Clonal Analysis of Myelodysplastic Syndromes: Evidence of Multipotent Stem Cell Origin

By Johannes W.G. Janssen, Michael Buschle, Mark Layton, Hans G. Drexler, John Lyons, Herman van den Berghe, Hermann Heimpel, Bernhard Kubanek, Enno Kleihauer, Ghulam J. Mufti, and Claus R. Bartram

Restriction fragment length polymorphisms (RFLPs) of the X-chromosome genes hypoxanthine phosphoribosyltransferase (HPRT) and phosphoglycerate kinase (PGK) were studied in 34 female patients with primary myelodysplastic syndromes (MDS). Twelve patients (35%) were heterozygous at the HPRT or PGK loci for BamHI or BglII RFLPs, respectively. In eight patients showing PGK polymorphisms, clonality was determined by X-chromosome inactivation analysis. These included patients from different morphologic subtypes: four with refractory anemia (RA), two with RA and ringsideroblasts (RARS), one patient with RA with excess of blasts (RAEB), and one with chronic myelomonocytic leukemia (CMML). A monoclonal pattern of X-chromosome inactivation was observed in seven cases. In a further case characterized by bone marrow hypoplasia, peripheral blood (PB) leukocytes were polyclonal in origin. Following low-dose cytarabine therapy, reversion to polyclonal hematopoiesis was observed in a case of RAEB indicating the presence of residual normal hematopoietic stem cells with the capacity for marrow reconstitution. The clonal relation of lymphoid and granulocyte/monocyte lineages was studied directly in two cases of CMML exhibiting somatic mutations of N-ras or Ki-ras oncogenes. By selective oligonucleotide hybridization to ras gene sequences amplified in vitro by the polymerase chain reaction, a mutated ras allele was demonstrated in PB granulocytes, monocytes, and B and T lymphocytes of both patients. We conclude that MDS arise from a multipotent hematopoietic stem cell with the potential for myeloid and lymphoid differentiation.

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detraction of G-6-PD isoenzyme analysis posed by the rarity of heterozygosity outside certain ethnic groups.

To directly study the involvement of specific cell lineages in MDS, we applied yet another strategy. Somatic mutations of ras oncogenes involving single nucleotide substitutions at codon 12 or 13 have recently been detected in a proportion of MDS cases.20-22 These genomic alterations serve as clonal markers and may be used to study the clonal composition of different cell populations. This novel strategy is facilitated by the ability to detect mutated ras genes in as few as 100 cells by first amplifying ras gene sequences several hundred-thousand-fold by the polymerase chain reaction.23,24 Hybridization to radiolabeled oligonucleotides enables the distinction between wild-type and mutant alleles.

Applying an X-linked DNA polymorphism and ras oncogene mutation as markers, we have studied clonality in ten cases of primary MDS and conclude that MDS arise as a consequence of somatic mutations affecting a pluripotent stem cell common to myeloid and lymphoid cell lineages.

**METHODS**

**Patients.** Thirty-four female MDS patients (aged 28 to 86) with primary MDS were screened for heterozygosity of X-linked RFLPs. According to FAB criteria, these cases were classified as RA (15 patients), RARS (five), RAEB (seven), RAEB-T (one), and CMML (six). In addition, we studied the clonal composition of peripheral blood cells in two transfusion-independent male CMML patients using mutated ras alleles as a marker. Peripheral blood or bone marrow samples as well as a skin biopsy of one patient were obtained with informed consent. The duration of disease at the time of analysis varied from 4 to 147 months. Clinical data of ten patients studied by clonal analysis are given in Table I.

**Southern blot analysis.** High molecular-weight DNA was prepared from bone marrow or peripheral blood cells obtained from all 34 MDS patients as well as a skin biopsy of one patient by standard technique of proteinase K digestion, phenol extraction, and ethanol precipitation.23 Protocols used for clonal analysis with X-linked genes have previously been described by Vogelstein et al.24 Briefly, for analyses with the PGK probe, 10 µg of respective DNAs were screened for a BglII RFLP characterized by 9.4 kilobase (kb) and 5.7 kb fragments. DNAs from females heterozygous for this RFLP were further used for clonal analysis. Twenty micrograms of DNA were digested with the restriction enzymes BstXI and PstI producing polymorphic fragments of 1.5 kb and 0.9 kb. Subsequently, the DNA was divided into equal aliquots; one aliquot was not digested further and the other was digested with the methyl-sensitive enzyme HpaII.

