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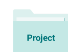
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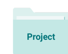
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Non-transferrin-bound iron in myelodysplastic syndromes: a marker of ineffective erythropoiesis?

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Introduction: Iron overload is usually observed in patients (even untransfused) with myelodysplastic syndromes (MDS), and contributes towards the generation of low molecular weight iron complexes or non-transferrin-bound iron (NTBI), which in turn favors oxidative DNA damage and consequent apoptosis.
Materials and methods: Levels of NTBI and lipid peroxidation were evaluated by means of free serum malondyaldehyde (MDA) in untransfused MDS patients and we tried to correlate them with ineffective erythropoiesis, apoptosis and the pattern of *in vitro* growth.
Results: NTBI levels were found to be significantly higher in low-risk than in high-risk MDS patients, as well as in patients with a lower myeloid/erythroid ratio. MDA was found to be uniformly higher in the MDS patients as a whole than in normal controls. The bone marrow progenitor cells in the MDS patients with high NTBI levels showed a higher degree of apoptosis, but this difference was not statistically significant. Patients with a leukemic growth pattern had lower NTBI levels than those with a non-leukemic pattern.
Conclusion: These data suggest that NTBI is related to the degree of ineffective erythropoiesis and that it contributes towards inducing apoptosis in MDS bone marrow precursors. The presence of leukemic growth is associated with low NTBI levels, probably due to increased iron consumption by blast cells.
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Keywords: non-transferrin-bound iron; myelodysplastic syndromes; dyserythropoiesis; apoptosis

Introduction

Iron overload, due to the presence of ineffective erythropoiesis and to the increased absorption following the anemic state, is usually observed in patients affected by myelodysplastic syndromes (MDS), even untransfused.[1] Jensen *et al*.[2] have demonstrated that iron chelation by desferrioxamine in transfusion-dependent MDS patients can improve hemoglobin levels by reducing transfusion need, thus indirectly suggesting that iron overload may worsen the ineffective erythropoiesis and the level of anemia. Excess body iron contributes towards the generation of low molecular weight iron complexes or non-transferrin-bound iron (NTBI), which in turn favors the formation of potentially toxic oxygen derivatives.[3,4] NTBI is more readily taken up by cells than transferrin-bound iron is,[5] and contributes towards increasing the labile pool of cytoplasmic iron (LIP).[6] The degree of cell damage induced by NTBI can be deduced from the level of lipid peroxidation, which is known to be a consequence of an increase in LIP.[3]

Increased bone barrow (BM) cell apoptosis is a common finding in MDS, particularly in patients at low risk of leukemic evolution.[7 10] Although many factors are likely to be involved in this process, such as an increased ratio of pro- *vs* anti-apoptotic proteins (bcl2 family),[11] the real etiopathogenesis of MDS is far from being completely understood. It has recently been reported that the high serum levels of tumor necrosis factor-α (TNF-α) observed in MDS patients may lead to apoptosis by inducing the generation of free radicals, which can induce oxidative DNA damage.[12 18] In particular, Peddie *et al*.[19] have demonstrated that

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MDS $CD34^+$ cells have an increased level of oxidized pyrimidine nucleotides.

On the basis of these observations, the levels of NTBI and malondyaldehyde (MDA), an index of lipid peroxidation, were evaluated in serum taken from untransfused MDS patients, with the aim of revealing their possible role in ineffective erythropoiesis and apoptosis.

Materials and methods

Patients

Thirty-three untransfused patients with primary MDS (36 to 88 years old, median age 70) and ten normal controls were enrolled after having given their informed consent. The patients were classified according to the FAB criteria as having refractory anemia (RA, 12 cases), RA with ring sideroblasts (RARS, nine cases), RA with excess of blasts (RAEB, 10 cases), and RAEB in transformation (RAEB-t, two cases). Patients with RA or RARS were classified as low risk (LR), and those with RAEB or RAEB-t as high risk (HR). The characteristics of the patients are summarized in Table 1.

