**Intramedullary apoptosis of hematopoietic cells in myelodysplastic syndrome patients can be massive: apoptotic cells recovered from high-density fraction of bone marrow aspirates**

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A higher percentage of apoptotic cells (apoptotic index or AI) is consistently found in bone marrow (BM) biopsies compared to BM aspirates of patients with myelodysplastic syndrome (MDS). Most studies have only investigated the low-density fraction (LDF) mononuclear cells from BM aspirates following density separation for AI determination. In the present study, both LDF and high-density fraction (HDF) cells for AI were examined by electron microscopy (EM) in 10 MDS patients and 4 healthy donors. Matched BM biopsies were subjected to AI detection by in situ end labeling (ISEL) of fragmented DNA. The results indicate that in LDF and HDF cells, AI is consistently higher in MDS patients (8.5% vs 1.5%, respectively; \( P = .039 \)) compared to healthy donors (27% vs 4%, respectively; \( P = .004 \)). The BM biopsy AI was also higher in MDS patients than in healthy donors (3+ vs 0+, respectively; \( P = .036 \)). In addition, in MDS patients, more apoptotic cells were found in HDF cells than in LDF cells (27% vs 8.5%, respectively; \( P = .0001 \)). All stages of maturation, ranging from blasts to terminally mature cells belonging to all 3 lineages, were represented in the dying cells in both compartments. Using EM, typical Pelger-Huett-type cells appeared to be apoptotic granulocytes. Both LDF and HDF cells should be examined for an accurate estimation of apoptotic cells because AI would be underestimated if only the LDF cells were studied. Ultrastructural studies consistently show a higher AI in BM biopsies compared to BM aspirates despite the correction factor of HDF cells provided by AI. This may represent the actual extant state, which could conceivably be due to a higher concentration of proapoptotic signals in the biopsies. (Blood. 2000;96:1388-1392)

**Materials and methods**

BM biopsies from 10 MDS patients and 4 healthy donors were studied as part of this protocol. Peripheral blood, BM aspirate, and BM biopsies were obtained and transported on ice to the laboratory. The donors were asked to donate BM for research purposes, and prior to the procedures, they signed consent forms for the protocol approved by the institutional review board of Rush-Presbyterian-St Luke’s Medical Center to study these samples.

**Studies conducted on BM aspirates**

We directly aspirated 15-20 mL BM into a syringe containing 3 cc of 2% sodium citrate. The aspirate was separated into LDFs and HDFs using Ficoll-Hypaque 1077 density gradient solution (Amersham Pharmacia Biotech, Piscataway, NJ). The LDF cells were fixed in 3% gluteraldehyde and subjected to routine transmission EM as described below. The HDF cells were further treated with sodium chloride to lyse the red blood cells. Subsequently the cells were fixed in 3% gluteraldehyde, postfixed in osmium tetroxide, dehydrated, and embedded according to the standard methods for routine EM.8

**Studies conducted on BM biopsies**

Long-core BM biopsies were obtained from every individual and placed in saline. Under aseptic conditions, these biopsies were cut in 2 segments. One-half was placed in Bouin’s fixative and embedded in plastic using...
glycol methacrylate; 2- to 3-μm-thick sections were placed on Alcian blue-coated coverslips for detection of apoptosis by ISEL. The second half was used for EM as described later.

**Measurement of apoptosis using ISEL of fragmented DNA**

ISEL was carried out on all BM biopsies. Briefly, the cells were pretreated with sodium chloride sodium citrate (SSC) solution at 80°C and 1% Pronase (1 mg/mL in 0.15 mol/L phosphate-buffered saline [PBS]) (Calbiochem, La Jolla, CA). The sections were then incubated at 18°C with a mixture of 0.01 mol/L deoxyadenosine, deoxythymidine, and deoxyguanosine 5′-triphosphate (dATP, dCTP, and dGTP) (Promega Company, Madison, WI); 0.001 mol/L biotinylated uridine 5′-triphosphate (bio-dUTP) (Sigma Chemical Co, St Louis, MO); and 20 U/mL DNA Polymerase I (Promega Company). Incorporation of bio-dUTP was finally visualized using an avidin-biotin-peroxidase conjugate (Vectastain Elite ABC Kit; Vector, Burlington, CA) and diaminobenzidine tetrachloride. Thus, cells labeled positively for ISEL showed brown staining in their nuclei under the light microscope. The controls for these experiments were carried out as described before.9

**Interpretation of slides.** All slides were observed on a televised screen by 3 different investigators. (A.R. was an investigator for each slide.) A subjective quantitative scale was formulated to determine the degree of positivity as follows: negative, low, intermediate, and high. 6 Negative or absent indicates that there were less than 15% ISEL-positive cells; low, 1-3% or 15%-30%; intermediate, 4-5% or 31%-75%; and high, 6-8% or greater than 75%.

