Dysmegakaryopoiesis in myelodysplastic syndromes (MDS): An immunomorphometric study of bone marrow trephine biopsy specimens

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Abstract

An immunohistochemical and morphometric analysis was performed on trephine biopsy specimens of the bone marrow in 40 patients (23 men and 17 women, mean age 62 years) with different subtypes of myelodysplastic syndromes (MDS) to determine dysmegakaryopoiesis, but particularly precursor cells—that is, pro- and megakaryoblasts. In 31 of the 40 patients the numbers of megakaryocytes were increased which was associated with a predominance of smaller cell forms (micromegakaryocytes). Compared with periodic acid Schiff, immunostaining with a formalin resistant monoclonal antibody against glycoprotein IIIa (Y2/51(CD61)) showed a clinically important proportion of immature elements. These could be designated pro- and megakaryoblasts by taking morphometric measurements on smears and bone marrow sections. There was a relevant increase in the number of promegakaryoblasts in 32 patients, consistent with uncontrolled expansion of the precursor pool. Seventeen repeated bone marrow biopsy specimens taken after chemotherapy largely showed a decrease in the numbers of megakaryocytes including the precursor cell population. Moreover, morphometric evaluation disclosed that micromegakaryocytes in MDS differ significantly from those in chronic myeloid leukaemia (CML) due to distinctive nuclear features and a disturbed nuclear:cytoplasmic ratio. These changes generate a more pleomorphic or atypical appearance of this cell population in MDS, compared with micromegakaryocytes in CML.

It is concluded that the disproportionate increase in megakaryocyte precursors and the grossly abnormal aspects of micromegakaryocytes in MDS are characteristics of the severe defect involving haematopoiesis in this disorder.

Myelodysplastic syndromes (MDS) include several morphological subtypes defined by cytological criteria.¹ MDS may precede acute myeloid leukaemia (AML), sometimes by many years, and are further characterised by a clonal evolution, an ineffective and abnormal haematopoiesis, and variable degrees of cytopenias.^{2,3} Among the various abnormalities

affecting bone marrow morphology dysmegakaryopoiesis—that is, the occurrence of immature and small megakaryocytes with hypolobulated nuclei—has been described as the most typical feature.^{1,4-10} Moreover, in vitro cultures of haematopoietic progenitor cells derived from bone marrow in patients with MDS¹¹⁻¹⁵ showed a striking defect of megakaryocyte colony formation.¹⁶ Although atypias of this cell lineage seem to have a major role in the evolution of this disorder, there is hardly any accurate information about the prevalence and size of megakaryocytes as well as pro- and megakaryoblasts. The introduction of a formalin resistant monoclonal antibody against glycoprotein (Gp) IIIa, termed Y2/51 (CD61), facilitates the evaluation of total megakaryocytopoiesis in routinely processed trephine biopsy specimens in addition to blood films and smears of marrow aspirates.^{17,18} The combination of Y2/51 immunostaining and morphometry yields an unusual opportunity to determine the number and size of megakaryocyte precursors in bone marrow sections or in situ.^{19,20} In this paper we analysed dysmegakaryopoiesis in MDS by studying the trephine biopsy specimens of 40 patients and by immunomorphometrical methods.

Methods

Accepted criteria for the definition of MDS^{2,3} were used in a total of 40 patients (23 men and 17 women, mean age 62 years). The classification of MDS according to the FAB criteria was as follows: refractory anaemia (RA) (n = 1); RA with ring sideroblasts (RARS) (n = 2); RA with excess blasts (RAEB) (n = 27); chronic myelomonocytic leukaemia (CMML) (n = 8); RAEB in transformation (RAEB-T) (n = 2).¹ In 17 of the 40 patients enrolled in this study repeated bone marrow biopsies were performed at intervals ranging from two to 41 months and after chemotherapy. Of the 40 bone marrow specimens, 25 displayed a hyperplastic, 13 a normocellular, and two a hypoplastic subtype of MDS. Bone marrow samples from 15 patients (eight men and seven women, mean age 56 years) without any haematological disorders and a thrombocyte count within the normal range ($150-345 \times 10^9/l$) served as controls.

Trephine biopsy specimens of the bone marrow were taken from the posterior iliac crest²¹ on admission and after chemotherapy. Fixation comprised aldehyde solution for 12 to 48 hours (2 ml 25% glutaraldehyde, 3 ml

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Figure 1A–F
Megakaryocytes in MDS after immunostaining with an antibody against glycoprotein IIIa (Y2/51 (CD61): comparison between smears of sternal aspirates (figs 1A–C) and sections of bone marrow tissue (figs 1D–F).
Smears: fig 1A cluster of micromegakaryocytes; fig 1B promegakaryoblast; fig 1C megakaryoblast.
Sections: fig 1D micromegakaryocytes; fig 1E promegakaryoblast (arrow head); fig 1F megakaryoblast (arrow head).

