$; $N=2$) vs. diseased ($0.29 \pm 0.07\%$; $N=8$) bone marrow aspirates. B. Microscopic analysis showed that purified cells were predominantly sideroblasts, with occasional siderocytes. Magnetically-purified cells were cytopun in calf serum, and stained with Perl's technique (potassium ferrocyanide and Kernechtrot nuclear fast red counterstain; left) or Wright-Giemsa technique (right) stained. x63 magnification with x1.6 optical zoom; white balance was set at 3200K; scale bar, 20 μm ; Pt, patient. Representative samples from 2 patients are shown. Arrow points to a ringed sideroblast; arrowhead points to a siderocyte. Occasional plasma cells were found in some fields, but represented less than 1% of the purified population, which was overwhelmingly erythroid progenitors.

Fig.2. Purified sideroblasts produced high levels of ROS, showed altered $\Delta\Psi_m$ and increased oxidative damage to proteins

A. Magnetic purification significantly enriched ROS-producing sideroblasts. ROS-production FACS assay; dot plots show the DCF (peroxide and mixed ROS sensitive fluorescein analogue) and DHE (superoxide sensitive dihydroethidine) intensity of FSC/SSC-gated marrow cells. Square quadrant allows measurement of DCF⁺ DHE⁺ double positive % gated cells (see results and discussion). B. Purified sideroblasts showed altered $\Delta\Psi_m$. $\Delta\Psi_m$ TMRM-FACS assay; plain curve and black line, CBF and SM cells stained with TMRM, respectively; dotted line, CBF cells incubated with TMRM and $\Delta\Psi_m$ inhibitor CCCP; overlaid histograms were smoothed and normalized. C. Purified sideroblasts showed increased oxidative damage to proteins. OxyBlot analysis of column fractions from 3 biospecimens. 15 μ g cell lysate protein was 2,4-dinitrophenylhydrazine (DNPH)-derivatized and separated on a 4–12% Bis-Tris Criterion XT precast gel (Bio-Rad Laboratories, Hercules, CA). Right scale: Approximate molecular weight in kDa. Pt, patient; SM, starting material (washed marrow); FT, column flow-through (iron⁻ fraction); CBF, column bound fraction (iron⁺ fraction). Dot plots and histograms are representative of 8 experiments; ***, $P < 0.0001$.