**Statistical analysis.** The nonparametric Mann-Whitney U test was used for comparison between the 2 parameters.

**Measurement of apoptosis using EM**

**Modified technique.** To avoid decalcification and cell deformation during the preparation process, a modified technique, which preserves the detailed morphology of the BM biopsy, was carried out. The procedure involved perfusion of the bone with 3% gluteraldehyde, further immersion for 15 minutes, and then careful teasing of the BM by decalcification. The processing for transmission EM (TEM) was carried out by using standard techniques.6 Briefly, after fixation the cells were postfixed in osmium tetroxide, treated with alcohol and propylene oxide, and embedded in Araldite (EM Sciences, Washington, PA) at 58°C for 48 hours. The biopsies were then processed for semi-thin and ultrathin sections. Semi-thin sections were postfixed in osmium tetroxide, treated with alcohol and propylene oxide, and embedded in Araldite (EM Sciences, Washington, PA) at 58°C for 48 hours. The processing for transmission EM (TEM) was carried out by using standard techniques.6 Briefly, after fixation the cells were postfixed in osmium tetroxide, treated with alcohol and propylene oxide, and embedded in Araldite (EM Sciences, Washington, PA) at 58°C for 48 hours. The biopsies were then processed for semi-thin and ultrathin sections. Semi-thin sections were stained with toluidine blue and evaluated by light microscopy. Ultrathin sections were contrasted with uranyl acetate and lead citrate and analyzed by TEM (JEOL 200, Japan).

**Table 1. Studies of apoptosis using BM aspirate and biopsy from MDS patients**

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>FAB classification</th>
<th>BM aspirate Apoptotic cells by LDF, %</th>
<th>BM biopsy ISEL</th>
<th>EM</th>
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<tr>
<td>1</td>
<td>Donor</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>4</td>
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<td>0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>2</td>
<td>5</td>
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</tr>
<tr>
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<td></td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
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<td>MDS</td>
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<td>22</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>RA</td>
<td>16</td>
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<tr>
<td>14</td>
<td>RARS</td>
<td>14</td>
<td>32</td>
<td>4</td>
</tr>
</tbody>
</table>

NA indicates not available; NE, not evaluable.

*Scale, 1 to 8.

**Figure 1. High incidence of apoptosis involving hematopoietic and stromal cells in the BM of MDS patients using ISEL.** Brown staining is noted (original magnification × 400).

**Table 2. Median AI in the different fractions of BM aspiration and biopsy from MDS patients and healthy donors**

<table>
<thead>
<tr>
<th>Median AI</th>
<th>MDS patients, no.</th>
<th>Donors, no.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM aspirate</td>
<td>10</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>LDF</td>
<td>8.5</td>
<td>1.5</td>
<td>.0399</td>
</tr>
<tr>
<td>HDF</td>
<td>27</td>
<td>4</td>
<td>.0046</td>
</tr>
<tr>
<td>BM biopsy ISEL*</td>
<td>3</td>
<td>0</td>
<td>.1</td>
</tr>
<tr>
<td>BM biopsy EM</td>
<td>4</td>
<td>0</td>
<td>.0363</td>
</tr>
</tbody>
</table>

*Indicates that these figures were measured subjectively on a scale of 0-8+.

**Results**

The present study was carried out using BM samples from 10 MDS patients and 4 healthy donors. The French-American-British (FAB) classification was used to identify the various subtypes of MDS. Of the 10 MDS patients, there were 7 cases of refractory anemia (RA), and 3 of these cases had RA with ring sideroblasts (RARS). Table 1 describes the details of the FAB type and apoptosis studies carried out on the different fractions of BM aspirate and biopsy using ISEL and EM.

**Measurement of apoptosis using ISEL**

ISEL of fragmented DNA was performed on plastic embedded BM biopsies. A distinct brown staining in the nucleus identified a cell as being engaged in DNA cleavage. Among the 10 cases studied, 2 cases showed high positivity; 2, intermediate; 4, low; and 1, no ISEL positivity. When comparing MDS patients to the healthy donors, an increase in apoptosis was noted in MDS patients (3+ in MDS patients vs 0+ in donors; P = .1). All 3 lineages of hematopoietic cells, including the myeloid, erythroid, and megakaryocytic cells, were found to be undergoing apoptosis. Furthermore, in almost every case, stromal cells were also found to be apoptotic (Figure 1).

