Studies on megakaryopoiesis in patients with myelodysplasia and idiopathic thrombocytopenic purpura
Houwerzijl, Ewout Johan

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Erythroid precursors from patients with low-risk myelodysplasia demonstrate ultrastructural features of enhanced autophagy

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Submitted for publication
ABSTRACT

The present study was designed to define underlying cell death pathways and their relationship to abnormal mitochondria in erythroblasts from low-risk myelodysplasia (MDS) patients. Immunohistochemistry of bone marrow MDS erythroblasts (n = 23) demonstrated no positivity for active caspase -3 and -8. Ultrastructurally (n = 9), abnormal and iron-laden mitochondria were abundant, but apoptosis was found in only 2 ± 2% of cells. However, 52 ± 16% of immature and mature MDS erythroblasts contained cytoplasmic vacuoles, partly double-membraned and positive for lysosomal marker LAMP-2 and mitochondrial markers, compatible with autophagic removal of dysfunctional mitochondria. In healthy controls only mature erythroblasts comprised these vacuoles (12 ± 3%). These findings were confirmed morphometrically showing an increased vacuolar surface in MDS erythroblasts compared to controls (P<0.0001).

When bone marrow mononuclear cells were cultured *in vitro* for 24 hours, 27 ± 7% of MDS erythroblasts were apoptotic versus 8% from controls. However, when erythroblasts were cultured in the context of their own microenvironment, i.e. from hematons, 6 ± 4% of MDS erythroblasts were apoptotic (P = 0.007). In summary, these data indicate that MDS erythroblasts show primarily features of enhanced autophagy, which is probably initiated to remove defective iron-containing mitochondria, and may switch to apoptosis when lacking an appropriate microenvironment.
INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal stem cell disorders characterized by an ineffective and dysplastic hematopoiesis. Especially in low-risk MDS, such as refractory anemia (RA) and refractory anemia with ringed sideroblasts (RARS), increased programmed cell death (PCD) of bone marrow hematopoietic cells has been described. This might be an important mechanism to explain the typical clinical findings of a hypercellular bone marrow and peripheral blood cytopenias. In particular, enhanced apoptosis has been reported in MDS. However, results on the degree of apoptosis are conflicting. This may have several reasons, including differences in patient characteristics, detection techniques and in bone marrow sample handling and preparation. An alternative explanation might be that nonapoptotic forms of cell death are taking place in certain MDS hematopoietic cells. In megakaryocytes of MDS patients nonapoptotic PCD characterized by the absence of chromatin condensation and caspase-3 and -8 activation has been demonstrated. Recent studies have indicated that besides apoptosis other types of PCD can be distinguished such as autophagic cell death and certain types of necrosis. In addition, cells are capable to switch between the different types of PCD dependent on their cellular context. For instance, inhibition of the apoptotic machinery, can trigger a switch from apoptosis to necrosis or autophagy. Loss of mitochondrial transmembrane potential plays an important role in these events, and the intensity of the stimulus and the cellular context often determine which type of cell death develops. Based on these findings it is conceivable that the recognized differences in the type of PCD in MDS might in part be related to whether cells are studied in in vitro culture assays or in the context of their own microenvironment.

Previous studies have shown that hematons can be isolated from the bone marrow light density fraction. These hematons are compact hematopoietic complexes containing several cell lineages, including mesenchymal cells, endothelial cells, and hematopoietic progenitor cells. In these hematons a high number of erythroblasts are located within their own microenvironment.

To investigate whether the type of PCD of MDS erythroid precursors is dependent on their cellular context, ultrastructural, cytomorphometric and immunohistochemical studies were performed on MDS erythroid precursors from the mononuclear cell fraction versus the hematon fraction. The results demonstrate that erythroid cells directly prepared from bone marrow hematons and MNC fraction show no ultrastructural and
immunohistochemical signs of apoptosis. Instead, the ultrastructural, immunogold labeling and morphometric results were compatible with the presence of autophagy. However, when mononuclear cells from MDS bone marrow were cultured for 24 hrs in vitro a relatively high proportion of apoptotic erythroid cells was noticed, which was not observed in erythroid cells from hematonic fraction. Instead, in the hematons the fraction of erythroblasts showing autophagic vacuoles strongly increased.

