



## Expression and activity of caspases 1 and 3 in myelodysplastic syndromes

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**Myelodysplastic syndromes (MDS) are characterized by abnormal growth of committed progenitors in clonogenic assay, with reduced number of colonies and decreased colony/cluster ratio. It has been suggested that excessive apoptosis is the cause of marrow failure in MDS. We studied the expression of caspase-1 (interleukin-1 $\beta$ -converting enzyme, ICE) and caspase-3 (CPP32/apopain) in marrow mononuclear cells, and the growth pattern of committed progenitors in a series of 83 MDS cases. The percentage of apoptotic cells as detected by TUNEL technique, and the percentage of caspase-3-positive cells were significantly higher in refractory anemia (RA) and RA with ringed sideroblasts (RAS) than in chronic myelomonocytic leukemia (CMML), refractory anemia with excess of blasts (RAEB) and RAEB in transformation (RAEB-T). Spontaneous growth of CFU-GM was associated with a higher percentage of blasts, and with a lower expression of caspase-3 and caspase-1. The yield of CFU-E, BFU-E, and CFU-GM (in the presence of growth factors) was decreased by comparison to normal marrow, but large individual differences were observed in all cytological categories. Inhibition of caspase-1 and caspase-3 activities by specific inhibitors resulted in a significant increase of the production of all types of colonies (up to 50-fold of control). In the presence of caspase-3 inhibitor, the number of BFU-E and CFU-E was in the range of normal values in most cases of RA and RAS. In addition, caspase-1 and -3 protease activities were detectable by fluorogenic assay in all cases studied. Western blot analysis confirmed the expression of caspase-3, including the cleaved (activated)-p17 form in most cases of RA/RAS analyzed. It is concluded that caspase-3 is implicated in the increased apoptosis observed in MDS and that inhibition of its activity can restore at least partially the growth of committed progenitors. *Leukemia* (2000) 14, 2045–2051.**

**Keywords:** myelodysplastic syndromes; apoptosis; caspase; committed progenitors

### Introduction

Myelodysplastic syndromes (MDS) are a group of clonal diseases characterized by inefficient hematopoiesis with morphological evidence of myeloid dysplasia and abnormal growth of marrow committed progenitor cells.<sup>1,2</sup> The pathogenesis of MDS is unclear. Because of the apparent contradiction between a frequently increased marrow cellularity and peripheral blood cytopenias, it has been hypothesized that an excess of programmed cell death or apoptosis might occur in MDS marrow cells.<sup>3,4</sup> Indeed, an increased rate of apoptosis has been observed in MDS cells by morphological,<sup>3,5</sup> DNA-labeling techniques<sup>6–9</sup> or Annexin V techniques,<sup>10</sup> although the percentage of apoptotic cells varies according to the technique and the type of cells studied. Interestingly, in one study apoptosis assessed by *in situ* end labeling (ISEL) was also observed in stromal cells.<sup>6</sup> The Fas-dependent pathway of apoptosis could be implicated as an excess of FAS expression on CD34<sup>+</sup> cell of MDS has been reported.<sup>11</sup>

Apoptosis is a complex process subject to regulation by a group of proteins known as Bcl-2 family proteins, after the name of the first described member.<sup>12,13</sup> Bcl-2-related proteins control the activation of specific proteins with cystein-protease activity. Now termed caspases (cystein aspartate specific protease),<sup>14</sup> these proteins appear to play a major role in the apoptotic process (reviewed in Ref. 15). This growing family of proteins includes interleukin-1 $\beta$ -converting enzyme (ICE) or caspase-1, also involved in the cleavage of pro-IL-1 $\beta$  into the active cytokine,<sup>16,17</sup> and CPP32/apopain or caspase-3,<sup>18,19</sup> which appears to be the key effector protein. Little is known about the expression and role of caspases in MDS. An increased synthesis of the active form of IL-1 $\beta$  by MDS mononuclear marrow cells in short-term culture has been reported, which was blocked by a specific inhibitor of caspase-1.<sup>20</sup> This suggests a possible role for this caspase in both the proliferation (by autocrine stimulation) and the apoptosis of progenitor cells.

In this paper, we investigated the effects of caspase inhibitors on the growth of committed progenitors from 83 MDS samples. In addition, expression of CD34, Bcl-2, and caspases-1 and -3 was studied by flow cytometry. Caspase activity was also assessed in some samples by a fluorometric study, together with the expression of activated (cleaved) form of caspase-3 by Western blot analysis.