**Measurement of apoptosis using EM**

Ultrastuctural studies were carried out on both the LDF and HDF cells of the BM aspirate in MDS patients and the donors for the
detection of apoptosis morphologically. Table 2 shows the median AI in the different fractions in MDS patients and donors. The AI was calculated by counting 500-1000 cells in randomly selected fields by EM. The overall incidence of apoptosis was found to be significantly higher in both the LDF and HDF cells in MDS patients as compared to normal BM cells. The median AI in the LDF of MDS marrows was 8.5% compared to 1.5% in healthy donors ($P = 0.0399$). The HDF cells in MDS patients showed an AI of 27% compared to 4% in donor marrows ($P = 0.0046$). However, when the rate of apoptosis between the 2 fractions within individual MDS patients was compared, a significantly higher rate of apoptosis was seen in the HDF cells compared to the LDF cells (27% versus 8.5%, $P = 0.0001$).

Morphologically, apoptosis was seen in all the lineages, with a predominance in the myeloid and erythroid lineages. In LDF cells, apoptosis was seen in the progenitor cells of the myeloid and erythroid lineages (Figure 2A,B). In HDF cells, the differentiated cells showed a higher degree of apoptosis (Figure 2C,D); however, both early and late stages of apoptosis were clearly recognizable under EM.

The normal nonapoptotic myeloblasts showed oval euchromatic nuclei, nucleoli were prominent, and a fair number of primary and secondary granules were readily found (Figure 2E). The dysplastic promyelocytes showed a low nuclear cytoplasmic ratio with the presence of Auer rods (Figure 2F). In HDF, the classical Pelger-Huet anomaly showing bilobed nucleoli was noted (Figure 2G). In the erythroid series, nonapoptotic polychromatophilic megaloblasts with the appearance of a few clumps among the chromatin beads were noted (Figure 2H).

In the apoptotic myeloblasts, initial changes in the chromatin condensation could be graphically seen (Figure 2I), thereby documenting the earliest unequivocal evidence of apoptosis. In apoptotic cells the proportion of the nuclei occupied by condensed chromatin varied with the cell type, with the early events occurring in the nucleus, followed by condensation of the cytoplasm and eventual vacuole formation. Apoptotic promyelocytes were seen with perinuclear vacuoles (Figure 2I). In HDF, apoptotic neutrophils were seen in abundance (Figure 2K), and similar observations were made in the apoptotic erythroid cells (Figure 2L). In summary, all stages of maturation were unequivocally represented among the dying cells, ranging from blasts to mature cells belonging to all the 3 lineages.
Pancytopenia, the hallmark of MDS, has long been attributed to an “ineffective hematopoiesis.” In 1990, Clark and Lampert1 published a study of 23 MDS patients whose BM biopsies demonstrated histological features of apoptosis by light microscopy in the erythroid and immature myeloid cell precursors. In 1992, Hatfill et al10 demonstrated intramedullary apoptosis in large numbers of megakaryocytes in the BM biopsies of 20 MDS patients. These observations were followed by the hypothesis of Yoshida2 in 1993 stating that apoptosis may be the mechanism for premature intramedullary cell death in MDS. The same year, we published our initial observations of excessive intramedullary apoptosis in the BM biopsies of MDS patients documented by using the ISEL technique;2 this was followed by several publications confirming the findings in larger numbers of patients over the next 2 years.11-13 Using the ISEL technique, we demonstrated large numbers of apoptotic cells in BM biopsies.11

However, we could not detect either ISEL-positive cells or low-molecular DNA fragments by conventional DNA laddering on gel electrophoresis when low-density BMMNCs from BM aspirates were studied.11 Leaving BMMNCs in short-term cultures in complete medium for 4 hours and then studying these samples for the presence of low-molecular weight DNA fragments showed an increase in AI.11 Starting with our very first publication,11 we have consistently pointed out this peculiar discrepancy between the percentage of apoptotic cells in BM aspirates and matched MDS biopsies. Ultrastructural confirmation of apoptosis in BM aspirates was provided by Bogdanovic et al15 in 1996.

