Anagrelide-induced bone marrow changes during therapy of chronic myeloproliferative disorders with thrombocytosis.

An immunohistochemical and morphometric study of sequential trephine biopsies

JUERGEN THIELE, HANS MICHAEL KVASNICKA, NINA FUCHS, KERSTIN BRUNNBAUER, NINA VOLKWEIN, ANNETTE SCHMITT-GRAEFF

Background and Objectives. Anagrelide is an agent with a significant platelet-lowering activity in humans. Contrasting the wealth of clinical data, bone marrow (BM) changes during therapy have been relatively rarely studied; information is particularly lacking regarding specific features of megakaryocytopenia.

Design and Methods. A study was performed on 15 patients with essential thrombocythemia and early stage chronic idiopathic myelofibrosis presenting with an elevated platelet count. These patients received anagrelide for 25 months resulting in a significant improvement of thrombocytosis. Evaluations were carried out by morphometry on sequential BM biopsies following enzym- and immunohistochemical stainings that also included those for proliferative capacity and apoptosis.

Results. No significant change in proliferation or apoptosis was recognizable during anagrelide treatment. The most conspicuous alterations were those of the CD61+ megakaryocytes. Megakaryocytopenia revealed an increase in promegakaryoblasts together with an enhancement of proliferating cell nuclear antigen activity. This feature is in keeping with an inhibitory effect on endoreduplication implying an arrest of polyploidization and maturation into platelet-shedding large megakaryocytes. On the other hand, a significant increase in the number of megakaryocytes was not detectable. Anagrelide failed to exert a stimulating influence on the progression of myelofibrosis or on the amount of CD34+ progenitor cells. Regarding angiogenesis, there was no increase in the density of BM vessels, but distension of the vascular lumina corresponded with a vasodilatory effect.

Interpretation and Conclusions. Anagrelide exerts a significant effect on endoreduplicative activity of megakaryocytes consistent with an inhibition of maturation and therefore, generates a relative predominance of precursor cells but fails to stimulate myelofibrosis.

Key words: anagrelide, megakaryopoiesis, proliferation, apoptosis, angiogenesis.

Haematologica 2003; 88:1130-1138
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Anagrelide hydrochloride (an imidazoquinazoline derivative) has been shown to exert a specific platelet-lowering activity in humans and animals. Therefore, this agent has been applied, with success, to control the thrombocytosis frequently complicating chronic myeloproliferative disorders (MPDs), in order to decrease the risk of fatal thrombosis and hemorrhage. Although there are a number of side effects with a drop-out rate of almost 9% in clinical studies comprising large number of patients, this drug is generally well-tolerated. Because essential thrombocythemia (ET) may present in younger (female) patients and has a chronic course it seems mandatory that thromboreductive treatment must be efficacious over a long time. Until now patients with ET have been treated with hydroxyurea, alkylating agents and radioactive phosphorus, all of which are potentially leukemogenic. This mutagenic risk has stimulated the search for drugs with a more favorable adverse effect profile. Promising results were obtained with interferon-α 2b and in particular anagrelide, which may be used safely and for a long time in MPDs accompanied by an excess in platelets. In contrast to the wealth of clinical data that have been accumulated in recent years concerning the efficacy of the latter agent, controversy and discussion continues about its exact pathomechanism of action. In vitro and in vivo studies suggest that anagrelide treatment decreases the quantity of platelet primarily by interfering with the maturation of megakaryocytes resulting in a left-shifting of this cell lineage. Contrasting this finding, another group postulated an additional reduction in the number of megakaryocytes associated with a concomitant reduction in size and ploidy of these cells. In this context it must to be explicitly mentioned that all these studies were focused on megakaryocytopenia, but the other constituents composing the bone marrow (BM) were not taken into consideration.

For this reason, the purpose of the present study was to obtain a greater insight into anagrelide-induced BM changes by performing a systematic immunohistochemical and morphometric investigation on sequential biopsy specimens in patients with MPDs and a significant elevation of platelet counts.