**PATIENTS, MATERIALS AND METHODS**

**Patients**
Bone marrow of low-risk MDS patients (n = 24) and healthy controls (n = 4) was aspirated from the iliac crest. The diagnosis of MDS was made according to the World Health Organization (WHO) classification. The international prognostic scoring system (IPSS) was used to define prognostic risk. Patients were grouped as having good, intermediate, and poor risk cytogenetics according to Greenberg et al. Transfusion dependency was defined if patients need one or more units of red blood cells per month. The institutional review board of the University Hospital Groningen approved the study protocol. All patients gave informed consent.

**Immunohistochemistry**
For immunohistochemical staining, serial 3-µm-thick sections were cut from paraffin-embedded bone marrow biopsies and mounted on aminopropyl-ethoxy-silan (APES; Sigma-Aldrich, Diesenhofen, Germany)–coated glass slides. After deparaffinization in xylene, antigen retrieval was performed using microwave heating at 700 W for 10 minutes in EDTA (ethylenediaminetetraacetic acid) buffer. Following blocking of endogenous peroxidase with 3% hydrogen peroxide for 30 minutes, the primary antibody was applied for one hour at room temperature. To identify activated caspase-3, immunostaining with a rabbit polyclonal antibody (1:100; New England Biolabs, Beverly MA) was used. Subsequently, the slides were incubated for 30 minutes with appropriate secondary and tertiary antibodies with streptavidin-conjugated peroxidase (DAKO, Glostrup, Denmark). Peroxidase activity was visualized with diaminobenzidine. Slides were counterstained with hematoxylin. A sample of colorectal carcinoma and bone marrow megakaryocytes from a patient with idiopathic thrombocytopenic purpura (ITP) served as positive controls. As negative controls, slides were immunostained in the
absence of the primary antibody. For the detection of activated caspase-8, the mouse monoclonal antibody 1C12 (catalog no. 9746; Cell Signaling Technology, Beverly, MA) was used, dilution 1:50. Antigen retrieval was performed by autoclave heating. A 2-step detection system (rabbit anti–mouse peroxidase/goat anti–rabbit peroxidase) was used. Diaminobenzidine was used as chromagen, and sections were counterstained with hematoxylin. Positive control was breast carcinoma; negative control, replacement of the primary antibody with phosphate-buffered saline (PBS).

**Electron microscopy**

**Bone marrow sample preparation.** Fresh bone marrow cells were washed in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium), pelleted, and subsequently fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 24 hours at 4°C. Cells were dehydrated, osmicated, and embedded in Epon 812 according to routine procedures. Semi thin sections (0.5 µm) stained with toluidine blue were inspected light microscopically to select erythroblasts. The ultrastructure of erythroid precursors was studied in both hematons and the mononuclear cell fraction from fresh bone marrow aspirate (Philips 201, Philips, Eindhoven, The Netherlands). Three stages of erythroblast development were distinguished: proerythroblast (stage I or early erythroblast), basophilic erythroblast (stage II or intermediate erythroblast), polychromatic erythroblast (stage III or late erythroblast). Hematons were prepared as previously described. Bone marrow hematons and mononuclear cell fractions were also studied after 24 hrs of culture in RPMI1640 and 5% fetal calf serum (FCS). A total of 100 erythroblasts per sample were examined.

**Immunogold labeling.** Bone marrow mononuclear cells and hematons were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde, pH 7.4 at 4°C for 30 minutes. For cryosectioning, the bone marrow samples were embedded in 5% gelatine and frozen in liquid nitrogen. Thin sections (80 nm) were cut at −100°C for immunoelectron microscopy. Cryosections were picked up in a 2.3M saturated sucrose solution. The sections were then labeled with polyclonal goat anti-lysosome-associated membrane protein (LAMP)-2 (Santa Cruz Biotechnology, Heidelberg Germany), rabbit anti-caspase-3 (Cell Signaling Technology, Danvers, MA), mouse anti-mitochondrial inner membrane (H6/C12) antigen (Santa Cruz Biotechnology, Heidelberg, Germany) and goat anti-cytochrome c (Santa Cruz Biotechnology, Heidelberg, Germany), which were detected with rabbit anti-goat-IgG- 5 nm gold, goat anti-rabbit-IgG-10 nm, goat anti-mouse IgG-10 nm, and rabbit anti goat IgG-10 nm, respectively. Double labelings were done by mixing the primary antibodies from different species and the corresponding secondary antibodies conjugated with different sized gold particles. The bigger sized gold
particles were used for the most abundant antigen. Sections were mounted in 1.5% methylcellulose containing 0.4% uranyl acetate and examined in detail with electron microscopy (Philips 201, Eindhoven, The Netherlands).

