

Myelodysplastic syndromes: immunohistochemical and morphometric evaluation of proliferative activity in erythropoiesis and endoreduplicative capacity of megakaryocytes

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Abstract. An immunohistochemical and morphometric analysis was performed on bone marrow trephine biopsies in 40 patients with primary myelodysplastic syndromes (MDS) to evaluate the proliferative activity in erythropoiesis and the endoreduplicative capacity of megakaryocytes. Control groups included normal bone marrow and marrow from cases presenting with pernicious anaemia. Double-immunostaining was applied with a monoclonal antibody (PC10) directed against proliferating cell nuclear antigen (PCNA), followed by antibodies against glycophorin C (Ret40f) or platelet glycoprotein IIIa (Y2/51-CD61) for the identification of the erythroid and megakaryocytic cell lineage. Comparison with normal bone marrow showed a reduction of erythropoiesis accompanied by an increase in atypical (micro-) megakaryocytes. Erythroid precursors displayed significant enhancement of PCNA-immunostaining. Megakaryocytes showed no increase in the relative frequency of PC10-positive cells (PCNA-labelling index). In pernicious anaemia, predominance of macrocytic-megaloblastoid erythropoiesis was associated with a striking increase in PCNA-labelling. Cell kinetic studies in this disorder revealed an abnormal arrest, particularly in S-phase which generates an over-expression of PCNA. Similar conditions were believed to be present in MDS with secondary folate deficiency. This mechanism explains the relatively high rate of positively-reacting pro- and erythroblasts which is not invariably accompanied by an increase in cell proliferation. Determination of megakaryocyte size and PCNA-staining capacity resulted in a significant increase in PC10-positive cells among micromegakaryocytes. Our findings on this cell lineage are in keeping with the assumption of a block in endoreduplicative activity at higher ploidy levels, asso-

ciated with an apparently not-deregulated endomitosis in small-sized megakaryocytes of lower ploidy stages.

Key words: Myelodysplastic syndromes – Erythroid precursors – Megakaryocytes – Proliferation – Endoreduplication

Introduction

In most patients with primary myelodysplastic syndromes (MDS) ineffective haematopoiesis with abnormalities of all three cell lineages dominates the bone marrow picture (Tricot et al. 1984; Fohlmeister et al. 1985; Frisch and Bartl 1986; Delacretaz et al. 1987; Kitagawa et al. 1989; Bartl et al. 1992; Maschek et al. 1992) and usually causes various degrees of bi- or pancytopenia (Jacobs 1985; Koeffler 1986). Most prominent is atypical morphology of the erythro- and megakaryopoiesis. Erythroid precursors frequently reveal a dissociation between nuclear and cytoplasmic maturation, nuclear budding and multinucleation with formation of intranuclear bridges and further megaloblastoid features (Delacretaz et al. 1987; Bartl et al. 1992). These changes in the red cell series are suggestive of secondary folate and/or vitamin B12-deficiency (Koeffler 1986; Bartl et al. 1992). Megakaryocytes are most often increased in number, display small sizes and a dysplastic appearance (Fohlmeister et al. 1985; Frisch and Bartl 1986; Delacretaz et al. 1987; Thiele et al. 1991). Cell-culture studies have repeatedly demonstrated that progenitor cells derived from bone marrow in the different subtypes of MDS are characterized by delayed maturation (Lidbeck 1980; Dörmer et al. 1987; Ruutu et al. 1984; Greenberg 1986; Partanen et al. 1986; Gebbia et al. 1989; Swolin et al. 1990). This maturation defect is assumed to account for the increased number of early precursor cells and is particularly expressed in the erythrocytic and megakaryocytic lineages (Koeffler et al. 1978, Cazzola et al. 1982, Chui and Clarke 1982, Ruutu et al. 1984; Juvonen et al.

