Interphase Cytogenetic Analysis of In Vivo Differentiation in the Myelodysplasia of Down Syndrome

By Alvin Zipursky, Hong Wang, Elizabeth J. Brown, and Jeremy Squire

In Down syndrome, acute megakaryoblastic leukemia (AMKL) occurs frequently during the first 4 years of life and is usually preceded by a period of myelodysplasia (MDS), often associated with chromosomal abnormalities. Archival peripheral blood and/or bone marrow films of six patients with Down syndrome and MDS whose leukemic cells contained monosomy 7 or trisomy 8 were studied to determine whether the abnormal precursors produce mature cells in vivo. Using fluorescence in situ hybridization (FISH) of interphase nuclei with chromosome-specific centromere probes for either chromosome 7 or 8, we were able to determine which cells had one, two, or three signals indicative of one, two, or three no. 7 or 8 chromosomes. In five patients with trisomy 8, 80% to 100% (94.5% ± 6.2%) of the megakaryoblasts had three signals using a chromosome 8 probe; in one patient with monosomy 7, 96.5% of the megakaryoblasts had one signal using a chromosome 7 probe. In all six patients, the myeloid and lymphoid series did not have evidence of the chromosomal abnormality present in the blasts. In three of five patients with trisomy 8, three signals were observed in 27%, 33%, and 41% of normoblasts, respectively. These data are evidence that the abnormal cell in MDS is a progenitor cell with the potential of forming cells of megakaryocyte and erythroid lineages.

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EUKEMIA OCCURS more frequently in children with Down syndrome. The majority of cases are acute megakaryoblastic leukemia (AMKL). Most cases of AMKL in Down syndrome are preceded by a preleukemic phase of myelodysplasia (MDS) characterized by thrombocytopenia, the presence of large numbers of dysplastic megakaryocytes in the bone marrow, and less than 30% blasts in the bone marrow. Frequently, chromosomal abnormalities are found in AMKL and MDS of Down syndrome, the most common of which are trisomy 8 and monosomy 7.

MDS associated with trisomy 8 and monosomy 7 also occurs in adults and children who do not have Down syndrome. Recently, it has been reported that the blasts in these cases have the potential, in vivo, of producing myeloid cells and macrophages, but not lymphocyte precursors. There have been reports that erythroid precursors also have been formed from these leukemic cells. The blasts in MDS/AMKL of Down syndrome have, in vitro, properties of a multilineage precursor cell with the potential for forming megakaryocyte, erythroid, and basophil/mast cell progenitors. Myeloid cells are not produced.

To determine the in vivo lineage potential of the abnormal precursors in the MDS of Down syndrome, we have used the technique of fluorescence in situ hybridization (FISH), which allows the chromosomes of cells in interphase nuclei to be visualized. For example, blast cells with trisomy 8 will have three distinct signals per nucleus and any progeny should also have three signals per nucleus. This technique can use fixed archival blood and bone marrow smears. FISH analysis of these smears can be combined with standard morphology (Fig 1) to determine whether cells of a particular lineage had arisen from the leukemic cells.

In the present study, we have examined blood and bone marrow films of Down syndrome patients with MDS and trisomy 8 or monosomy 7, to determine whether, in vivo, the abnormal precursors form cells of myeloid, lymphoid, or erythroid lineages. Our findings provide direct evidence of the in vivo lineage potential of the progenitor cell in the MDS of Down syndrome.

MATERIALS AND METHODS

The clinical and hematologic features of these cases are outlined in Table 1. Cases no. 2 through 6 were associated with trisomy 8. Patient no. 1 had monosomy 7. Patients no. 1, 3, 4, and 6 developed acute megakaryoblastic leukemia. Patient no. 6 was not treated and died, and patient no. 1 died despite chemotherapy. Patients no. 3 and 4 are in remission on therapy. Patients no. 2 and 5 were treated while myelodysplastic and are in remission. Patient no. 6 was not treated and died. The patients were diagnosed as myelodysplasia on the basis of thrombocytopenia, abnormal megakaryocytes in bone marrow aspirates, and increased numbers of megakaryoblasts in bone marrow aspirates. In all cases, the percentage of blasts in the marrow was less than 30%, in keeping with the French-American-British (FAB) classification of myelodysplasia.