### Materials and methods

#### *Patients and cell preparation*

Bone marrow aspirates were obtained from 83 consenting adult patients with MDS. According to the French–American–British (FAB) recommendations,<sup>21</sup> diagnoses were: refractory anemia with excess of blasts (RAEB) in 27 cases; RAEB in transformation (RAEB-T) in six cases; chronic myelomonocytic leukemia (CMML) in eight cases; refractory anemia (RA) in 21 cases; and refractory anemia with ringed sideroblasts (RAS) in 21 cases. Controls were performed on normal samples obtained with their consent from allogeneic marrow transplant donors.

Mononuclear cells (MNC) were separated by Ficoll sedimentation, washed twice with phosphate-buffered saline (PBS), resuspended in RPMI 1640 (Eurobio, Les Ulis, France) and incubated for 2 h at 37°C. Non-adherent cells were then recovered.

#### *Clonogenic assays*

Non-adherent MNC ( $0.2 \times 10^5$ ) were mixed with 0.7 ml methylcellulose (0.8%) supplemented with StemGEMbio1d medium containing IL-3, IL-6, SCF, erythropoietin, G-CSF, GM-CSF and IL-11, (StemGEM, Villejuif, France) and plated on to 24-well bottom plates (Nunc, Naperville, IL, USA). Cultures

were incubated for 7 or 14 days at 37°C in fully humidified air containing 5% CO<sub>2</sub>. Colonies (>50 cells) and clusters (5–50 cells) were enumerated by two independent observers. Caspase inhibition studies were performed by adding to the culture medium 5 μmol/l of caspase-1 inhibitor (Cell permeable YVAD-cho; BIOMOL, Plymouth Meeting, PA, USA) or 5 μmol/l of caspase-3 inhibitor (cell permeable DEVD-cho; BIOMOL).

### Apoptosis assay

Genomic DNA strand breaks characteristic of apoptosis were labeled by terminal deoxynucleotidyl transferase (TdT) using an *in situ* cell death detection kit (ApoDETEK; ENZO Diagnostics, Farmingdale, NY, USA) according to the manufacturer's instructions. Briefly, cytocentrifuge samples containing 50 000 cells were fixed in acetone. After washing in PBS, two drops of equilibration buffer were added and incubated for 5 min at 37°C. The cells were then exposed to TUNEL reaction mixture containing the label reagent (Bio-16-dUTP in reaction buffer) and TdT enzyme, for 60 min at 37°C in a humidified chamber. The ENZO Simply Sensitive Alkaline Phosphatase-NBT/BCIP test was used for the detection of biotin-labeled nucleic acids. Cells were incubated with the streptavidin-biotinylated alkaline phosphatase complex for 20 min, washed and newly incubated with the NBT/BCIP reaction mixture for 15 min. After washing, the red counterstain was added for 2 min. Positive reactivity was indicated by blue–purple deposits.

### Immunostaining and flow cytometry

The staining of intracellular antigens was performed with the Cell Permeabilization Kit (Harlan Sera-lab, Loughborough, UK). Cells ( $0.5 \times 10^6$ ) were resuspended in PBS and incubated for 15 min at room temperature with 100 μl of the fixative reagent A. After washing, the cells were incubated with 100 μl of the permeabilizing reagent B and 10 μl of the antibody at the appropriate titration for 40 min and washed twice with PBS-bovine serum albumin (1%). Cells were then incubated at room temperature with a polyclonal rabbit antibody to caspase-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a monoclonal mouse antibody (MoAb) to caspase-3 (Transduction Laboratories, Lexington, KY, USA). Labeled cells were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit (Caltag Laboratories, San Francisco, CA, USA) or anti-mouse (Silenus AMRAD, Melbourne, Australia) antibodies for 15 min at room temperature and washed twice. Finally the cells were resuspended in 1% paraformaldehyde prior to flow cytometry analysis.

Controls were performed with an isotypic MoAb (caspase-3) and with rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) (caspase-1).

Bcl-2 expression was analyzed using a FITC-conjugated anti-Bcl-2 monoclonal antibody (Dakopatts) after the same permeabilization procedure.

CD34 expression was assessed by direct immunofluorescence (IF) using FITC or phycoerythrin (PE)-conjugated MoAb (581, Immunotech, Marseille, France).

Flow cytometry analysis was performed with an Epics XL cytometer (Coulter, Hialeah, FL, USA). At least 10 000 events were analyzed. The results were expressed as percentage of positive cells, and as fluorescence intensity, assessed as mean

equivalent of soluble fluorescence (MESF), by reference to calibrated beads (Immunobrite; Coulter).

