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Anagrelide does not exert a myelodysplastic effect on megakaryopoiesis: a comparative immunohistochemical and morphometric study with hydroxyurea

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Summary. A comparative immunohistochemical and morphometric study was performed on megakaryocytes in 20 patients presenting with initial-early stage chronic idiopathic myelofibrosis and accompanying thrombocythemia to elucidate histological features developing after hydroxyurea (HU) versus anagrelide (ANA) therapy. Representative pre-and posttreatment bone marrow biopsies were involved including the monoclonal antibody CD61 for the identification of precursor and mature stages of megakaryopoiesis. An elaborate morphometric evaluation was in keeping with a left-shifting showing a more frequent occurrence of promegakaryoblasts and microforms in both therapy groups. However, contrasting ANA, HU generated defects of differentiation consistent with significant dysplastic changes. In conclusion, concern about a possible leukemogenic capacity following long-term HU therapy is supported by our findings.

Key words: Anagrelide, Morphometry, Hydroxyurea, Megakaryopoiesis, Megakaryocyte dysplasia, Immunohistochemistry

Introduction

Controversy and discussion continues to arise when referring to the leukemogenic potential of cytoreductive agents like hydroxyurea (HU) that is usually applied as standard therapy (Liozon et al., 1997; Finazzi and Barbui, 1999; Fruchtman, 2004) in chronic myeloproliferative disorders (CMPDs). Several studies have suggested the mutagenic potential of HU, but this issue remains an open question because so far no randomized clinical trials have been conducted (Barbui, 2004; Fruchtman, 2004). In this context, it was reported that significantly expressed myelodysplastic changes of the bone marrow (BM) may precede leukemic transformation (Löfvenberg et al., 1990; Weinfeld et al., 1994; Higuchi et al., 1995; Furgerson et al., 1996; Sterkers et al., 1998; Randi et al., 2000). Especially in CMPDs with associated thrombocythemia and the ensuing risk for thromboembolic complications and bleedings, HU was frequently used as the mainstay treatment option in patients that apparently later developed an increased frequency of acute leukemia (Higuchi et al., 1995; Furgerson et al., 1996; Liozon et al., 1997; Finazzi and Barbui, 1999). The putative mutagenic risk has stimulated the search for drugs with a more favorable effect profile, because thromboreductive treatment must be efficacious over a long time (Storen and Tefferi, 2001; Fruchtman, 2004). In this regard promising results were obtained in particular with anagrelide hydrochloride (ANA), a drug that, according to an increasing amount of clinical data accumulated in the last years, may be used safely in CMPDs accompanied by an excess in platelets (Anagrelide Study Group, 1992; Balduini et al., 1992; Mazzucconi et al., 1992; Petitt et al., 1997; Tefferi et al., 1997; Petrides et al., 1998; Brooks et al., 1999; Silverstein and Tefferi, 1999; Laguna et al., 2000; Pescatore and Lindley, 2000; Storen and Tefferi, 2001; Andersson, 2002; Gilbert, 2002; Birgegard et al., 2004; Dingli and Tefferi, 2004; Mazzucconi et al., 2004; Petrides, 2004; Steurer et al., 2004). It is well-known that abnormalities of megakaryocyte features (atypical micromegakaryocytes, defects of nuclear-cytoplasmic maturation) are characteristics of myelodysplasia and may possibly herald leukemic transformation (Tricot et al., 1984; Delacretaz et al., 1987; Fox et al., 1990; Rios et al., 1990; Thiele et al., 1991; Bartl et al., 1992; Mangi and Mufti, 1992). For this reason, we performed a comparative immunohistochemical and morphometric study to elucidate whether and to what extent morphological features of $CD61^+$ megakaryopoiesis are altered following administration of these two agents for

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more than one year.

