

Original Article

Progression of Bone Marrow Fibrosis in Patients with Essential Thrombocythemia and Polycythemia Vera During Anagrelide Treatment

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Abstract

Anagrelide is a second-line option for reduction of thrombocythemia in patients with chronic myeloproliferative disorders (CMPDs). A multicenter, open, phase II study of anagrelide treatment in 60 patients during 2 yr was performed by the Swedish Myeloproliferative Disorder Study Group. Adequate bone marrow biopsies were obtained from 53 of the CMPD patients [36 essential thrombocythemia (ET), 16 polycythemia vera (PV), 1 chronic idiopathic myelofibrosis (CIMF)] before treatment and compared with biopsies from 30 healthy volunteers and 34 patients with acute myeloid leukemia (AML). Higher reticulin and hyaluronan (HYA) scores were found before anagrelide therapy in the CMPD patients than in the normal controls ($p < 0.001$ and $p < 0.001$, respectively) and AML patients ($p < 0.001$ and $p = 0.011$, respectively). At the end of the study 30 CMPD patients were still on anagrelide treatment and in 19 of these patients, all diagnosed as ET ($n = 16$) or PV ($n = 3$), pretreatment bone marrow biopsies were compared with follow-up samples. After 2 yr of anagrelide therapy the reticulin and HYA scores were significantly higher than before treatment ($p = 0.02$ and $p = 0.002$, respectively). The cellularity was significantly higher ($p = 0.014$), although the number of megakaryocytes did not change significantly. The increase of reticulin and HYA in the bone marrow after 2 yr of treatment with anagrelide indicated progression of fibrosis. Although anagrelide is a valuable drug for reduction of platelet levels, it seems unable to stop progression of bone marrow fibrosis and hypercellularity in ET and PV.

Key Words: Anagrelide; hyaluronan; reticulin; fibrosis; essential thrombocythemia; polycythemia vera.

Introduction

Fibrosis is an important bone marrow finding overlapping the various chronic myeloproliferative

disorder (CMPD) entities. Most patients with chronic idiopathic myelofibrosis (CIMF) are diagnosed in the fibrotic state. In polycythemia vera (PV), reticulin fibrosis in the bone marrow has been reported in 11% of the patients at diagnosis and fibrosis is regularly seen before and during the spent phase of the disease, whereas fibrosis is rarely seen in essential thrombocythemia (ET)

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patients at diagnosis (1). Megakaryocyte proliferation and interaction with the bone marrow microenvironment are important components in the pathogenesis of myelofibrosis. It is believed that the interaction between the clonal megakaryocytes and the reactive fibroblasts is mediated by various fibrogenic growth factors, like transforming growth factor beta (TGF-beta), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and calmodulin, and through involvement of specific integrin receptors (2,3). Essential components of the myelofibrotic stroma are collagen types I, III (reticulin), IV, V, and VI, proteins like laminin and fibronectin, and the glycosaminoglycans (GAGs), with hyaluronan (HYA) being the most abundant.

HYA is a large non-sulfated polymer composed of alternating units of *N*-acetyl-glycosamine and D-glucuronic acid. The molecule has not only structural properties in joints and connective tissues but also important biological functions during embryonic development, healing processes, inflammation, and tumor development (4). Several growth factors, e.g., PDGF, TGF-beta, FGF-2, and epidermal growth factor (EGF), have been reported to stimulate the synthesis of HYA in cell culture (5,6). We have earlier reported a correlation between HYA and reticulin staining in bone marrow in patients with acute myeloid leukemia (AML) and in healthy individuals (7,8). The established method to evaluate bone marrow fibrosis is reticulin and collagen staining. However, the dynamics and velocity of the fibrotic process cannot be studied with this method. As HYA is synthesized as a "forerunner" to collagen production, HYA deposition may be the earliest sign of impending fibrosis (9).