### Western blot

Studies were performed as previously described.<sup>22</sup> U937 cells treated with 50 μM etoposide for 6 h were used as positive control for caspase-3 activation. After Ficoll separation, cells were washed twice in PBS, lysed in boiling lysis solution (1% SDS, 1 mM Na-vanadate, 10 mM Tris pH 7.4), in the presence of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 μg/ml pepstatin, 10 μg/ml aprotinin, 5 μg/ml leupeptine). The viscosity of the samples was reduced by several passages through a 26-gauge needle. Thirty microgram proteins were incubated in a loading buffer (125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol and 0.003% bromophenol blue), separated by 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electroblotted to PVDF membrane (BioRad, Ivry sur Seine, France). After blocking non-specific binding sites overnight by 5% non-fat milk in TPBS (PBS, Tween-20 0.1%), the membrane was incubated for 2 h at room temperature with anti-human procaspase-3 monoclonal antibody (Transduction Laboratories) or anti-human caspase-3-p17 rabbit polyclonal antibody (kindly provided by Dr D Nicholson, Merck Co, Quebec, Canada) which was used especially to detect procaspase-3 activation in apoptotic cells. After two washes in TPBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min at room temperature and then washed twice in TPBS. Immunoblot was revealed using enhanced chemiluminescence detection kit (Amersham, Les Ulis, France) by autoradiography.

### Fluorogenic assays

Ficoll-separated cells were washed twice with PBS. A pellet of  $5 \times 10^6$  cells was resuspended in 200 μl of buffer lysis solution consisting of 25 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF (Sigma, St Louis, MO, USA) and 20 μg/ml of cocktail inhibitor proteases (Boehringer Mannheim, Mannheim, Germany), and homogenized. Cells were lysed by four cycles of freezing in liquid nitrogen for 30 s and thawing at 37°C for 1 min. After centrifugation at 17 000 g for 45 min at 4°C, the resulting supernatant was used as the soluble cytosolic fraction and stored at –80°C in multiple aliquots. The protein concentration was estimated by the DC BIO-RAD assay kit according to the manufacturer's instructions (BIO-RAD, Hercules, CA, USA).

Caspase-3 protease activity was measured by cleavage of a model fluorescent substrate Ac-DEVD-AMC (Acetyl-Asp-Glu-Val-Asp-7-amino-4 methylcoumarin) (Bachem, Bobendorf, Switzerland) that mimics the known cleavage site of PARP (DEVD/G) for which caspase-3 shows the highest affinity.<sup>19</sup> Caspase-1 protease activity was detected in the same conditions using the fluorogenic tetrapeptide Ac-YVAD-AMC (Bachem), for which this caspase shows the highest affinity.<sup>23</sup> Fluorescence was measured in a Kontron fluorometer (Kontron Instruments, Everett, WA, USA) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Aliquots containing 100 μg of cytosolic protein were incu-

bated with 100  $\mu\text{M}$  of either fluorogenic substrate for 15 min at room temperature in a 1 ml final buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA and 1 mM EGTA. Fluorescence was then measured each minute for 30 min. The amount of fluorochrome released was determined by comparison to a 0–1400 pmoles standard curve prepared in the same buffer. The caspase activity was finally defined in pmoles/min/mg of proteins.

To test the *in vitro* effects of caspase inhibitors, some cell lysates were preincubated for 30 min at room temperature with Ac-YVAD-cho or Ac-DEVD-cho (BIOMOL) at 100  $\mu\text{M}$  (final concentration) before measuring the proteases activities with YVAD-AMC or DEVD-AMC.

### Statistical analysis

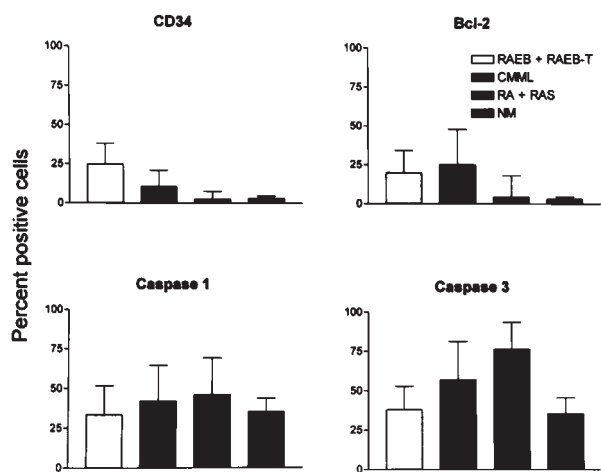
Differences between mean percentages of positive cells were compared by non-parametric tests (Mann–Whitney or Kruskal–Wallis tests according to the number of groups). Paired tests were used for assessing the effects of caspase inhibitors.

