Evidence for Nonclonal Hematopoietic Progenitor Cell Populations in Bone Marrow of Patients With Myelodysplastic Syndromes

By Haruhiko Asano, Haruhiko Ohashi, Masatoshi Ichihara, Tomohiro Kinoshita, Takashi Murate, Miya Kobayashi, Hidehiko Saito, and Tomomitsu Hotta

Clonality of marrow hematopoietic progenitor cells in myelodysplastic syndromes (MDS) was analyzed by X-chromosome inactivation pattern using polymerase chain reaction (PCR). Five female patients were included in this study; two with refractory anemia (RA) and three with RA with excess blasts (RAEB). They were heterozygous for BstXI restriction fragment length polymorphisms (RFLP) of the X-chromosome-linked phosphoglycerate kinase (PGK) gene. Each patient, erythroid and nonerythroid colonies, grown in the presence of erythropoietin and granulocyte-macrophage colony-stimulating factor (GM-CSF), exhibited no remarkable difference in clonal constitution. Two patients showed only one methylation pattern, suggesting the monoclonal origin of hematopoietic progenitor cells. Colonies of two other patients exhibited predominant and minor methylation patterns in PGK gene, indicating that nonclonal progenitor cells remain a minor population. The bone marrow of one patient appeared to contain a greater proportion of nonclonal progenitors. Stem cell factor (SCF), a potent colony-stimulating factor, enhanced both erythroid and nonerythroid colony formation. However, it did not notably alter the clonal constitutions. We conclude that nonclonal hematopoietic progenitor cells persist in a substantial number of MDS patients.

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THE MYELODYSPLASTIC syndromes (MDS) are a morphologically defined heterogeneous group of stem cell disorders characterized by maturational defects of marrow hematopoietic cells, resulting in peripheral blood cytopenias.1,2 Clonal hematopoiesis is another characteristic in MDS.3 Analyses using X-linked isozyme system of glucose-6-phosphate dehydrogenase (G6PD) revealed the clonal origin of peripheral blood cells of MDS patients.4,6 These studies have provided valuable information on the clonality of hematologic disorders because it is applicable for the analysis of erythrocytes and platelets lacking nuclei. However, the rarity of females with heterogeneity of G6PD has limited further study. Cytogenetic analyses7-13 and studies of nonspecific gene mutations,14 further clarifying the clonal nature of MDS, have been powerful tools for direct demonstration of the clonal population. If the karyotypic (genetic) changes occurred after the clonal evolution, the data of such analyses might not reflect the real clonality in the patient. For that reason, reports on the mosaicism of normal and abnormal karyotypes in hematopoietic progenitor cells16,17 do not necessarily provide evidence for the presence of normal clone in MDS. In any event, investigators have targeted mainly mature peripheral blood cells for clonal analysis. Adequate information on the clonality of marrow progenitor cells has not been available in MDS.

Vogelstein et al18 established an elegant method for clonality analysis by means of DNA polymorphisms at X-linked loci, which uses random X-chromosome inactivation in females.19 This strategy has the great advantage of general applicability for clonal studies, even for patients with no genetic markers. Using the method, many investigators disclosed the clonal nature of MDS,19,17,20 as well as other neoplastic disorders.15,21 Because the X-inactivation pattern is sustained in the daughter cells in a highly stable manner,20 clonal analysis of individual colonies grown in culture should show the clonality of hematopoietic progenitor cells. Inclusion of polymerase chain reaction (PCR) in the genetic analysis has made it possible to examine the clonality of a small cell population.22 In this study, we wish to clarify the clonal origin of MDS marrow hematopoietic progenitor cells. X-inactivation patterns of single erythroid (burst-forming units–erythroid [BFU-E]) and nonerythroid colonies were analyzed using PCR. Results suggest that nonclonal progenitor cells remain in a substantial number of MDS patients, which might be important implication for the therapy of MDS.

