

## More cell death in refractory anemia with excess blasts in transformation than in acute myeloid leukemia

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**Refractory anemia with excess blasts in transformation (RAEB-T) is a subgroup of myelodysplastic syndrome (MDS) in which the bone marrow blast count ranges from 20% to 30%. The recently proposed World Health Organization Classification of Hematologic Malignancies eliminated this category from MDS by lowering the blast count cutoff for acute myeloid leukemia (AML) from 30% to 20%. However, MDS is distinguished from AML by a significant increase in apoptosis. To investigate the difference in apoptosis between RAEB-T, AML, and other categories of MDS, we prospectively analyzed fresh bone marrow samples using the Annexin V and mitochondrial potential assays. There was a significantly higher level of apoptosis in RAEB-T than in AML according to both assays, while no significant differences between RAEB-T and other categories of MDS were noted. The data suggest that RAEB-T is more likely to be an advanced stage of MDS and biologically different from AML.**

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### Introduction

Based on the French–American–British (FAB) classification, refractory anemia with excess blasts in transformation (RAEB-T) is an advanced stage of myelodysplastic syndrome (MDS) in which the blast count ranges from 20% up to 30%.<sup>1</sup> The recently proposed World Health Organization Classification of Hematologic Malignancies eliminated RAEB-T from MDS, however, and changed the blast-count cutoff of acute myeloid leukemia (AML) from 30% to 20%.<sup>2</sup> The rationale for this change was based on the fact that the prognosis for patients having a blast count of 20% to 30% is similar to that for patients having a blast count greater than 30%. While response to therapy is an important criterion in a disease classification system, there may be a fundamental difference between the biologic features of RAEB-T and those of AML. There is a considerable body of data suggesting that MDS is distinguished from AML by a higher degree of apoptosis.<sup>3–7</sup> While a number of studies have shown the difference in apoptosis between MDS and AML, only a few have specifically compared RAEB-T with other categories of MDS and AML, producing conflicting results.<sup>8–10</sup> Therefore, in the present study, we compared the degree of apoptosis in RAEB-T with that in refractory anemia (RA), with ringed sideroblasts (RARS), RAEB, and AML using the Annexin V<sup>11</sup> and mitochondrial potential assays.<sup>12</sup>

### Materials and methods

#### *Patients and specimens*

Fresh bone marrow aspirates were obtained from 25 previously untreated patients having RAEB-T, 133 patients having AML (8 M0, 31 M1, 49 M2, 33 M4/M5, 8 M6 and 3 M7) and 121 patients having 'other MDS' (33 RA/RARS, 51 RAEB, and 37 chronic myelomonocytic leukemia). Sixty eight percent of AML had no antecedent hematologic disease (AHD). Thirteen patients having anemia and 13 patients having solid tumors without bone marrow involvement were included, as controls. All samples were collected with consent form and the Institutional Review Board approved the protocol.

#### *Measurement of apoptosis using Annexin V*

Bone marrow samples were collected in ethylenediaminetetraacetic acid tubes and processed within 5 h from collection. The cells (a minimum of 10<sup>6</sup>) were isolated via a double-density gradient centrifugation technique using both Histopaque 1119 and 1077. Polymorphonuclear leukocytes and mononuclear cells were mixed and washed with phosphate-buffered saline. The cells were then incubated with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V antibodies (Trevigen, Gaithersburg, MD, USA) and for PerCP-CD14 and APC-CD34 (Becton Dickinson, San Jose, CA, USA) for 15 min at room temperature. After washing, the cells were acquired into a FACSCalibur (Becton-Dickinson) within 5 min of staining. Stained cells were analyzed using the CellQuest software program (Becton-Dickinson). PI-positive cells were excluded from further analysis. The percentage of positivity for Annexin V was determined in all cells, as well as in CD34<sup>+</sup> cells.