Table 1 Patient characteristics

Diagnosis	*Patient*	*Sex*	*Caryotype*	*%Blasts*
RA	1	M	46XY	3.5
	2	F	46XX	2
	3	M	n.v.	2
	4	F	n.v.	3.75
	5	F	46XX	2
	6	F	46XX	1.75
	7	F	45XX, 7	2.25
	8	F	47XX, +8	1
	9	F	46XX	1
	10	M	46XY	4.25
	11	M	46XY	5
	12	F	46XX	3
RARS	13	F	46XX	3.5
	14	M	46XY	0.25
	15	M	46XY	1.25
	16	M	46XY	2.25
	17	M	46XY	1
	18	M	46XY	1.25
	19	F	46XX	3.75
	20	M	n.v.	1.5
	21	F	46XX	3.5
RAEB	22	F	46XX	11.25
	23	M	46XY	7
	24	M	46XY	13
	25	F	46XX, 5q	6
	26	F	46XX	6
	27	F	n.v.	13.75
	28	M	46XY	17
	29	F	n.v.	6.75
	30	M	46XY	20.25
	31	F	47XX, +8	5.75
RAEB t	32	F	46XX	28
	33	M	46XY	26.75

F female; M male; RA refractory anemia; RAEB refractory anemia with excess of blasts; RAEB t refractory anemia with excess of blasts in transformation; RARS refractory anemia with ring sideroblasts.

NTBI levels

Peripheral blood serum was obtained by centrifugation at 3000 r.p.m. Serum aliquots were stored at −20°C until their NTBI levels were evaluated using the chromatographic method.[20] Briefly, 450 μl of serum was added to 50 μl of nitrilotriacetic acid (NTA) 800 mM (pH 7.0) and allowed to stand for 20 min. The solution was then ultrafiltered using Amicon Centricon 30 microconcentrator units (Amicon Millipore Corporation, Bedford, MA, USA), and the ultrafiltrate (20 μl) was injected directly into the HPLC system using a Perkin Elmer Series 200 IC titanium pump.

The chromatographic conditions were the following: flow rate 1.5 ml/min; mobile phase isocratic containing 20% acetonitrile and 80% sodium phosphate buffer, 5 mM (pH 7.0) containing 3 mM CP22; visible detection, 450 nm. A standard curve was generated by injecting different concentrations of iron prepared in a 100-fold excess of NTA. The standards were routinely run at 0 to 10 μM, although absorbance was linear up to 40 μM. Under these conditions, the 0 μM standard corresponds to 80 mM of NTA. The addition of 80 mM of NTA to the serum of normal individuals always results in negative NTBI values. These values are less than the 0 μM standard, presumably because some iron is donated from NTA to transferrin.

The NTBI level was considered relevant at concentrations of +1 μM or higher.[21]

MDA assay

Free MDA was evaluated by means of a colorimetric assay for lipid peroxidation using a Bioxytech LPO-586 kit (Oxis International Inc, Portland, OR, USA).

Clonogenic assay

The BM samples were collected in preservative-free heparin. The BM mononuclear cells (BMMNC) were separated by means of gradient centrifugation using Ficoll Lymphoprep (Nicomed Pharma As, Oslo, Norway), and 1×10^5 cells/ml in IMDM were cultured in 1 ml of a mixture containing 20% FCS, 0.3% agar, GM-CSF 200 U/ml, IL-3 100 U/ml and SCF 8 U/ml (Genzyme, Cambridge, MA, USA). The plates were incubated in humidified air with 5% CO_2 at 37°C for 14 days. Aggregates containing >50 cells were scored as colonies, whereas those containing <50 cells were scored as clusters. All of the cultures were set up in quadruplicate.

Definition of growth pattern

The agar colony assay was used on the fresh BMMNC in order to classify the MDS patients into two groups (leukemic or non-leukemic) according to their pattern of clonal growth:[22] (1) normal; (2) absent or reduced growth; (3) small number of colonies with a large number of leukemic clusters; (4) normal or large number of colonies with a large number of leukemic clusters. Patterns 1 and 2 were considered non-leukemic, while

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patterns 3 and 4 with a colony/cluster ratio of <5 were considered typical of a leukemic growth.[23]

Apoptosis

Apoptosis was evaluated by means of a TdT/dUTP assay using the commercially available "In Situ Cell Death Fluorescein Detection Kit" (Roche Diagnostic, Mannheim, Germany). Briefly, $1-2\times10^6$ cells were fixed in cold ethanol 70% in PBS, and stored at 4°C until use. Before staining, cells were washed in PBS, permeabilized with 100 μl Triton X-100 0.1% in sodium citrate 0.1% for 2 min on ice, and again washed twice in PBS. After incubation for 1 h at 37°C with dUTP FITC, with and without TdT, cells were washed again in PBS, resuspended in 500 μl of PBS and made ready for cytofluorimetric analysis. A total of 1×10^4 events were analyzed.

Statistical analysis

The between-group correlations and differences were respectively evaluated using Spearman's Rank correlation coefficient and the Mann-Whitney test. A P value of <0.05 was considered statistically significant.