MATERIALS AND METHODS

Patients. Five female patients with primary MDS were included in this investigation (Table 1): two with refractory anemia (RA) and three with RA with excess blasts (RAEB) according to the French-American-British (FAB) nomenclature.23 They were heterozygous for BstXI restriction fragment length polymorphisms (RFLP) of the X-chromosome gene phosphoglycerate kinase (PGK) and received neither chemotherapy nor bone marrow transplantation before the study. Their marrow and peripheral blood samples were obtained with informed consent. As a control, two healthy female volunteers who were heterozygous for the RFLP were also included in this study, after informed consent was obtained.

Cell preparation. Light-density mononuclear cells (MNC) were obtained from marrow and peripheral blood samples by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Marrow MNC were washed and resuspended in RPMI 1640 (GIBCO, Grand Island, NY) with 20% fetal calf serum (PCS, Boehringer Mannheim, Mannheim, Germany) at 1 × 10^7/mL and incubated for 2 hrs at 37°C in a fully humidified atmosphere of 5% CO2, then nonadherent cells were collected. Polymorphonuclear cells (PMN) were recovered from the peripheral blood erythrocyte pellet by removal of lysed red cells after incubation in Tris-NaHCl buffer. The purity of the PMN obtained was 94.0% ± 3.5% (mean ± SD) by morphologic examination. In some patients, T lymphocytes were separated from peripheral blood MNC by agglutination with sheep
red blood cells (Kyokuto, Tokyo, Japan). Immunohistochemical analysis showed 93.7% ± 1.5% of the cells obtained were CD2+.

**Hematopoietic growth factors.** Recombinant human erythropoietin (EPO) and stem cell factor (SCF) were generous gifts from Kirin Brewery Co (Tokyo, Japan). They were used at a concentration containing 100 ng/mL and 4 U/mL, respectively. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 ng/mL) was a generous gift of Sumitomo Pharmaceutical (Hyogo, Japan).

**Cell culture.** Cells from MDS and control marrow samples were grown at 5 × 10^7/mL in 1 mL of Minimum Essential Medium-a (MEM-a, GIBCO) containing 1% methylcellulose supplemented with 20% FCS, 1% dialyzed bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO), 5 × 10^-3 mol of 2-mercaptoethanol (2-ME; Nacalai Tesque, Kyoto, Japan), EPO, and GM-CSF, either with or without SCF in a 35-mm culture dish (Nunc, Naperville, Ill) for 14 days at 37°C in a fully humidified atmosphere of 5% CO2. After 14 days of incubation, cell groups composed of more than 40 cells were scored as colonies.

**DNA extraction and processing.** Individual red-colored (erythroid) and other (nonerythroid) colonies were randomly plucked by hand with a micropipette and immediately placed in 200 µL buffer containing 1% sodium dodecyl sulfate (SDS), 2 mmol/L EDTA, 20 µmol/L Tris (pH 7.4), and 1 mg/mL proteinase K (Boehringer Mannheim) followed by incubation for 12 hours at 37°C. High-molecular weight DNA, extracted from each colony with phenol/chloroform24 using 44 µg of glycogen (Boehringer Mannheim) as a carrier, was digested by 40 U of HpaII, methylation-sensitive restriction endonuclease (all restriction enzymes except BstXI were purchased from New England Biolabs, Beverly, MA) at 55°C for more than 6 hours, then electrophoresed in 2% agarose gel and stained with ethidium bromide.

**Results**

**Table 1. Clinical and Hematologic Data of Patients at the Time of Study**

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age/Sex</th>
<th>Diagnosis*</th>
<th>Time After Diagnosis (mo)</th>
<th>WBC (10^9/L)</th>
<th>Blast (%)</th>
<th>Hb (g/dL)</th>
<th>Platelets (10^9/L)</th>
<th>Bone Marrow Blast (%)</th>
<th>Karyotype</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>46/F</td>
<td>RA</td>
<td>13</td>
<td>2.5</td>
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<td>12.1</td>
<td>9</td>
<td>&lt;1</td>
<td>46,XX</td>
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<tr>
<td>2</td>
<td>71/F</td>
<td>RA</td>
<td>9</td>
<td>3.3</td>
<td>0</td>
<td>7.1</td>
<td>6</td>
<td>&lt;1</td>
<td>46,XX</td>
</tr>
<tr>
<td>3</td>
<td>71/F</td>
<td>RAEB</td>
<td>26</td>
<td>2.9</td>
<td>2</td>
<td>8.7</td>
<td>42</td>
<td>4</td>
<td>46,XX</td>
</tr>
<tr>
<td>4</td>
<td>75/F</td>
<td>RAEB</td>
<td>66</td>
<td>3.8</td>
<td>2</td>
<td>6.6</td>
<td>99</td>
<td>8</td>
<td>46,XX</td>
</tr>
<tr>
<td>5</td>
<td>68/F</td>
<td>RAEB</td>
<td>13</td>
<td>1.1</td>
<td>0</td>
<td>4.7</td>
<td>43</td>
<td>12.7</td>
<td>46,XX</td>
</tr>
</tbody>
</table>