## Results

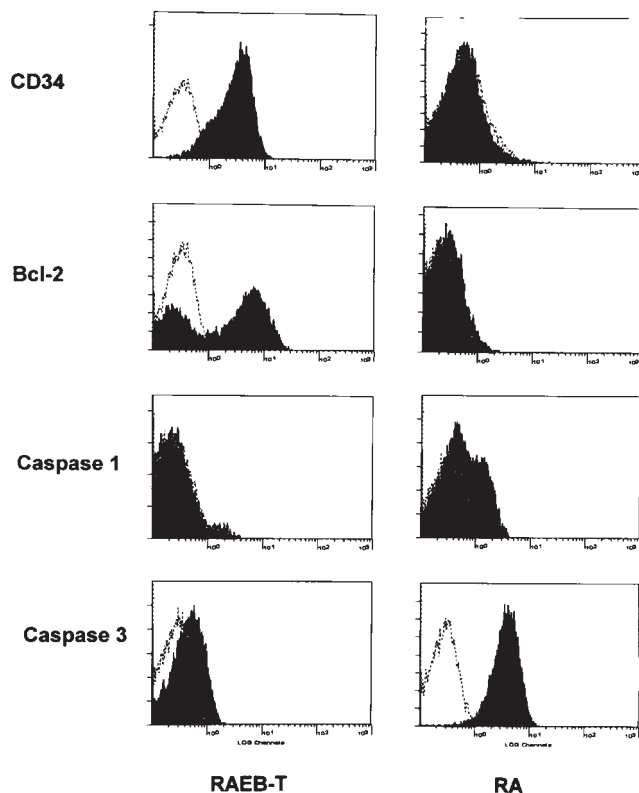
### Expression of caspases-1, 3 and of Bcl-2 and CD34

A large heterogeneity was observed regarding the percentage of positive cells and the intensity of fluorescence. In the total population, caspases were usually detected in a significant percentage of cells (mean number of positive cells: 41% for caspase-1, 59% for caspase-3) whereas Bcl-2 and CD34 expression was more heterogeneous (mean 12% and 12.5% positive cells, respectively).

The distribution of the Bcl-2, CD34 and caspases (assessed as percent positive cells) according to FAB subtype is presented in Figure 1. Representative cytograms of two cases are shown in Figure 2. Bcl-2 and CD34 were detected in a higher percentage of cells in RAEB and RAEB-T when compared to RA and RAS ( $P < 10^{-5}$ ), or to normal marrow. Caspase-3 was expressed in a significantly higher percentage of cells in RA and RAS, and CMML than in RAEB and RAEB-T



**Figure 1** Expression of Bcl-2, CD34 and caspase-1 and caspase-3 by flow cytometry, in mononuclear cells of normal bone marrow and MDS according to FAB classification. Protein expression is assessed by percent of positive cells.



**Figure 2** Cytofluorimetric analysis of Bcl-2, CD34, and caspase-1 and -3 expression in two representative cases of MDS. Isotypic controls are shown by dotted lines.

( $P < 0.005$ ), or normal marrow. Accordingly, the percentages of blasts and of CD34-positive cells were inversely correlated with those of caspase-3-positive cells ( $r^2 = 0.35$  and  $0.25$ , respectively,  $P < 10^{-4}$ ).