_Ultrastructural definitions of programmed cell death and autophagy._ Apoptosis and autophagy/autophagic cell death were defined according to recent recommendations of the Nomenclature Committee on Cell Death.\(^\text{10}\) In brief, apoptosis and autophagy are distinct morphological entities. The presence of cytoplasmic double membrane-bound vacuoles containing degenerating cytoplasmic organelles or cytosol was essential for the definition of autophagy. Autophagolysosomes were defined by electron microscopy as vacuoles containing both molecular markers of lysosomes (LAMP-2) and molecular markers of organelles (mitochondria) or morphological remnants of organelles (iron deposits were considered as mitochondrial remnants).

_Morphometry._ A Quantimet 520 Image Analysis System (Cambridge Instruments, Cambridge, UK) was used to analyze the area of cytoplasmic vacuoles and the total cellular area of erythroid precursors in each section. A total of 30 cells per sample were studied.

Statistical analysis

Data are reported as mean ± standard deviation (SD) and as median (range). Statistical analysis was performed using the unpaired t-test and the Mann Whitney test.

RESULTS

Patients

A total of 24 patients with refractory anemia (RA; \(n = 18\)) and RA with ringed sideroblasts (RARS; \(n = 6\)) were studied. Table 1 shows the patient characteristics. Cytogenetics were classified as ‘good’ in 15 patients, ‘intermediate’ in 6 and ‘poor’ in 2 patients. IPSS could be scored in 23 patients. According to this classification the patients were categorized as low risk (\(n=3\)), intermediate risk-1 (\(n=18\)), and intermediate risk-2 (\(n=2\)).

Composition of bone marrow aspirate of MDS patients and healthy controls

As reported previously,\(^\text{11}\) the number of hematons in MDS bone marrow aspirates was reduced compared to normal bone marrow. However, in all 9 patients in whom ultra-
Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>RA</th>
<th>RARS</th>
<th>All</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>18</td>
<td>6</td>
<td>24</td>
<td>—</td>
</tr>
<tr>
<td>No. male/female</td>
<td>12/6</td>
<td>4/2</td>
<td>16/8</td>
<td>—</td>
</tr>
<tr>
<td>Age, years</td>
<td>61 ±17</td>
<td>73 ± 8</td>
<td>64 ± 16</td>
<td>—</td>
</tr>
<tr>
<td>Hemoglobin, mmol/L</td>
<td>6.4 ± 1.2</td>
<td>5.9 ± 1.1</td>
<td>6.2 ± 1.1</td>
<td>F:7.4-10/M:8.6-11</td>
</tr>
<tr>
<td>WBC, × 10⁹/L</td>
<td>3.9 ± 1.9</td>
<td>4.2 ± 2.7</td>
<td>4 ± 2.1</td>
<td>4.0-10</td>
</tr>
<tr>
<td>Platelets, × 10⁹/L</td>
<td>89 ± 87</td>
<td>201 ± 161</td>
<td>117 ± 117</td>
<td>150-350</td>
</tr>
<tr>
<td>MCV, fl</td>
<td>103 ± 10</td>
<td>99 ± 6</td>
<td>102 ± 9.3</td>
<td>82-96</td>
</tr>
<tr>
<td>Karyotype⁹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>11</td>
<td>4</td>
<td>15</td>
<td>—</td>
</tr>
<tr>
<td>Intermediate</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>Poor</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>IPSS</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>—</td>
</tr>
</tbody>
</table>

⁹Karyotype: good points to normal karyotype, sole:–Y, del 5q, del 20q; intermediate = others; poor = complex (≥3 chromosomal abnormalities) and/or chromosome 7 anomalies. F, female; IPSS, international prognostic scoring system; M, male; MCV, mean corpuscular volume; RA, refractory anemia; RARS, RA with ringed sideroblasts; —, not applicable. Results are expressed as mean ± SD.

