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Evidence for Involvement of Tumor Necrosis Factor-α in Apoptotic Death of Bone Marrow Cells in Myelodysplastic Syndromes

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We previously reported excessive apoptosis and high levels of tumor necrosis factor-alpha (TNF-α) in the bone marrows of patients with myelodysplastic syndromes (MDS), using histochemical techniques. The present studies provide further circumstantial evidence for the involvement of TNF-α in apoptotic death of the marrow cells in MDS. Using our newly developed in situ double-labeling technique that sequentially employs DNA polymerase (DNA Pol) followed by terminal deoxynucleotidyl transferase (TdT) to label cells undergoing apoptosis, we have characterized DNA fragmentation patterns during spontaneous apoptosis in MDS bone marrow and in HL60 cells treated with TNF-α or etoposide (VP16). Clear DNA laddering detected by gel electrophoresis in MDS samples confirmed the unique length of apoptotic DNA fragments (180–200 bp). Surprisingly, however, phenotypically heterogeneous population of MDS cells as well as the homogenous population of HL60 cells showed three distinct labeling patterns after double labeling—only DNA-Pol reaction, only TdT reaction, and a combined DNA Pol + TdT reaction, albeit in different cohorts of cells. Each labeling pattern was found at all morphological stages of apoptosis. MDS mononuclear cells, during spontaneous apoptosis in 4 hr cultures, showed highest increase in double-labeled cells (DNA Pol + TdT reaction). Interestingly, this was paralleled by TNF-α–induced apoptosis in HL60 cells. In contrast, VP16 treatment of HL60 cells led to increased apoptosis in cells showing only TdT reaction. The double-labeling technique was applied to normal bone marrow and peripheral blood mononuclear cells after treatment with known endonucleases that specifically cause 3′ recessed (*Bam*HI), 5′ recessed (*Pst*I), or blunt ended (*Dra*I) double-stranded DNA breaks. It was found that the DNA-Pol reaction in MDS and HL60 cells corresponds to 3′ recessed DNA fragments, the TdT reaction to 5′ recessed and/or blunt ended fragments, and a combined ''DNA Pol + TdT reaction'' corresponds to a copresence of 3′ recessed with 5′ recessed and/or blunt ended fragments. Clearly, therefore, apoptotic DNA fragments, in spite of a unique length, may have differently staggered ends that could be cell (or tissue) specific and be selectively triggered by different inducers of apoptosis. The presence of TNF-α–inducible apoptotic DNA fragmentation pattern in MDS supports its involvement in these disorders and suggests that anti–TNF-α (or anticytokine) therapy may be of special benefit to MDS patients, where no definitive treatment is yet available. Am. J. Hematol. 60:36–47, 1999.

Key words: human disorders; myelodysplastic syndromes (MDS); HL60cells; apoptosis; DNA fragmentation; in situ labeling; tumor necrosis factor-α (TNF-α); etoposide (VP16); hematopoietic disorders

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INTRODUCTION

In the past we showed that hematopoietic as well as stromal cells in the bone marrows (BMs) of patients with myelodysplastic syndromes (MDS) demonstrate excessive apoptotic death, thus forming the basis for ineffective hematopoiesis observed in these disorders [1–3]. Others have confirmed our observation of high intramedullary apoptosis in MDS [4,5]. Such a widespread incidence of apoptosis affecting all types of bone marrow cells was indicative of a possible involvement of cytokine(s) with a broad range of target cells. Using immunohistochemistry, we indeed detected high levels of tumor necrosis factor-alpha (TNF-α) correlating significantly with the levels of apoptosis detected in situ in the same BM biopsies of MDS patients [3]. In our attempts to further understand the association between TNF-α and apoptosis in MDS, in the present studies we examined the patterns of DNA fragmentation during spontaneous apoptosis in MDS and in TNF-α–induced apoptosis in HL60 cells.