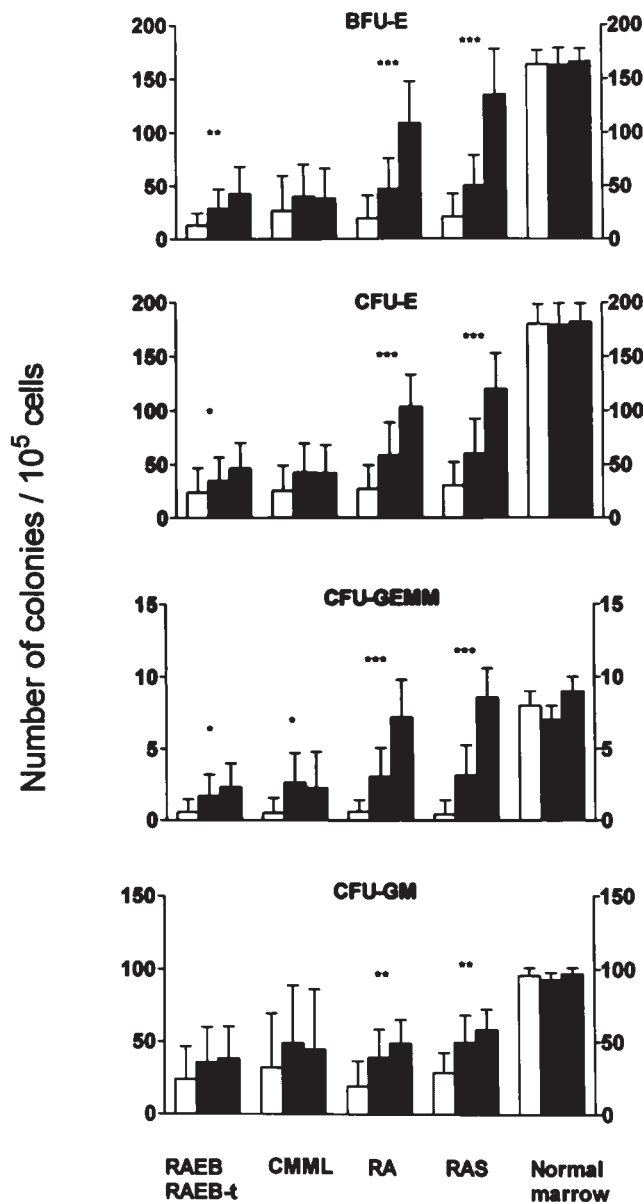
Karyotype was available in 68 cases, and normal in 35 cases. There was no significant correlation of Bcl-2 proteins or caspase expression with normal/abnormal karyotype, or with any specific anomaly, including 5q– (seven cases), 20q– (four cases) or other anomalies of chromosomes 5 or 7 (14 cases).

### Clonogenic assays

A large heterogeneity between the number of each type of progenitor was observed, in all FAB subtypes. Spontaneous growth (in the absence of colony-stimulating factor) of CFU-GM was observed in 41 samples, while no spontaneous growth was observed in 42 cases. This phenomenon was more frequent in RAEB or RAEB-T (20 and five cases, respectively, vs seven and one without spontaneous growth) than in RA and RAS (seven and six cases vs 14 and 15,  $P < 0.001$  by Fisher's exact method). The percentage of CD34 and Bcl-2-positive cells was therefore higher in cases with ( $18.6 \pm 20.5$  and  $18.3 \pm 15.9$ , respectively) than in those without spontaneous growth ( $6.2 \pm 9.3$  and  $5.9 \pm 9$ ,  $P < 10^{-3}$ ), whereas it was lower for caspase-3 ( $49.3 \pm 21.8$  vs  $69.3 \pm 24$ ,  $P < 10^{-3}$ ).

In the presence of growth factors, there was no particular pattern of colony growth associated with FAB subtype, cytogenetics or apoptosis proteins expression, but the number of any type of colony was usually low, with a decreased colony/cluster ratio. The effects of addition of caspase-1 and

-3 inhibitors are presented in Figure 3. The number of all types of progenitors was significantly increased in RAEB, RAEB-T, RA and RAS in the presence of caspase inhibitors, when compared to control. Moreover, in RA and RAS, the increase in CFU-E, BFU-E and CFU-GEMM was significantly greater in the presence of caspase-3 inhibitor than in the presence of caspase-1 inhibitor ( $P < 10^{-2}$ ). The ratio colony/cluster at day 7 was also significantly increased ( $P < 10^{-3}$ ) (Table 1). Caspase inhibitors had no significant effect on CMML samples, but the number of cases studied may be too small to reach a conclusion. There was also no detectable effect of caspase inhibitors on the number of any type of colonies recovered from normal marrow.



**Figure 3** Number of colonies CFU-E, BFU-E, CFU-GEMM, CFU-GM after 14 days of clonogenic cultures for normal and MDS mononuclear cells bone marrows, in three different experimental conditions. Open boxes: without caspase inhibitor; hatched boxes: with caspase-1 inhibitor YVAD-cho; black boxes: with caspase-3 inhibitor DEVD-cho.

**Table 1** Mean colony/cluster ratio  $\pm$  s.d. in normal marrow and FAB MDS sub-types (RAEB and RAEB-T pooled) in the presence of caspase inhibitors. CFU-GM colonies ( $>50$  cells) and clusters (5–50 cells) were enumerated at day 7