Results

NTBI levels and FAB classification

MDS serum NTBI levels (Figure 1) were significantly higher than those observed in the normal controls ($+0.81\pm0.26$ *vs* -0.63 ± 0.15 μM, $P<0.01$). NTBI

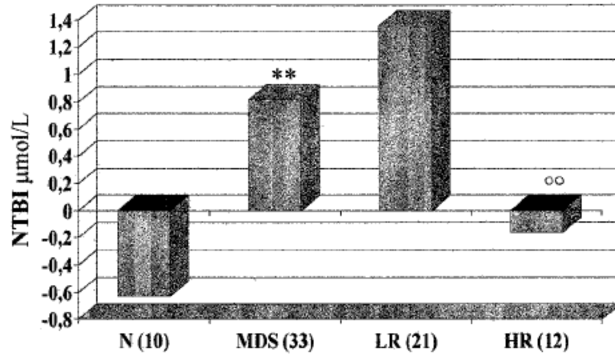

Figure 1 NTBI levels in samples from normal individuals and MDS patients. $^{**}P$ value <0.01 *vs* normals; $^{\circ\circ}P$ value <0.01 *vs* LR.

Table 2 Hematological parameters of MDS patients

Dioagnosis	*Patient*	*NTBI* μM	*M/E ratio*	*Hb g/dl*	*Ferritin* μg/l	*Reticulocytes* $\times 10^3$	*Fe* μg/dl	*Trf sat.%*
RA	1	2.09	0.3	12.7	215	17.1	138	57
	2	1.71	2	9	602.9	25.2	126	47
	3	0.43	1.9	14	198.2	14.1	224	36
	4	1.53	1.9	12.1	13.5	12.3	112	24
	5	0.81	3.9	11.7	24.8	8.6	79	23
	6	1	1.1	10	316	13.2	94	39
	7	2.9	1.2	11.4	480	19	47	17
	8	0.41	6.8	11.4	8.5	18.7	75	20
	9	0.49	1.5	10	323	25.9	140	48
	10	3.94	1.07	12.4	243	9.6	96	37
	11	0.89	3.8	12.1	121.6	23.7	105	36
	12	0.27	0.6	10.5	80.8	14.4	101	32
RARS	13	4.16	0.3	8.5	703.3	12.9	92	40
	14	3.81	0.9	8.9	562	16	81	40
	15	0.54	0.3	11.2	231	16.6	119	51
	16	0.99	0.7	9.2	459	10.8	92	45
	17	1.26	0.52	11.2	1160.1	8.5	183	82
	18	3.38	0.7	8.1	676	8	156	59
	19	0.27	1.7	10.6	792.2	10.6	103	46
	20	1.22	1.1	15.5	394	12.7	124	54
	21	1.85	0.8	10.6	460	11.7	115	54
RAEB	22	0.53	4	10.1	378.7	15.2	50	24
	23	0.47	0.66	8.3	275.7	15	81	30
	24	1.05	3.2	9.2	750	41	115	53
	25	0.02	1.7	12	103.3	11.9	104	45
	26	0.88	2.3	11.3	218.4	11.2	67	26
	27	0.35	0.7	12.1	267.7	18.4	106	41
	28	1.07	0.6	11	393	18.6	99	34
	29	0.27	1.3	12.7	100	10.1	119	39
	30	0.27	1.2	10.8	347.8	15.8	131	47
	31	0.67	3.4	13.9	407.2	8.4	85	36
RAEB t	32	0.28	10.8	9.1	61.7	36	71	26
	33	0.81	1.24	8.5	109	9.3	87	58

M/E ratio was defined as number of leucocytes (without granulocytes)/number of erythroblasts. M/E myeloid/erythroid; NTBI non transferrin bound iron. For other abbreviations see Table 1.

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was particularly high in the LR subgroup and significantly higher than those in the HR subgroup (+1.36±0.34 *vs* −0.16±0.19 μM, $P<0.01$). Only four out of 21 LR patients had negative NTBI values, whereas none of the HR patients had NTBI levels of >1 μM.

NTBI did not correlate with hemoglobin (Hb), reticulocyte count, serum iron (Fe), ferritin or transferrin (Trf) saturation (Table 2).

NTBI levels and bone marrow M/E ratio

There was a significant correlation ($P<0.05$) between NTBI and the myeloid/erythroid (M/E) ratio among the MDS samples. Patients with NTBI levels of >1 μM had a M/E ratio of 0.99±0.15, whereas those with NTBI levels of <1 μM had a M/E ratio of 2.44±0.54 ($P<0.05$) (Figure 2).

NTBI levels and pattern of growth

Patients with an *in vitro* leukemic growth pattern had significantly lower levels of NTBI than those with a non-leukemic pattern (0.5±0.38 *vs* 1.18±0.33, $P<0.05$) (Figure 3). All four LR patients with NTBI levels of <1 μM had an *in vitro* leukemic growth pattern.