#### *Measurement of mitochondrial membrane potential*

An aliquot of fresh bone marrow samples containing a minimum of 1 × 10<sup>6</sup> cells was first lysed with ammonium chloride for 3 min and then washed twice with a phosphate-buffered saline solution. DePsipher reagent (5,5', 6,6', tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl carbocyanin iodide; Trevigen) was added to the washed cells, and the mixture was incubated at 37°C in 5% CO<sub>2</sub> for 20 to 30 min. After being stained with DePsipher, the cells were washed and analyzed immediately using a FACSCalibur and the CellQuest software program. The degree of change in mitochondrial membrane potential was assessed by comparing the percentage of cells having green fluorescence in patients having RAEB-T, other MDS, or AML with that in marrow samples obtained from patients not hav-

ing MDS. Analysis was performed first in all cells and subsequently in the polymorphonuclear cells alone by gating based on light scatter. To exclude the possibility of red cell lysing step affecting the mitochondrial membrane potential, we compared the results of 26 samples processed by density gradient, as well as by lysis. We found no statistical difference between the methodologies and the rest of the samples were analyzed using lysing method.

### Statistical analysis

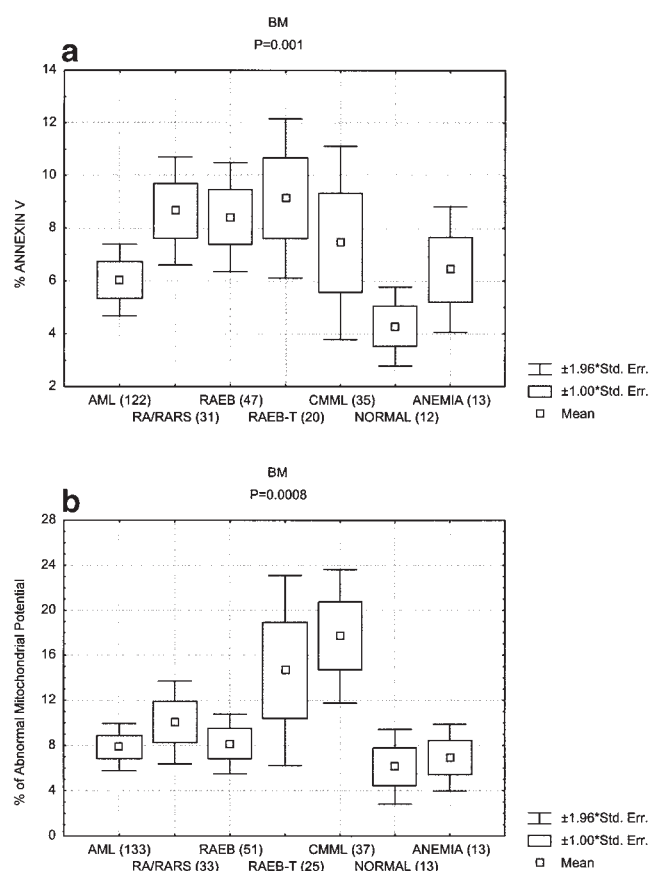
The Kruskal–Wallis test was used to compare categorical differences in the groups. Correlation was performed using the Spearman correlation test.