structural studies were performed, hematons could be isolated. The composition of the hematons in regard to the relative numbers of different stages of erythroid precursors was not significantly different from the MNC fraction (Figure 1), although there was a trend towards more immature stages, especially stage II erythroblasts: percentage of erythroblasts in hematon vs MNC fraction: stage I: 25% ± 3% vs 14% ± 5% (P = 0.15); stage II: 56% ± 4% vs 40 ± 6 (P = 0.06); stage III: 20% ± 7% vs 46% ± 10% (P = 0.06).

**MDS erythroblasts from fresh bone marrow samples display no features of enhanced apoptosis**

To evaluate whether a caspase-dependent type of PCD could be demonstrated in erythroid cells of low-risk MDS patients (n=23), bone marrow biopsies of patients with RA (n=17) and RARS (n=6) were stained for activated caspase-3 and -8. In none of the MDS patients and healthy controls (n= 4), erythroid cells stained positive for activated caspase-3 and/or -8. It is very unlikely that this is due to an improper technique since megakaryocytes from bone marrow samples of ITP patients were positive. The bone
Ultrastructural studies were performed on freshly prepared hematons in a subgroup of these patients (n = 9; for patient characteristics see Table 2). By ultrastructural analysis characteristics of apoptosis were found in a very low percentage of MDS erythroid cells (2 ± 2%, median 2%, range 0-6%) compared to no apoptotic erythroblasts in healthy controls (n = 4), underscoring the results of the immunohistochemical staining.

**MDS erythroid precursors from fresh bone marrow samples demonstrate an increased level of autophagy**

However, the ultrastructural studies of erythroid cells in all patients (n = 9) demonstrated that in a significantly higher fraction of erythroblasts cytoplasmic vacuolization was present (52 ± 16% vs 12 ± 3% of the erythroblasts in healthy controls, P=0.003). These vacuoles were present in all stages of MDS erythroblasts, whereas in the normal counterparts only erythroblasts of stage III demonstrated these vacuoles.
Table 2. Characteristics of patients studied with electron microscope.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age, y</th>
<th>Sex, M/F</th>
<th>WHO</th>
<th>Hb, mmol/L</th>
<th>WBC, $\times 10^9$/L</th>
<th>Platelets, $\times 10^9$/L</th>
<th>MCV, fL</th>
<th>Transfusion dependency</th>
<th>Karyotype</th>
<th>IPSS</th>
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<tbody>
<tr>
<td>1</td>
<td>68</td>
<td>F</td>
<td>RA</td>
<td>7.1</td>
<td>3.1</td>
<td>75</td>
<td>99</td>
<td>Yes</td>
<td>Del 20q</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>F</td>
<td>RA</td>
<td>5.4</td>
<td>1.0</td>
<td>108</td>
<td>104</td>
<td>Yes</td>
<td>Normal</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>M</td>
<td>RA</td>
<td>7.9</td>
<td>5.2</td>
<td>342</td>
<td>—</td>
<td>No</td>
<td>+8</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>F</td>
<td>RARS</td>
<td>5.6</td>
<td>9.5</td>
<td>490</td>
<td>98</td>
<td>Yes</td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>67</td>
<td>M</td>
<td>RARS</td>
<td>7.6</td>
<td>4.2</td>
<td>115</td>
<td>105</td>
<td>Yes</td>
<td>-Y</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>M</td>
<td>RARS</td>
<td>5.0</td>
<td>3.2</td>
<td>199</td>
<td>105</td>
<td>Yes</td>
<td>+8</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>84</td>
<td>F</td>
<td>RARS</td>
<td>5.9</td>
<td>2.4</td>
<td>68</td>
<td>92</td>
<td>No</td>
<td>-5</td>
<td>1.0</td>
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<tr>
<td>8</td>
<td>72</td>
<td>M</td>
<td>RARS</td>
<td>4.6</td>
<td>3.1</td>
<td>261</td>
<td>101</td>
<td>Yes</td>
<td>Normal</td>
<td>0.5</td>
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<tr>
<td>9</td>
<td>68</td>
<td>F</td>
<td>RARS</td>
<td>6.5</td>
<td>2.8</td>
<td>70</td>
<td>92</td>
<td>No</td>
<td>-Y</td>
<td>0.5</td>
</tr>
</tbody>
</table>