37% formaldehyde, 1.58 g calcium acetate and distilled water per 100 ml), and further processing included decalcification for three to four days in 10% buffered EDTA, pH 7.4, paraffin wax embedding, and use of several staining methods.²²

The monoclonal antibody Y2/51 (CD61), which is directed against Gp IIIa, was purchased from Dako-Diagnostica GmbH (Hamburg, Germany). Before staining, all slides of the paraffin wax embedded marrow specimens were predigested with pronase (1 mg per 1 ml TRIS-buffered saline) for about 30 minutes at 37.5°C. Thereafter samples were stained using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique, with neo-fuchsin as the alkaline phosphatase substrate.^{23 24}

Morphometric evaluation was performed using a manual optic planimeter (MOP-A-MO1-Kontron) with a standard program set (Kontron software) on trephine biopsy specimens with an artefact-free marrow area of 6.9 (SD 2.5) mm² after immunostaining with Y2/51 (CD61) and periodic acid Schiff (PAS).

Total count for megakaryocytes per square millimetre was obtained at ×500 magnification by calculating the evaluable marrow area in the trephine biopsy specimen (excluding areas of haemorrhage and distortion as well as cortical and trabecular bone) and the total number of the corresponding PAS and

glycoprotein IIIa (Y2/51) positive elements. In this way only nucleated megakaryocytes were sought—that is, no pyknotic—degenerative forms or naked (bare) nuclei—and no (anuclear) cytoplasmic fragments, to avoid erroneous counting of giant platelets and marginal sections of megakaryocytes.

As in previous studies^{19 20} methods to characterise megakaryocyte precursors in smears and sections of bone marrow are only briefly described. For quantitative assessment of this cell population we performed a pilot study on smears of sternal aspirates stained with Y2/51 (CD61) from five patients. This preliminary investigation disclosed that, compared with the micromegakaryocytes of MDS (fig 1A), the most immature elements clearly recognisable as members of the megakaryocyte series—that is, promegakaryoblasts^{25–27}—had a diameter of 10.3 (0.6) μm (size 69.6 (1.3) μm²) (fig 1B). In corresponding tissue sections, in addition to the micromegakaryocytes (fig 1D), the most immature, glycoprotein IIIa (Y2/51) positive precursors (promegakaryoblasts) had a size of less than 50 μm,² a diameter about 8 μm, a round nucleus with dispersed chromatin, and a small rim of cytoplasm (fig 1E). On bone marrow smears there is a considerable flattening of megakaryocytes, so this decrease in size in tissue sections amounting up to 30% (spreading factor 1.3 to 1.5) is understandable.²⁸ Accordingly, on smear preparations megakaryoblasts, consist-

Table 1 Morphometric features (means and standard deviations) of megakaryocyte precursor cells in MDS after immunostaining with Y2/51 (CD61)

		Promegakaryoblasts	Megakaryoblasts
Size (μm^2)	Cell	39.6 (3.8)	73.4 (4.3)
	Nucleus	16.2 (2.9)	27.2 (3.8)
Diameter (μm)	Cell	7.5 (0.8)	11.6 (0.8)
	Nucleus	5.1 (0.6)	6.6 (0.5)
Form factor ($\times 10^{-3}$)	Cell	751 (13)	718 (5)
	Nucleus	762 (13)	737 (5)
Nuclear:cytoplasmic ratio ($\times 10^{-2}$)		40.2 (6.7)	37.2 (4.8)

tent with maturation stage I (fig 1C) were defined by a diameter of $19.6 (0.8) \mu\text{m}$ (size $110.4 (5.4) \mu\text{m}^2$), which agrees with published findings.²⁶⁻²⁹ In tissue sections of trephine biopsy specimens, these cells were characterised by a size ranging between 50 and $100 \mu\text{m}^2$ (diameter about $13 \mu\text{m}$), a round or slightly indented nucleus, and a not very extended portion of cytoplasm (fig 1F). The form factor or circular deviation (CD) of the megakaryocytes and their nuclei was defined as $\text{CD} = 4\pi A/C^2$ (C = circumference and A = area), giving the value $1000 \times 10^{-3} (1.0)$ for a

circular shape and a lower factor indicating an ellipsoid outline or increased irregularity. For morphometric calculation of this early subpopulation and the more mature, small to medium-sized and large megakaryocytes corresponding with maturation stages II to IV,²⁹ the total area of the trephine biopsy specimens was divided into five segments of the same size. More than 20 randomly selected megakaryocytic elements were measured in each field at a magnification of 1250 times with determination of area, diameter, circumference and circular deviation. Morphometric features of pro- and megakaryoblasts are summarised in table 1.