The blasts in all cases appeared similar on Wright-stained, air-dried smears, varying in size from 15 to 20 μm in diameter, with one to two nucleoli, a small amount of deeply basophilic cytoplasm, and usually distinct cytoplasmic blebbing.

Bone marrow biopsies in three of five cases studied indicated that megakaryocytes were increased in number in the marrow, but were small and abnormal in appearance with either small single nuclei or unusual nuclear structure. These findings are characteristic of the myelodysplasia of Down syndrome. Immunohistochemical staining of slides showed that these large cells stained positively for factor 8 and MB2 antigens, consistent with megakaryocytic lineage. Myelofibrosis was observed in three of five cases.

In case no. 1 marrow cells were studied by electron microscopy. The majority of blast cells were considered to be megakaryoblasts by the presence of the surface antigen CD61 (glycoprotein [Gp] IIIa) as determined by immunogold labeling. There was ultrastructural evidence of megakaryocyte differentiation, including demarcation.
cells contained megakaryocyte organelles or showed evidence of surface blebbing. However, when studied in liquid culture, the cells produced megakaryocyte precursors, as described elsewhere. These observations, together with the finding of trisomy 8 in these cells, suggested that these cells were primitive megakaryoblasts with no evidence of differentiation.

All studies of patients and control material were performed on stained peripheral blood or bone marrow films. Control bone marrows were obtained from the Hematology Laboratory of The Hospital for Sick Children and were from patients whose marrows were considered to be normal. Most of the marrow films in normals and patients were less than 3 months old, but in some patients, films older than 1 year were successfully used. Films to be studied were first examined by light microscopy. In some cases, photographs were taken of areas that were to be studied subsequently for evidence of interphase labeling. In this way, specific cells (e.g., leukemic cells, erythroblasts, etc.) could be identified by light microscopy and subsequently studied for evidence of interphase labeling.

**FISH.** The technique used was adapted from that described by Trask and Pinkel. Cover slips were removed by placing the slides in xylene for 30 minutes. Thereafter, the slides were fixed and decolorized in methanol/acetic acid (3:1) for 1 hour. Some slides were treated with RNase (100 μL RNase [100 μg/mL in 2× SSC] for 1 hour at 37°C). SSC contains 0.15 mol/L sodium citrate, pH 7. Other slides did not receive RNase treatment and there did not appear to be a significant difference in the results.

For those slides treated with RNase, the slides were subsequently rinsed extensively in 2× SSC, three times for 3 minutes each, and dehydrated in an ethanol series (80%, 90%, and 100%).

The DNA was denatured by immersion of the slides in 70% formamide–2× SSC, pH 7, at 70°C for 2.5 minutes. After denaturation, the slides were immediately and sequentially dehydrated in ethanol (70%, 80%, 90%, and 100% at 4°C) and then air-dried.

Chromosome 7– or chromosome 8–specific probes (D7Z1 and D8Z1, respectively) are alpha satellite DNA probes (Oncor, Gaithersburg, MD). The hybridization mixture contained biotin-labeled D8Z1 or D7Z1/hybrisol VI (1.15 μg/mL probe DNA). The probe mixtures were denatured in 72°C water bath for 5 minutes then quickly placed on ice. Thirteen microliters of the hybridization mixture were added to each slide and sealed with a glass cover slip rimmed with rubber cement. Then the slides were incubated in a moist chamber at 37°C for 14 to 16 hours. After hybridization, the cells were washed three times by placing them in Coplin jars containing 50% deionized formamide 2× SSC at 45°C. The slides were then washed in 2× SSC (pH 7) three times for 3 minutes each at 45°C. Signals were detected by fluorescein-labeled avidin (Oncor). As a counterstain, we used a mixture of propidium iodide/antifade.