Design and Methods

Patients

A retrospective evaluation of clinical records and BM trephine biopsies was performed in 15 patients (medi-
an age 49 years, range 27 to 71 years) with MPDs associated with thrombocytosis in excess of 600×10^9/L, for more than 3 months. Eligibility for inclusion in this evaluation included a clear-cut diagnosis according to the WHO-criteria. Hence 5 of these patients were regarded as having ET32 and 10 patients as having chronic idiopathic myelofibrosis (CIMF) mostly at early, either prefibrotic or mild fibrotic stages.30 All patients had a history of short-term therapy with hydroxyurea, although this drug had not been able to control the thrombocytosis. Following anagrelide (Agyrlin39 therapy, in the absence of concurrent treatment with other agents, a significant thrombocytoreductive effect was seen in all patients, with a decrease in platelet count from 1,104±265 (first biopsy) to 485±179×10^9/L (last biopsy). A total of 36 sequential BM trephine examinations (1 in 3 patients, 2 in 4 patients, 3 in 7 patients and 4 in 1 patient) were carried out during the follow-up, with the median interval between first and last examination being 25 months (range 8 to 70 months). Treatment with anagrelide was based on the thrombocyte count and the average maintenance dose ranged between 1.5 to 2.5 mg/day. Bone marrow samples from 20 age-matched patients without known hematological disorders served as controls.

**Bone marrow biopsies**

Representative BM trephine biopsies were taken from the posterior iliac crest of our 15 patients. The mean size of the biopsy was 18.5±2.9 mm. The specimen was fixed 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 to make 100 mL). Further processing included decalcification for 3–4 days in 10% buffered ethylene-diamine tetra-acetic acid (EDTA), pH 7.2, paraffin embedding, and staining, employing various techniques, including hematoxylin-eosin (H&E), Giemsa, PAS (periodic acid Schiff reagent), naphthol-AS-D-chloroacetate esterase, Perls’ reaction for iron and Gomori’s silver impregnation method. Immunohistochemistry with monoclonal antibodies was applied to identify CD34+ progenitor cells and CD61+ megakaryocytes correctly.31,32 Staining procedures for proliferating cell nuclear antigen (PCNA) activity33,34 followed modified methods reported in detail in previous communications.35 Apoptosis was visualized by using the specific monoclonal antibody (dilution 1:100) anti-ss DNA/apostatin (Bender MedSystems, Vienna, Austria). Morphometric evaluation of all BM features was carried out using two planimeters (MOP-A-MO1-Kontron and VIDAS-Zeiss-Kontron) with a standard program set on large trephine biopsies with an artefact-free BM area of 13.4×1.9 mm². All evaluations were performed independently by three hematologists without prior knowledge of patients’ data or end-points of biopsies. Frequencies of CD34+ progenitor cells, CD61+ megakaryocytes — including atypical microforms and precursors, PCNA-reactive — and apoptotic cells per mm² were determined at 500× magnification by calculation of the total evaluable hematopoietic area of the trephine biopsy. Measurements characterizing angiogenesis included not only the incidence of CD34+ vascular (endothelial) structures (microvessel density), but also the vascular area (luminal dispersion — µm²), the maximal length of vessels (µm) and their deviation from a circular perimeter (form factor) to obtain a closer insight into tortuosity and branching, both reflecting perfusion. Determination of cellularity explicitly considered the age-related changes in this population. Grading of BM fibrosis (reticulin and collagen fiber density) followed a generally acknowledged semiquantitative scoring system36 modified by corresponding morphometric data on the density of arcyophilic fibers.37 Accordingly, for purpose of practicality the following scoring was applied: 0 — no increase from the normal, showing a very few dispersed thin reticulin fibers, 1 — minimal to mild, often focal increase in reticulin, 2 — marked and diffuse increase in reticulin throughout the section plus some bundles of collagen, 3 — coarse collagen and reticulin fibrosis, usually associated with varying degrees of osteosclerosis (endophytic bone formation).

Megakaryocyte precursor cells, i.e. promegakaryoblasts and megakaryoblasts, were identified in accordance with former descriptions.38,39 Briefly, according to relevant studies of immature elements in normal megakaryocytogenesis on smear preparations or cell suspensions derived from BM particles or stem cell cultures, promegakaryoblasts were characterized as mononuclear, lymphoid-like elements with a diameter ranging between 7 and 14 µm.40-43 The smallest elements clearly recognizable as members of the megakaryocyte series, i.e. promegakaryoblasts41 had a diameter of 9.3±1.8 µm (area 61.7±14.8 µm²). In corresponding tissue sections the most immature CD61+ precursors had an area of less than 50 µm², a diameter of about 7 µm and a round nucleus with dispersed chromatin containing a prominent nucleolus and a small rim of cytoplasm.32,39 Since there is considerable flattening of cells in BM smears, this decrease in size of as much as 30% in tissue sections is comprehensible.44 Consequently, on smear preparations megakaryoblasts (maturation stage I) had a diameter of 15.9±0.6 µm (area 94.4±5.4 µm²).45,46 On the other hand, in tissue sections these CD61+ cells were smaller, with an area between 50 and 100 µm² and a diameter of about 11 µm; they also had a round or slightly indented nucleus with a frequently recognizable nucleolus and a fine chromatin pattern as well as relatively scanty cytoplasm. Finally, CD61+ and PAS+ cells exceeding 100
µm² in size in tissue sections and with increasing lobulation of the nucleus were consistent with promegakaryocytes (maturation stage II — size ≤ 150 µm² on smears). Larger, easily recognizable (mature) platelet-shedding (granular) megakaryocytes indicated maturation stages III to IV.44,45