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1986; Dörmer et al. 1987). Although in-vitro growth patterns and differentiation have a predictive value in assessing the progression of MDS to acute non-lymphocytic leukaemia (Greenberg and Mara 1979; Cazzola et al. 1982; Francis et al. 1983; Montecucco et al. 1983; Peters et al. 1986; Raymakers et al. 1991), there are hardly any data available regarding proliferative capacity of erythropoiesis or the endoreduplicative activity of megakaryocytes in-vivo. To investigate whether deregulated proliferation of both cell lineages is also present in bone marrow tissue, we performed a morphometric analysis in combination with immunohistochemistry on trephine biopsies. As easily-applicable cell-cycle marker an antibody (PC10) against nuclear proliferating antigen (PCNA) was used on formalin-fixed and paraffin wax-embedded marrow specimens (Keim and Hanash 1992; McCormick and Hall 1992). Moreover, because of the conspicuous macrocytic-megaloblastoid features of erythropoiesis in MDS (Jacobs 1985; Koeffler 1986; Delacretaz et al. 1987; Bartl et al. 1992), a comparative study on a small number of patients presenting with pernicious anaemia (vitamin B12 deficiency) was also carried out.

Materials and methods

Forty patients with MDS (23 men and 17 women; median age 66 years) were enrolled into this study. Diagnosis of this disorder was based on accepted criteria (Jacobs 1985; Koeffler 1986). The classification of MDS according to the French, American, British scheme (FAB) (Bennett et al. 1982) was as follows: refractory anaemia (RA) $n=1$, RA with ring sideroblasts (RARS) $n=2$, RA with excess of blasts (RAEB) $n=27$, chronic myelomonocytic leukaemia (CMML) $n=7$ and finally RAEB in transformation (RAEB-T) $n=3$. Of the 40 bone marrow specimens 25 displayed a hyperplastic, 11 a normocellular and four a hypoplastic subtype of MDS. Two other groups were also entered into this study: bone marrow specimens from four patients (1 man and 3 women, median age 74 years) with the clinical findings of pernicious anaemia due to vitamin B12 deficiency and samples from 15 individuals (5 men and 10 women; median age 59 years) with osteoporosis, but without haematological disorder (control group).

With informed consent, representative trephine biopsies of the bone marrow were performed from the posterior iliac crest as initial examinations. Fixation was carried out in an aldehyde solution for 12 to 24 h and further processing included decalcification for 3–4 days in 10% buffered EDTA, paraffin embedding and employment of several staining techniques for routine diagnosis (Schaefer 1984). Briefly, sequential double-immunostaining was performed using the biotin-streptavidin or LAB method (Guesdon et al. 1987) followed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (Cordell et al. 1984). All monoclonal antibodies were purchased from Dako-Diagnostika (Hamburg, FRG). The corresponding sections were incubated with PC10 as primary antibody against PCNA (1:100, 18 h at 0–4°C). The activity of the streptavidin labelled alkaline-phosphatase was visualized with nitro-blue-tetrazolium. After completion of immunostaining with the first antibody (PC10) according to the LAB method, sections were predigested with pronase. As second antibodies either Ret40f (anti-glycophorin C; 1:100, 48 h), or Y2/51-CD61 (1:15, 72 h) were applied (Gatter et al. 1988). Incubation with rabbit anti-mouse antibody and the APAAP complex (1 h) was repeated for 30 min each. The alkaline phosphatase was detected by the new fuchsin method (Stein et al. 1985). A weak nuclear counterstain with hae-

matoxylin was finally applied to identify the nucleated precursors of the erythroid lineage.

Following double-immunostaining with PC10 and Ret40f or Y2/51 (CD61) morphometric evaluation was carried out by a manual optic planimeter (MOP-A-MO1-Kontron) on trephine biopsies with extensive artefact-free marrow areas. As a parameter for cellularity the amount of haematopoiesis (absolute and relative value) was measured by determination of the total marrow area minus the area occupied by fat cells. Assessment of erythro- and megakaryopoiesis was undertaken by dividing the biopsy into five segments of approximately the same size. Following this procedure we counted about 100 Ret40f-positive nucleated cells (pro-, erythro-, and normoblasts) and in a similar way Y2/51 (CD61)-reacting cells (megakaryocytes) at 500 \times magnification in each field. Using this method at least 500 erythroid precursors and megakaryocytes were evaluated per marrow specimen. The PC10-reacting cells were recorded separately and calculated as absolute numbers per area of haematopoiesis and percentages of the total erythro- and megakaryopoiesis. Sizes of megakaryocytes were measured at a magnification of 1250 \times and regarded approximately 100 Y2/51 (CD61)-positive, randomly selected cells in each specimen. Statistical evaluation included Student's *t*-test to calculate possible differences between mean values of morphometric variables.