The FISH specimens were viewed with an epifluorescence microscope (Zeiss Standard Research Microscope) using a single fluorescein isothiocyanate (FITC) filter emitting at 525 nm or a dual-band FITC/Texas red emitting at 525 nm and 615 nm. First, the areas photographed were found so that specific cells that had

![Image of FISH of interphase nuclei in a bone marrow film of a Down syndrome patient with MDS. (A) Wright's stain showing three large megakaryoblasts (b), a single neutrophil, two normoblasts (n), two lymphocytes (l), and two myeloid precursors (m). (B) The same area of the film, treated by the FISH technique with a chromosome 8 probe; three signals are seen in each of the megakaryoblasts and two signals in the other cells. The signals of one normoblast were out of the plane of focus and are not shown.](image)

Table 1. Clinical and Hematologic Features of Six Cases of Myelodysplasia in Down Syndrome

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (mo)/Sex</th>
<th>Hemoglobin (g/L)</th>
<th>Platelets (× 10^10/L)</th>
<th>Bone Marrow Blasts (%)</th>
<th>Cytogenetics</th>
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<tbody>
<tr>
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<td>43/F</td>
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<td>91</td>
<td>11</td>
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<td>39/F</td>
<td>113</td>
<td>113</td>
<td>24</td>
<td>48XX,+8,+21</td>
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<tr>
<td>3.</td>
<td>22/M</td>
<td>93</td>
<td>44</td>
<td>5</td>
<td>48XY,+8,+21</td>
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<tr>
<td>4.</td>
<td>8/F</td>
<td>122</td>
<td>53</td>
<td>20</td>
<td>48XX,+8,+21</td>
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<tr>
<td>5.</td>
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<td>25</td>
<td>48XY,+8,+21</td>
</tr>
<tr>
<td>6.</td>
<td>23/F</td>
<td>130</td>
<td>40</td>
<td>28</td>
<td>49XX,+8+14+21</td>
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</table>
been identified and photographed, using bright-field microscopy, could be reexamined for analysis of interphase FISH signals. Therefore, a random series of cells were directly analyzed by epifluorescence microscopy to determine the number of chromosome specific signals per nucleus. It was possible on such specimens to recognize blasts, myeloid elements, lymphocytes, and erythroid precursors. Hybridization signals were detected as green spots within the nucleus with the FlTC filter or, using the FITC/Texas red filter, the cells exhibited a red membrane fluorescence and the hybridization signals were detected as yellow spots. For each specimen, a series of nucleated cells were randomly selected using different random fields of view.

RESULTS

Controls

Eleven bone marrow films from six subjects were studied. Using the chromosome 7 probe, a mean of 91, 45, and 32 myeloid, erythroid, and lymphoid cells, respectively, were counted on these slides (Table 2). Myeloid cells include promyelocytes, myelocytes, metamyelocytes, and neutrophils. No signals were found in (mean ± SD) 1.6% ± 2.5%, 9.6% ± 10.3%, and 1.9% ± 4.6%; one signal was detected in 3.4% ± 2.9%, 7.8% ± 6.1%, 5.6% ± 4.6%; and two signals in 95% ± 4.2%, 82.5% ± 14.3%, and 92.5% ± 8% of myeloid, erythroid, and lymphoid cells, respectively.

Using the chromosome 8 probe, eight slides of five subjects were studied (Table 3 and Fig 2). A mean of 84, 65, and 48 myeloid, erythroid, and lymphoid cells, respectively, were counted on these slides. No signals were seen in (mean ± SD) 0%, 2% ± 3.9%, and 0% ± 2.7%; one signal in 1.6% ± 2.0%, 2.4% ± 2.1%, and 2.2% ± 2.7%; two signals in 98% ± 3.1%, 95.6% ± 3.4%, and 97% ± 2.9%; and three signals in 0.4% ± 0.5%, 0% and 0.8% ± 1.1% of myeloid, erythroid, and lymphoid cells, respectively.

Myelodysplasia

Blasts. In the one case of monosomy 7 (case no. 1), 54 of 56 cells counted had one signal and the other two had no signal. In four cases of trisomy 8 (Fig 1), 92% to 100% of the blasts showed three signals, with the remainder of the cells containing two signals. In one case (case no. 6), 80% of the blasts had three signals, 13% two, 2.6% one, and 3.9% none.