Thrombocytopenia can cause both bleeding and thromboembolism and is always present in patients with essential thrombocythemia (ET) and may also occur in the other CMPDs. One aim of CMPD treatment is to reduce the risks associated with increased platelet levels. Anagrelide is a non-cytostatic drug with a selective effect limited to reducing the platelets by inhibiting the megakaryocyte development (10). Anagrelide has been introduced as an option for treatment of thrombocytopenia in CMPD, although hydroxyurea is generally considered to be the drug of choice (11). We have recently reported

the results of a prospective study investigating clinical toxicity and efficacy of anagrelide in patients with CMPD (12). The aim of the present study, which was performed on biopsies from the same patients, was to investigate the effect of anagrelide on the bone marrow, evaluating the matrix and the cellular elements with histological analysis and the extent of fibrosis with reticulin and HYA staining.

Material and Methods

Patients

A multicenter, open, prospective, phase II study of anagrelide treatment in 60 patients with thrombocytopenia due to chronic myeloproliferative disorders was performed by the Swedish Myeloproliferative Disorder Study Group (12). The trial was designed to assess the feasibility of anagrelide treatment, i.e., clinical effects, short- and long-term tolerability, and patient management. No comparison with other treatment was intended and therefore no control group was recruited. Anagrelide was administered orally and the starting dose was 0.5 mg given twice a day. If there was no response, the daily dose was increased by 0.5 mg per week. The dose limit was 2.5 mg for a single dose. No patient exceeded 5 mg/d, and the mean maintenance dose was 2.3 ± 0.15 mg/d. The observation time was 2 yr and at the end of the study 50% ($n = 30$) of the patients continued anagrelide treatment (12).

Bone marrow trephine biopsies were collected at the start of the study and adequate samples were obtained in 53 patients with median age 55.1 yr, range 28.3–78.3 yr. The median age of the 31 females was 4.1 yr lower than that of the 22 males, 54.8 and 58.9 yr, respectively. The diagnosis was established according to the diagnostic criteria of Pearson et al. for PV (13) and Kutti and Wadenvik for essential ET (14). Diagnoses and gender distribution are shown in Table 1. Twenty-nine of the patients had no previous treatment, 19 had hydroxyurea, 1 had hydroxyurea and alpha-interferon, 1 had hydroxyurea and busulphan, and 3 had alpha-interferon before anagrelide treatment. Biopsies were taken before the start of anagrelide treatment, after 6 mo, and after 2 yr on treatment. At the end of the study there were 19 patients with adequate biopsies both from start and at 2 yr, with a median age of

Table 1
Diagnoses and Gender Distribution for Patients with Bone Marrow Biopsy at Start of the Study (n, %)

Diagnosis	Females	%	Males	%	Total	%
ET	23	74	13	59	36	68
PV	8	26	8	36	16	30
CIMF	0	0	1	5	1	2
Total	31	100	22	100	53	100

ET: essential thrombocythemia; PV: polycythemia vera; CIMF: chronic idiopathic myelofibrosis.

52.6 yr, range 34–74 yr. Ten of these were females (median age 51.0 yr) and 9 were males (median age 55.1 yr). Diagnoses and gender distribution are shown in Table 2. Eleven of these patients had no previous treatment, 6 had hydroxyurea, 1 had hydroxyurea and alpha-interferon, and 1 had alpha-interferon before start of anagrelide.

Normal Controls and AML Patients

Bone marrow biopsies from 30 healthy volunteers, 10 males and 20 females, median age 29.5 yr (range 18–60 yr), served as normal controls (7). Bone marrow biopsies from 34 patients with newly diagnosed AML with no antecedent CMPD, 19 males and 15 females, median age 64.5 yr (range 29–81 yr), were also used in the analysis (8).

Bone Marrow Biopsies

Serial sections of bone marrow biopsies were mounted on glass slides for hematoxylin–eosin staining, silver impregnation for visualization of reticulin fiber content according to Laidlaw or Gordon-Sweet (15,16), and histochemical localization of HYA. Isolation and biotin labeling of the hyaluronan-binding protein (HABP) have been described previously (7,17). The HABP was a kind gift from Corgenix (CO, USA). The histochemical staining procedure for HYA was performed in the same manner as described earlier (7,18).