F, female; Hb, hemoglobin; IPSS, International Prognostic Scoring System; M, male; MCV, mean corpuscular volume; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; WBC, white blood cell; —, not done; WHO, World Health Organization.
A median of 13% (range 2-25) of the vacuoles were double membraned, which is a main characteristic of autophagosomes (Figure 2). The late step in autophagy involves the fusion of autophagosomes and lysosomes. Therefore, to further underscore the presence of autophagy immunogold double-labeling was performed for molecular markers of lysosomes (LAMP-2) and organelles that may be present in autophagosomes, including mitochondria. Detection of mitochondria or remnants of mitochondria can be assessed with an antibody against a mitochondrial inner membrane antigen and cytochrome c. Immunogold labeling for LAMP-2 and cytochrome c demonstrated that both were present and co-localized in the vacuoles (Figure 3A). In addition, co-localization of LAMP-2 and the mitochondrial inner membrane antigen and iron deposits was demonstrated in the vacuoles (Figure 3B). Based on the morphological findings of double membranes, the presence of LAMP-2 in conjunction with cytochrome c, the mitochondrial inner membrane antigen or iron, we concluded that our observations are compatible with autophagy in part of mitochondria.

Iron-laden mitochondria were present in a large percentage of erythroblasts of all patients (MDS vs healthy controls: 37 ± 19% vs 0%, \( P = 0.03 \)). This fraction was higher in patients with RARS than with RA (48 ± 14% vs 19 ± 7%, \( P = 0.04 \)). More than 50% of these iron-laden mitochondria were abnormally shaped mitochondria, especially in patients with RARS. As expected, the mitochondrial inner membrane antigen and cytochrome c were present in mitochondria. Cytochrome c was also present in moderate amounts in the cytoplasm, suggesting that a fraction of the MDS mitochondria were defective and undergoing mitochondrial outer membrane permeabilization.

As indicated the presence of vacuoles was not restricted to MDS erythroblasts. Also in normal erythroblasts vacuoles were present, but only in mature erythroblasts of stage III. These vacuoles contained LAMP-2, cytochrome c and the mitochondrial inner membrane antigen, underscoring that autophagy occurs at a late time point in the normal erythroid differentiation program (Figure 3C).

To quantify the observed morphological differences between normal and MDS erythroid cells, morphometry of these cells was performed. No differences were observed in cell volume and the ratio of area of mitochondria/cytoplasm, but the ratio area of vacuoles/cytoplasm in stage II and III erythroblasts was significant higher in MDS samples (stage I, 0.001 vs 0.0048 (\( P = 0.17 \)); stage II, 0.002 vs 0.011 (\( P <0.0001 \)); stage III, 0.003 vs 0.013 (\( P <0.0001 \)) (Figure 4). A significant difference in the ratio of iron-accumulated mitochondria/cytoplasm was also found (healthy controls vs MDS: 0.000 vs 0.0212 (stage I), 0.000 vs 0.0266 (stage II), 0.000 vs 0.0233 (stage III) (\( P<0.0001 \)).
Figure 2. MDS erythroblast showing morphological features of autophagy. The cytoplasm of this erythroblast contains enlarged and abnormal mitochondria with iron deposits (arrows). There are several double-membranated autophagic vacuoles (asterisks). The double membrane appears to be in continuation with the smooth endoplasmic reticulum (arrowheads). Apart from autophagosomes with granular contents, electron lucent single membranated cytoplasmic vacuoles (indicated by ×) are present.
Figure 3. Autophagic vacuoles in erythroblasts from healthy controls and MDS patients.

A. MDS erythroblast: LAMP-2 (5 nm gold) (arrowhead) co-localizes with cytochrome c (10 nm gold) (arrows) in autophagic vacuoles.

B. MDS erythroblast: co-localization of LAMP-2 (5 nm gold) (arrowhead) and mitochondrial inner membrane protein (10 nm gold) (arrows) in autophagic vacuoles.