Because all patients had received minimal pretreatment with hydroxyurea and a pilot study had failed to display differences of the major histological features under consideration, both cohorts (patients with ET and those with CIMF) were grouped together in the final evaluation of data (Table 1). A paired-samples t-test with a significance level of 0.05 was used to compare mean differences of morphologic variables before and after treatment with anagrelide.

**Results**

After at least 8 months of anagrelide therapy (median 25 months), no relevant changes in cellularity could be detected in either cohort of patients, in accordance with the insignificant differences in proliferation and apoptosis (Table 1). On the other hand, striking alterations of megakaryocytopoiesis were always a conspicuous feature. Many of the large megakaryocytes characteristic of these disorders (Figure 1a,b) disappeared and a more pleomorphic pattern of sizes emerged, including a greater number of medium-sized as well as dwarf forms of this cell lineage (Figure 1c,d). However, no relevant change in the total number of CD61+ megakaryocytes was observable. Immunohistochemistry and morphometric evaluations were consistent with this impression and revealed an increased population of promegakaryoblasts during treatment (Table 1). Despite this rise in the fraction of very early precursors, no significant decrease in cell sizes or nuclear sizes was detectable (Table 1). This phenomenon is probably related to the relatively small size characterizing this immature cell population and its only slight increase in frequency (22% versus 30% of the total CD61+ megakaryocytopoiesis). A more detailed analysis of megakaryocyte parameters and maturation stages in comparison with the normal BM showed a pronounced shift to the left with predominance of immature, i.e. apparently non-platelet shedding, low-ploidy classes presenting megakaryocytes (Figure 2a,b). In this context, overall proliferative capacity according to PCNA staining of all BM cells and apoptosis (Figure 2c) did not show a significant imbalance during therapy (Table 1). This finding is consistent with the failure to demonstrate relevant changes in the total number of hematopoietic cells (cellularity). A slight enhancement of PCNA labeling was observed after treatment with anagrelide:

*Paired-samples t-test: p ≤ 0.05.*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before anagrelide therapy</th>
<th>After anagrelide therapy</th>
<th>Normal bone marrow</th>
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</thead>
<tbody>
<tr>
<td>Cellularity (%)</td>
<td>86±3</td>
<td>89±5</td>
<td>51±14</td>
</tr>
<tr>
<td>CD6+ megakaryocytes total megakaryopoiesis frequency (mm²)</td>
<td>127±117</td>
<td>136±53</td>
<td>25±5</td>
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<tr>
<td>cell size (µm²)</td>
<td>447±125</td>
<td>432±150</td>
<td>277±175</td>
</tr>
<tr>
<td>nuclear size (µm²)</td>
<td>144±44</td>
<td>139±63</td>
<td>78±4.0</td>
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<tr>
<td>circular deviation (×10² score)</td>
<td>0.85±0.05</td>
<td>0.88±0.03</td>
<td>76±3.2</td>
</tr>
<tr>
<td>nuclear/cytoplasmic ratio</td>
<td>0.32±0.02</td>
<td>0.31±0.04</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td>promegakaryoblasts frequency (µm²)</td>
<td>28±26*</td>
<td>41±31</td>
<td>1.9±1.2</td>
</tr>
<tr>
<td>CD34+ progenitor cells frequency (mm²)</td>
<td>10.5±2.8</td>
<td>12.3±4.2</td>
<td>7±3</td>
</tr>
<tr>
<td>Proliferating cell nuclear antigen (PCNA) reactivity (%)</td>
<td>17.5±10.3</td>
<td>22.5±9.8</td>
<td>17.8±4.9</td>
</tr>
<tr>
<td>Apoptosis (% x 10⁻¹)</td>
<td>3.5±0.7</td>
<td>3.0±0.9</td>
<td>1.1±1.2</td>
</tr>
</tbody>
</table>

This was, however, probably due to the increased number of megakaryocyte precursors. Regarding megakaryocytopoiesis, PCNA reactivity demonstrated a prevalence of positively stained nuclei in the smaller (immature) megakaryocytes and rarely in the large or giant ones showing hyperlobulated (staghorn-like) nuclei (Figure 2b). A comparative assessment regarding size (stage of maturation) and PCNA positivity revealed a significant association between megakaryocyte precursors and nuclear labeling in the control group and in the patients before treatment. Following anagrelide therapy the number of PCNA+ promegakaryoblasts and megakaryoblasts increased significantly, consistent with a pronounced left-shifting of this lineage in both groups of patients (Figure 3a,b).