Microscopy

Hematoxylin–eosin stained sections were used for evaluation of morphology, cellularity, and number of megakaryocytes/mm². At least four marrow spaces with high quality had to be present in a biopsy for inclusion and evaluation. All samples were coded and examined in a blinded fashion. Bone marrow

Table 2
Diagnoses and Gender Distribution for Patients with Bone Marrow Biopsies at Both Start and End of the Study (n)

Diagnosis	Females	Males	Total
ET	10	6	16
PV	0	3	3
Total	10	9	9

ET: essential thrombocythemia; PV: polycythemia vera.

reticulin was quantified using the following scoring system described by Bauermeister (19): 0, no reticulin fibers demonstrated; N, occasional fine individual fibers only; 1, occasional fine individual fibers plus foci of fine fiber network; 2, fine fiber network throughout most of the section with no coarse fibers demonstrated; 3, diffuse fiber network and scattered thick, coarse fibers but no true collagen; 4, diffuse, often coarse, fiber network with areas of collagenization. According to Bauermeister the upper limit for normal biopsies is 2. The content of HYA in bone marrow was graded according to intensity in a four-graded scale: 1, sparse; 2, weak; 3, moderate; 4, intense. The same grading scale was used for the control group and AML patients. Sparse and weak was considered normal (7,8). Reticulin and HYA staining of two bone marrow samples are shown in Figs. 1 and 2.

Statistics

The reticulin grading, HYA score, cellularity, and number of megakaryocytes/mm² in the consecutive bone marrow samples were compared using the Wilcoxon signed rank test. The reticulin grading and

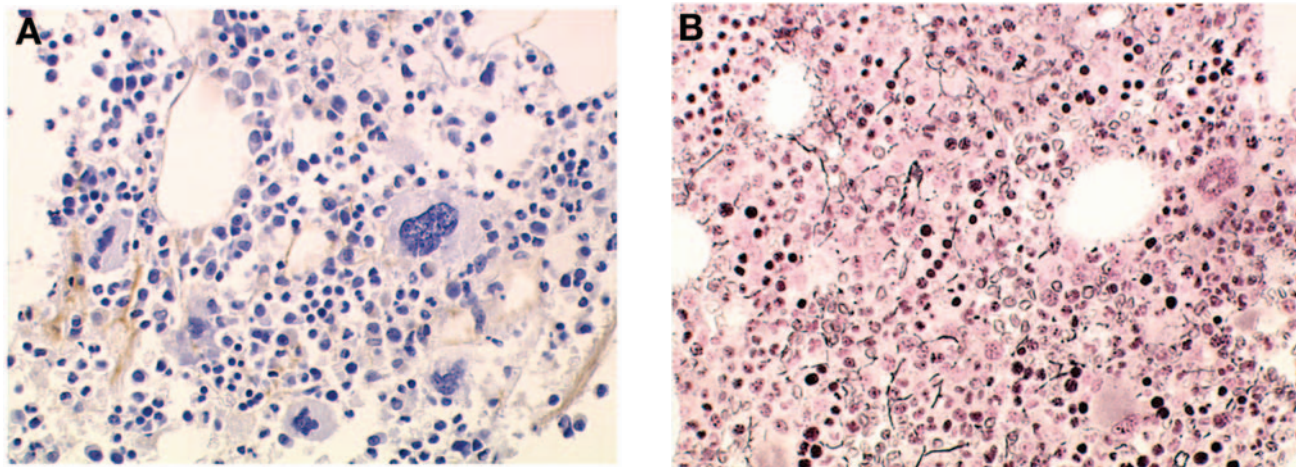


Fig. 1. Bone marrow biopsy from patient A with essential thrombocythemia. (A) HYA staining (brown) is sparse in scattered areas and graded 1. Magnification $\times 360$. (B) Reticulin staining showing occasional fine individual fibres and small foci with fine fibre network, graded 1. Magnification $\times 360$.