Material and methods

Patients

A retrospective comparative evaluation of clinical records and BM biopsy samples was performed in two groups of patients with the diagnosis of early stage chronic idiopathic myelofibrosis (CIMF), according to the WHO classification (Thiele et al., 2001) associated with a sustained platelet count that exceeded $600x10^9$/l. The first cohort consisted of 10 patients that received ANA at an average maintenance dose between 1.5 to 2.5 mg/day for a median of 18 months. The second cohort comprised also 10 patients treated with HU (20 to 40 mg/day) for a median period of 13 months. Following monotherapy in the absence of any preceding or concurrent agents, there was a significant thromboreductive effect observable in all patients with a decrease in the platelet count ranging from 1,340-1,100 $x10^9$/l at presentation to 450-385$x10^9$/l during follow-up.

Bone marrow biopsies

Representative trephine biopsies of the BM (length 14.9±1.7 mm) were performed in all patients from the posterior iliac crest at diagnosis. Fixation was carried out in an aldehyde solution for 12-48 hours (2 ml 25% glutaraldehyde, 3 ml 37% formaldehyde, 1.58 g anhydrous calcium acetate, and distilled water per 100 ml). Further processing included decalcification for 3-4 days in 10% buffered ethylene-diamine tetra-acetic acid (EDTA), pH 7.2 and paraffin embedding. Immunohistochemistry with a monoclonal antibody was applied for the proper identification of $CD61^+$ megakaryocytes including precursor cells like pro- and megakaryoblasts (Gatter et al., 1988). Monoclonal antibodies and other reagents were purchased from Dako-Diagnostica GmbH, Hamburg, Germany. Details of staining procedures (APAAP-method) were reported in previous communications (Cordell et al., 1984; Thiele et al., 1999).

Morphometry

Following immunostaining with CD61, morphometric analysis was performed by two manual optic planimeters (VIDAS-Zeiss-Kontron) and a specially developed program set (Optimas software) on large, representative trephine biopsies with an artefact-free mean marrow area of 15.4±4.7 mm^2. In each sample we regarded the total marrow area and no patient showed a megakaryocyte count below 250. Computer-assisted planimetric measurements of megakaryocyte size and allied 21 standardized parameters for each individual cell (amongst others form factor, aspect ratio, roundness, perimeter, maximal length, width) was carried out and involved a total of 15,619 $CD61^+$ megakaryocytes. To elucidate the possible effect of therapy (ANA versus HU), all morphometric results concerning megakaryopoiesis were pooled for each of the pre- and post-treatment groups. Statistical analysis included the Mann-Whitney-U test with $p<0.01$.

Results

Compared to the first (pretreatment) BM biopsy samples with early stage CIMF that showed a prominent and abnormally clustered megakaryopoiesis (Fig. 1a), after therapy with HU and ANA, even more pronounced changes were detectable. It is noteworthy that HU caused an impressive enhancement of cellular abnormalities of $CD61^+$ megakaryocytes, occasionally generating a bizarre appearance (Fig. 1b,d). Additionally, a 5 to 7 fold increase in the frequency of

Table 1. Planimetric evaluations of $CD61^+$ megakaryopoiesis - cytoplasmic parameters during different therapeutic regimens

	ANAGRELIDE (ANA)			HYDROXYUREA (HU)		
	before treatment	after treatment		before treatment	after treatment	
No. of megakaryocytes evaluated	4,523	4,295		3,621	3,180	
Size (μm^2)	470±294	393 ±2 89	*	439±288	370±257	*
micromegakaryocytes (< 150 μm^2)	7.6 %	17.3 %	*	11.4 %	17.7 %	*
normal megakaryocytes (> 250 μm^2)	76.3 %	60.0 %	*	71.7 %	60.5 %	*
Perimeter (µm)	79±26	72±27	*	77±27	72±27	*
Maximal length (µm)	29.4±10.3	26.7±10.7	*	28.5±10.8	27.6±11.3	*
Roundness $x10^2$	65.9±14.7	65.5±14.5		64.2±15.4	60.4±16.7	*
Circularity	14.7±2.1	14.7±2.2		15.0±2.4	15.7±3.3	*
Aspect ratio $x10^{-1}$	14.7±4.0	14.8±3.9		15.2±4.4	16.4±5.8	*
Width $x10^{-2}$ (µm)	1.9±0.6	1.6±0.6	*	1.8±0.6	1.5±0.6	*
Form factor x 10^2	86.8±9.7	87.0±9.9		85.4±10.6	82.5±12.6	*

*: $p < 0.05$ before and after treatment for therapy group.

