Essential Thrombocythemia in Two Sisters Originating From Different Stem Cell Levels

By Johannes W.G. Janssen, Bernd R. Anger, Hans G. Drexler, Claus R. Bartram, and Hermann Heimpel

We report the rare occurrence of essential thrombocytenia (ET) in two sisters. In one patient, the clinical phenotype of the disease evolved from ET to polycythemia vera (PV) after 4 years of follow-up. Clonal hematopoiesis was established in both cases by X-chromosomal inactivation analysis using a DNA polymorphism of the phosphoglycerate-kinase (PGK) gene. Cell separation studies suggested a common ancestor for granulocytes, monocytes, and T lymphocytes in one patient; however, in her sister, monoclonality could only be demonstrated convincingly for the granulocyte fraction. Our data indicate that ET may originate from heterogenous stem cell levels.

E S S E N T I A L  T H R O M B O C Y T E M I A (ET) is a rare chronic myeloproliferative disorder characterized by sustained elevation of the thrombocyte count, hyperplasia of the bone marrow with excessive proliferation of megakaryocytes, and a high incidence of thromboembolic complications. 1,2 Since an elevated platelet count is a common feature of various infectious, inflammatory or neoplastic disorders, and is also frequently associated with other chronic myeloproliferative syndromes such as polycythemia vera or idiopathic myelofibrosis, ET is diagnosed by exclusion, generally following the criteria of the Polycythemia Vera Study Group. 2

The clonal nature of the disease has been previously established by cytogenetic analyses demonstrating nonrandom yet variable chromosomal abnormalities, 6 and by studies using glucose-6-phosphate dehydrogenase (G-6-PDH) isoenzymes. 5,7,8 Moreover, G-6-PDH analyses indicated that granulocytes, monocytes, thrombocytes, erythrocytes, and B lymphocytes share a common ancestral origin in the few ET patients investigated thus far. The latter approach, however, is limited by the rarity of G-6-PDH heterozygosity outside certain ethnic groups. This major detraction of G-6-PDH analysis is overcome by the use of X-linked DNA polymorphisms, as recently described by Vogelstein et al. 10,11 The DNA technique allows clonality to be studied in about 50% of females and is comprised of two steps: (1) the two copies of an X-linked gene are distinguished through a restriction fragment length polymorphism (RFLP), and (2) active and inactive alleles are then differentiated by subsequent analysis of their methylation pattern. The method has been used to demonstrate the clonal nature of acute myeloid leukemia, 14 and chronic myeloproliferative disorders. 15,16

The present report describes the application of this approach to clonal analyses in two sisters with ET. Our data confirm the present view of ET as a clonal hematopoietic disease and suggest, moreover, that this chronic myeloproliferative disorder might originate from different stem cell levels.

PATIENTS AND METHODS

Case Histories

Patient 1. Elevated thrombocyte and leukocyte counts were first documented when the patient underwent hysterectomy at the age of 56 years. Thrombocytosis was sustained with counts varying between 800 and 1,200 x 10^9/L at follow-up. The patient was still asymptomatic 2 years later, and the spleen was palpable. The white blood cell count was 17.7 x 10^9/L; erythrocyte count, 4.9 x 10^12/L; hematocrit level, 44%; thrombocyte count, 1,200 x 10^9/L; and the alkaline neutrophil phosphatase score was 129 (normal, 10 to 100). A bone marrow core biopsy showed an enhanced cellularity due to panhyperplasia of myelopoiesis, erythropoiesis, and atypical megakaryocytes. Moderate diffuse myelofibrosis was seen. Cytogenetic analysis from peripheral blood showed a normal karyotype. Southern blot analysis showed a germline configuration of the BCR gene.