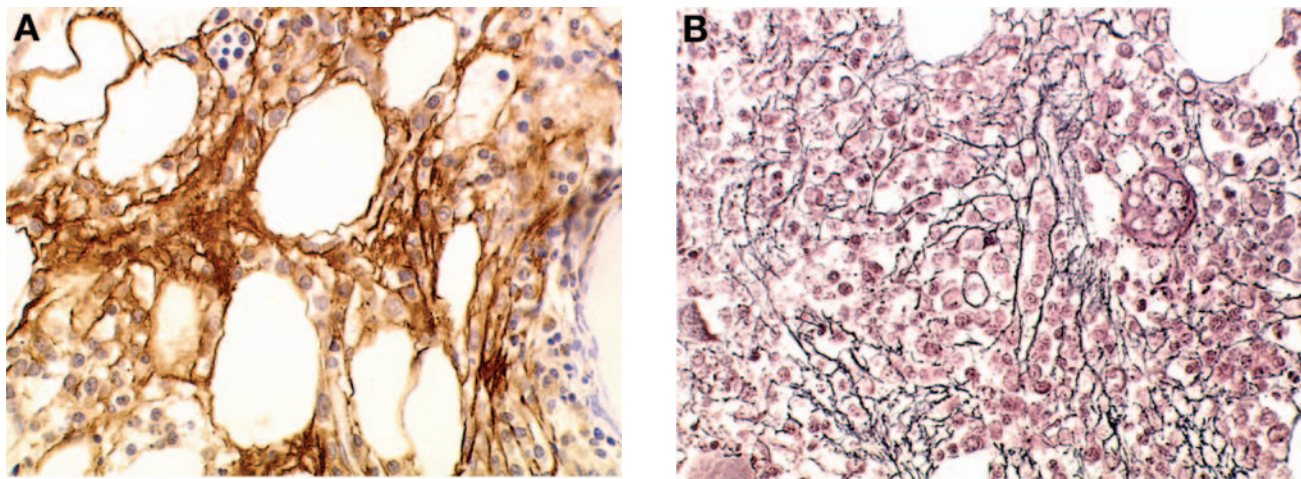


Fig. 2. Bone marrow biopsy from patient B with essential thrombocythemia. (A) HYA staining (brown) is intense and graded 4. Magnification $\times 360$. (B) Reticulin staining with diffuse fibre network and scattered thick coarse fibers, graded 3. Magnification $\times 360$.

HYA scores were compared between normal controls, AML and CMPD patients using Kruskal–Wallis and Mann–Whitney U test. The relation between reticulin grading and HYA score was analyzed using Pearson's chi-square test. Differences were considered significant when the p value was below 0.05.

Ethics

The study was conducted according to the Declaration of Helsinki and approved by the Research Ethical Committee of Uppsala University.

Results

In 19 patients with ET and PV, three consecutive bone marrow samples with adequate morphology were obtained before anagrelide treatment, at 6 mo and 2 yr (end of study). Reticulin grading, HYA score, cellularity, and number of megakaryocytes/ mm^2 were compared at start and after 2 yr treatment with anagrelide. At the end of the study the reticulin scores were significantly higher than at the start ($p = 0.02$) (Fig. 3A). The reticulin score increased in 6 patients