It is noteworthy that the number of CD34+ progenitor cells was not significantly altered. A more refined evaluation of angiogenesis (Table 2) showed
that there was no change of microvessel density (MVD) or tortuosities. On the other hand, the microvascular area (MVA) did increase significantly (Figure 2d,e) consistent with an apparent luminal distension (Table 2).

Finally, none of the patients with ET developed myelofibrosis even after treatment with anagrelide, which a few of them received for more than 5 years. The 8 patients with early stage CIMF (fibre scores 0 and 1) failed to show a relevant progression of fibrosis during the observation period and only one patient with manifest myelofibrosis (fibre score 2) developed overt collagen fibrosis (fibre score 3).

Discussion

Megakaryocytopoiesis

According to our data on megakaryocytopoiesis during anagrelide treatment, this drug’s thrombocytopoietic effect is achieved by interference with endoreduplicative capacity, thus impairing maturation to large platelet-shedding end-stages of the lineage. This pathomechanism generates a conspicuous number of megakaryocyte precursors (so-called left-shifting). This finding of an increased number of immature megakaryocytes confirms previous data derived from H&E–stained marrow specimens.26,27 On the other hand, the total amount of

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Figure 1a–d. Megakaryocytopoiesis before anagrelide therapy reveals large megakaryocytes in ET (a) and early stage CIMF with thrombocytosis (b). Following treatment there is no decrease in the number of megakaryocytes but a conspicuous reduction in the size of most cells, which exhibit a pleomorphic appearance in ET (c) and CIMF (d). (a–d CD61-immunostaining; a–d ×180)
Figure 2a-e. Size and maturation stage of megakaryocytes during anagrelide therapy showing large to giant megakaryocytes without PCNA labeling surrounded by stained myeloid cells (dark nuclei) before treatment (a). This feature contrasts with the small and immature megakaryocytes and nuclei labeled by PCNA seen after treatment (b). Programmed cell death (apoptosis) is easily recognizable during anagrelide therapy (c). There is a conspicuous alteration of microvascular structures, exhibiting small vessels before anagrelide treatment (d) and a striking distension of vascular lumen, but no increase in number, after therapy (e). (a, b: CD61-immunostaining, c immunostaining with anti-ss DNA/apostatin, d, e CD34-immunostaining; a, b × 38, c × 1100, d, e × 180).
CD61+ megakaryocytes is not reduced, which contrasts with information gained by flow-cytometry of CD41-marked megakaryocytes. Overall, the significant increase in small-sized, PCNA+ megakaryocyte precursors during anagrelide therapy warrants comment. A striking relationship between cell and nuclear size, nuclear lobulation and maturity with stages of ploidy has been established by scrutinized in vitro studies. Moreover, treatment with anagrelide has been shown to increase the number of immature megakaryocytes, implying left-shifting. This result is in keeping with our data on the quantity of promegakaryoblasts and megakaryoblasts occurring after administration of anagrelide (Table 1). Moreover, the shift to lower ploidy classes is also in line with this alteration. It is generally accepted that

<table>
<thead>
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<th>Before anagrelide therapy</th>
<th>After anagrelide therapy</th>
<th>Normal bone marrow</th>
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</thead>
<tbody>
<tr>
<td>MVD (mm²)</td>
<td>70±28</td>
<td>89±45</td>
<td>77±27</td>
</tr>
<tr>
<td>MVA (µm²)</td>
<td>126±65</td>
<td>214±55*</td>
<td>76±25</td>
</tr>
<tr>
<td>Tortuosity of microvessels (maximal length in µm)</td>
<td>19±5</td>
<td>26±4</td>
<td>14±5</td>
</tr>
<tr>
<td>Form factor</td>
<td>0.53±0.07</td>
<td>0.48±0.05</td>
<td>0.70±0.06</td>
</tr>
</tbody>
</table>