	Control	Caspase-1 inhibitor (YVAD-cho 5 $\mu$ M)	Caspase-3 inhibitor (DEVD-cho 5 $\mu$ M)
Normal marrow	13.3 $\pm$ 3.5	12.7 $\pm$ 5	12.2 $\pm$ 6.8
RAEB(T)	1.6 $\pm$ 3.2	2.4 $\pm$ 3.5	3.9 $\pm$ 5.5 <sup>a</sup>
CMML	1.3 $\pm$ 2	5.3 $\pm$ 7.7	7.2 $\pm$ 15.2 <sup>a</sup>
RA	0.8 $\pm$ 1.2	2.5 $\pm$ 2.1 <sup>a</sup>	4.7 $\pm$ 3.5 <sup>a</sup>
RAS	0.6 $\pm$ 0.2	5.3 $\pm$ 8.4 <sup>a</sup>	11.8 $\pm$ 14.5 <sup>a</sup>

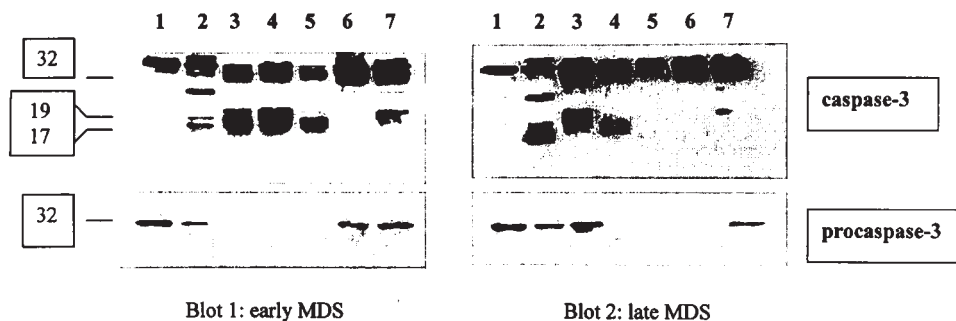
<sup>a</sup>Significantly different ( $P < 10^{-3}$ ) from control.

#### Detection of apoptotic cells

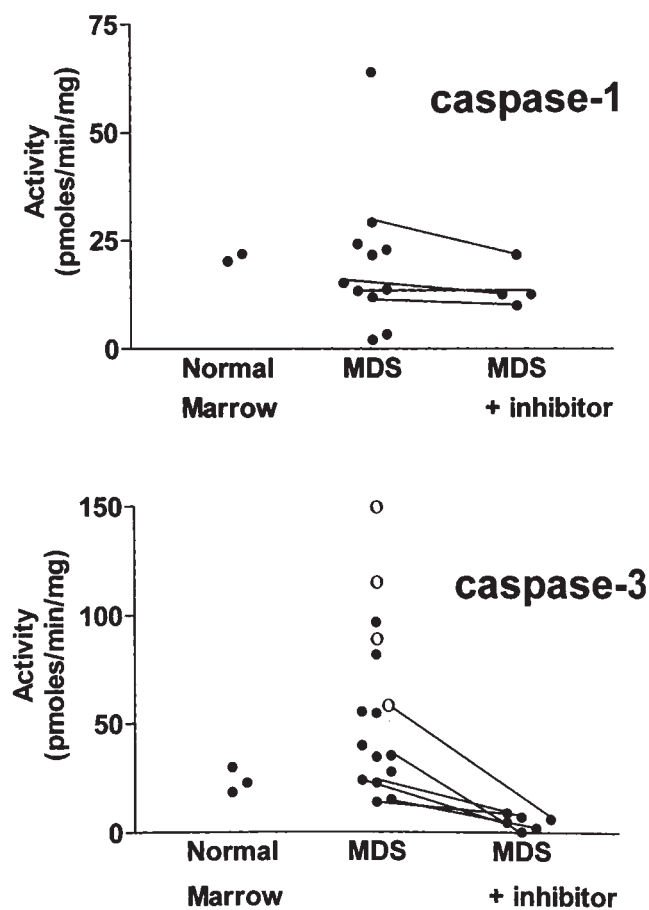
Data regarding the percentage of apoptotic cells were available in 56 MDS and four normal marrow samples. A small percentage of apoptotic cells was detected in normal marrow ( $7 \pm 2$ ). In MDS, the percentage ranged from 1 to 80 with a median of 15%. Twenty-six of the 56 MDS cases exhibited a percentage of apoptotic cells higher than 25%. The percentage of apoptotic cells was significantly higher ( $29 \pm 23$ ) in RA and RAS (pooled together) than in CMML ( $24 \pm 18$ ) and in RAEB and RAEB-t ( $11 \pm 11$ ) ( $P < 0.005$ ), and also in cases without spontaneous growth as compared to cases with spontaneous growth ( $10 \pm 10$  vs  $3 \pm 4$ ,  $P < 0.001$ ). However, we did not observe any significant correlation between this percentage and the expression of CD34, Bcl-2 or caspase-1 and -3. Interestingly, the effect of caspase-3 inhibitor on the number of CFU-GEMM, CFU-E and BFU-E remained very significant ( $P < 10^{-3}$ ) for the sub-group exhibiting a percentage of apoptotic cells  $\geq 20\%$ , whereas it was much smaller and not statistically significant when there was less than 20% apoptotic cells in the sample.