Results

Following immunostaining in most subtypes of MDS a Ret40f-positive erythropoiesis was recognizable which frequently revealed a macrocytic-megaloblastoid appearance (Fig. 1a). Y2/51 (CD61)-stained megakaryocytes were increased in number and occasionally clustered and, in comparison with the normal marrow (Fig. 1b), consisted either of atypical microforms or small-sized cell elements (Fig. 1c). Distinctive nuclear labelling could be detected easily in some erythroid precursor cells and also in megakaryocytes displaying PCNA-reactivity (Fig. 1a, c). In comparison with a control group morphometric evaluation of MDS revealed a reduction in erythropoietic cell elements, particularly in the subtypes CMML and RAEB-T (Table 1) and an increase in the number of megakaryocytes (Table 2). In contrast to a quantitative reduction, erythropoiesis exhibited a significant increase in PCNA-immunostaining (Table 1). With regard to the normal bone marrow, in MDS no difference of the PCNA-labelling index of the megakaryocytes was calculable (Table 2). However, a remarkable prevalence of microforms of this series (Fig. 1c) was found in the latter disorder, which is also evident from the corresponding size distribution curves (Fig. 2). Moreover, these curves indicate that PCNA-positive megakaryocytes were characterized by a shift to the left (significantly reduced cell sizes, Fig. 2). In pernicious anaemia erythroid and megakaryocytic cells were characterized by a striking increase in PCNA-staining capacity (Table 3). In this context, markedly small megakaryocytes (micro-megakaryocytes) were noticeable (Table 3).

Discussion

The results of our histochemical and morphometric evaluations are compatible with the commonly observed characteristics of MDS. These have been described as