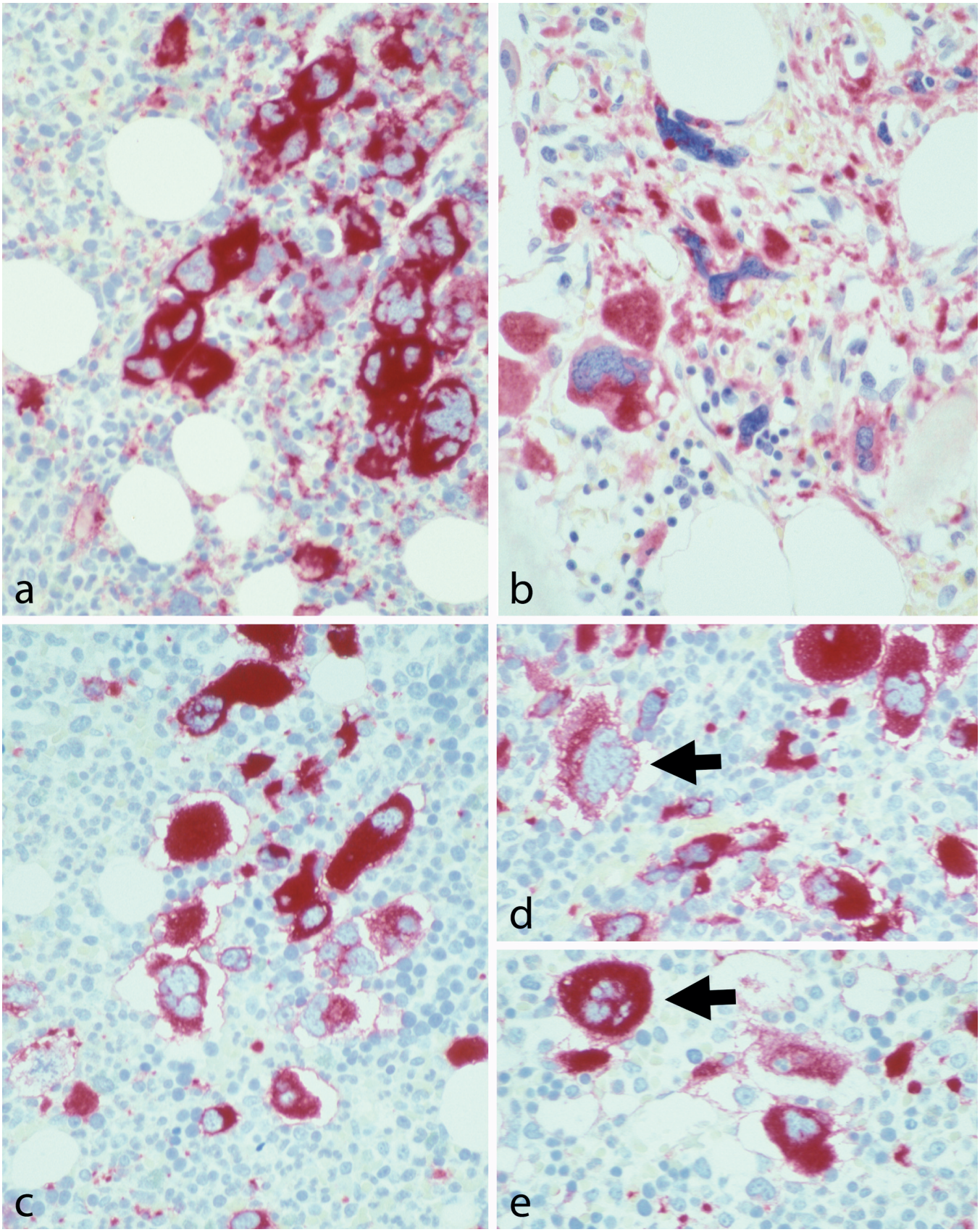

Fig. 1. Megakaryopoiesis in chronic idiopathic myelofibrosis with associated thrombocythemia following hydroxyurea (HU) or anagrelide (ANA) therapy. **a.** Pretreatment specimens reveal a dense clustering of large to medium-sized megakaryocytes with moderate abnormalities of differentiation. **b.** Following HU treatment, megakaryopoiesis shows a bizarre aspect and several dense naked nuclei. **c.** ANA causes some abnormal differentiation including the occurrence of micromegakaryocytes. **d.** Marked anomalies, i.e. a dysplastic appearance (arrow) is detectable in megakaryocytes after HU treatment. **e.** On the other hand, following ANA therapy, a number of megakaryocytes displays only minor to moderate anomalies (arrow) of maturation. a-e, CD61 immunostaining; a-c, x 380; d, e, x 420

naked and almost denuded nuclei with condensed chromation pattern was observable (Fig. 1b). Contrasting this finding, ANA generated a pleomorphous aspect with small and large megakaryocytes (Fig. 1c) that lacked a striking abnormality of differentiation (Fig. 1e). Morphometric evaluation was in keeping with significant alterations occurring especially after HU treatment and included not only cell size with a left-shifting, i.e. increase in microforms (dwarf megakaryocytes), but further anomalies concerning features of megakaryocytes (Table 1) and their nuclei (Table 2). On the other hand, following ANA therapy, an increase in the quantity of micromegakaryocytes was also detectable (Table 1), however, no significant changes indicating a prodigious disturbance of differentiation were detected (Table 2). Comparison of the cytoplasmic and nuclear features of $CD61^+$ megakaryopoiesis between the two cohorts of patients exhibited significant differences concerning the effect of ANA versus HU as outlined in more detail in Tables 1 and 2. Taken together, although both agents generated an increase in the occurrence of micromegakaryocytes (Table 1), HU treatment caused a conspicuous anomaly of cytoplasmic and nuclear parameters (Tables 1, 2) that were consistent with a dysplastic