Clonal Granulocytes and Bone Marrow Cells in the Cellular Phase of Agnogenic Myeloid Metaplasia

By Hans Kreipe, Kai Jaquet, Jörg Felgner, Heinz-J. Radzun, and M. Reza Parwaresch

Myelofibrosis with myeloid metaplasia (MMM; also known as agnogenic myeloid metaplasia) may arise during the course of chronic myeloproliferative disorders (CMPD) such as chronic myeloid leukemia (CML) and polycythemia vera (PV). While it occurs rather rarely in CML and PV, one subtype of CMPD characterized by a predominant proliferation of megakaryocytes and granulocytes has a considerably greater tendency to develop osteomyelofibrosis. Because of the increase in megakaryocytes this disease also has been named megakaryocytic myelosis. The fully developed fibrosis of the bone marrow is preceded by a hypercellular phase of variable duration. Because no characteristic blood cell findings exist, the diagnosis of MMM depends on the demonstration of atypical megakaryocytes and an increase of fibers within bone marrow biopsies and the exclusion of other CMPD such as CML.

Although consistent chromosomal abnormalities could not be established, the neoplastic nature of MMM was assumed early. In other CMPD such as PV and essential thrombocytopenia, a probably neoplastic origin at the stem cell level could be shown by demonstration of clonal blood cells. Until now only two cases of MMM, both at a final stage of the disease, have been investigated for clonal hematopoiesis. As a consequence, it has been argued that the number of patients in whom clonal growth has been proven is too small to draw the firm conclusion of an underlying stem cell disease.

In this study we analyzed granulocytes and bone marrow cells at various stages of MMM, including the cellular phase and advanced myelofibrosis, to determine whether a monoclonal hematopoiesis is detectable in all stages of MMM, including the cellular phase, which has minimal bone marrow fibrosis.

MATERIALS AND METHODS

Blood cells. Blood was obtained from patients and healthy volunteers after informed consent. Thirty-nine female patients with the diagnosis of MMM were included in the study (mean age 63.2 years, median 64, range 26 to 85 years). Diagnosis was based on peripheral blood cell findings, bone marrow cytology, and bone marrow biopsies obtained from the iliac crest. In addition, 15 hematologically normal females or with a reactive myeloproliferation, and 10 females with the diagnosis of CML were analyzed and served as controls. Peripheral granulocytes were separated by density gradient centrifugation (d = 1.077), whereas bone marrow cell DNA was obtained from stored smears as described elsewhere. Purity of separated granulocytes was controlled by cytochemistry and electron microscopy. Peripheral granulocytes DNA was purified after lysis of the cells by 10 mmol/L guanidine thiocyanate and 0.5% N-lauroylsarcosine, followed by cesium chloride density gradient centrifugation and extraction with phenol-chloroform. Archived air-dried and unstained bone marrow cell smears were used for the extraction of DNA. After scraping off the cells from three to five slides with a scalpel the resulting powder was lysed with 0.1% Nonidet P 40 (Sigma, Steinheim, Germany). The nuclear pellet obtained after short centrifugation was lysed with 2% sodium dodecyl sulfate and proteinase K (2 mg/μL) in 10 mmol/L tri-HCl pH 8.0 for 6 to 12 hours at 37°C. DNA was extracted with phenol-chloroform. After extensive dialysis against 10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 7.2, DNA was digested with one of the restriction enzymes BglII, BstXI, PstI, or HpaII for clonality analysis as outlined by Vogelstein et al or with BglII, HindIII, EcoRI, BamHI, and XbaI for rearrangement detection according to the manufacturers’ recommendations (Boehringer-Mannheim, Mannheim, Germany; Bethesda Research Laboratories, Egggenstein, Germany; New England Biolabs, Schwabach, Germany). Restriction fragments were separated by electrophoresis through 0.8% agarose gels and transferred to nylon membranes as described by Southern. Prehybridization, hybridization, washings, and exposure of membranes to X-ray films were performed as previously described. The hypoxanthine phosphoribosyltransferase probe (pHPRT-600) and the phosphoglycerate kinase gene (pSP72/PGK) were generous gifts from Dr Bert Vogelstein (Baltimore, MD). The 2-kb BglII/HindIII 5′ fragment and the 1.2-kb HindIII/BglII 3′ fragment of the bcr gene cloned into the pUC vector were generously provided by Dr C.R. Bartram (Ulm, Germany). Appropriate inserts were electrophoresed from the gels, purified by precipitation, and radiolabeled with 32P by the oligolabeling method (Multiprime, Amersham, Germany). After washing four times with 1X SSC (0.15 mol/L NaCl,

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Table 1. Clinical Findings and Histopathology of Bone Marrow in 12 Patients With Megakaryocytic Myelosis