For analysis with the HPRT probe, 10 µg of DNAs were screened for a BamHI RFLP showing 24 kb and 12 kb fragments. Subsequently, 20 µg of DNA from patients heterozygous for this RFLP were digested with BamHI and PvuII demonstrating 18 kb and 12 kb HPRT fragments and were either not digested further or digested with HpaII. Restriction enzymes were obtained from Boehringer Mannheim (FRG) with the exception of BstXI (New England Biolabs, Beverly, MA). Electrophoresis on 1.5% (PGK analysis) or 0.6% (HPRT analysis) agarose gels, Southern transfer, and hybridization to PGK and HPRT sequences were performed as described.25 The following probes (kindly provided by Drs. J. Singer-Sam, Duarte, C.A., and J. Respess, La Jolla, CA) were used: a 0.85 kb MspI/PstI insert from pPB 1.7 containing sequences from the 5' region of the HPRT gene,26 and pSP71/PGK containing a 0.8 kb EcoRI/BamHI fragment from the 5' end of the PGK gene.27

**Cell separation.** Mononuclear cells and granulocytes were separated from 50 mL heparinized peripheral blood of two CMML patients by Ficoll-Hypaque density gradient centrifugation. T and B lymphocytes and monocytes were fractionated by positive selection with immunomagnetic beads.28 Mononuclear cells were incubated with lineage-specific monoclonal antibodies (MoAbs) at excess concentration. MoAbs with the following specificities were used: T cells RFT-1 (CD3), RFT-2 (CD7), RFT-3 (CD4), and RFT-8 (CD8); B cells B4 (CD19), B1 (CD20), RFB-6 (CD11a), and RFB-4 (CD22); monocytes MCS-2 (CD13) and

<table>
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<th>Case No.</th>
<th>FAB Type</th>
<th>Karyotype (No. of Metaphases)</th>
<th>Age (yr)</th>
<th>Disease Duration (mo)</th>
<th>Follow-Up After Molecular-Generic Analysis (mo)</th>
<th>Source of DNA</th>
<th>Peripheral Leukocyte Count (x 10⁹/L)</th>
<th>Total</th>
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<th>L</th>
<th>M</th>
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RFLP analyses were performed in cases 1 through 8, mutated ras alleles served as clonal markers in cases 9 and 10.

Abbreviations: G, granulocytes; L, lymphocytes; M, monocytes; BL, blasts; BM, bone marrow; PB, peripheral blood; NE, not evaluated at time of DNA analysis; NM, no analyzable metaphases.

*Died due to cerebrovascular accident.
†Further follow-up not documented.
‡Case 7A and B indicate before and after cytarabine therapy.
§Differential count of BM sample NE.
VIM-D2 (CD14-like). After washing, cells were incubated with excess amounts of magnetic beads (Dynabeads M-45; Dianova, Hamburg, FRG) coated with goat anti-mouse IgG. Rosette cells were separated by magnet (Dianova).

In order to determine the degree of contamination, samples were stained with MoAbs using the indirect immunofluorescence technique in suspension in microtiter plates.29 Cells were labeled with RFT-8a (CD8) + RFT-12 (CD6), RFB-7 (CD20), or VIM-D5 (CD15) as T cell, B cell, or myelomonocytic markers, respectively. Positivity was evaluated under a fluorescence microscope. Less than 1% of contaminating cells could be detected in the monocyte or B cell fractions. The T cell fractions of both patients included approximately 5% and 10% VIM-D5 (CD15) positive myeloid cells, respectively.

The RF-MoAbs, MCS-2, and VIM-D2/VIM-D5 were generous gifts from Dr George Janossy (London, UK), Dr Jun Minowada (Okayama, Japan), and Dr Walter Knapp (Vienna, Austria). BI, B4, and Leu-4 were purchased from Coulter Immunology (Hialeah, FL) and Becton Dickinson (Mountain View, CA).

Dot blot assay for detection of ras gene mutations. Polymerase chain reaction (PCR) was performed directly on cell lysates derived from individual peripheral blood subsets in two patients with CMML. Cell lysates were incubated with 100 ng of amplifier complementary to sequences upstream and downstream of codon 12 of N-ras and Ki-ras in the presence of Taq polymerase (Biolabs, New England, MA). Sequences of respective amplimers used in these experiments have been published elsewhere.30 The reaction mixture included 1 mmol/L of each deoxynucleotide triphosphate, 67 mmol/L Tris-HCl, pH 8.8, 6.7 mmol/L MgCl2, 16.6 mmol/L (NH4)2SO4, 10% DMSO, 10 mmol/L β-mercaptoethanol, and 6.7 µmol EDTA, in a total volume of 50 µL. PCR was performed by a modification of the method described by Saiki et al. The mixture was incubated at 95°C for five minutes to denature double-stranded DNA, then cooled at 37°C for five minutes to allow primer annealing. Primer extension was initiated by the addition of 4 units of Taq polymerase and allowed to proceed at 65°C for five minutes. Fifteen cycles of amplification with an outer set of primers were followed by 15 cycles with an inner set to enhance specificity after the addition of 5 units of Taq polymerase.