C. Normal mature erythroblast: both LAMP-2 (5 nm gold) (arrowhead) and cytochrome c (10 nm gold) (arrows) are present in autophagic vacuoles.
Figure 4. A significantly higher fraction of erythroblast cytoplasm is vacuolated in MDS patients than in healthy controls. Both in stage II and III erythroblasts the ratio of the surfaces of vacuoles versus total cytoplasm is approximately 5 times higher in MDS than in normal controls. Data are presented as mean ± SEM (standard error of the mean).

MDS erythroblasts from the bone marrow mononuclear cell fraction undergo enhanced apoptosis after short-term culture

To evaluate whether cell processing methods and interaction with the microenvironment affects the type of PCD, erythroblasts from hematons and MNC fraction were ultrastructurally analyzed after being prepared freshly and after culturing for 24 hrs.

*MNC vs hematons from healthy controls.* Compared to immediately fixed erythroblasts, the cultured cells from the MNC fraction of healthy controls (n = 4) showed a higher level of apoptosis (11 ± 3% vs 0%, *P* = 0.003), and cytoplasmic vacuolization (62 ± 8% vs 14 ± 8%, *P* = 0.009) and a smaller fraction of morphologically intact erythroblasts (26 ± 5% vs 86 ± 5%, *P* = 0.0001). The cultured hematons of healthy controls (n = 4) demonstrated comparable results: a slightly higher percentage of apoptosis (5 ± 2% vs 0%), a strongly elevated level of cytoplasmic vacuolization (68 ± 8% vs 12 ± 3%, *P* = 0.002) and less intact erythroblasts (28 ± 6%, vs 88 ± 3%, *P* = 0.002) compared to directly prepared samples (Figure 5).
Figure 5. Distribution of erythroblasts showing apoptosis and autophagic vacuoles in hematos versus mononuclear cell (MNC) fraction from healthy controls and low-risk MDS patients. After culturing hematos for 24 hours (depicted as 24) the level of apoptotic erythroblasts is slightly higher than in directly fixed cells (depicted as 0) in both healthy controls and MDS patients, whereas the fraction of apoptotic erythroblasts from the MNC fraction of MDS after short-term culture is distinctly elevated compared to zero time. Data are presented as means, see text for exact figures.

MNC vs hematos from MDS samples. After culturing, a significantly higher fraction of erythroid cells from the MNC fraction (n = 4) than from the directly fixed MNC samples demonstrated features of apoptosis (27 ± 7% vs 3 ± 2%, P = 0.03), based on typical ultrastructural characteristics and immunogold labeling for activated caspase-3. This coincided with a decrease in morphologically intact erythroblasts (36 ±19% vs 12 ± 12% [median 6.5 (6-30)%), P = 0.08). The percentage of erythroblasts showing cytoplasmic vacuolization remained relatively unchanged during culture (61 ± 19% vs 61 ± 17%, P = 1.0) (Figure 5). Approximately 55% of the apoptotic erythroblasts showed a mixed morphology of apoptotic nuclei and extensive cytoplasmic autophagic vacuoles after culture (Figure 6 and 7).

In contrast, in the hematos of the same MDS patients there was only a very small non-significant increase in erythroblasts undergoing apoptosis after culture (6 ± 4% vs
ENHANCED AUTOPHAGY IN ERYTHROBLASTS FROM LOW-RISK MDS PATIENTS

2 ± 2% in directly fixed samples), whereas the fraction of erythroblasts showing cytoplasmic vacuolization strongly increased (75 ± 8% vs 32 ± 5%, \( P = 0.001 \)). Cells with both apoptotic and autophagic characteristics were not found in the hematocrit fractions.

DISCUSSION

The present study was designed to investigate the type of premature PCD of bone marrow erythroblasts in low-risk MDS patients. The results demonstrate that apoptosis was present in a limited number of the erythroblasts. The main abnormalities were defects compatible with autophagy. Ultrastructural examination of the directly fixed samples demonstrated an approximately five times increase in the number of cytoplasmic vacuoles in MDS erythroblasts compared to healthy controls. A proportion of the vacuoles were double-membraned. In addition, immunogold labeling for LAMP-2 revealed that lysosomes were present in these vacuoles. The majority of these lysosomal structures was containing endogenous mitochondrial proteins or was iron-laden, indicating enhanced autophagic degradation of mitochondria.