In 1996, Lepelley et al12 published their results of 40 MDS patients in whom apoptosis was determined using the terminal deoxynucleotidyl transferase (TdT) incorporation of nucleotides on the 3‘-ends of DNA (TUNEL technique [TdT–mediated dUTP nickend-labeling]) in BM aspirates. These authors found no evidence of increased apoptosis in the LDF cells of MDS patients compared to donor marrows. However, when both LDF and HDF cells of BM aspirates from 7 of 40 MDS patients were subjected to short-term cultures (18 hours), a higher incidence of apoptosis was seen by the TUNEL technique in both LDF cells (4 of 7 patients) and HDF cells (1 of 7 patients). Because this percentage of apoptotic cells ranged from 4%-7% in LDF cells and 7%-24% in HDF cells and because our results in BM biopsies had yielded more than 75% of apoptotic cells in half the MDS patients studied, Lepelley et al12 raised the question of whether apoptosis is indeed a “massive” process in MDS. This question should have only been raised if the authors had examined matched BM biopsies of their MDS patients using the same techniques that we did and had found that the biopsies yielded results similar to the aspirates.

Others who have examined BM biopsies of MDS patients have confirmed our findings precisely. For example, both Hellstrom-Lindberg et al14 and Parcharidou et al15 found more than 50% apoptotic cells in BM aspirates and matched MDS biopsies. The same year, we published our initial observations of excessive intramedullary apoptosis in the BM biopsies of 20 MDS patients. These observations were followed by the hypothesis of Yoshida2 in 1993 stating that apoptosis may be the mechanism for premature intramedullary cell death in MDS. The same year, we published our initial observations of excessive intramedullary apoptosis in the BM biopsies of MDS patients documented by using the ISEL technique;2 this was followed by several publications confirming the findings in larger numbers of patients over the next 2 years.11-13 Using the ISEL technique, we demonstrated large numbers of apoptotic cells in BM biopsies.11

Ultrastructural details were very well preserved in the normal BM biopsies. Using the modified technique described earlier, the branching sinus network and intersinal hematopoietic tissue were seen. Remnants of fat cells were also noted. Both the stromal and parenchymal cells were clearly identified (Figure 3A). In contrast, the MDS marrow showed an abundance of apoptotic cells belonging to all lineages. Again, apoptosis was predominantly noted in the myeloid and erythroid lineages. The stromal cells showed minimal apoptosis by morphology. Throughout the BM biopsy, macrophages engulfing apoptotic red cells were noted (Figure 3B).

Discussion

Pancytopenia, the hallmark of MDS, has long been attributed to an “ineffective hematopoiesis.” In 1990, Clark and Lampert1 published...
patients. A lower AI could have a similar etiology as well because PBMCNs from MDS patients are not only nonapoptotic, but they have actually been shown to be more resistant to apoptosis than normal PBMCNs. Increased apoptosis and proliferation of hematopoietic cells in the biopsy compartment compared to BM aspirates could be because the levels of cytokines are much higher in biopsies than in aspirates.

Another interesting observation is that the recovery of BMMNCs is not usually high in MDS patients even though the BM is generally hypercellular. For example, it is not uncommon to recover 50-80 million BMMNCs from approximately 20 cc of citrated BM aspirate obtained from healthy donors, while the majority of BM aspirates from MDS patients yield between 20-30 million BMMNCs. Taken together, the discrepancy between the AI of BM aspirates and biopsies as well as a low recovery of BMMNCs following density separation on Ficoll-Hypaque gradient appear to indicate that the unknown but fairly substantial number of cells are unaccounted if only the LDF is examined. This study was undertaken to examine the possibility that the AI of BMMNCs was low because the apoptotic cells had migrated to the HDF cells. Indeed, that is what we found. The present study clearly demonstrates the presence of large numbers of apoptotic cells in the BM aspirates of MDS patients.

In fact, there are many more apoptotic cells in the HDF (median AI, 27% ) compared to the MNCs obtained from the LDF (median AI, 8.5%; P = .0001) in MDS patients. One of the possible reasons could be that the apoptotic cells have a tendency to shrink and sink to the bottom of the HDF. So it is very important to look at both the LDF and HDF in BM aspirate to get an accurate estimation of the AI.

References

2. Yoshida Y. Apoptosis may be the mechanism responsible for the premature intramedullary cell death in myelodysplastic syndromes. Leukemia. 1993:7:144-146.
Intramedullary apoptosis of hematopoietic cells in myelodysplastic syndrome patients can be massive: apoptotic cells recovered from high-density fraction of bone marrow aspirates

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