Abbreviations: UPN, unique patient number; Hb, hemoglobin.
* French-American-British (FAB) classification.

**RESULTS**

**Clonality of erythroid progenitors.** In the presence of EPO and GM-CSF, erythroid (BFU-E) colonies were formed in all five MDS patients, as well as two control individuals (Fig 2A), and were randomly plated. Although each colony from patients was generally small-sized, DNA was effectively amplified by PCR (data not shown). The clonal origin could be determined even if the colony was composed of less than 100 cells.

In two patients (UPN 3 and 4), all the erythroid colonies...
examined had the same X-inactivation pattern (Table 2), yielding a dominant band at 530 bp (pattern A) as illustrated in Fig 3A. Colonies of two other patients (UPN 1 and 2) had extremely skewed inactivation patterns; most of them exhibited pattern A, while four of 25 colonies in UPN 1 and two of 22 in UPN 2 produced a dominant band at 433 bp (pattern B). In UPN 5, five of 18 colonies examined were pattern A and other 13, pattern B (Table 2 and Fig 3B). In control individuals (C1 and C2), the ratios of pattern A to B of the colonies were 8:11 and 8:21, respectively (Table 2). Representative band patterns in C2 are shown in Fig 3C.

**Clonality of nonerythroid progenitors.** Nonerythroid colonies grown in the presence of EPO and GM-CSF (Fig 2B) were randomly plucked and their clonal origin was determined. In patients UPN 3 and 4, all of the nonerythroid colonies had only one inactivation pattern (Table 2 and Fig 4A). The colonies of UPN 1 and 2 gave three band patterns: pattern A, pattern B, and ambiguous. The ratios were skewed

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**Fig 1.** Band patterns of PGK gene from artificial cell-mixtures for determining the clonal origin of individual colonies. Because of the formation of heteroduplex, the intensity of upper bands is high.

**Fig 2.** Erythroid (A) and nonerythroid (B) colony growth in each patient and normal individual. Adherent cell-depleted marrow mononuclear cells were stimulated by both EPO and GM-CSF, and either (■) with or (□) without SCF. Data represent mean colony number grown from 10⁵ mononuclear cells. The error bars depict 1 SD from the mean.
Table 2. X-Chromosome Inactivation Pattern of Individual Colonies

<table>
<thead>
<tr>
<th>UPN</th>
<th>FAB</th>
<th>SCF*</th>
<th>Pattern of BstXI RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Erythroid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>RA</td>
<td>–</td>
<td>21</td>
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<td></td>
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<td>25</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>RA</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>RAEB</td>
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<tr>
<td></td>
<td>+</td>
<td>7</td>
<td>11</td>
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<tr>
<td>Control (C1)</td>
<td>–</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Control (C2)</td>
<td>–</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7</td>
<td>23</td>
</tr>
</tbody>
</table>

Clonality analysis of erythroid and nonerythroid colonies grown in the presence of both EPO and GM-CSF, and either with or without SCF. The clonal origin of each colony was determined on the basis of the ratio of amplified DNA. The ratio of between 40:60 and 60:40 in artificial cell-mixtures (Fig 1) was regarded as ambiguous, and the others as having the inactivated allele without the BstXI site (pattern A) or with the site (pattern B). The ambiguous pattern may result from contamination by cells having another clonality. The diffuse nature of the nonerythroid colonies presumably leads to the plucking of cells with a different clonal origin.

* Symbols + and – represent the stimulation with and without the factor, respectively.