Internucleosomal DNA fragmentation constitutes one of the most salient features of apoptotic cell death. The four endonucleases most prominently implicated in apoptotic DNA fragmentation are Ca^{++} Mg^{++} dependent, Mg^{++} dependent, Mn^{++} dependent, and acidic endonuclease. These endonucleases have been shown to maintain the property of internucleosomal cleavage [6–8]. No specific differences with respect to substrate requirement or products formed have been noted among different apoptotic endonucleases. The DNA fragments formed by such endonucleolytic activity that typically appear to have 5′-P and 3′-OH terminals [9–11], could be detected using two specific enzymatic reactions that label the ends of DNA fragments. One of these techniques uses DNA Polymerase (DNA Pol) or Klenow fragment of DNA Pol [10] whereas the other uses terminal deoxynucleotidyl transferase (TdT) for this purpose [9]. We have extensively used these enzymatic reactions to detect apoptosis in situ in a variety of clinical samples [1–3,12,13]. In one of these studies, we compared the labeling of apoptosis by the two enzymatic reactions performed separately in serial sections of different types of solid tumors. It was found that some tissues like breast tumors or primary brain tumors did not show labeling with DNA Pol, but showed positivity with TdT labeling [12,13]. On the other hand, tissues like nonHodgkin's lymphoma or head and neck squamous cell carcinoma showed comparable labeling by the two enzymes in serial biopsy sections [12]. Gold et al. [14] in 1994 reported that whereas DNA Pol preferentially labels necrosis, TdT was more specific for apoptosis. Contrastingly, we found that both methods label apoptosis as well as necrosis except that necrosis is labeled with an extremely low intensity by both methods [12,15].

To explain the tissue-specific differences in labeling with DNA Pol and TdT noted in our studies, we hypothesized that the apoptotic DNA fragments may have a unique length of integral multiples of 180–200 bp, but have differently staggered ends—3′ recessed, 5′ recessed, or blunt—perhaps reflecting the specificity of the endogenous endonuclease(s) involved. To test this postulate we developed an in situ double-labeling technique in which the DNA-Pol reaction is performed first, followed by the TdT reaction. Using this technique, as described in the present paper, we have now been able to confirm our hypothesis and characterize and compare the ends of apoptotic DNA fragments in spontaneous apoptosis in MDS with that induced by TNF-α in HL60 cells. The findings of these studies reported in the present paper highlighting their therapeutic implications suggest a novel approach to the treatment of MDS.

MATERIALS AND METHODS

Patients

Sixteen bone marrow aspirate specimens from 15 patients with a confirmed diagnosis of MDS [diagnosed according to the French-American-British classification as refractory anemia (RA)-7, RA with ringed sideroblasts (RARS)-3, RA with excess of blasts (RAEB)-3, RA with excess of blasts in transformation (RAEBt)-1, and Chronic myelomonocytic leukemia (CMMoL)-1] were studied for the incidence of spontaneous apoptosis. One MDS patient (RARS) was studied on two occasions. Six normal bone marrows from healthy donors were studied for comparison. The protocols, MDS 90 02 and MDS 95 01, under which clinical specimens were obtained, were approved by the local Institutional Review Board (IRB) and informed consent was obtained from the donors. Bone marrow aspirates were subjected to Ficoll-Hypaque density gradient centrifugation to separate mononuclear cells. After confirming the viability by trypan blue dye exclusion test, cells were suspended (1×10^6 cells/ml) in RPMI-1640 medium (GIBCO-BRL Life Technologies Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM/l glutamine (complete medium). One aliquot was used for 0 hr tests and the other was incubated for 4 hr at 37°C in the presence of 5% CO_2. At both time points, cells were washed and divided into two aliquots. One was fixed on alcian blue coated coverslips using 4% buffered paraformaldehyde (pH 7.1) overnight at 4°C and stored in 70% ethanol until in situ tests for detection of apoptosis were performed. The other aliquot was used to extract DNA following cell lysis in guanidine isothiocyanate (GITC) as described previously [12].