Two years later, at the time of the present examination, the patient presented with a phenotypic change of her chronic myeloproliferative disorder, which now resembled polycythemia vera: erythrocyte count and hematocrit level rose to 7.2 x 10^12/L and 58%, respectively; the thrombocyte count was 850 x 10^9/L; and the white blood cell count was 15.3 x 10^12/L with 72% neutrophils, 6% eosinophils, 1% basophils, 2% monocytes, and 26% lymphocytes; erythrocyte count was 4.4 x 10^12/L; hematocrit level, 38%; and thrombocyte count, 1,400 x 10^9/L. The score of the alkaline neutrophil phosphatase was normal. A bone marrow core biopsy showed an enhanced cellularity with hyperplasia of atypical megakaryocytes. Moderate diffuse myelofibrosis was seen. Since there was a dry tap on bone marrow aspiration, cytogenetic analysis was not possible. Southern blot analysis established a germline configuration of the BCR gene. Currently the patient is well and followed without treatment.

Essential thrombocythemia was diagnosed in both patients according to the criteria of the Polycythemia Vera Study Group. 7 Requirements were as follows: a sustained thrombocytosis above 600 x 10^9/L, enhanced cellularity of the marrow with hyperplasia of immature megakaryocytes, normal or elevated score of the alkaline...
neutrophil phosphatase and absence of the Philadelphia chromosome, exclusion of reactive thrombocytosis due to infectious, inflammatory or neoplastic disease, no blood loss or iron deficiency, no erythrocytosis, and no high-grade myelofibrosis.

The family history of the two sisters, having no additional siblings, is inconspicuous. Both live separately with their own families.

Cell Separation Analyses

Heparinized peripheral blood and a skin biopsy were obtained from the patients with informed consent. Lymphocytes, monocytes, and granulocytes were separated from 200 mL peripheral blood by Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway) density gradient centrifugation. Granulocytes were purified from erythrocytes by dextran sedimentation. T lymphocytes were further isolated by neuraminidase-treated sheep erythrocyte rosetting, followed by Ficoll density gradient centrifugation. Monocytes were enriched by adherence to plastic dishes (90 minutes at 37°C). Purity of the cell populations was examined by morphologic inspection of May-Grünwald-Giemsa-stained cytospin slide preparations and indirect immunofluorescence staining using the microtiter plate method with cell lineage-specific monoclonal antibodies (MoAbs). The following MoAbs were used: MY7 (CD13) as a marker of myeloid cells; Leu-9 (CD7) and OKT3 (CD3) for T lymphocytes; B4 (CD19) and M1 (CD14) for monocytes (MoAbs MY4, MY7, B1, and B4 from Coulter Immunology, Hialeah, FL; OKT3 from Ortho Diagnostics, Raritan, NJ; Leu-9 from Becton-Dickinson, Mountain View, CA). Positivity was analyzed by flow cytometry using an EPICS C cell sorter (Coulter Electronics, Hialeah, FL).

A fibroblast cell line was established from a skin biopsy obtained from the right leg of patient 2.

Southern Blot Analysis

High molecular weight DNA was prepared from leukocytes and skin fibroblasts by standard techniques. For clonal analysis, we adopted the protocols of Vogelstein et al., as described previously by us. For investigation with the hypoxanthin phosphoribosyltransferase (HPRT) probe, 10 μg of DNA was screened for a BamHI RFLP showing 24 kb and 12 kb fragments; for analysis with the PK probe, respective DNAs were screened for a Bgl I RFLP characterized by 9.4 kb and 5.7 kb fragments. DNAs of both patients were only heterozygous for the PGK RFLP that was subsequently used for clonal analysis. DNA (20 μg) was codigested with BstXI and Pst I, producing polymorphic fragments of 1.05 kb and 0.9 kb, and either not digested further or cleaved with Hpa II, which, in the case of the PGK gene, identifies the unmethylated active allele. Restriction enzymes were obtained from Pharmacia LKB (Uppsala, Sweden), with the exception of BstXI (New England Biolabs, Beverly, MA). Restriction enzymes were used at a concentration of 5 units/μg of DNA. Electrophoresis on 1.5% (PGK) or 0.6% (HPRT) agarose gels, Southern transfer to nylon membranes (Nytran; Schleicher & Schuell, Dassel, FRG), hybridization, and washing of filters was performed as described. Filters were exposed to Kodak Xomat-S films for 12 hours to 7 days at -70°C using intensifying screens. The following probes were kindly provided by Drs J. Singer-Sam (Duarte, CA) and J. Resseps (La Jolla, CA): a 0.85 kb MspI/Pst I insert from pPB 1.7 containing sequences from the 5' end of the HPRT gene and sPST/PGK containing a 0.8 kb EcoRI/BamHI fragment from the 5' region of the PGK gene.