Amplified DNA (5 ng) was spotted onto nylon filters (Gene Screen Plus; New England Nuclear, Boston) and fixed by UV illumination. The filters were prehybridized overnight at 50°C in 5 x SSPE (10 mmol/L sodium phosphate, pH 7.0, 0.18 mol/L NaCl, and 1 mmol/L EDTA), 7% SDS, 100 µg/mL sonicated, denatured salmon sperm DNA and 5% Denhardt's solution, and hybridized for three hours at 50°C in the presence of 6 ng γ32P-labeled oligomer probe. Oligomers specific for the wild-type and mutant alleles in conjunction with a negative control were used.31 Posthybridization filters were washed twice in 2 x SSPE, 0.1% SDS for five minutes at room temperature, and once in 5 x SSPE, 0.1% SDS for 30 minutes at 50°C. A final high stringency wash in 5 x SSPE, 0.1% SDS was performed for ten minutes at 63°C. Filters were exposed to Kodak XAR film for 12 hours at ~70°C using intensifying screens.

RESULTS

Studies using X-linked RFLPs. Thirty-four de novo MDS patients were screened by a strategy recently described by Vogelstein et al16-18 using RFLPs and methylation patterns of X-chromosome genes for clonal analysis. Ten patients were found to be heterozygous for the PGK and another two cases for the HPRT RFLPs, respectively. Thus 35% of all MDS patients were suitable for further clonal analyses. This prevalence corresponds to similar percentages reported by others17,18 and to observations in our laboratory during the analysis of 26 AML cases, of whom 14 were heterozygous for one of both polymorphisms (data not shown). Clonal analysis was undertaken in eight patients. In six cases peripheral blood and in two cases bone marrow was the source of DNA analyzed. Clinical data of these cases and composition of the samples used for DNA analyses were presented in Table 1. With the exception of case 4, all patients exhibited a monoclonal pattern of X inactivation in which one allele was lost completely following digestion with HpaII (Fig 1A) indicating most cells in the samples analyzed to share a common clonal origin. In contrast, a polyclonal pattern of X inactivation in which the signal intensity of each allele was reduced approximately 50% following methylation sensitive cleavage, was seen in case 4 (Fig 1A) and normal controls (not shown).

The RFLP methylation strategy is based on the assumption that the ratio of active maternal X chromosomes to active paternal X chromosomes in normal female tissue is approximately 1:1. However, because of the random nature of X-chromosome inactivation this ratio is normally distributed.16,19 Ideally therefore DNA derived from a constitutive source should be used to determine the normal pattern of X-chromosome inactivation in a given individual. In one patient (case 2) DNA obtained from a skin biopsy served as an internal control confirming the pattern of X inactivation to be polyclonal in nature (Fig 1A, case 2F). Following treatment of case 7 with low-dose cytarabine, reversion to polyclonal hematopoiesis was seen in the bone marrow (Fig 1B) confirming the random nature of X-chromosome inactivation and indicating the reemergence of normal hematopoietic stem cells.

In other cases the complete loss of one allele after HpaII digestion strongly supports the monoclonal nature of the samples analyzed. The alternative possibility that imbalanced mosaicism was responsible for the apparently monoclonal pattern seems unlikely. Furthermore, even X-inactivation ratios of 80:20 result in faint hybridization signals of the under-represented allele after HpaII digestion (Janssen JWG, Bushche M, Bartram CR, unpublished results).18

Restoration of polyclonality following cytarabine therapy. One patient (case 7) with RAEB was treated three months after diagnosis with low-dose cytarabine (10 mg/m2) twice daily subcutaneously for 21 days at 6-week intervals. A clinical and cytogenetical remission with restoration of normal hemoglobin, platelets, and leukocyte counts as well as disappearance of a clone characterized by trisomy 8 (Table 1) was achieved after three courses and has been maintained for 9 months by intermittent low-dose cytarabine therapy. While a bone marrow sample taken before therapy revealed a monoclonal pattern for the PGK RFLP after HpaII digestion (Fig 1A, case 7), analyses 5 months (not shown) and 8 months after the beginning of treatment showed a polyclonal pattern (Fig 1B).