HL60 Cell Cultures

HL60 cells were maintained in RPMI-1640 complete medium supplemented with 20% FBS. Freshly harvested cells, after confirming viability by trypan blue dye exclusion test, were resuspended (1×10^6 cells/ml) in complete medium and treated with human recombinant TNF-α (0.01 ng/ml; Promega Inc., Madison, WI) for 8 hr or with VP16 (etoposide—35 μM/l; Bristol Laboratories, Princeton, NJ) for 4 hr. As both these agents were dissolved in RPMI 1640, cells suspended in plain complete medium and incubated for a maximum interval, i.e., 8 hr served as controls. The dose of TNF-α was chosen based on the ED_{50} dose (0.016 ng/ml) suggested by the manufacturer and according to our initial dose response experiments, which showed a linear apoptotic response at 0.01 ng/ml until 8 hr (data not shown). The concentration and incubation time of VP16 was based on our previous studies [12]. Cells were fixed on alcian blue coated coverslips using 4% paraformaldehyde before and after the designated time of incubation, and stored in 70% ethanol for in situ detection of apoptosis. Experiments were repeated four times and the results represent the average of four experiments.

Single Labeling of Apoptosis With DNA Pol I or TdT

These techniques to detect apoptotic cell death in situ have been described in detail in previous reports by us [1–3,12] and others [9,10]. Briefly, cells were rehydrated, postfixed in 0.23% periodic acid, and pretreated with SSC solution (30 mM/l sodium citrate + 0.3 M/l sodium chloride, pH 7.0) at 80°C for 20 min. The cells were then treated with either a mixture of deoxyribonucleotides, one of which was biotinylated (11 bio-dUTP; Sigma, St. Louis, MO) and E. coli DNA Pol I (Promega, Madison, WI) or with 11 bio-dUTP and TdT (Promega). Incorporation of labeled nucleotide was visualized using avidin-biotin-horseradish peroxidase (Vectastain Elite ABC kit, Vector, Burlingame, CA) and diaminobenzidine tetrahydrochloride (DAB). Dark brown nuclear staining indicated cells that were undergoing apoptotic death.

Double Labeling With DNA Pol I and TdT

Cells were rehydrated, postfixed with 0.23% periodic acid, and pretreated with SSC solution. Subsequently, cells were washed in buffer A, pH 7.5 (50 mM/l Tris hydrochloride, 5 mM/l magnesium chloride, 10 mM/l β mercaptoethanol, and 0.005% bovine serum albumin, fraction V) (Sigma) and treated with a cocktail of four deoxynucleotides, one of which was biotinylated (bio dUTP) and DNA Pol I prepared in buffer A [0.01 mM/l of dATP, dCTP, and dGTP (Promega) + 0.001 mM/l bio dUTP (Sigma) and 20 U/ml *Escherichia coli* DNA Pol I (Promega)] at 18–19°C for 2 hr. The DNA-Pol reaction was visualized with ABC and DAB giving brown nuclear staining. Subsequently, cells were washed with buffer B, pH 6.8 (100 mM/l sodium cacodylate, 0.1 mM/l dithiothreitol, 5 mM/l cobalt chloride) (Sigma) and treated with a mixture of TdT (10 U/ml) and 0.001 mM/l digoxigenin-dUTP (Boehringer Mannheim, Germany) prepared in TdT buffer. The reaction was performed at 37°C for 1 hr and was terminated by immersing the coverslips in SSC solution. Incorporation of digoxigenin-dUTP was detected using antidigoxigenin Fab fragment conjugated with alkaline phosphatase (1:100 diluted; Boehringer Mannheim, Germany). The cells were then immersed in a solution freshly prepared as follows: Naphthol As-Mx Phosphate (20 mg; Sigma) was dissolved in 2 ml of N-N′-dimethylformamide (Sigma) and this was added to 100 ml of 0.1 M/l Tris buffer, pH 8.2, at 20°C. Next, 0.1 ml of 1 M/l levamisole was added to the solution to inhibit endogenous alkaline phosphatase, followed by 100 mg of Fast blue BB salt (Sigma). This mixture was stirred for 2 min and then filtered. The coverslips were then immersed in this solution for 10–12 min. The coverslips were finally washed in distilled water and mounted in fluoromount. Positive TdT reaction thus stained nuclei blue.

Controls for In Situ Labeling Procedures

Specimens treated with the in situ end labeling reaction mixtures prepared in respective buffers with labeled nucleotide (and nucleotides for DNA Pol), but devoid of the enzyme (DNA Pol or TdT), served as negative assay controls.