Results

An overview of bone marrow tissue from patients with MDS and stained by PAS shows that there was a remarkable clustering and predominance of pleomorphic small megakaryocytes (figs 2A and C). After immunostaining with Y2/51 (CD61) these features are most

Figure 2A–F Bone marrow tissue in MDS, comparison between PAS reaction (figs 2A, C, and D) and immunostaining (figs 2B, E, and F) with an antibody against glycoprotein IIIa (Y2/51 (CD61)).

Figure 2A Survey showing clusters of pleomorphic megakaryocytes.

Figure 2B Immunostaining shows dislocation of very small to medium sized megakaryocytes along the trabecular area (arrow heads).

Figure 2C Atypical micromegakaryocytes with hypolobulated dense nuclei and relatively small areas of cytoplasm.

Figures 2D and E Large megakaryocytes of the control group for comparison, displaying horseshoe-like nuclei surrounded by an extensive portion of cytoplasm.

Figure 2F Pleomorphic appearance of megakaryocytes disclosed by immunostaining.

Table 2 Prevalence and size of megakaryocytes (mean and standard deviation) in MDS (normal values of control group are given in parentheses)

Stain	Prevalence per mm ²	Size (µm ²)
PAS	30.4 (11.1) (10.6 (3.1))	297.4 (157.5) (338.6 (47.7))
Y2/51 (CD61)	69.9 (38.3) (22.8 (5.5))	226.0 (179.7) (277.3 (174.8))

prominently expressed and the appearance of micromegakaryocytes is conspicuous (figs 2B and F). An atypical localisation at the endostal border, normally reserved for neutrophilic granulocytopenia, was often demonstrable (fig 2B). Compared with the megakaryocytes of the control group (figs 2D and E), in MDS micromegakaryocytes show a relatively large, but hypolobulated nucleus surrounded by a not very extensive area of cytoplasm (fig 1D; figs 2C and F). Furthermore, immature elements (pro- and megakaryoblasts) are often disclosed (figs 1E and F) which are not or only partially (such as megakaryoblasts) identifiable by conventional staining methods. An increase in precursor cells as well as mature megakaryocytes (stages II to IV)²⁹ was often, but not significantly, associated with an increase in reticulin (argyrophilic) fibres. Collagen fibrosis was not present.

In only nine of the 40 patients with MDS the numbers of megakaryocytes fell within the normal range, independent of bone marrow cellularity (hyper-, normo-, or hypocellular subtypes). The overall prevalence and size distribution of megakaryocytes are given in table 2. In this survey cases of MDS were compared with normal bone marrow specimens after a PAS reaction as well as immunostaining with Y2/51 (CD61). The differences were significant. Discordant measurements in size distribution are illustrated in fig 3. A pronounced left-shift, which results in a predominance of small, probably immature elements of the megakaryocytopenia after Y2/51 staining was evident. Detailed data on the prevalence of pro- and megakaryoblasts are given in table 3. Compared with normal bone marrow, a disproportionate expansion of the precursor pool of megakaryocytes (relative numbers) is common in many patients with MDS. In 25 of 40 patients an excess of the upper limit of normal for promegakaryoblasts was calculated, and correspondingly, for megakaryoblasts in 32 patients. Chemotherapy generated a significant decrease in the number of megakaryocytes and their precursor elements in 13 of 17 patients investigated (table 3).

Figure 3 Size distribution of megakaryocytes in MDS after conventional PAS reaction and immunostaining with a monoclonal antibody against glycoprotein IIIa (Y2/51 (CD61)).

Discussion

Immunomorphological techniques were used to obtain an exact determination of megakaryocytopenia, thus extending the descriptive findings of cytological features in MDS. Moreover, in most patients an uncontrolled increase in pro- and megakaryoblasts was noticeable in bone marrow tissue compared with normal specimens. Dysmegakaryopoiesis was further characterised by the occurrence of abnormal micromegakaryocytes, highlighting obvious defects of maturation in comparison with CML specimens.

Exact quantitation of the numbers of megakaryocytes is only feasible by examining bone marrow biopsy specimens.³⁰ For this reason studies based on smears which report a reduction in the numbers of cells of this lineage in MDS^{31, 32} should be treated with caution. Significant differences may be generated by methodology.⁶ Megakaryocytes are known to have a close affinity with marrow reticulin, and a varying degree of fibrosis is a common finding in MDS.^{6-10, 32} It is doubtful, therefore, whether aspirates yield a representative insight into the amount of these marrow elements. Furthermore, the numbers of megakaryocytes were related to bone marrow cellularity.³² This result was not possible from our study because only two cases had a hypocellular subtype of MDS. A recently published immunohistochemical study of bone marrow trephines in MDS which also used Y2/51 (CD61) showed a significant increase in numbers of megakaryocytes, confirming our findings.³³

Previous studies on routinely stained smear preparations in patients with suspected “pre-leukaemia” showed a large number of micromegakaryocytes with a mean size of 618 (219)

