Clonal Studies in the Myelodysplastic Syndrome Using X-Linked Restriction Fragment Length Polymorphisms

By A. Tefferi, Stephen N. Thibodeau, and Lawrence A. Solberg, Jr

We used the X-linked restriction fragment length polymorphism (RFLP)-methylation strategy to study the clonal basis of the myelodysplastic syndrome (MDS) in seven patients. RFLP-methylation analysis was performed on cell populations from bone marrow (BM) aspirates and peripheral blood using probes specific for the hypoxanthine phosphoribosyltransferase (HPRT) or phosphoglycerate kinase (PGK) gene regions. Density gradient centrifugation methods were used to separate granulocytes and monocytes, and T lymphocytes were positively selected by CD2 (a pan-T marker) immunoconjugated magnetic beads. Cell populations from BM aspirates in 6 of the 7 patients with MDS showed a monoclonal pattern of X-inactivation. The neutrophilic and T-lymphocytic cell fractions were analyzed in 4 of the 6 patients, and the monocytic cell fraction in one of these, and all fractions analyzed showed a similar monoclonal pattern. In 2 of the latter 4 patients, both of whom had normal karyotypes, DNA from a skin biopsy showed a polyclonal pattern. Our data suggest that MDS is a clonal disorder, even in the absence of detectable cytogenetic abnormalities, and that the abnormal clone is capable of myeloid, monocytic, and lymphoid differentiation.

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CLONAL STUDIES IN MYELODYSPLASTIC SYNDROME

Patient

1
BM
a
b

2
BM
a
b

3
BM
a
b

4
BM
a
b

5
BM
a
b

6
BM
a
b

7
BM
a
b

Fig 1. RFLP-methylation analysis on specimens from the BM (patients 1 through 7), neutrophilic cell fraction (N) (patients 2, 4, 6, and 7), T-lymphocytic cell fraction (T) (patients 2, 4, 6, and 7), monocytic cell fraction (M) (patient 4), and skin tissue (S) (patients 6 and 7) of patients with myelodysplastic syndrome. In each column, (a) represents bands resulting from digestions with restriction enzymes detecting polymorphisms, and (b) represents the absence of additional digestion with a methyl-sensitive enzyme. The particular patterns are indicated in Table 1.

restriction enzymes were purchased from New England Biolabs (Beverly, MA), and the conditions of digestion were according to the manufacturer’s recommendations. The DNA probes pHRT-800 and pSPT/PGK were kindly provided by Dr. B. Vogelstein (Johns Hopkins Medical School, Baltimore, MD).

Defining clonality from the pattern of X-inactivation. The radiographic bands obtained from the HpaII undigested and digested DNA aliquots were compared visually. A monoclonal pattern of X-inactivation was defined as a greater than 80% signal loss by one of the paired alleles accompanied by a less than 40% signal loss by the other. Because of occasional differences in the amount of DNA in the two aliquots containing HpaII digested and undigested DNA, we also considered a pattern monoclonal if there was a greater than 50% disproportionate signal loss between the two alleles after digestion with the methyl-sensitive restriction enzyme. A polyclonal pattern was defined as a near-equivalent degree of signal reduction between the two alleles.

RESULTS

The results of both RFLP-methylation analysis and cytogenetic analysis performed on specimens from seven patients with MDS are shown in Fig 1 and summarized in Table 1. The results indicate the presence of a clonal hemopathy in these patients even in the absence of a detectable karyotypic abnormality. They also suggest a multipotent origin for the abnormal clone, which may involve neutrophils, monocytes, and T lymphocytes.

DISCUSSION

Clonal studies with both G-6-PD isoenzyme analysis and the RFLP-methylation strategy rely on differences in the pattern of X chromosome inactivation between normal and neoplastic tissues. The former approach has been invaluable in defining the clonal nature of several myeloid disorders. Despite its enormous contributions to the understanding of hematopoietic stem cell diseases, the use of G-6-PD isoenzyme analysis has been limited due to the rarity within the indigenous female population of G-6-PD heterozygosity.

The RFLP-methylation strategy analyzes DNA polymorphisms rather than protein polymorphisms and has a wider applicability. In our survey of 133 patients, the incidences of heterozygosity to an RFLP within the HPRT, PGK, or either one or the other gene regions, were 20%, 32%, and 45%, respectively. The corresponding incidences from the study by Vogelstein et al. were similar to our own (29%, 33%, and 53%). In contrast, the incidence of BamHI polymorphism in another survey of 80 females was only 11%. The discrepancy may reflect differences in allele frequencies among different geographic regions. Nevertheless, the assay is potentially applicable for approximately half the North American female population using the aforementioned restriction enzymes and DNA probes.