*Paired-samples T-test, p < 0.05.
megakaryocytopenesis proceeds initially through a phase of mitotic divisions of lineage-restricted progenitor cells followed by endomitotic reduplication that occurs in a non-dividing (2N) promegakaryoblast terminating in the mature (granular) polyploid megakaryocyte. However, because maturation and polyplidization appear to be closely linked in the normal BM, the platelet-shedding megakaryocytes were found to range between 16N and 32N. Taking this feature in account, evaluation of size and proliferation activity allows a better insight into the state of endoreduplication associated with maturation and polyplidization. The enhanced PCNA staining of smaller-sized, apparently immature megakaryocytes after anagrelide treatment provides support to a former hypothesis on the dynamics of this complex process. According to elaborate experimental data, differences were postulated in the duration of single cell-cycle phases at successive ploidy levels. These variations in distinctive cell-cycle periods imply a prolongation of the resting phases (G1/G2-phases) in higher ploidy classes. The finding of a relative reduction of PCNA staining in large and giant megakaryocytes in reactive lesions as well as in ET and CIMF is opposed to the increased positivity displayed in the small precursors. Finally, PCNA activity is mainly limited to the S-phase of the cell cycle. It may be argued that some of the very large and giant hyperpolyploid megakaryocytes in MPDs could have already reached the end-stage of endomitotic activity and therefore, may escape into the (PCNA-negative) G0-phase of the cell cycle. In a clinical study 13 patients with CIMF were followed for about 2 years (range 6 months to 4 years) during anagrelide therapy. However, no relevant treatment benefit was demonstrable and although the number of platelets decreased, in most patients there was an increase in the quantity of BM megakaryocytes. In contrast to our study it is noteworthy that in this trial only 3 of the 13 patients with advanced CIMF and accompanying myeloid metaplasia revealed a platelet count in excess of 500×10^9/L. Moreover, the frequency of megakaryocytes was determined only by routine staining methods (H&E) and low magnification (×160) of the examined field.

Bone marrow stroma

Finally, concern has been expressed that anagrelide may exert a profound effect on the development of myelofibrosis, especially considering its interference with megakaryocyte maturation and the generally acknowledged cytokine-mediated fibrillogenesis. In this regard, a clinicopathologic study involving patients with overt myelofibrosis (CIMF) and associated myeloid metaplasia failed to demonstrate any relevant progression of the fibro-osteosclerotic BM process. This is a general agreement with the corresponding cytokine levels (platelet-derived growth factor, transforming growth factor β, basic fibroblast growth factor) after anagrelide treatment. However, it can be presumed that evaluations of prefibrotic or early fibrotic stages of IMF, rather than a focus on advanced stages of the disorder, are best suited to elucidate this point. In our cohort of patients with early (hypercellular) CIMF according to the BM fiber content, significantly enhanced progression of fibrosis was not detected. This finding supports data derived from large series of patients with repeated BM biopsies during comparable periods of observation. Moreover, a careful scrutinized evaluation of angio- genesis showed a significant increase in the vascular area, corresponding to a relevant luminal distension. This feature fits well with the clinical data recording a vasodilatory effect of anagrelide.

In conclusion, anagrelide therapy exerts a significant effect on the endoreduplicative capacity of megakaryocytes (arrest of polyplidization – maturation) yielding an increased number of precursor cells (promegakaryoblasts and megakaryoblasts). However, the drug does not seem to have a relevant influence on the progress of myelofibrosis in patients with CIMF and associated thrombocytosis. Finally, it has no effect on the number of CD34+ progenitor cells, but does increase the distension of vascular lumina (vasodilatation) in the BM stroma.

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Pre-publication Report & Outcomes of Peer Review

Contributions
JT: principal author; HMK: conception and design; NF, KB and NV: assessment of morphometric data and collection of clinical and laboratory data including follow-up. JT, HMK and ASG: revision of bone marrow biopsies. JT and HMK: designed the study and analyzed and interpreted the findings and helped to produce a draft of the paper. Tables 1 and 2 were created by HMK. Figures 1 to 3 were created by JT.

The authors are greatly indebted to Mrs. B. Rosenbach, Mrs. A. Seccin and Mr. G. Simons for their excellent technical assistance.

Funding
This work was supported by a grant from Shire Comp., Germany, Cologne, Germany.

Disclosures
Conflict of interest: none. Redundant publications: no substantial overlapping with previous papers.

Manuscript processing
This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received August 8, 2003; accepted September 2, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic:
Anagrelide has been shown to be effective in controlling the thrombocytosis of chronic myeloproliferative disorders, but the mechanisms of its action are unclear.

What this study adds
Anagrelide appears to inhibit endoreduplicative activity of megakaryocytes.