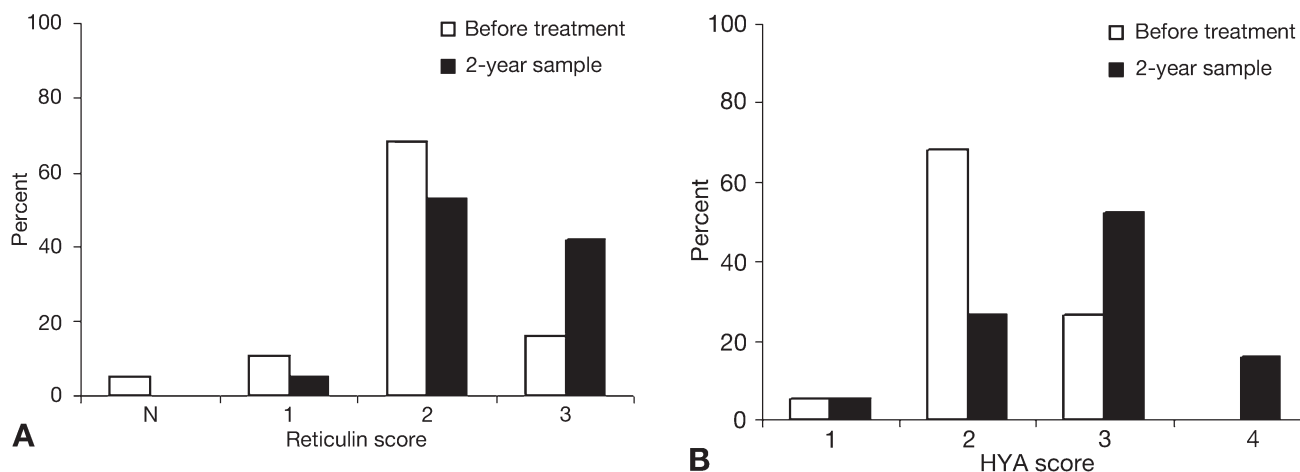


Fig. 3. (A) Reticulin scores in bone marrow biopsies from patients with CMPD ($n = 19$) at start of the study (“Before treatment”) and after 2 yr of treatment with anagrelide (“2-year sample”), as percentages. No sample was scored 0 or 4. More patients had higher scores in the 2-yr samples and the change was significant ($p = 0.02$). (B) HYA scores in bone marrow biopsies ($n = 19$) at start of the study (“Before treatment”) and after 2 yr of treatment with anagrelide (“2-year sample”), as percentages. More patients had higher scores in the 2-yr samples and the change was significant ($p = 0.002$).

(5 with ET, 1 with PV), but in 13 subjects (11 with ET, 2 with PV) no change was seen. There were no significant differences concerning age, gender, diagnoses, reticulin score, and HYA score before treatment, platelet levels (at start and during treatment), and previous medical treatment between the groups with stable and increasing reticulin scores (data not shown). The HYA scores was also significantly higher ($p = 0.002$) after 2 yr of treatment (Fig. 3B). Ten patients (8 with ET, 2 with PV) had a higher HYA score at the end of the study, but in 9 cases (8 with ET, 1 with PV) no change was found. No significant differences were seen in gender, diagnoses, reticulin score, and HYA score before treatment, platelet levels (at start and during treatment), and previous medical treatment between the groups with stable and increasing HYA scores (data not shown). However, the average age was higher for the patients with increased HYA levels than in those with no change, 57.0 and 48.3 yr, respectively ($p = 0.028$). Among the six patients with increased reticulin score after 2 yr of treatment, four also had increased HYA values.

The mean bone marrow cellularity increased significantly from 61.1% to 69.5% ($p = 0.014$). The mean number of megakaryocytes/ mm^2 in the bone marrow samples was 48.8 at start and 54.0 after 2 yr, but the change was not significant ($p = 0.198$). In the

bone marrow biopsies taken at 6 mo, the HYA and reticulin scores, cellularity, and number of megakaryocytes/ mm^2 showed intermediate values between the ones found in the samples before treatment and the 2-yr samples (data not shown).

Bone marrow samples were obtained from all patients at the start of treatment, but only 53 could be adequately analyzed regarding reticulin staining and HYA score. The results were compared with the same analysis performed on bone marrow samples from the healthy control group and patients with AML (Fig. 4). The reticulin score was lower ($p = 0.005$) and the HYA score was higher ($p = 0.003$) in AML patients than in controls (8). Significantly higher reticulin staining scores ($p < 0.001$) and HYA scores ($p < 0.001$) were found in the patients with CMPD compared to the controls. Furthermore, significantly higher reticulin staining ($p < 0.001$) and HYA scores ($p = 0.011$) were seen in the CMPD patients compared with the AML patients.

Previous analysis had shown that HYA and reticulin scores were correlated in normal controls ($p < 0.001$) and in AML patients ($p = 0.005$) (7,8). When all bone marrow samples from the normal controls, AML and CMPD patients were included in the analysis a highly significant correlation was seen between HYA and reticulin scores ($n = 117$, $p < 0.001$).