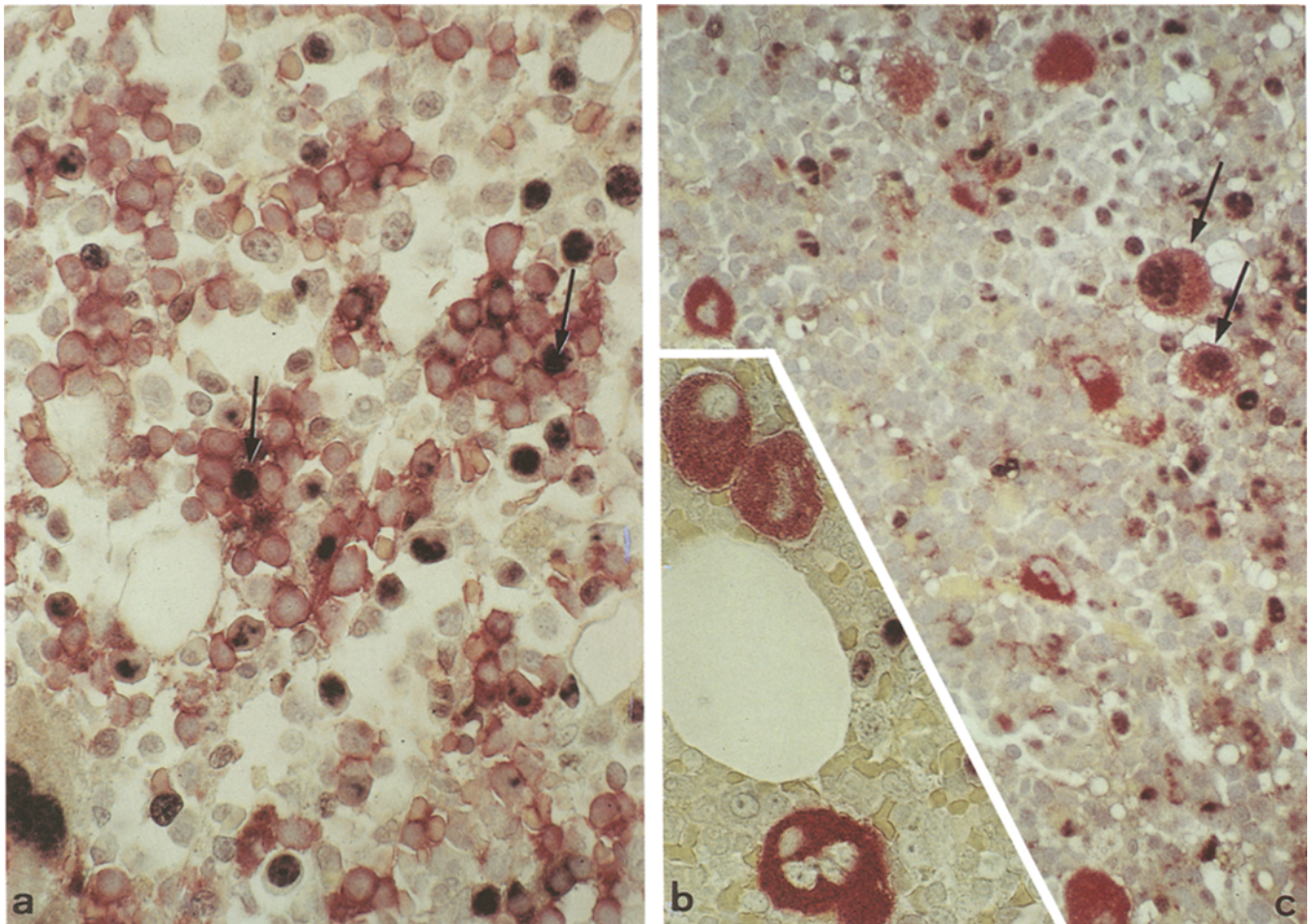


Fig. 1a–c. Erythro- and megakaryopoiesis in myelodysplastic syndrome – refractory anaemia with excess blasts – (MDS) following double immunostaining. **a** Ret40f (anti-glycophorin C) reveals large clusters of positively-reacting erythro-normoblasts with a macrocytic-megaloblastoid appearance. The nuclear labelling (arrows) in two cells by PC10 is consistent with PCNA-expression. **b** Medium-

sized megakaryocytes of the control group show a distinctive cytoplasmic staining by Y2/51 (CD61), but in this area no nuclear labelling by PC10. **c** Micromegakaryocytes in MDS disclose nuclear reactivity, indicative for PCNA expression (arrows), in addition to cytoplasmic staining (Y2/51-CD61), **a–c** $\times 650$

Table 1. Morphometric characteristics of erythropoiesis including proliferative activity in 40 patients with different subtypes of MDS. Comparison with corresponding normal bone marrow findings reveals significant differences

Erythroid precursors	MDS	Controls	
per mm ² total marrow area $\times 10^2$	9.1 \pm 2.4	14.6 \pm 2.7	$p \leq 0.0001$
per mm ² haematopoiesis $\times 10^2$	16.7 \pm 12.3	26.3 \pm 5.6	$p \leq 0.0007$
PCNA-positive cells per mm ²	32 \pm 14	44 \pm 24	$p \leq 0.0078$
PCNA-labelled cells per total erythropoiesis %	2.5 \pm 0.8	1.7 \pm 0.8	$p \leq 0.0001$

Table 2. Morphometric characteristics of megakaryopoiesis (including endoreduplicative activity and size) in 40 patients with different subtypes of MDS. With exception of the PCNA-labelling index comparison with normal bone marrow findings reveals significant differences

Megakaryocytes	MDS	Controls	
per mm ² total marrow area	74.2 \pm 16.1	20 \pm 6	$p \leq 0.0001$
per mm ² haematopoiesis	117.6 \pm 62.9	39.6 \pm 12	$p \leq 0.0001$
PCNA-positive cells per mm ²	1.5 \pm 1.3	0.3 \pm 0.3	$p \leq 0.0001$
PCNA-labelled cells per total megakaryopoiesis %	1.3 \pm 0.6	1.4 \pm 1.1	$p = 0.6185$
Size (μm^2)	182 \pm 78	277 \pm 75	$p \leq 0.0001$