Myeloid cells. Eight slides of five patients with trisomy 8 were studied. A mean of 81 cells per slide were examined. In six slides, no cells with three signals were seen, and in two 0.67% (one of 149) and 1.42% (one of 70) cells had three signals. These are within the control range (1.4% ± 3.1%). In case no. 1, with monosomy 7, none of 68 cells had a single signal.

Erythroid cells. Eight slides of five patients with trisomy 8 were studied. A mean of 81 cells were studied per slide. In patient no. 2, no cells with three signals were seen; in patient no. 6, five of 135 cells had three signals. In patients no. 3, 4, and 5, 28%, 33%, and 41%, respectively, of the erythroid precursors had three signals (Fig 1). Cases 3, 4, and 5 are clearly higher than the control values, and even case no. 6 is slightly above the controls in which no normoblasts had three signals.
MYELODYSPLASIA IN DOWN SYNDROME

In case no. 1, no erythroid cells were seen, and in case no. 2, all 60 erythroid cells had two signals.

**Lymphocytes.** Eight slides of five patients with trisomy 8 were studied with the chromosome 8 probe. A mean of 42 cells were studied per slide. Three signals were observed in 1.33% ± 1.27% of cells, a value not significantly different from the control value (0.8% ± 1.1%).

Using the chromosome 7 probe, two of 76 cells (2.6%) in case no. 1 had one signal, a value within the control range.

**DISCUSSION**

Down syndrome AMKL and its preleukemic state of MDS are frequently associated with trisomy 8 or monosomy 7. These chromosomal abnormalities occur also in MDS in patients who do not have Down syndrome. There is evidence that these chromosomal disorders in Down syndrome patients differ from those occurring in normal children in that in the former there is a strong association with AMKL and the bone marrow is characterized by an abnormal and selective proliferation of dysplastic megakaryocytes. Also, the cure rate with chemotherapy is high in the Down syndrome patients compared with low rate in the other group.

The leukemic cells in the Down syndrome patients are considered to be megakaryoblasts because of their ultrastructural and antigenic features; however, these cells also have features of erythroid and basophil/mast cell precursors. In culture, the leukemic cells produce not only megakaryocyte precursors, but also basophils/mast cells. Also, the blasts contain erythroid antigens such as glycoporphin. Recent studies of the abnormal cells in non-Down syndrome MDS with trisomy 8 or monosomy 7 indicate that these disorders involve the myeloid and megocytic series and possibly an earlier stem cell. Thus, Gerritsen et al. reported a series of eight cases (five <15 years of age) with monosomy 7 and MDS. Using FISH analysis of interphase nuclei, they found monosomy 7 in myeloid and monocytic cells, but not in lymphoid cells. Parlier et al. described one case of refractory anemia with ringed sideroblasts and trisomy 8. They found trisomy 8 in myeloid, erythroid, and megakaryocyte precursors. Anastasi et al. studied three adults with refractory anemia associated with trisomy 8. They found evidence of trisomy 8 in monocytes, granulocytes, eosinophils, and basophils. No lymphocytes had evidence of trisomy 8 and erythroid precursors were not studied. Kibbel and L. made similar observations. Indeed, in all reported cases of MDS associated with trisomy 8 or monosomy 7, there is evidence that the leukemic cell has the potential of forming myeloid precursors.

In contrast to the above findings, our observations in patients with Down syndrome and MDS indicate that the myeloid cells were not of leukemic cell origin in that there was no evidence of trisomy 8 or monosomy 7 in the myeloid series. However, there was evidence that the erythroid series was derived from the leukemic line in three of five patients with trisomy 8, since the normoblasts had the same chromosomal abnormality as the leukemic cells. In no case was there evidence of involvement of the lymphoid series.

We conclude therefore that MDS in Down syndrome is a unique disorder characterized by the proliferation of a progenitor cell, that has the potential of forming cells of megakaryocyte and erythroid lineages.

**REFERENCES**


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