Table 3 Morphometric features (mean and standard deviation) of megakaryocytopenia in MDS after immunostaining with Y2/51 (CD61)

	Myelodysplastic syndromes			
	Control group	Total	Sequential biopsies	
			First trephine	Second trephine
Megakaryocytes (total per mm ²)	22.8 (5.5)	69.9 (38.3)	83.4 (46.9)	64.0 (31.4)
Promegakaryoblasts (per mm ²)	1.3 (0.5)	6.3 (7.2)	9.6 (9.1)	6.6 (8.1)
Percent of total megakaryocytopenia	5.7 (2.1)	9.1 (6.9)	10.0 (6.8)	8.8 (9.2)
Megakaryoblasts (per mm ²)	2.1 (0.8)	13.5 (15.3)	22.3 (19.1)	18.2 (13.7)
Percent of total megakaryocytopenia	9.4 (3.2)	19.2 (12.3)	23.7 (12.2)	26.9 (15.9)

Table 4 Comparison of (micro)-megakaryocytes in CML and MDS with control group after immunostaining with Y2/51 (CD61) (mean and standard deviation)

	CML (n=41)	MDS (n=40)	Controls (n=15)
Cell			
Size (μm^2)	193.5 (122.3)	226.0 (179.7)	277.3 (174.8)
Form factor ($\times 10^{-3}$)	787 (152)	728 (143)	778 (140)
Nucleus			
Size (μm^2)	42.9 (30.2)	75.2 (66.3)	77.3 (55.7)
Form factor ($\times 10^{-3}$)	574 (255)	625 (202)	589 (225)
Nuclear:cytoplasmic ratio ($\times 10^{-2}$)	25.0 (12.3)	35.5 (16.6)	29.3 (15.3)

Data on CML are partially derived from a previous study.¹⁹ Only nucleated elements were sought—that is, excluding cytoplasmic fragments and naked (bare) and pyknotic nuclei.

μm^2 .³⁴ Provided that the spreading factor of about 30%²⁸ is taken into account in addition to the high standard deviation, this value falls in the range of measurements (bone marrow sections) reported in table 2. Qualitative abnormalities included in addition to normal sized megakaryocytes, a large number of immature and small cells with a compact or incompletely lobulated nucleus, and a hypogranulated cytoplasm—that is, micromegakaryocytes.^{14-10 33 34} In the immunohistochemical study already referred to,³³ all Y2/51 (CD61) positive "mononuclear cells" with a diameter of less than 15 μm were regarded as micromegakaryocytes. This cell population comprised about 25% of the total megakaryocytopoiesis which was apparently evaluated by calculation of five high power fields per case (n = 11). As these determinations were entirely based on size (diameter) and not maturity, it is evident that this fraction included also pro- and megakaryoblasts with a diameter ranging between 7.5 to 11.6 μm (table 1).

Because micromegakaryocytes are a prominent histopathological feature of CML,^{19 34-42} it is intriguing to compare this peculiar cell population with the small and pleomorphic megakaryocytes observed in MDS. In this context, immunostaining with Y2/51 (CD61) seems to be the most appropriate method of identifying all the elements of megakaryocytopoiesis. Morphometric characteristics of megakaryocytes in CML, which were partially derived from a previous study,¹⁹ permit such an evaluation. The results of this calculation are

Figure 4 Histomorphometric characteristics of megakaryocyte maturation stages—that is, dysmegakaryopoiesis in CML and MDS compared with the normal differentiation of a control group^{26 29} shown by immunostaining with Y2/51 (CD61).

summarised in table 4 and schematically illustrated in fig 4. Although there was no significant difference in size for both subtypes of micromegakaryocytes, in MDS a more irregular cell shape and a larger and more rounded nucleus were demonstrable, generating a greater deviation of the nuclear:cytoplasmic ratio (fig 4). These variables may account for the more abnormal—that is, dysplastic aspect—of the micromegakaryocytes in MDS compared with those of CML.

Analysis of dysplastic hematopoiesis in bone marrow cultures disclosed abnormal behaviour patterns, with common defects including absent, reduced, or even increased cluster formations of myeloid progenitor cells,¹¹⁻¹⁴ but particularly after long term culture experiments.¹⁵ The ability megakaryocyte progenitors (CFU-M) have for colony formation is of special interest when considering the increase in promegakaryoblasts in many patients enrolled in this study (table 3). In a comprehensive investigation¹⁶ nine out of 10 patients with MDS displayed defective growth of CFU-M colonies, similar to findings for erythroid progenitor cells.¹² It is tempting to speculate that this unruly expansion of the megakaryocyte precursor pool is indicative of a severe defect involving haematopoiesis in MDS which grossly impairs the normal regulation and maturation of this cell line.^{27 43 44}

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