Autophagy is connected with several cellular processes. It is a natural degradative process in which cytoplasmic material, including organelles and macromolecules, is segregated into double membraned vesicles (autophagosomes), which are subsequently degraded in a lysosomal dependent manner. A low basal level of constitutive autophagy is critical for clearance of misfolded proteins. In addition, autophagy functions as a physiologic cytoprotective response to nutrient and/or growth factor depletion, by producing amino acids that support ATP generation necessary for maintaining cell viability, but it is also linked to PCD.\(^{14-17}\)

In normal erythropoiesis autophagy is present during the process of enucleation. At this stage the autophagic process contributes to the removal of organelles, such as mitochondria.\(^{18}\) In rat studies autophagosomes occupied 0.2% of the cytoplasm of differentiated erythroblasts\(^{19}\), which is consistent with the results of the present study. In MDS erythroblasts, however, this fraction is approximately 5 times higher. Moreover, the autophagic vacuoles were not only present in mature, but also in immature erythroblasts, indicating that autophagy in MDS is more pronounced and occurs earlier in the differentiation program. A recent study of Tehranchi et al\(^{20}\) demonstrated that MDS CD34\(^+\) cells have an enhanced expression of genes encoding for the erythroid lineage. Based on these and the present findings it is conceivable that intrinsic defects in the
Figure 6. Ultrastructure of MDS erythroblasts after short-term culture.

A. Example of an apoptotic erythroblast with a spherical nucleus containing condensed chromatin at the margin. The cytoplasm is clear of vacuoles.

B. Erythroblast from the MNC fraction with an apoptotic nucleus comparable to the one shown in A, except that the nuclear membrane shows frequent double membraned blebs. In the cytoplasm there is a marked increase in vacuoles.

C. Erythroblast from the MNC fraction, identified by the dense ribosomal distribution and featuring several apoptotic bodies and blebbing of the outer cell membrane. In addition, the cytoplasm is filled with numerous vacuoles containing profiles with electron dense and lucent material.
Figure 7. Immunoelectron microscopy of MDS erythroblast after culture. Immunogold labeling shows presence of active caspase-3 in an erythroblast from the MNC fraction.

Erythroid differentiation program are responsible for the early initiation of the autophagic process. A consequence of the early initiation of autophagy might be that relevant proteins which are critical for the normal erythroid differentiation are degraded, resulting in a hampered erythroid differentiation program.

Besides these cellular defects, an increased number of dysfunctional and iron-laden mitochondria have been noticed in MDS. Several studies\textsuperscript{20-25} have demonstrated that mitochondrial dysfunction exists in MDS, which may result in increased oxidative stress, an impaired cellular ATP production, and an increased release of apoptosis-inducing factors in the cytoplasm.\textsuperscript{26,27} The present data show that these damaged and dysfunctional mitochondria are degraded by autophagy probably as a protective mechanism in order to restore the cellular bioenergetic state and decrease ROS accumulation.\textsuperscript{28} A recent \textit{in vitro} study demonstrated that this repair mechanism is in particular operational if caspase activation is blocked and if glycolysis is elevated as a result of sustained levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).\textsuperscript{29} Cells subsequently survived, even though mitochondrial outer membrane permeabilization and cytochrome c release was present. The present study demonstrates that these
prosurvival signals might be provided from the microenvironment. MDS erythroblasts disconnected from the microenvironment undergo enhanced apoptosis after short-term culture, whereas those from hematons, in which the interaction with stromal cells is preserved, did not. So, the process of autophagy may switch to apoptosis when the MDS erythroblasts detach from their microenvironment. This process of apoptosis in MDS erythroblasts might be more pronounced due to impairments in the PI(3)K signaling pathway that has been demonstrated in granulocytes and CD34+ cells from MDS patients.30,31

In summary, these data indicate that MDS erythroblasts show features of enhanced autophagy at an earlier stage of the erythroid differentiation program, and that this process may switch to apoptosis when the appropriate microenvironment is lacking. The enhanced autophagy might be considered as a cell protective mechanism to remove defectively formed and iron-laden mitochondria, but can have consequences for the erythroid differentiation program due to the premature autophagic degradation of proteins relevant for the maturation process.

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