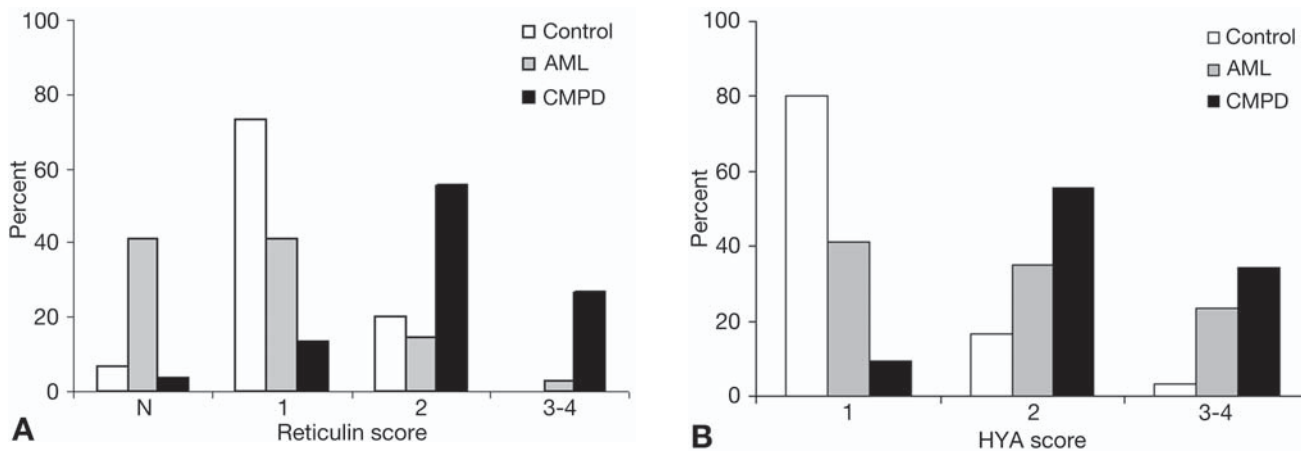


Fig. 4. Bone marrow biopsies from patients with CMPD ($n = 53$) at start of the study compared to normal controls ($n = 30$) and AML patients ($n = 34$), as percentages. **(A)** CMPD cases had significantly higher reticulin scores than controls ($p < 0.001$) and AML patients ($p < 0.001$). No sample was scored 0. **(B)** The HYA score was significantly higher in CMPD patients than in controls ($p < 0.001$) and AML patients ($p = 0.011$).

However, a trend but no significant correlation was seen between reticulin staining and HYA score among the 53 CMPD patients ($p = 0.187$).

Discussion

Patients with ET and PV have many morphological and clinical features in common. Increased numbers of megakaryocytes is a consistent finding in the CMPDs. The megakaryocytes are part of the clonal hematopoietic stem cell disorder and produce functionally defective platelets. Thrombocytopenia is regularly seen in patients with CMPD and is the dominating finding in ET. The hazards associated with thrombocytopenia are thromboembolic incidents as well as bleeding. Cytoreductive treatment aims at reducing the number of platelets, thus reducing the risk of thromboembolic events and bleeding, and hopefully also the development of marrow fibrosis. Anagrelide has been introduced as a new, non-cytostatic drug that has been shown to be effective in reducing platelet numbers. Although the majority of patients had a complete platelet response to anagrelide treatment (12), the fibrotic development, evaluated with both reticulin and HYA histology, showed no decrease in either of these components during the study. On the contrary, a significant increase was seen in the reticulin score with 6 out of 19 patients

having more fibrosis after 2 yr of anagrelide treatment, although no patient developed collagen fibrosis. Furthermore, HYA staining, suggested to be one of the earliest signs of an impending fibrosis, significantly increased during the 2 yr follow up. The reason for the higher average age of the patients with increased HYA scores than for the group with stable scores is unknown and the relative small number in the groups might affect the outcome. In previous studies, focusing mainly on CIMF patients, no significant progression of bone marrow fibrosis was seen in the entire cohorts during anagrelide therapy, although some patients had higher fibrosis scores after treatment (20,21). Furthermore, transformation to clinical myelofibrosis was more prevalent in patients treated with anagrelide than with hydroxyurea in the recently published PT1 study comparing hydroxyurea with anagrelide in patients with ET (11). We and others have previously reported regression of bone marrow fibrosis during hydroxyurea therapy in patients with CMPD on treatment with hydroxyurea (22, 23).

