Clonal Granulocytes and Bone Marrow Cells in the Cellular Phase of Anergic 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 thrombocythemia, 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 in Pappenheim-stained cytospin preparations and usually exceeded 95%.

Molecular-hybridization studies. High molecular weight DNA was purified after lysis of the cells by 4 mol/L guanidine thiocyanate and 0.5% N-lauroylsarcosine, followed by cesium chloride density gradient centrifugation and extraction with phenolchloroform. 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 μg/μL) in 10 mmol/L tris-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 deletion rearrangement detection according to the manufacturers' recommendations (Boehringer-Mannheim, Mannheim, Germany; Bethesda Research Laboratories, Egggenstein, Germany; New England Biolabs, Schwalbach, 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 (pSP710/PKG) 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 "P by the oligolabeling method (Multiprime, Amersham, Germany). After washing four times with 1X SSC (0.15 mol/L NaCl,
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RESULTS

In 12 patients with MMM and 4 patients with CML, a heterozygous genotype of the PGK gene or the HPRT gene, respectively, could be found. The frequency of heterozygous patients was 30% for the PGK gene and 8% for the HPRT gene. The clinical and histopathologic findings in heterozygous patients with the diagnosis of MMM are listed in Table 1. In nine cases a thrombocytosis exceeding 6 x 10^9/L could be noticed. Six cases showed a leukocytosis with an increase of neutrophilic granulocytes above 8 x 10^9/L. In one case a considerable number of blasts occurred in the peripheral blood (case 3). None of the patients showed ring sideroblasts or had a prior history of radiation or chemotherapy. All patients had in common the proliferation of atypical megakaryocytes in the bone marrow. In nine cases the proliferation of atypical megakaryocytes was accompanied by a moderate diffuse increase in fine argyrophilic fibers, single or in loose bundles (grade +, Table 1).

In two specimens an increase in thick fibers creating a diffuse network with unchanged or moderately reduced hematopoiesis occurred (grades ++ and ++++, respectively; Table 1). Mild to pronounced broadening of osteoid trabeculae (osteosclerosis) was found in six bone marrow specimens. In 50% of patients the spleen was enlarged (Table 1).

All heterozygous granulocyte samples obtained from different stages of MMM showed a monoclonal inactivation pattern of X-chromosomal genes as demonstrated by restriction with methylation sensitive HpaII and hybridization with either the PGK or the HPRT gene (Table 2). Clonal granulocytes could be found in cases with mild bone marrow fibrosis (eg, case 3, Fig 1) as well as in cases with advanced myelofibrosis (eg, case 5, Fig 1). In control granulocytes obtained from hematologically normal donors an inactivation pattern affecting both alleles of the PGK or HPRT gene was found (Fig 1, control). In all CML granulocyte samples (n = 4) the results were identical to those from MMM (Fig 1).

Because nuclei containing cells of the erythropoiesis and thrombopoiesis are restricted to the bone marrow, total bone marrow cells were analyzed in three cases of MMM. This was done to show that not only the granulopoiesis but the complete myeloid hematopoiesis was clonal in origin. In all samples analyzed a monoclonal inactivation pattern could be found, whereas controls obtained from reactive myeloproliferation showed methylation of both alleles, which showed slight asymmetry in some cases (Fig 2). In one case granulocytes as well as bone marrow cells were analyzed and found to be clonal (case 7, Table 2).

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Abbreviations: Gra, granulocytes; BM, bone marrow; GL, germline.
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 hemophiliac females. Although lyonization may
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 myeloproliferation 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.

REFERENCES
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