Clonal acquisition analyzed by peripheral blood cells. It is necessary to know if the clonal nature of MDS marrow hematopoietic progenitor cells was the result of acquired clonal evolution or of extreme lyonization. Clonality of peripheral blood PMN and either T lymphocytes or MNC was analyzed by means of PCR, and the results are shown in Table 3 and Fig 5. In PMN of three patients (UPN 1, 3 and 4), DNA precut with HpaII produced only an upper (530-bp) band, indicating PMN were of monoclonal origin. The T-lymphocyte DNA gave rise to extremely skewed (oligoclonal) band patterns in UPN 1 and 3, which were composed of dominant upper (530-bp) and minor lower (433-bp) bands, suggesting that their T lymphocytes included a small nonclonal population. Such a difference in clonality of PMN and T lymphocytes indicated that acquired clonal events were going on in their hematopoietic system. In UPN 4, the T-lymphocyte DNA also yielded only the upper band, indicating monoclonal origin. The X-inactivation pattern of PMN from UPN 2 was oligoclonal, but the MNC DNA produced an unskewed polyclonal band pattern, which im-

Fig 2. X-chromosome inactivation pattern of individual erythroid colonies. Representative band patterns are illustrated. All bands in H(+) lanes were derived from DNA precut with HpaII. (A) An RAEB patient (UPN 3). DNA in H(+) lanes yielded a band at 530 bp, indicating inactivated X-chromosomes of cells in the colonies lacked BstXI site of PGK gene. (B) An RAEB patient (UPN 5). A dominant band at 433 bp is observed in each H(+) lane, indicating inactivated X-chromosomes contained the BstXI site. (C) A normal individual (C1). Both patterns are noticed.
plied that acquired clonal evolution in hematopoiesis took place in the marrow. UPN 5, who had a greater proportion of nonclonal marrow hematopoietic progenitor cells than any other patients, showed almost the same polyclonal patterns in PMN and MNC.

Thus, despite the presence of nonclonal hematopoietic progenitor cells, peripheral blood PMN of UPN 1 and 2 showed monoclonal and oligoclonal appearances, respectively. To clarify this discrepancy, we further performed clonal analysis of PMN of these patients by Southern blotting. PMN of UPN 1 appeared to be monoclonal and those of UPN 2 had an extremely skewed clonal constitution (Fig 6), which were similar to the results of PCR-based clonal analysis.

DISCUSSION

To know the clonal constitution of MDS marrow hematopoietic progenitor cells, we performed a PCR-based clonal analysis for colonies grown in culture. X-chromosome inactivation patterns were determined on individual erythroid and nonerythroid colonies. Nonclonal hematopoietic progenitor cells were present in four out of five patients tested. In patients UPN 3 and 4, few, if any, nonclonal progenitor cells were detected; in UPN 1 and 2, nonclonal cells formed a small progenitor cell population, while UPN 5 had more of these cells. These clonal constitutions of marrow hematopoietic progenitor cells were well reflected in the clonality of circulating PMN. PMN exhibited monoclonal or extremely skewed (oligoclonal) band patterns in UPN 1, 2, 3, and 4, while PMN of UPN 5 exhibited a polyclonal band pattern. It is worth noting that, despite the presence of nonclonal marrow progenitors, PMN of UPN 1 showed a monoclonal band pattern. This apparent discrepancy could be caused by the sensitivity of our PCR-based clonal analysis or by selective release of monoclonal cells into the peripheral blood. Specifically, the amplification of DNA from artificial cell-mixtures containing 85% cells whose X-chromosomes lacked the BstXI site apparently yielded only one band at 530 bp (Fig 1). It is very probable that if the nonclonal hematopoietic progenitor cell population is small, the circulating PMN show a monoclonal band pattern. Our results indicated that in MDS the monoclonal appearance of PMN by PCR\(^\text{20,22}\) or by Southern blotting\(^\text{15,17-20}\) would not necessarily represent the total marrow replacement by clonal progenitor cells.

We demonstrated that erythroid and nonerythroid progenitors of MDS had a similar clonal constitution, which indicates that the partially committed or earlier stem cells capable of differentiating into at least both erythroid and myelomonocytic lineages were affected. In MDS, clonal involvement of erythroid cells, whose clonality was not fully studied because of the anucleated nature of erythrocytes, has been reported to be always accompanied by myeloid cell involvement.\(^\text{4,6,10,11}\) This was confirmed again in the present study.