We found the assay useful in readily identifying clonal excess in the BM, lymph node, and peripheral blood of patients with known clonal hematologic neoplasms. We have,

Table 1. Results of RFLP-Methylation Analysis and Bone Marrow Cytogenetic Studies of Patients With Myelodysplastic Syndrome

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>Karyotype</th>
<th>BM</th>
<th>Neut</th>
<th>T-Lym</th>
<th>Mono</th>
<th>Skin</th>
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<td>1</td>
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<td>NN</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>RA</td>
<td>AN (4/20)</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>RA</td>
<td>AN (4/20)</td>
<td>P</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>RARS</td>
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<td>ND</td>
</tr>
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</tr>
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<td>P</td>
</tr>
<tr>
<td>7</td>
<td>RA</td>
<td>NN</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>ND</td>
<td>P</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent ratios of abnormal to total number of metaphases studied.

Abbreviations: RFLP, restriction fragment length polymorphism; BM, bone marrow; Neut, neutrophil fraction of peripheral blood; T-Lym, T-lymphocyte fraction of peripheral blood; Mono, monocytic fraction of peripheral blood; RA, refractory anemia with ringed sideroblasts; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; NN, normal; AN, both normal and abnormal metaphases present; M, monoclonal; P, polyclonal; ND, not done.
in general, elected to use relatively pure tumor samples because in a separate mixing experiment performed to investigate the effect of normal tissue contamination, we found that in the presence of greater than 50% normal tissue in the test material, we could not appreciate a distinct pattern visually different from that derived from pure normal tissue (Fig 2). This limits the use of the assay to tissues with a relatively pure clonal representation. As such, the assay may not be helpful in detecting minimal residual disease or confirming complete remissions. However, the inability to detect minor clonal populations has its own benefits. For example, in studying specific cell lineages, minor contamination with other cells may not affect the resultant X-inactivation pattern, which is representative of the majority cell population. Similarly, in a tissue sample comprised of different cell types where the percentage of any one cell type does not exceed 50%, the demonstration of a clonal excess implies multilineage clonal involvement.

The assay was particularly helpful in determining the clonal basis of MDS in seven patients. In two of these patients (both with refractory anemia [RA]), a monoclonal pattern of X-inactivation was demonstrated in the BM, granulocytic, and T-lymphocytic components of the peripheral blood, while a polyclonal pattern was obtained from corresponding skin tissue. This suggests a multipotent stem cell origin of the neoplastic clone, which is capable of myeloid and lymphoid differentiation. Furthermore, clonal dominance was indicated in these patients with MDS in the absence of a detectable cytogenetic abnormality, suggesting that the acquisition of a cytogenetic abnormality may not occur with the initial clonal development. This interpretation is supported by the demonstration by G-6-PD isoenzyme analysis of clonal, Ph' negative B lymphocytes in a patient with Ph' positive CGL. However, the recognition of early clonal dominance does not necessarily indicate neoplasia and may be caused by stem cell depletion or damage. Four of the remaining five patients with MDS showed a monoclonal pattern in their BM samples, and of these two showed a similar monoclonal pattern in the neutrophilic and T-lymphocytic cell fractions, and one also in the monocytic cell fraction.

The only polyclonal pattern of X-inactivation was seen in a patient with RA (Table 1, patient 3), despite the presence of a cytogenetically abnormal marker in 4 of 20 analyzed metaphases. The discrepancy is probably due to the inability of the RFLP-methylation assay to detect minor clonal populations, as shown by our mixing experiments (Fig 2). Interestingly, another patient with RA (Table 1, patient 2) also had karyotypic abnormality in 4 of 20 analyzed metaphases, but displayed a monoclonal pattern of X-inactivation. These observations suggest heterogeneity in the clonal composition of a hematopoietic tissue among patients with MDS belonging to the same disease subgroup as defined by the French-American-British (FAB) cooperative group, independent of their karyotypic status. This may indicate different stages of clonal evolution that may have prognostic significance.

The occasional occurrence of extreme lyonization may result in a monoclonal pattern of X-inactivation in the absence of a neoplastic clone. Thus, clonal dominance should be validated, whenever possible, by the demonstration of a polyclonal pattern from a corresponding normal tissue. The use of corresponding normal tissue as an internal control presupposes positive correlations between the mosaic compositions of different tissues within an individual female. This was found to be the case by a previous study of G-6-PD isoenzyme ratio determination in different tissues from single heterozygote females. We have further confirmed this by comparing X-inactivation patterns, using RFLP-methylation analysis, in corresponding normal blood and colon tissue in heterozygous females. This consistency in X-inactivation ratios within an individual female allows corresponding
normal tissues to be used as internal controls in the assessment of clonality in a given cell population.

In conclusion, the results of our clonal studies of patients with MDS parallel those of a recent report, and suggest a multipotent stem cell origin of the neoplastic clone, which may exist in the absence of a detectable cytogenetic abnormality, and may involve neutrophils, T lymphocytes, and monocytes. Furthermore, heterogeneity in the clonal composition of a hematopoietic tissue was demonstrated among patients with RA carrying a similar degree of karyotypic abnormality, and the neoplastic clone was not always dominant over a coexisting normal clone.

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Clonal studies in the myelodysplastic syndrome using X-linked restriction fragment length polymorphisms

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