### Results

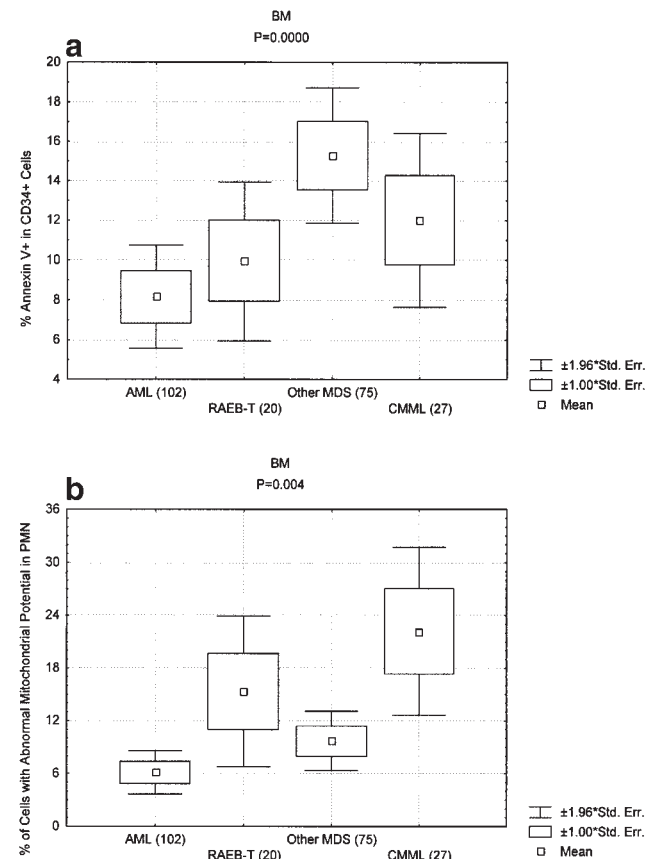
A higher degree of apoptotic cell death was observed in patients having all categories of MDS, including RAEB-T, as compared with those having AML, normal controls and patients having anemia as measured using the two independent assay methods. According to the Annexin V assay, a median of 4.7% (range, 4.0%–34.9%) of cells in patients having RAEB-T showed apoptosis compared with a median of 2.4% of cells (range, 0.1%–55.5%) in patients having AML ( $P=0.001$ ) (Figure 1a). In contrast, there was no significant

difference between patients having RAEB-T and other MDS (median, 3.9%; range, 0.0%–63.6%) ( $P=0.22$ ). Furthermore, the median percentage of apoptotic cells in patients having anemia and those having solid tumors without marrow involvement was 3.5% (range, 0.1%–15.5%) and 2.4% (range, 0.3%–9.4%), respectively. When only CD34<sup>+</sup> cells were analyzed, the percentage of apoptotic cells was still significantly higher in patients having RAEB-T (median, 9.5%; range, 0%–75%) than in patients having AML (median, 3.5%, range, 0%–68.8%;  $P=0.03$ ), while no significant difference between patients having RAEB-T and those having other MDS was noted (median, 9.4%, range, 0%–75%;  $P=0.97$ ) (Figure 2a). Furthermore, there was no significant correlation between the percentage of CD34<sup>+</sup> cells, which represents blasts, and the percentage of apoptotic cells measure by Annexin V assay ( $R=0.041$ ,  $P=0.41$ , Spearman correlation). Examples of dot plots showing the expression of CD34 and Annexin V are illustrated in Figure 3.

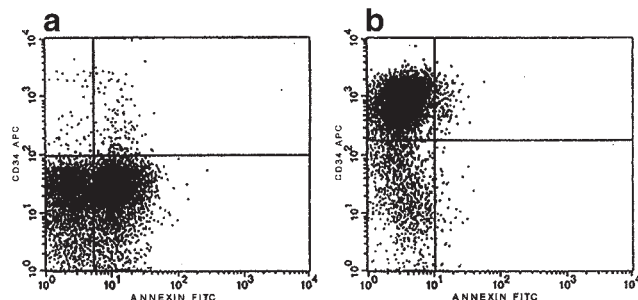
Flow cytometric analysis of mitochondrial potential using the DePsipher assay also showed a greater percentage of apoptotic cells in patients having RAEB-T (median, 3.7%; range, 0.1%–84.6%) than in those having AML (median, 2.5%; range, 0%–55.7%;  $P=0.05$ ), but did not show a significant difference between patients having RAEB-T and other MDS (median, 4.6%; range, 0.1%–84.4%;  $P=0.41$ ) (Figure 1b). The same pattern was demonstrated when polymorphonuclear cells were analyzed alone (Figure 2b). The median



**Figure 1** The percentage of apoptotic cells in different diseases. Box plots showing the percentage of apoptotic cells in bone marrow sample as measured using the Annexin V (a) and mitochondrial potential assays (b).