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (y)</th>
<th>Erythrocytes*</th>
<th>Neutrophilic granulocytes†</th>
<th>Eosinophilic granulocytes</th>
<th>Basophilic granulocytes</th>
<th>Lymphocytes†</th>
<th>Monocytes†</th>
<th>Thrombocytes†</th>
<th>Immature precursor cells†</th>
<th>Blasts†</th>
<th>Splenomegaly</th>
<th>Bone marrow fibrosis</th>
<th>Osteosclerosis</th>
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<td>4.6</td>
<td>3.6</td>
<td>0.7</td>
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<td>2.5</td>
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<td>+</td>
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<td>2</td>
<td>63</td>
<td>3.9</td>
<td>21.7</td>
<td>0.8</td>
<td>0.6</td>
<td>2.5</td>
<td>2.2</td>
<td>843</td>
<td>0.3</td>
<td>-6</td>
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<td>-</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>1.8</td>
<td>3.1</td>
<td>-</td>
<td>0.2</td>
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<td>1.3</td>
<td>1,000</td>
<td>1.5</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4</td>
<td>60</td>
<td>3.3</td>
<td>3.7</td>
<td>-</td>
<td>0.1</td>
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<td>1.5</td>
<td>440</td>
<td>0.2</td>
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<td>7.6</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>2.2</td>
<td>243</td>
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<td>-</td>
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<td>8.3</td>
<td>0.1</td>
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<td>-</td>
<td>2.4</td>
<td>991</td>
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<td>-</td>
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<td>-</td>
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<tr>
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<td>-</td>
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<td>-</td>
<td>1.6</td>
<td>977</td>
<td>4.9</td>
<td>-</td>
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Abbreviations: BM, bone marrow; GL, germline.
Fig 1. Monoclonality of granulocytes in MMM. Ten MMM patients (cases 3 through 12; panels 3 through 12) heterozygous for the BstXI or BglII RFLP within the X-chromosomal PGK gene (lane a) were analyzed. After restriction with BstXI and the methylation-sensitive enzyme HpaII one allele of the PGK gene represented by either a 1.05-kb or a 0.9-kb fragment was completely digested (lane b), whereas the remaining fragment rendered an autoradiographic signal of almost identical intensity when compared with BstXI digestion alone (lane a). Patients 11 and 12 were restricted with BglII instead of BstXI, yielding polymorphic fragments of 8.2 and 5.1 kb. Hypermethylation of only one allele of the X-chromosomal PGK gene pointed to a clonal origin of all granulocytes in MMM. Granulocytes obtained from CML (panels 1 and 2) also showed a clonal inactivation pattern in this analysis, whereas granulocytes from normal controls (Contr.) displayed a nonclonal pattern without loss of one allele after methylation-sensitive restriction.

DISCUSSION

Clonality of tumor cells represents one of the cornerstones of the mutation theory of carcinogenesis. In CMPD a hematopoietic stem cell itself appears to be the neoplastic tumor cell. Because the ability to differentiate is retained, mature blood cells are produced. But, in contrast to normal mature blood cells, those obtained from CMPD are clonal in origin. Clonality can be demonstrated by glucose-6-phosphate dehydrogenase isoenzyme analysis, detection of clonal somatic mutations, or by X-linked RFLP analysis. We chose the latter system to determine whether the cellular phase of MMM represents a clonal proliferation. Up to now only two cases of MMM, both at a final stage of the disease, have been analyzed for clonality of blood cells. Our results show that all stages of the disease reveal monoclonal granulocytes and, specifically, that monoclonality of marrow cells is present in the cellular phase (Figs 1 and 2). In earlier stages of the disease autonomous proliferation affects primarily the granulocyte and megakaryocyte lineages of differentiation, but all bone marrow cells in the cellular phase of the disease seem to be derived from a single stem cell clone (Fig 2). Although hematopoietic tissue from the spleen itself has not been investigated, the monoclonality of granulocytes demonstrated in MMM patients with splenomegaly would be consistent with clonal hematopoiesis in the spleen.

Detection of monoclonal granulocytes could be of diagnostic value in differentiating reactive thrombocytosis from putatively neoplastic proliferation of megakaryocytes in MMM. However, in individual cases the method applied in this study requires controls of nonhematopoietic tissue. Vogelstein et al reported that in 3 of 81 normal females an asymmetric X-chromosomal inactivation pattern exceeding a ratio of 80:20 was found. Moreover, a nonrandom distribution of X-chromosomal methylation has been described in hemophilic females. Although lyonization may
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![Fig 2. Monoclonality of total bone marrow cells in MMM. When total bone marrow cells of MMM patients (cases 1 and 2; panels 1 and 2) were analyzed for methylation of the X-chromosomal PGK gene a clonal inactivation pattern could be found. The results corresponded to those obtained with peripheral granulocytes (Fig 1). Controls (Contr.) derived from patients with reactive myeloproliferative syndrome showed a nonclonal methylation pattern, whereas bone marrow cells from CML (panel 3) also yielded complete loss of one allele of the X-chromosomal PGK gene. Lane a, BstXI digestion; lane b, BstXI + HpaII digestion.](image)

cause relative differences in the intensity of the two alleles (Fig 2, control), we found that all of the heterozygous MMM samples \( n = 12 \) exhibited a monoclonal inactivation pattern with a complete digestion of one allele (Figs 1 and 2). Complete methylation-sensitive digestion could be observed in none of the normal controls \( n = 5 \) but occurred, besides MMM, in all informative CML granulocyte samples \( n = 4 \). Therefore, clonality of granulocytes in MMM seems to be the most probable explanation for the results obtained, whereas a constitutive asymmetric X-chromosomal methylation in all patients with MMM appears highly unlikely.

Another aim of this study was to investigate whether peripheral granulocytes in MMM are suitable for the detection of specific genetic aberrations because myelofibrosis may prevent the harvest of bone marrow cells for cytogenetic or molecular analysis. We have demonstrated that granulocytes from 10 of 10 informative females were clonally derived in MMM. Peripheral cells thus provide a readily accessible source of DNA for analysis of specific mutations that might give rise to the disease.

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REFERENCES

2. Ward HP, Block MH: The natural history of agnogenic myeloid metaplasia (AMM) and a critical evaluation of its relationship with the myeloproliferative syndrome. Medicine 50:357, 1971
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