The cellularity increased in the bone marrows analyzed during treatment with anagrelide. In a previous report, no significant change in bone marrow cellularity was recorded after approx 2 yr of anagrelide therapy (21). However, in that study the majority of patients had CIMF and the mean cellularity was 86%

before anagrelide treatment. In the present study the cellularity was much lower at the start of the study (61.1%) and the patients followed for 2 yr had ET or PV, none had CIMF. The differences in diagnoses and cellularity at the beginning of treatment with anagrelide could thus be one explanation for the diverging results. The cause of the increasing cellularity is not fully understood. It could reflect the natural progression of the CMPD and thus be independent of anagrelide treatment, but the opposite pattern with reduced cellularity has previously been described in CMPD patients treated with hydroxyurea and alpha-interferon (22,23). Both these drugs have a general bone marrow suppressive effect influencing all cell lines and a reduction in cellularity is not surprising. Anagrelide, on the other hand, seems to be specific for the megakaryocytic lineage (24). In the present study, the number of megakaryocytes was slightly higher after anagrelide treatment but the difference was not significant. This is in accordance with previous data about CIMF and ET patients, and the proposed effect of anagrelide on the megakaryocytes is not a reduction of cells but rather a left-shifted maturation pattern of the megakaryopoiesis (20,21). Anagrelide exerts its effect by influencing the late stages of megakaryocyte development, reducing differentiation (25,26). In contrast, hydroxyurea acts on the early phase of megakaryopoiesis, reducing proliferation. This could also be the basis for the more pronounced dysplastic morphology described in the megakaryopoiesis in hydroxyurea-treated bone marrows in comparison with anagrelide-treated samples (27).

Increased bone marrow fibrosis is common in CMPD and, not surprisingly, significantly higher reticulin and HYA levels were found in the 53 biopsies from CMPD patients at the start of the study than in normal controls and AML patients. A correlation between HYA and reticulin has previously been shown in normal bone marrows as well as in AML, and HYA has been suggested as a marker for impending fibrosis (7–9). Combined analysis of all normal controls, patients with AML, and the 53 patients with CMPD showed a strong correlation between HYA and reticulin scores. However, when only the CMPD patients were studied, a trend but no significant association was found. The reason for that discrepancy is elusive. The reticulin scores were higher in CMPD patients than in AML cases and

normal controls, and one possible explanation could be that if HYA is an early sign of fibrosis, patients with a history of several years of increased reticulin could have lost some previously existing HYA, especially if there is no or slow progression of the fibrosis. This is, however, merely a hypothesis and future studies of HYA, reticulin fibres and the process of fibrosis might elucidate this question.

Anagrelide therapy has been shown to significantly reduce platelet levels and thus possibly reduce the risk of thromboembolic events as well as bleeding. Only a few studies have focused on the effects of anagrelide in the bone marrow of patients with CMPD (20,21,27). The data of the present study show that anagrelide treatment does not stop or reverse the progression of bone marrow fibrosis in patients with ET and PV because both the HYA and reticulin scores increased during anagrelide therapy. In the absence of a group with different treatment for comparison, it is not possible to say whether anagrelide may have any causative role in fibrosis development. The same conclusion was, however, reached even in the large randomized PT1 study, where a higher number of transformations to myelofibrosis were found in the anagrelide arm than in the hydroxyurea arm, but a causative role for anagrelide could not be concluded (11). It would be of interest to include analysis of reticulin and HYA deposition for early evaluation of impending fibrosis in future larger studies of CMPD patients in order to further explore the effects of anagrelide and other pharmacological substances such as alpha-interferon and hydroxyurea on development of bone marrow fibrosis.

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