**Figure 2** The percentage of apoptotic cells in AML, RAEB-T and other MDS. Box plots showing the percentage of apoptotic cells in bone marrow samples as measured using the Annexin V (a) and mitochondrial potential assays (b). Other MDS includes RA/RARS, RAEB and CMML.



**Figure 3** Dot plots showing CD34 and Annexin V by dual parameter display. (a) 51% of total cells and 60% of CD34<sup>+</sup> cells showing apoptosis in a patient with MDS. (b) A patient with AML showing only 2% apoptosis while CD34 is expressed in 85% of total cells.

percentage of apoptotic cells in patients having anemia and those having solid tumors without marrow involvement according to this assay was 2.7% (range, 0.1%–19%) and 3.6% (range, 0.3%–9.4%), respectively. There was no significant correlation between the percentage of CD34<sup>+</sup> cells and the percentage of apoptotic cells measured by DePsipher assay ( $R = -0.007$ ,  $P = 0.89$ , Spearman test). Examples of dot plots showing no significant apoptosis (a) and higher degree of apoptosis (b) by DePsipher assay are illustrated in Figure 4.

## Discussion

Numerous studies have demonstrated a significant increase in apoptosis in patients having MDS using different methodologies.<sup>7–10</sup> The frequency and percentage of cells showing apoptosis in these patients vary depending on the methodology used and cell population analyzed. Apoptotic cell death can be measured by various methods including terminal dUTP nick-end labeling (TUNEL), caspase, annexin V and mitochondrial membrane potential. Each assay method measures a different component or event of apoptotic process. In this study, we used mitochondrial membrane potential measurement and annexin V assay as representative for early and advanced apoptosis.<sup>11–13</sup> Mitochondria has been shown to play a pivotal role in apoptosis in a number of ways, including the release of caspase activators, changes in electron transport and loss of mitochondrial transmembrane potential.<sup>12,13</sup> DePsipher is a dye that enters the mitochondria, polymerizes when the mito-

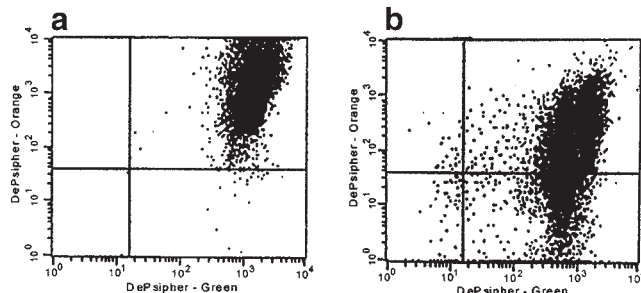
chondrial membrane potential is intact, and emits orange fluorescence; when the mitochondrial membrane potential is disturbed, the dye does not polymerize and emits green fluorescence.<sup>14</sup> These two fluorescence colors can be analyzed by flow cytometry, and this assay can be used to detect early apoptosis. Annexin V allows identification of cell surface changes that occur during the apoptotic process. The binding of annexin V to phosphatidylserine on the damaged cell surface can be measured by flow cytometry. It is important to distinguish apoptotic cells from damaged cells as a result of non-apoptotic cell injury. In this study, we used PI in the four-color staining incubation mix to exclude cells that have lost membrane integrity, such as necrotic cells that may not reflect apoptotic process. Relatively lower estimates of apoptotic cells in this study compared to other studies that did not incorporated PI in the assay may be due to the exclusion of PI-positive cells in our annexin V assay. Furthermore, some studies<sup>7</sup> used frozen samples and annexin V is significantly higher in frozen samples as compared with fresh samples.