#### Western blot analysis of caspase-3 expression (Figure 4)

Western blot was performed on cell lysates from eight MDS and two control normal bone marrows samples using two different antibodies: a rabbit polyclonal antibody that recognizes both caspase-3 proenzyme and its p19 and p17 subunits,<sup>22</sup> and a monoclonal antibody more specific of the procaspase-3 form. In U937 cells, etoposide exposure (50  $\mu$ M, 6 h) induced a decrease of the proenzyme form procaspase-3 and its cleavage/activation into a p19 and a p17 fragments (p17 corresponds to the active form of caspase-3). In RA samples, we observed a low expression or in most instances an absence of procaspase-3 using the monoclonal antibody, while p19 and especially p17 fragments (three cases out of four) were detected by the polyclonal antibody (Figure 4, blot 1). In the RAEB samples studied, we also observed a low expression of the proenzyme form (as compared to normal marrow), but the p17 fragment was detected in only one case (Figure 4, blot 2). Moreover, the cases which expressed cleavage products exhibited a higher caspase-3 protease activity in fluorometric assay (Figure 5). In normal bone marrow, only procaspase-3 and the p19 inactive fragment could be detected.



**Figure 4** Analysis of procaspase-3 expression (32 kDa) and its cleavage products p19 and p17 kDa, in mononuclear cells of early (blot 1) and late MDS (blot 2). In each blot, a control of caspase-3 activation was performed, consisting of U937 cells, untreated (lane 1) or treated with 50  $\mu\text{M}$  etoposide for 6 h (lane 2). Lane 7 is a control normal marrow. Lanes 3 to 6 correspond to RA samples (blot 1) and RAEB samples (blot 2). Procaspase-3 was more specifically detected using a monoclonal antibody (bottom lanes) while p17 and p19 fragments were detected using the polyclonal antibody (top lanes). Cases 3, 4, 5 in blot 1 and case 4 in blot 2 correspond to samples exhibiting a high caspase-3 activity (open circles in Figure 5).



**Figure 5** Caspase protease activity (in pmoles/min/mg protein) assessed by fluorometric study with specific substrates Ac-YVAD-AMC (caspase-1-like activity) (top figure), and Ac-DEVD-AMC (caspase-3-like activity) (bottom figure) in normal marrows and MDS marrows, with or without inhibitor. Values obtained in a sample without or with preincubation with inhibitor are connected by a solid line. Open circles refer to the four cases presenting cleaved p17 and p19 fragments of caspase-3 in Figure 4.

### Caspase protease activity

Fluorometric study was performed in 11 MDS specimens and two control bone marrows for caspase-1 activity and in 16

MDS cases and three control bone marrows for caspase-3 activity (Figure 5). Low caspase-1-like and caspase-3-like activities were observed in normal marrow. The caspase-3-like activity measured was higher in MDS as compared to control (mean 56.8 pmoles/min/mg vs 23.8). A caspase-1-like activity was also measured in MDS samples, but was not different from that of control marrows.

To confirm the *in vitro* effect of caspase-1 inhibitor YVAD-cho and caspase-3 inhibitor DEVD-cho, protease activities were measured after preincubation with each inhibitor. In the six cases studied, DEVD-cho at 100  $\mu\text{M}$  inhibited more than 80% of caspase-3-like activity (mean activity 27.4  $\pm$  10.7 vs 5  $\pm$  3.2). In contrast, YVAD-cho blocked only between 5 and 17% of caspase-1-like activity. Moreover, DEVD-cho at 100  $\mu\text{M}$  exhibited a better inhibition potential on caspase-1 activity than YVAD-cho (data not presented).