Cell separation studies using mutated ras genes as clonal markers. As an alternative molecular genetic approach to study the underlying clonality of hematopoiesis in MDS, we used mutated ras genes in two cases of CMML. Both male patients have been included in a previous study in which we demonstrated mutations at codon 12 of the Ki-ras and N-ras
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3

-1.05
-0.90

5

a

b

6

a

b

7

a

b

8

a

b

-1.05
-0.90

Fig 1. Clonal composition of blood cell samples from eight MDS patients (cases 1 through 8) and fibroblasts of case 2 determined by X-chromosome inactivation analysis. A PGK RFLP characterized by 1.05 kb and 0.9 kb fragments in BstXI/PstI digests distinguishes the material from the paternal allele, and methylation differences, as shown by HpaII digestion distinguish between active and inactive X chromosomes. For this analysis 20 µg of DNA obtained from each patient were digested with BstXI and PstI; subsequently the DNA was divided into two equal aliquots; one was not digested further (lanes b) and the other was digested with HpaII (lanes a). (A) With the exception of case 4, a complete loss of one allele is demonstrated in blood cell samples from all patients after HpaII digestion (lanes a) indicating a monoclonal cell population. However, analysis of the fibroblast sample from case 2 (F) shows a polyclonal pattern. (B) Clonal analysis of bone marrow from a RAEB patient (case 7) after low-dose cytarabine therapy also reveals a polyclonal cell population.

Fig 2. Detection of mutated ras sequences in different hematopoietic cell lineages of two CMMML patients. Five nanograms of amplified DNA obtained from the patients' peripheral blood (a), separated granulocytes (b), monocytes (c), B lymphocytes (d), and T lymphocytes (e) were hybridized to oligomers representing wild-type and mutation-specific ras sequences. An analysis of case 9 (A) revealed a point mutation at KI-ras codon 12 substituting aspartic acid (asp) for the normal amino acid glycine (gly), while in case 10 (B) a point mutation at N-ras codon 12 substitutes the sequence GGT (alanine) for the wild-type codon GGT (glycine). In both cases wild-type alleles are present in addition to the mutated ras versions on the second allele. Two other mutation-specific ras oligomers were used as negative hybridization controls.

oncogenes in three of 34 cases of primary MDS. Clinical data of the two CMMMLs as well as make-up of the samples used for DNA studies are shown in Table 1. Using a dot-blot procedure based on a combination of in vitro amplification of target sequences by the polymerase chain reaction (PCR) and hybridization to mutation-specific oligonucleotide probes, we demonstrated that peripheral blood cells of patient no. 9 were characterized by a mutation at codon 12 of KI-ras resulting in substitution of GAT (aspartic acid) for GGT (glycine) (Fig 2A). Peripheral blood cells of case 10 exhibited a mutation at codon 12 of N-ras substituting alanine (GCT) for glycine (GGT) (Fig 2B).

Since PCR permits rapid analysis of ras mutations from less than 1,000 cells (Janssen JWG, Lyons J, Bartram CR, unpublished results), we separated the peripheral blood cells of both patients into granulocyte, monocyte as well as B and T lymphocyte fractions. As shown in Fig 2, ras gene mutations could be established in all four cell lineages of both patients. The limit of sensitivity of our dot-blot assay permits the detection of ras mutations only if present in at least 10% of cells. We wish to emphasize that the monocyte and B-cell fractions were pure as determined by morphologic or immunologic methods. The T-cell samples contained 5% (case 10) and 10% (case 9) contaminating myeloid cells, respectively; these contaminants could induce very faint hybridization signals at the most. However, the strong and equal autoradiographic signals observed for all cell fractions of both cases rather indicate that ras mutations are present at
least in the vast majority, if not all, cells of the different lineages analyzed.

DISCUSSION

The occurrence of nonrandom chromosome abnormalities in the majority of MDS together with the few data obtained by G-6-PD studies have convincingly established the clonal nature of MDS. However, the extent of cell lineages involved in these disorders, i.e., the nature of the stem cell level of which MDS originates, remains a matter of controversy. In the present study we approached this problem by two independent molecular genetic strategies. RFLP analysis of X-linked genes demonstrated the clonal relationship of bone marrow or peripheral blood cells in seven of eight MDS patients. The involvement of the lymphocyte lineage in five cases can be inferred from the fact that the percentage of lymphocytes in the samples analyzed exceeded 40% in all cases. The presence of polyclonal cells in such numbers would undoubtedly be detectable in Southern blot analysis. However, in all cases a clear-cut pattern of monoclonality was observed.