RESULTS

After cell separation, the degree of contaminating cells was 7% and 4% for the granulocyte fraction as well as 3% and 2% for T lymphocytes of patients 1 and 2, respectively (Table 1). The content of residual cells, mainly T lymphocytes, was rather high in the monocyte samples of both cases; ie, 33% and 29%, respectively.

Both patients were suitable for clonal analyses due to heterozygosity at the PGK locus. After Hpa II digestion, peripheral, blood leukocytes showed the characteristic pattern of monoclonal hematopoiesis, as indicated by the loss of one allele represented by the 0.9 kb fragment in patient 1 and the 1.05 kb fragment in patient 2 (Fig 1A and B, lane 1). A monoclonal pattern is defined as decrease in autoradiographic intensity by over 80% for one allele and less than 40% for the other allele after Hpa II digestion.

Marked differences between both cases became obvious in investigating the separated cell fractions. In patient 1, only the granulocytes showed a clear-cut monoclonal pattern (Fig 1A). In contrast, all hematopoietic cell lines analyzed in patient 2 (granulocytes, monocytes, and T lymphocytes) exhibited a clonal composition (Fig 1B). The possibility that imbalanced mosaicism simulated the monoclonal pattern of the latter case could be ruled out unequivocally, since analysis of the patient's fibroblasts established a 1:1 ratio of active maternal to active paternal X-chromosomes (Fig 1B, lane 5).

Table 1. Characterization of Separated Cell Fractions by Immunologic and Morphologic Examination

<table>
<thead>
<tr>
<th></th>
<th>Myeloid (CD13)</th>
<th>Monocytic (CD14)</th>
<th>T Cells (CD7 + CD3)</th>
<th>B Cells (CD19 + CD20)</th>
</tr>
</thead>
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<tr>
<td>Granulocytes</td>
<td>96</td>
<td>&lt;1*</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Conventional</td>
<td>93*</td>
<td>&lt;1*</td>
<td>7*</td>
<td>1</td>
</tr>
<tr>
<td>T cells</td>
<td>1</td>
<td>1</td>
<td>97</td>
<td>1</td>
</tr>
<tr>
<td>Monocytes</td>
<td>71</td>
<td>29</td>
<td>&lt;1*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>67*</td>
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<td></td>
<td>88</td>
<td>&lt;1*</td>
<td>5</td>
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<td>96*</td>
<td>&lt;1*</td>
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<td>77</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>71*</td>
<td>29</td>
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Values express percentage of positive cells.
Abbreviation: ND, not done.
* Morphologic evaluation of May-Grünwald-Giemsa-stained cytopsins.
ESSENTIAL THROMBOCYTHEMIA IN SISTERS

Fig 1. (A) Clonal composition of peripheral blood leukocytes (1), as well as separated monocytes (2), granulocytes (3), and T lymphocytes (4) of patient 1. A PGK RFLP defined by 1.05 kb and 0.9 kb fragments in BatXI/Pst I digests distinguishes the maternal from the paternal allele, and methylation differences, as shown by further Hpa II digestion, distinguish between active and inactive X-chromosomes. DNA (20 µg) was digested with BatXI/Pst I. Subsequently, the DNA was divided into equal aliquots; one was not digested further (b lanes), and the other was digested with Hpa II (a lanes). A monoclonal pattern indicated by loss of one allele was observed in peripheral blood leukocytes (1) and granulocytes (3), while a polyclonal pattern emerged for monocytes (2) and T lymphocytes (4). (B) Clonal composition of peripheral blood cell subpopulations and skin fibroblasts of patient 2, revealing a monoclonal pattern in peripheral blood leukocytes (1), monocytes (2), granulocytes (3), and T lymphocytes (4). A polyclonal pattern is observed in skin fibroblasts (5).