appearance. These changes are schematically outlined in Fig. 2. Regarding the other cell lineages, although morphometric evaluations were not carried out, following HU therapy, abnormalities of histopathological features were also observed. Maturation defects consisted mainly of a relative predominance of neutrophil granulopoiesis that showed a prevalence of less mature cells (so-called left-shifting) and an arrested maturation of erythropoiesis with a macrocytic appearance of the nucleated erythroid precursors in some of the patients.

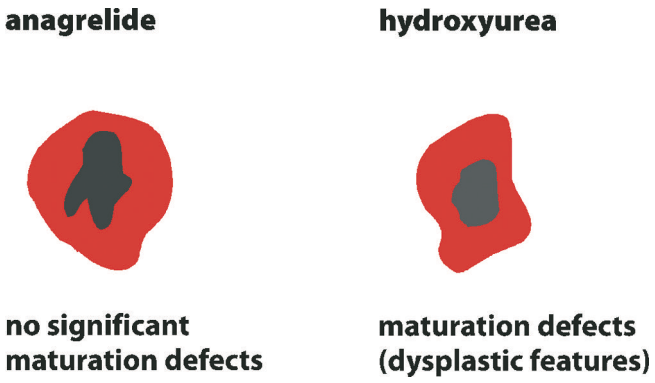

Fig. 2. Schematic presentation of megakaryocyte morphology summarizing morphometric data in both therapy groups.

Discussion

One of the most prevalent clinical challenges in the treatment of CMPDs is thrombocythemia which may frequently be present in all subtypes and presents the causative patho-mechanism for fatal thromboembolic complications as well as bleedings (Schafer, 2004). Altogether, therapeutic strategies are aimed at reducing thrombohemorrhagic incidences and should accomplish this goal without increasing the risk of leukemic transformation (Barbui, 2004). Currently, the main options for platelet lowering treatment are HU, interferon-α (IFN), in some countries pipobroman and ANA (Gilbert, 2002; Fruchtman, 2004). With respect to its potency for leukemic conversion, there is still controversy regarding the adverse long-term effects of HU (Barbui, 2004). Several studies documenting an increased frequency of leukemic transformation (Löfvenberg et al., 1990; Weinfeld et al., 1994; Higuchi et al., 1995; Furgerson et al., 1996; Liozon et al., 1997; Sterkers et al., 1998) have been criticized for an obvious bias, due to the fact that besides small series under investigation, patients with more aggressive disease are more likely to receive cytostatics (Finazzi and Barbui, 1999; Barbui, 2004; Fruchtman, 2004). However, an increasing concern about the possible long-term leukemogenic effect of this, in particular in patients who have already been treated by busulfan drug (Tefferi, 2002; Barbui and Finazzi, 2003), cannot be overlooked