Many cytokines have been reported to improve the cytopemias of MDS patients.\(^\text{25-28}\) Whether they can bring about qualitative improvement (polyclonal hematopoiesis) in MDS is a matter of consideration. In the present study, we added SCF to our culture system and analyzed the clonal origin of resulting colonies. SCF, or designated as either mast cell growth factor (MGF) or c-kit ligand (KL), acts relatively early in the scheme of normal hematopoiesis.\(^\text{29,30}\) In addition, it improves colony formation by MDS marrow cells in synergy with GM-CSF\(^\text{31}\) or EPO,\(^\text{32}\) although the responses vary among patients.\(^\text{33}\) In the present study, SCF enhanced colony formation two to six times more in each patient as well as normal individuals, but it did not alter the clonal constitution of either erythroid or nonerythroid progenitor cells. Thus, the additional colonies stimulated by SCF were not the result of the preferential stimuli to nonclonal progenitor cells. It is difficult to expect SCF will restore normal (polyclonal) hematopoiesis in MDS patients. However, it cannot be ex-

![Fig 4. X-inactivation pattern of individual nonerythroid colonies. Representative band patterns are illustrated. All bands in H(+) lanes were derived from DNA precut with HpaII. (A) An RAEB patient (UPN 4). DNA in H(+) lanes yielded a band at 530 bp. (B) An RAEB patient (UPN 5). A dominant band at 433 bp is observed in each H(+) lane. (C) A normal individual (G2). Three band patterns are noticed. *Denotes ambiguous band pattern. A dominant 433-bp band in each H(+) lane is often accompanied by a faint 530-bp band, which may be explained either by the contamination of nonclonal cells or by the escape from restriction endonuclease digestion. Slight contamination of alleles containing the BsrX1 site by the site-lacking alleles might yield the 530-bp band, which was strengthened by the formation of heteroduplex.\(^\text{22}\)](https://www.bloodjournal.org/content/128/14/592)
eluded that other cytokines, or their combinations, preferentially act on residual nonclonal progenitor cells, resulting in qualitative improvement in MDS hematopoiesis. Present clonal analysis is useful in evaluating the cytokine effects in vivo as well as in vitro.

Whenever X-inactivation is used in clonal analysis, extreme lyonization should be considered. To rule out such lyonization, we analyzed clonality of either T lymphocytes or MNC from peripheral blood and compared the clonality with that of PMN. Among three patients whose PMN showed a monoclonal band pattern, two (UPN 1 and 3) had T lymphocytes of oligoclonal band patterns. MNC of one patient with oligoclonal PMN (UPN 2) yielded a polyclonal band pattern. In these three cases, acquired clonal evolution may have occurred in marrow. In UPN 4, because her T lymphocytes showed a monoclonal band pattern identical to PMN, we could not rule out completely the possibility that clonal hematopoiesis resulted from extreme lyonization. However, buccal mucosa of UPN 4 were composed of polyclonal cell populations (data not shown), which might indicate pluripotent hematopoietic stem cell involvement in the patient. Circulating PMN and MNC of UPN 5 showed a similar polyclonal band pattern. Therefore, we could not ascertain that acquired clonal evolution had caused the skewed clonal constitution of marrow progenitor cells.

As monoclonal hematopoiesis has been considered one of the characteristics in MDS, the polyclonal appearance in UPN 5 may seem extraordinary. However, this case may not be so exceptional because polyclonal blood-cell populations, other than lymphocytes, have been reported in some MDS patients. Here, we demonstrated that polyclonal hematopoiesis can persist in MDS marrow. In addition, the clonal composition of hematopoietic progenitor cells may be different from patient to patient. Thus, the heterogeneity of MDS might be true also in the hematopoietic progenitor cell clonality.

Finally, it is of great interest whether the mosaicism of clonal and polyclonal progenitor cells is stable or not with disease progression and whether remaining polyclonal progenitors affect the outcome with chemotherapy and cytokine stimulation. Studies in this area are in progress in our laboratory.

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