Both mitochondrial potential and annexin V assays showed higher degree of apoptosis in RAEB-T as compared to AML. The possibility that the increase in apoptosis in MDS and RAEB-T cases could be due to increased percentage of mature and maturing cells should be considered and for this reason, we analyzed apoptosis in CD34<sup>+</sup> cells separately from mature neutrophils. Higher apoptosis was observed in both CD34<sup>+</sup> cells, as well as in CD34<sup>-</sup> cells by annexin V assay. Similarly, higher apoptosis was present in both polymorphonuclear cells and mononuclear cells assessed by mitochondrial potential assay. Thus, apoptosis is not merely a function of the percentage of blasts in the bone marrow. Our findings are consistent with those of Raza *et al*,<sup>5</sup> who demonstrated a high degree of apoptosis in bone marrow biopsies from patients with MDS.

We observed particularly increased apoptosis in CMML using mitochondrial potential. Overall, there was significant correlation between mitochondrial potential and annexin V in detecting apoptosis in CMML, as well as in all other subgroups. The disturbance in the mitochondrial potential is the earliest change in the apoptotic pathway. In contrast, annexin V positivity is a late phenomenon. The relatively higher apoptosis in CMML, which is detected by mitochondrial potential may reflect difference in the stage of apoptosis and how apoptotic cells are cleared.

Measurement of apoptosis is particularly influenced by the method of processing, time to processing, and other physiologic factors. Although we have made every effort to process samples in a similar fashion as soon as possible and many samples were analyzed in duplicate, we cannot rule out the possibility of variation due to the methodology of the assays. The high number of cases analyzed and the consistent results obtained using two different methodologies, support our conclusions.

Most published studies did not separate RAEB-T from other subgroups of MDS in their analysis. Specifically, one study that grouped RAEB-T with secondary AML (MDS-AML) showed a significantly lesser degree of apoptosis in patients having RAEB-T/MDS-AML compared with those having RA/RARS and RAEB.<sup>9</sup> Our group recently reported a significantly higher degree of apoptosis in patients having RAEB-T compared with those having AML as assessed according to their caspase 3 activity ( $P = 0.0001$ ).<sup>10</sup> In that study, the caspase 3 activity in RAEB-T patients did not differ significantly from that in patients having other MDS. In addition, RAEB-T was distinguished from AML with respect to several laboratory and clinical parameters. In particular, RAEB-T patients had a



**Figure 4** DiPsipher assay showing examples of no apoptosis in a patient with AML (a) and presence of significant apoptosis in a patient with AML (b). 99% of cells emit orange fluorescence indicating intact mitochondrial membrane potential (a), while 37% of cells showing only green without orange fluorescence indicating disturbed mitochondrial potential in a patient with MDS.

higher proliferating rate and tend to have a lower platelet count and bone marrow cellularity as compared with AML patients.<sup>10</sup>

The data presented here support the concept that MDS disease is a disease of ineffective hematopoiesis and the reason for the ineffective hematopoiesis is increased cell death in bone marrow. In contrast, AML is a disease of proliferation of immature cells (blasts). Both diseases are clonal. In MDS, leukemic cells are capable of differentiating while in AML, usually leukemic cells do not differentiate. There is certainly some overlap between MDS and AML in the level of apoptosis. Patients classified as RAEB-T may include those having AML who were detected at an early stage, and similarly some AML patients with a blast count higher than 30% may have higher degree of apoptosis, therefore biologically closer to MDS. Our data suggest that perhaps neither 20% nor 30% blasts is a magic cut-off point to distinguish AML from MDS, but overall RAEB-T is biologically closer to MDS than AML. Irrespective of the cut-off point, therapy should take into consideration the biological and cellular abnormalities in AML and MDS and exploit these abnormalities either to kill the leukemic cells or correct the environment that allow these cells to dominate. Currently, outcome of RAEB-T patients may not be significantly different from that of AML, however, as we develop new therapies that specifically target the biological and molecular abnormalities in leukemic cells, we may start seeing differences between AML and MDS. Elimination of the RAEB-T category by lumping it in with AML does not achieve any goal and does not advance our understanding or management of AML nor MDS.

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