Table 2. Planimetric evaluation of $CD61^+$ megakaryopoiesis - nuclear parameters during different therapeutic regimens.

	ANAGRELIDE (ANA)			HYDROXYUREA (HU)		
	before treatment	after treatment		before treatment	after treatment	
Size (μm^2)	124±106	111±97	*	126±105	100±87	*
Perimeter (μm)	52±30	49±30	*	50±27	44±25	*
Maximal length (μm)	18.4 ±10.8	17.5 ±16.4	*	18.2±9.9	16.4±9.2	*
Roundness $x10^2$	50.7±20.9	50.6±20.8		52.0±20.6	50.8±20.5	
Circularity	17.3±5.1	17.1±4.9		16.6±4.5	16.7±4.4	
Aspect ratio $x10^{-1}$	15.2±3.9	15.2±3.8		15.3±4.2	16.1±5.0	*
Width $x10^{-2}$ (μm)	7.8±3.5	7.3±3.3	*	7.9±3.5	6.9±3.0	*
Form factor $x10^2$	63.4±24.9	63.6±24.7		66.2±23.9	66.9±23.5	
Nuclear/cytoplasmic ratio $x10^2$	29.9±0.7	32.6±4.5	*	33.6±6.3	30.8±6.0	*

*: $p<0.05$ before and after treatment for therapy group.

(Finazzi et al., 2000; Nielsen and Hasselbalch, 2003). On the other hand, unwanted toxic side effects related to ANA therapy are in need of monitoring, especially of elderly patients, concerning heart failure and pulmonary hypertension (Dingli and Tefferi, 2004; Mazzucconi et al., 2004; Petrides, 2004; Steurer et al., 2004).

Altogether, a conflict of opinion still persists about the mutagenic capacity of HU which is mostly due to the fact that no randomized prospective trial has been conducted on a large series of patients with prolonged follow-up to validate this important issue (Sterkers et al., 1998; Fruchtman, 2004). There is general consent that maturation defects of the megakaryocytic cell lineage are prominent features of myelodysplastic syndromes (Thiele et al., 1991; Vardiman, 2003) and may be the prelude to leukemia. It is noteworthy that, contrasting IFN treatment, HU generated significantly expressed maturation defects (dysplasia) in the megakaryo- as well as erythropoiesis in CIMF comparable to the alkylating agent busulfan (Thiele et al., 2003b). The alterations described in megakaryocytes in this study are in keeping with this observation and underline the adverse influence of this drug on the differentiation of this cell lineage. In addition to these adverse effects, the striking left-shifting with prevalence of promegakaryoblasts indicates an inhibition of the endomitotic (endoreduplicative capacity) that is comparable with the changes after ANA therapy. Therefore, it seems to be reasonable to assume that both, left-shifting and gross disturbances of maturation, are the causative pathomechanims for the platelet-lowering efficacy of HU, while ANA acts predominantly by interfering with the normal process of megakaryocyte differentiation (Solberg et al., 1997; Yoon et al., 1999; Thiele et al., 2003a). In this context, it is noteworthy that the thromboreductive effect is not accomplished by a decrease in the number of megakaryocytes or stimulation of programmed cell death, as has been previously suggested (Tomer, 2002).

In conclusion, in contrast to ANA, HU generates significantly expressed maturation defects on the megakaryopoiesis consistent with dysplastic changes, besides a left-shifting with predominance of promegakaryoblasts. Therefore, the concern of a leukemogenic potential of HU, especially following long-term therapy is supported by morphology.

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