Treatment of normal human bone marrow and peripheral blood mononuclear cells with known endonucleases. Normal bone marrow was collected from a resected rib procured during a thoracic surgical procedure and peripheral blood was obtained from a healthy donor under IRB approved protocols and with donors' written consent. Mononuclear cells were separated by density centrifugation and fixed on alcian blue coated coverslips with 4% paraformaldehyde and stored in 70% ethanol. The cells were rehydrated, postfixed with 0.23% periodic acid and pretreated with SSC solution as described above. At this point cells were subjected to differential treatments with known sequence specific endonucleases (Boehringer Mannheim) in respective reaction buffers provided along with the enzymes and at the overdigestion concentrations recommended by the manufacturer as follows:

1. *Bam*HI (causes 3′ recessed breaks with 5′ overhangs—5′-G↓GATCC-3′) at 40 U/100 μl for 16 hr at 37°C.
2. *Pst*I (causes 5′ recessed breaks with 3′ overhangs—5′-CTGCA↓G-3′) at 120 U/100 μl for 16 hr at 37°C.

3. *Dra*I (causes blunt ended breaks—5′-TTT↓AAA-3′) at 120 U/100 μl for 16 hr at 37°C.
4. *Pst*I (120 U/100 μl) for 16 hr at 37°C followed by *Bam*HI (20 U/100 μl) for 2 hr at 37°C.
5. Mixture of *Pst*I + *Dra*I (120 U/100 μl each) for 16 hr at 37°C.

At the end of treatments, cells were washed thoroughly with phosphate buffered saline and continued with double labeling as described above. Experiments were repeated to confirm the results.

Determination of labeling index. Every specimen was carefully observed under light microscopy and 1–2 thousand cells from several randomly selected 100× objective fields were counted to determine the percent of labeled cells in each case. The significance of differences in the mean percent labeling indices in various test groups was determined by the Student's *t* test. Also, the paired *t* test was used to determine the significance of differences in relative percent increase in labeling index of individual labeling patterns within each study group.

RESULTS

Spontaneous Apoptosis in MDS

Our earlier studies showed an excessive incidence of spontaneous apoptosis in the bone marrows of MDS patients in which a significant number of mononuclear cells from the bone marrow aspirates of these patients underwent apoptosis in 4-hr cultures in complete medium containing 10% serum [1,2]. Therefore, in the present study comparative labeling of Ficoll-separated mononuclear cells from 16 MDS aspirates and six normal aspirates, with DNA Pol and TdT was examined at 0 and 4 hr following incubation in vitro in a complete medium. Staining was performed after paraformaldehyde fixation at each time point. As shown in Figure 1, the mean labeling index of MDS cells at 4 hr by either enzymes was significantly higher than that at 0 hr. Furthermore, the labeling by the two enzymes at each time point was comparable (0.8% ± 0.4% at 0 hr vs. 3.9% ± 0.9% at 4 hr, n = 16, $P = 0.004$, by DNA Pol, and 1.3% ± 0.4% at 0 hr vs. 3.8% ± 1.0% at 4 hr, n = 10, $P = 0.046$, by TdT). Interestingly, normal cells also showed a marginal but significant increase in labeling index in 4 hr (0.1% ± 0.07% at 0 hr vs. 0.8% ± 0.1% at 4 hr, n = 6, $P = 0.001$, by DNA Pol, and 0.2% ± 0.08% at 0 hr vs. 1.5% ± 0.2% at 4 hr, n = 5, $P = 0.005$, by TdT). It is evident that at 4 hr, the labeling indices of MDS cells were 2–3 times higher than those of normal cells ($P = 0.003$ for DNA Pol and $P = 0.05$ for TdT), hence confirming the increased propensity of MDS bone marrow cells to undergo spontaneous apoptosis in vitro.