Table 3. Morphometric characteristics of erythro- and megakaryopoiesis in pernicious anaemia including proliferative capacity of erythroid precursors and endoreduplicative activity and size of

megakaryocytes. Comparison with corresponding normal bone marrow findings reveals significant differences (* $p \leq 0.0001$) between these variables (see also Tables 1 and 2)

Pernicious anaemia	Erythroid precursors	Megakaryocytes
per mm ² total marrow area	721.9 ± 448.5*	67.8 ± 10.6*
per mm ² haematopoiesis	1312.6 ± 395.4*	89.5 ± 10.4*
PCNA-positive cells per mm ²	73.6 ± 32.7*	3.1 ± 0.7*
PCNA-labelled cells per cell lineage %	5.5 ± 1.5*	3.5 ± 0.9*
size (µm ²)	—	154.2 ± 23.9*

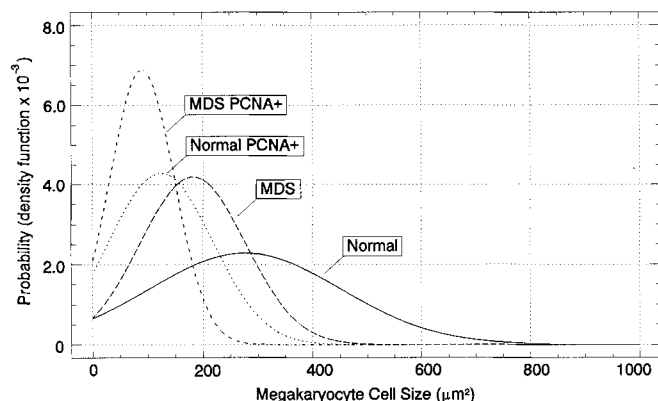


Fig. 2. Size distribution of megakaryocytes in the normal bone marrow and MDS with differentiation between proliferating cell nuclear antigen (PCNA)-positive fraction and total megakaryopoiesis. PCNA-positively reacting megakaryocytes are characterized by a significant shift to the left (smaller cell forms)

defective colony growth and maturation of haematopoietic precursors, generation of a megaloblastoid appearance to erythropoiesis and atypical micromegakaryocytes (Jacobs 1985; Koeffler 1986). However, comparison of both entities under study revealed that some of these features may be also encountered in pernicious anaemia and consequently could be related to the same underlying cause, that is to say, a vitamin B12/folate deficiency or disturbance of DNA-synthesis.

Problems arising with the exact quantification of single marrow elements in MDS are often associated with the different subtypes of the FAB classification (Bennett et al. 1982), the various degrees of cellularity and, finally the presence or absence of reticulin fibrosis. Usually, hyperplastic erythropoiesis and an increase in atypical (dysplastic) megakaryocytes are regarded as the most distinctive marrow features (Tricot et al. 1984; Fohlmeister et al. 1985; Frisch and Bartl 1986; Delacretaz et al. 1987; Kitagawa et al. 1989; Bartl et al. 1992). It is tempting to speculate that the general impression of erythroid hyperplasia may be partly simulated by its prominent macrocytic-megaloblastoid appearance. Consequently, on semi-quantitative evaluation of a non-selected series of 352 patients with primary MDS, including all subtypes of the FAB classification and different grades of myelofibrosis, about 50% of all cases displayed a normal to markedly decreased amount of this cell lineage (Maschek

et al. 1992). These data on erythropoiesis were in agreement with the results of our morphometric analysis showing a reduction of the mean value in a randomly selected cohort of patients (Table 1). Similar findings were recorded for megakaryopoiesis with a normal to increased frequency ranging between 80 to 90% of all specimens (Maschek et al. 1992). These semi-quantitative gradings corroborated data derived from a previous morphometric study (Thiele et al. 1991) and were also in keeping with the present evaluation.