### Discussion

Evidence of increased apoptosis in MDS has been now established by a number of studies, using morphological criteria, DNA staining techniques and Annexin V. Raza *et al*<sup>6</sup> demonstrated by ISEL massive apoptosis (>75% cells) in most MDS patients, involving the three myeloid lineages but also stromal cells. By a double staining technique, this group also showed that apoptosis was associated with increased proliferation, and that a large proportion of cells in S-phase were also apoptotic.<sup>7</sup> Our data obtained by TUNEL confirm an increased apoptosis in most MDS cases, but to a lesser extent. Using a similar technique, Lepelley *et al*<sup>8</sup> detected a moderate increase of apoptotic cells in only six of 34 cases. However, after a short-term liquid culture, the percentage of apoptotic cells increased to up to 39% of cells. The differences observed between the study of Lepelley and our study may be due to technical reasons, but also to the fact that our series included more cases of RA and RAS. Indeed, we observed a higher proportion of apoptotic cells in RA and RAS than in CMML, RAEB and RAEB-T, which is in accordance with most published data.<sup>5,6,8,9</sup>

The expression of apoptosis-controlling proteins in our study was consistent with the fact that 'early' MDS (RA and RAS) exhibited more apoptotic cells than 'late' MDS (RAEB and RAEB-T).<sup>8</sup> We observed a higher expression of Bcl-2 in RAEB and RAEB-T than in other types. The percentage of Bcl-2-positive cells correlated well with the percentage of blasts

and that of CD34-positive cells. In acute myeloid leukemia, high expression of Bcl-2 has been associated with 'stem cell' (CD34<sup>+</sup>) phenotype and poor response to chemotherapy.<sup>24–26</sup> Overexpression of Bcl-2 has also been associated with late MDS types or progression to overt leukemia,<sup>27,28</sup> as were high ratios of Bcl-2/Bax, Bcl-2/Bad, or Bcl-2/myc in CD34<sup>+</sup> cells.<sup>8</sup> High ratios of pro-apoptotic (Bax and Bad) to anti-apoptotic (Bcl-2, Bcl-x) proteins were also reported in early MDS CD34<sup>+</sup> cells, as compared to more advanced disease or secondary AML.<sup>10</sup> Moreover, we observed a significant association between Bcl-2 expression and autonomous growth of committed progenitors, as already described in AML.<sup>29</sup> Taken together, these findings confirm that although excessive apoptosis can be observed in all MDS types, an excess of blasts is associated with increased expression of anti-apoptotic proteins and probably reduced apoptosis in this subset of cells, correlated in our study with a lower caspase-3 expression and activity.

Few data are available regarding the expression of caspases in MDS. An increased production of caspase-1/ICE by MDS mononuclear cells maintained in short-term liquid culture has been described.<sup>20</sup> More recently, the same group reported that caspase-1 mRNA was detectable in normal and MDS mononuclear cells, whereas caspase-3 mRNA was detected only in MDS cells.<sup>30</sup> We could not demonstrate here that the expression of caspase-1 was increased in MDS, although there was a trend to higher expression in RA and RAS. On the contrary, caspase-3 was detected by flow cytometry in a higher percentage of mononuclear cells in RA and RAS as compared to RAEB and RAEB-T and normal marrow. Immunoblotting confirmed the expression of caspase-3, with the presence in several early MDS cases of the active p17 fragment. Accordingly, an increased caspase-3-like activity was detected with a fluorogenic specific substrate, which could be readily inhibited by incubation with DEVD-cho. By contrast, caspase-1-like activity was not elevated, and was incompletely blocked by YVAD-cho. Moreover, DEVD-cho exhibited a better inhibition potential on caspase-1 activity than YVAD-cho. This is consistent with the findings of Nicholson *et al*<sup>19</sup> on the overlapping effects of these inhibitors. Margolin *et al*<sup>31</sup> also reported that DEVD-cho is a better inhibitor than YVAD-cho, including on caspases other than caspase-3.

Abnormal growth in clonogenic assay, even in the presence of appropriate growth factors, is one of the characteristics of MDS, including reduced number of colonies and abnormal cluster/colony ratio.<sup>2</sup> In one report the addition of a specific caspase-1 inhibitor to culture medium inhibited the apoptosis of MDS mononuclear cells.<sup>20</sup> In our study, caspase inhibitors and particularly DEVD-cho permitted a significant improvement in the growth pattern of myeloid progenitors. This effect was especially dramatic in most cases of 'early' MDS, with an increase of all types of colonies CFU-E/BFU-E/CFU-GM (up to 50-fold of control). Again a higher efficiency of DEVD-cho was noted. Our findings are different to those recently reported by Bouscary *et al*.<sup>32</sup> In this study as in ours, an enhanced caspase-3 activity was observed in early stage MDS, which was inhibited by Z-VAD-fmk. However, clonogenic assays in the presence of this inhibitor did not result in increased colony formation. These discrepancies may result from the use of different inhibitors (Z-VAD-fmk vs DEVD and YVAD-cho), at different concentrations.

In conclusion, this paper confirms that increased apoptosis can be observed in MDS which seems more marked in 'early' sub-types. It also demonstrates by different approaches the involvement of caspase-3 in the apoptotic process.

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