We subsequently performed our newly developed enzymatic double labeling with DNA Pol/diaminobenzidine (brown staining) and TdT/fast blue (blue staining) systems, at the two time points on nine MDS and five normal specimens. Three distinct labeling patterns were observed both in MDS and normal specimens—cells with nuclei stained only brown (only DNA-Pol reaction), only blue (only TdT reaction), and double labeled (DNA Pol + TdT reaction). Interestingly, as depicted in Figure 2, each labeling pattern was found at all phases of apoptosis ranging from the early-stage nuclear margination to intermediate-stage chromatin condensation and clumping, to the end-stage karyorrhexis, indicating the maintenance of labeling pattern throughout the process of apoptosis at a single cell level. The increase in mean labeling index in 4 hr, for each individual pattern was significant in MDS (Fig. 3a). In contrast, normal cells did not show a significant increase in individual patterns, but the increase in total index reached statistical significance (Fig. 3b).

Parallel to these experiments, DNA fragmentation was also studied by agarose gel electrophoresis (MDS, n = 8 and normal, n = 2). As illustrated in Figure 4, at 0 hr neither the MDS specimen (lane 2) nor the normal cells (lane 5) showed low molecular weight DNA fragments. However, after 4 hr MDS cells showed an intensely

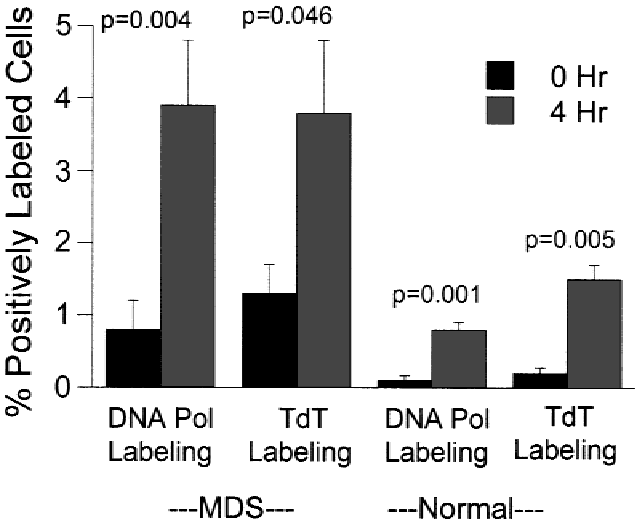

Fig. 1. Comparative detection of apoptosis in MDS and normal cells using DNA Pol or TdT single labeling: Bone marrow aspirate mononuclear cells from MDS and normal subjects were incubated in RPMI 1640 containing 10% FBS for 4 hr. Spontaneous apoptotic death was detected in these cells following fixation in 4% buffered paraformaldehyde, using single labeling with DNA Pol or TdT and bio-dUTP. Percent positively labeled cells (dark brown staining in the nuclei) were determined in each case. By either technique, the labeling index was significantly higher at 4 hr in MDS as well as in normal, but those in the former were 2–3 times higher than the latter, indicating higher propensity of MDS cells to undergo spontaneous apoptosis. No significant difference was noted in the labeling efficiencies of the two enzymes at either time points within each group.