In patients with MDS a variable degree of divergence from the normal growth pattern has been repeatedly observed in cell culture studies. Ineffective erythropoiesis was demonstrated by relevant ferrokinetic studies (Cazzola et al. 1982; Dörmer et al. 1987; Howarth et al. 1991). DNA content and cell-cycle analysis were consistent with the finding of erythroblasts arrested through their transit in the S- and G₂-phase (Wickramasinghe et al. 1968a; Montecucco et al. 1983; Peters et al. 1986). This prolongation of the cell-cycle due to a block in the S-phase was strikingly comparable with features occurring in vitamin B12 and folate deficiency anaemia (Wickramasinghe et al. 1968b). A similar defect of cell kinetics may be also encountered in megakaryopoiesis. In this lineage decreased or absent colony formation was reported as the most constant finding associated with impairment of erythroid growth (Lidbeck 1980; Greenberg 1986; Juvonen et al. 1986; Partanen et al. 1986; Gebbia et al. 1989).

A significant relationship between megakaryocyte size, nuclear size, degree of nuclear lobulation and ploidy is well-established (Levine et al. 1982; Williams and Levine 1982). In keeping with morphometric analysis (Thiele et al. 1991, 1992), MDS is characterized by a predominance of (dysplastic) hypolobulated and very small megakaryocytes, so-called atypical micromegakaryocytes (Fohlmeister et al. 1985; Frisch and Bartl 1986; Delacretaz et al. 1987; Bartl et al. 1992). Megakaryocytes of similar appearance may also be encountered in pernicious anaemia (Table 3). Small size and hypolobulation is consistent with a low ploidy level and therefore impairment of endoreduplicative capacity (Queisser et al. 1971; Kobayashi et al. 1993). The disturbance of this process by a cell-cycle block has been shown to be effective, particularly in pernicious anaemia (Queisser et al. 1971) and was also demonstrated to be present in MDS (Queisser et al. 1974; Kobayashi et al. 1993). It is noteworthy however, that in MDS regulation of en-

doreuplicative activity does not seem to be grossly altered at the lower ploidy levels. This assumption may be derived from the fact that PCNA-expression is significantly increased in small-sized megakaryocytes, comparable with normal megakaryopoiesis (Fig. 2). In the normal bone marrow a rhythmic polyploidization of this cell lineage occurs. Allowing the same time for DNA synthesis (S-phase), and identical lengths of resting periods (G1/2-phases) at the different ploidy stages would result in a decrease of megakaryocytes in the G-phases, due to their influx into maturation compartments (Queisser et al. 1971). Because of a progressive increase of cells in various ploidy stages as well as maturation stages (up to 16N in the normal bone marrow), there must be a difference in duration of cell-cycle phases in successive ploidy levels, with a significant prolongation of G1 and/or G2-phases. According to the modalities of PCNA-production lower ploidy levels should be associated with an increased expression of this marker protein, which has been demonstrated in our study (Fig. 2).

PCNA has been characterized as an auxiliary protein for polymerase-delta in DNA synthesis, is present in the nuclei throughout in the cell-cycle and produced in relevant amounts in late G1- and S-phase (Keim and Hanash 1992; McCormick and Hall 1992). In a previous paper it has been reported that erythroblasts and normoblasts derived from patients with pernicious anaemia or MDS showed marked PCNA-expression (Wilkins et al. 1992). In MDS an undue over-expression of PCNA was found in erythropoietic cell elements (Table 1), most probably caused by secondary folate deficiency. The latter defect was thought to generate an abnormal arrest of the cell-cycle predominantly of the S-phase (Wickramasinghe 1968a). Prolongation of the S-phase consequently increased the PCNA-labelling and was severe in vitamin B12 anaemia (Table 3); this feature may be used for differentiation from MDS. In both entities macrocytic-megaloblastoid erythropoiesis associated with small (dysplastic) megakaryocytes is a common morphological characteristic (Jacobs 1985; Koeffler 1986). Without further knowledge of laboratory data and clinical history, a clear-cut diagnosis may prove to be difficult. In these cases PC10-immunostaining could be a valuable aid. Although there are similar pathological mechanisms involved, a significant or excessive increase in the number of positively-reacting nuclei of haematopoietic cell elements provides arguments for pernicious anaemia rather than MDS, where these features are less conspicuously expressed.

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