Serum MDA values

MDS patients had significantly higher levels of free MDA (Figure 4) than the normal subjects (0.95±0.08 *vs* 0.4±0.08 μM, $P<0.01$), but there was no significant difference between the HR and LR subgroups (0.86±0.1 *vs* 1±0.11 μM, $P=0.7$). Moreover, in samples from these patients there was no correlation between MDA levels, NTBI or M/E ratio.

NTBI levels and apoptosis

Apoptosis was evaluated in 24 MDS and 10 normal BMMNC samples. Apoptosis was found to be significantly greater in the MDS patients as a whole than in the normal controls (2.65±0.41 *vs* 1.49±0.27, $P<0.05$), and was also greater in the LR than in the HR subgroup (3.04±0.54 *vs* 1.6±0.39, $P<0.05$).

Patients with high NTBI levels (>1 μM) showed increased apoptosis in comparison with those with low NTBI levels, although this difference was not statistically significant (3.4±0.99 *vs* 2.25±0.35, $P=0.4$) (Figure 5).

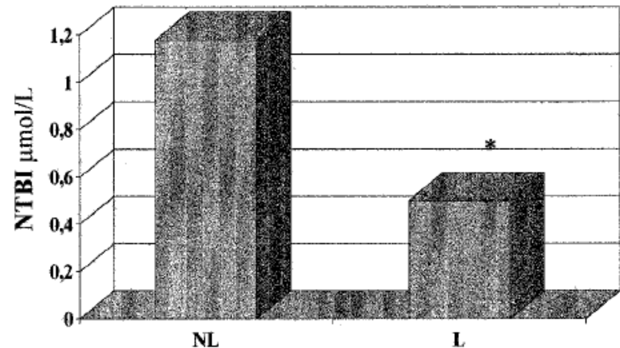

Figure 3 NTBI levels and pattern of growth. NL non leukemic growth; L leukemic growth. *P value <0.05.

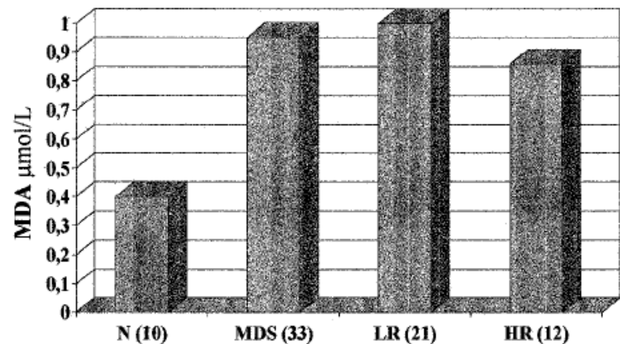

Figure 4 MDA levels in samples from normal individuals and MDS patients.

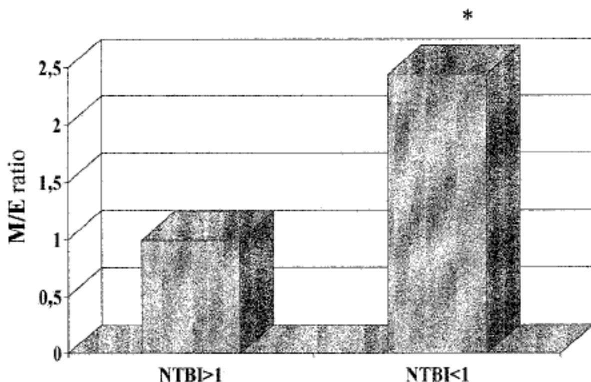

Figure 2 M/E ratio in patients with low (<1 μM) and high (>1 μM) levels of NTBI.

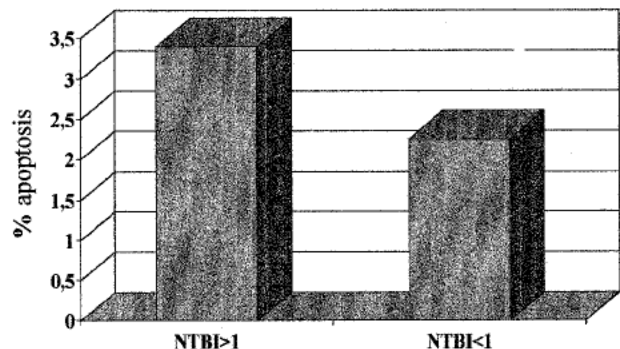

Figure 5 Apoptosis in patients with low (<1 μM) and high (>1 μM) levels of NTBI.

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