Patient 1. However, the inequality of the two autoradiographic bands after Hpa II digestion indicates in all likelihood that the weaker 0.9 kb fragment stems from admixed polyclonal lymphocytes, thus masking a clonal monococyte cell population (Fig 1A, lane 2).

DISCUSSION

Essential thrombocythemia is the least common chronic myeloproliferative disorder, with an annual incidence of approximately 0.1 in 100,000.1-3 To our knowledge, the development of ET in siblings has previously been reported only once.20 Familial occurrence, however, is known in polycythemia vera (PV), another myeloproliferative disorder closely linked to ET. In the large series of the Polycythemia Study Group, approximately 6% of the patients had family members with the same disease.21 The close relationship between ET and PV can be derived from the observation that a proportion of patients with ET convert to PV.1,4 This clinical course is also noticed in patient 1; her clinical phenotype changed after 4 years of follow-up from ET to PV. The molecular basis, however, for both the transition in clinical manifestation and the familial occurrence of these diseases remains to be elucidated.

The fact that up to 5% of PV and ET cases evolve into acute leukemia might indicate the premalignant nature of these disorders.3,4,20,22 Along this line, clonal analysis based on X-chromosome inactivation studies convincingly established the clonal nature of hematopoiesis in both patients and thus confirmed the clinical diagnosis of ET. We would like to emphasize that this type of investigation is applicable to 50% of female patients and may have potential as a diagnostic tool in the differential diagnosis between clonal myeloproliferative disorders and reactive processes.1,5,16

Interestingly, the transformation event in both patients apparently occurred at different stem cell levels. In patient 2, granulocytes, monocytes, and T lymphocytes were clonally related, while skin fibroblasts showed the expected polyclonal X-inactivation pattern. Therefore, in this patient, transformation must have affected a very early hematopoietic progenitor cell. In patient 1, only the granulocyte fraction exhibited a clear-cut monoclonal pattern, while T lymphocytes were by definition of a polyclonal nature. The pattern of the monocytic fraction was also polyclonal. However, the latter finding most likely reflects the admixture of polyclonal lymphoid contaminants to a monoclonal monococyte population. Although clonal myeloproliferation without involvement of the monocytic lineage has previously been reported in a unique ET patient,7 our interpretation is in keeping with the current view that a disease that is multipotent for at least megakaryocytes, red blood cells, and granulocytes should also include monocytes as part of the clone. In any case, the disease originated in this patient from a more committed progenitor cell without capacity to differentiate into the lymphoid lineage.

Heterogeneity at the affected stem cell level has been demonstrated in other hematopoietic disorders, such as acute myeloid leukemia (AML), Ph-positive leukemia, and lymphoma.23-26 These biologic differences might bear clinical significance. A case in point is the recent observation of Fialkow et al that in AML patients after chemotherapy, cases originating from a multipotent stem cell may evolve into a clonal remission, in contrast to leukemias derived from a granulocyte progenitor cell.23

Thus far, the meaning, if any, of differences at the stem cell level to the clinical evolution of ET or other chronic myeloproliferative disorders remains obscure. This issue merits further studies.

NOTE ADDED IN PROOF

We recently performed X-inactivation analyses in 24 heterozygous c-MPD patients (17 PV, 4 ET, 3 IMF). All but one case exhibited clonal hematopoiesis. Cell separation analysis in one ET and three PV patients demonstrated in all four cases a monoclonal pattern of the granulocyte fraction, while T lymphocytes of these patients were of nonclonal origin.27

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