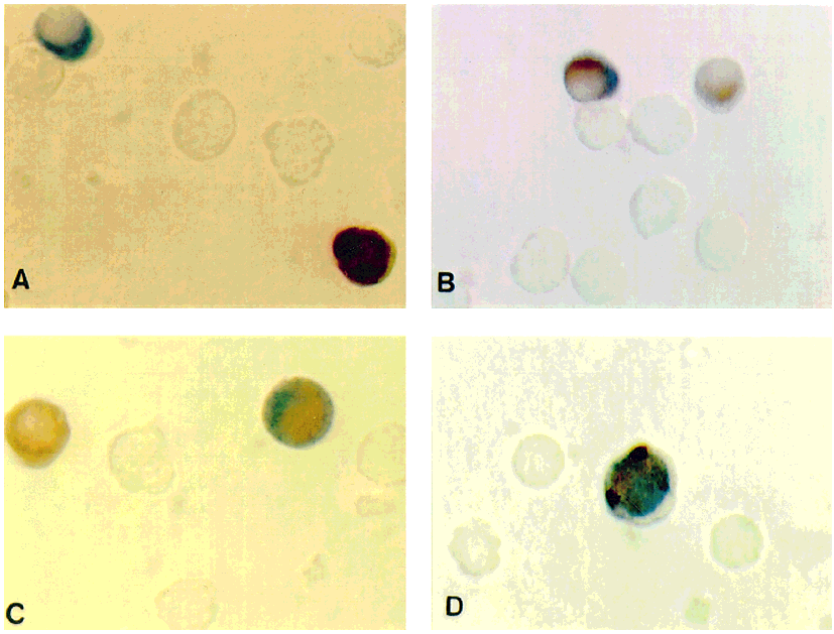

Fig. 2. Double labeling of bone marrow aspirate mononuclear cells with DNA Pol/biotin-dUTP/diaminobenzidine (brown staining) and TdT/digoxigenin-dUTP/Fast blue (blue staining): As depicted here, both in MDS and normal specimens, three distinct labeling patterns were recognized: only-brown, only-blue, and double labeled. Furthermore, interestingly enough, each pattern was seen at early as well as late stages of apoptosis ranging from nuclear margination at early stage to larger clumping and fragmentation at late stages, e.g., double-labeled cells at early (B), intermediate (C), and late (D) stages or brown labeling at early (C) and late (A) stages seen in these micrographs. Original magnification, ×1000.

stained, characteristic ladder of low molecular weight DNA fragments (lane 3), whereas the two normal specimens studied showed only a faint ladder (lane 6). One of the MDS specimens studied showed laddering at 0 hr also. The presence of clear laddering and lack of smearing in each case shows the absence of necrosis and confirms apoptosis.

TNF-α Vs. VP16-Induced Apoptosis in HL60 cells

DNA fragmentation patterns were also studied in HL60 cells induced to undergo apoptosis by treatment with TNF-α (0.01 ng/ml for 8 hr) or VP16 (0.35 μM/l for 4 hr). Cells incubated with vehicle only (culture medium) for the highest incubation period of 8 hr, served as controls. The experiments were repeated four times. Controls however were available from three experiments. DNA Pol/TdT double labeling was performed after paraformaldehyde fixation of cells before and after treatment in each experiment. The results described here represent the average of four experiments. Surprisingly, like MDS or normal cells, even the HL60 promyelocytic cells showed three distinct labeling patterns in each group. In the untreated group, during the 8-hr incubation, there was a slight increase in cells with only-DNA Pol reaction, a notable increase in cells with only-TdT reaction, and virtually no increase in double-labeled cells (Fig. 5a). On the other hand, TNF-α treatment induced an appreciable increase in the number of cells with only-DNA Pol reaction, virtually no increase in cells with only-TdT reaction, and a significant increase in double-labeled cells (DNA Pol + TdT reaction) and in total labeling index (Fig. 5b). In contrast, VP16 treatment showed the highest labeling indices with a remarkable increase in cells with only-TdT reaction and only a marginal increase in the other two patterns (Fig. 5c), with a significant increase in the total labeling index. Thus, at the end of the designated period of incubation, among individual labeling patterns, untreated cells and VP16-treated cells showed the highest labeling indices in cells with only-TdT reaction, whereas TNF-α treated cells showed the highest indices in double-labeled cells (DNA Pol + TdT reaction).

Comparison of Relative Percent Increase in Different Labeling Patterns in MDS, Normal, and HL60 Cells

Considering the total net increase in percent positively labeled cells as 100%, relative percent increase in individual pattern was calculated in each case. Figure 6 compares the relative percent increase in individual labeling patterns during spontaneous apoptosis of MDS and normal cells, and in HL60 cells with or without treatment. Normal cells showed a comparable increase in the three labeling patterns (only-DNA Pol reaction—30.6% ± 19.5%; only-TdT reaction—39.8% ± 18.8%, and double labeled (DNA Pol + TdT reaction)—29.6% ± 11.2%, $n = 5$). On the other hand, MDS cells showed twice as much increase in double-labeled cells and in cells with only-DNA Pol reaction as compared with the cells with only-TdT reaction (39.2% ± 6.8%, $P = 0.08$ and 38.6% ± 5.3%, $P = 0.06$ vs. 22.3% ± 6 % respectively, $n = 9$). Surprisingly, TNF-α–treated HL60 cells exhibited a similar pattern to spontaneous apoptosis in MDS, showing the highest increase in double-labeled cells and the

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