It is of interest that the patient showing a polyclonal X-inactivation pattern (case 4) represents the only case in our study showing a hypoplastic marrow. Hypoplastic variants of MDS are rare and may be difficult to distinguish from aplastic anemia. The absence of demonstrable clonality despite the presence of trilineage dysplasia in this particular case illustrates this problem. Clonal analysis may help to clarify the biologic relationship between these different entities and perhaps prove to be of diagnostic value.

Another strategy to investigate the clonal nature of MDS takes advantage of the fact that a portion of these cases exhibit a mutation within the ras gene family. While the significance of this genomic alteration in terms of pathogenesis of MDS or transition into overt ANLL remains to be elucidated, ras mutations are certainly powerful markers for clonal analysis. Moreover, in vitro amplification of DNAs via PCR considerably facilitates investigations of small cell numbers as observed in the majority of cytopenic patients. We were able to perform cell separation analyses in two male CMML patients identified in a previous study, both of whom were characterized by mutations at codon 12 of N-ras and Ki-ras, respectively. In fact, all cell lineages tested (granulocytes, monocytes, B cells, and T lymphocytes) contained mutated ras alleles. These results are in agreement with a previous report using the same technique in which we also demonstrated involvement of the lymphocyte lineage in another case of CMML. As to the relationship between ras gene mutations and chromosomal aberrations, Hirai et al recently reported on two MDS patients in whom abnormalities in chromosomes 5 preceeded acquisition of N-ras mutations. Karyotypic changes observed in a minority of metaphases in our CMML patient characterized by a Ki-ras mutation would suggest that the ras mutation had been established before those chromosomal abnormalities. These data indicate that ras mutations may occur at different stages during the course of MDS. Unfortunately, other tissues of both patients were not available for molecular analysis. Thus we cannot unequivocally prove the somatic nature of the ras mutations. However, the alternative possibility of germline mutations in both patients appear to be rather unlikely, since mutations affecting codons 12, 13, or 61 have thus far never been observed in normal human or animal cell samples.

Molecular genetic approaches to clonality confer important advantages over cytogenetic analyses. This is illustrated by the absence of a detectable karyotypic maker in seven of ten cases in which we performed chromosomal analysis (Table 1). In those patients showing clonal karyotypic abnormalities these were confined to a portion of analyzed cells only. In distinction, X-inactivation analysis showed the vast majority of cells to be derived from a common clonal progenitor. These data suggest that karyotypic changes occur late in the evolution of MDS. Our findings may be reconciled with studies applying cytogenetic and RFLP techniques that show myeloid but not lymphoid cells to be involved in MDS. This suggests that chromosomal abnormalities may affect a hematopoietic progenitor that is relatively committed with respect to differentiation potential. The initial event in MDS, however, appears to involve a stem cell common to lymphoid and myeloid lineages.

The relation between clonal expansion and disease progression in MDS remains unclear. Although the vast majority of peripheral leukocytes appeared clonal, none of our patients have developed ANLL thus far. Whether residual normal stem cells exist in a state of regression in MDS is of fundamental biologic importance and may also be relevant to their therapy. The restoration of polyclonality in marrow cells following treatment with low-dose cytarabine clearly establishes the existence of normal stem cells capable of repopulating the marrow. Studies of clonality may also help to clarify the mechanism of action of low-dose cytarabine, the use of which in MDS is based on the differentiating effect on leukemic cell lines in vitro. We recently reported the disappearance of a ras gene mutation following treatment of a case of RAEB with low-dose cytarabine. This observation is consistent with previous evidence from cytogenetic and isoenzyme studies. Similarly, X-inactivation analysis may shed light on the mode of action of differentiating agents and potentially other biologic response modifiers. The reversion to polyclonal hematopoiesis seen in one patient (case 7, Fig 1B) following low-dose cytarabine treatment lends further support to the notion that even at low dosages cytarabine acts primarily as a cytotoxic agent.

In conclusion, our results provide direct and indirect evidence that the majority of MDS, irrespective of the morphologic subtype, originate from a multipotent stem cell capable of myeloid and lymphoid differentiation. Moreover, our findings confirm the value of recombinant DNA strategies as powerful tools for understanding the clonal basis of human neoplasias.

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Clonal analysis of myelodysplastic syndromes: evidence of multipotent stem cell origin

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