Cytogenetic Clonality in Myelodysplastic Syndromes Studied With Fluorescence In Situ Hybridization: Lineage, Response to Growth Factor Therapy, and Clone Expansion

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Clonality in myelodysplastic syndromes (MDS) has been studied with various techniques including glucose-6-phosphate dehydrogenase (G6PD) isoenzyme and cytogenetic analyses, and with molecular techniques such as gene deletion studies and the analysis of restriction fragment-length polymorphisms (RFLP) of X-linked genes. In this study, we investigated the use of fluorescence in situ hybridization (FISH) with a chromosome-specific probe to examine cytogenetic clonality in peripheral blood (PB) cells from three patients with MDS. In each case, trisomy 8 was shown by conventional cytogenetic analysis at the time of the initial diagnosis. By using FISH with a probe for the centromere of chromosome 8, we identified the trisomy in individual PB cells from Wright-stained smears. With this technique, we could determine the cell lineage involved by the trisomy, and through several therapies we could assess the response of the clonal and nonclonal cells to growth-factor therapy, and the expansion of the trisomic clone over time. In each of the three cases, various proportions of granulocytes, monocytes, eosinophils, and basophils showed trisomy 8 by FISH analysis. In none of the cases did we detect trisomy 8 in lymphocytes. By analysis of PB cells before and during therapy with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF), we found that GM-CSF stimulated both trisomic and disomic cells. During a 1-year period of sequential study, we detected an abrupt increase in the percentage of trisomic cells in one patient, a stable percentage in another, and a slowly increasing percentage in the third. The abrupt increase in the first patient preceded a transformation to a more acute phase by 2 months. We conclude that FISH analysis of PB cells of patients with MDS offers an additional approach to the study of clonality in this disorder. In some cases this analysis may provide a useful and simple means of assessing response to therapy and progression of disease.

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MYELODYSPLASTIC syndromes (MDS) are clonal disorders that result in ineffective hematopoiesis and peripheral blood cytopenias. 1,4 The nature of the hematopoietic stem cell affected has been the subject of debate. Some reports indicate that a multipotent stem cell capable of both myeloid and lymphoid differentiation is involved, 1,2,5,7 whereas other studies point to a more restricted progenitor as the origin of the clone. 8,9 Nearly two thirds of patients with MDS die as a result of bleeding or infection, whereas the remaining patients die after evolution to acute leukemia. 10,11 The development of recombinant colony-stimulating factors (CSF) has offered a new therapeutic approach to myelodysplastic patients with low peripheral blood (PB) counts, and a number of studies have demonstrated the effectiveness of treatment with such factors. 12,13 However, whether growth factors stimulate the nonclonal cells, clonal cells, or both, and whether they may act to prevent, prolong, or hasten the development of acute leukemia is questions that still need to be resolved.

In this study we have investigated the use of fluorescence in situ hybridization (FISH) analysis to identify trisomy 8 in individual PB cells of two patients who had refractory anemia with excess blasts (RAEB), and one patient who had refractory anemia with multilineage dysplasia (RAD); the disease in each patient was characterized by trisomy 8. The analysis has allowed us to study the lineage involved by the trisomy, the response of the clonal cells to growth factor therapy, and the apparent expansion of the trisomic clone over time. Our work shows that FISH offers a useful tool for both the study and clinical management of patients with MDS.

CASES

Case 1

This 64-year-old woman presented in April 1990, with a 3-month history of increasing fatigue. The finding of anemia prompted a bone marrow (BM) examination that showed changes consistent with RAEB. The patient was treated with transfusions, and then with a brief trial of fluoxymesterone and iron, to which she did not respond. She was referred to the University of Chicago in August 1990, for growth-factor therapy. Just before the start of treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF), the white blood cell (WBC) count was 1,500/µL with 165 neutrophils/µL; hemoglobin (Hb), 7.8 g/dL; and platelets, 579,000/µL. Cytogenetic analysis showed a normal cell line as well as a cell line characterized by a gain of chromosome 8 (47,XX+8[11 cells, 52%];46,XX[10 cells, 48%]). GM-CSF therapy was initiated at a dose of 180 µg/d (3 µg/kg), and 4 weeks later the WBC count had increased to 12,800/µL, with 10% neutrophils, 31% bands, 7% myelocytes, 4% metamyelocytes, 8% promyelocytes, and 6% blasts. After 3 months at the initial dose, she was placed on a maintenance regimen of 90 µg every other day, but adjustments were made over the next few months for maintenance of an optimal granulocyte response. In April 1991, GM-CSF therapy was discontinued because of an increased percentage of blasts (17%) in the blood. This finding did not regress after cessation of therapy. A BM specimen obtained 3 weeks after discontinuation of GM-CSF showed that the patient had evolved to RAEB in transformation (RAEB-T). The BM was 100% cellular and it showed 18% blasts and absent erythroid precursors. Because of persistent, severe, transfusion requirements, it was decided to initiate acute leukemia therapy with high-dose cytarabine. The patient became pancytopenic and died of BM failure 2 months later.
cytogenetic studies showed that 95% of the metaphase cells examined from a marrow specimen had trisomy 8; the remaining metaphase cells were normal (48,XY, +Y, +8 [19 cells, 95%]/46,XY [1 cell, 5%]). The patient received no therapy until January 1988, when a complete blood count (CBC) showed Hb 8.3 g/dL, mean corpuscular volume (MCV) 117 fl., WBC 2,700/μL, and platelets, 146,000/μL. A BM aspirate at that time showed 35% cellularity and multilineage dysplasia but, only 2% blasts. The patient was treated with transfusions until October 1988, when he was placed on 230 μg GM-CSF daily (3 μg/kg). This dose was reduced after 1 month because of marked neutropenia and eosinophilic leukocytosis and was reduced again in January 1989, when he was placed on a maintenance dose of 58 μg/d for the first 14 days of each 4-week period for 32 months. During this time, the patient became increasingly thrombocytopenic, but maintained a neutrophilic response to the GM-CSF. Just before his death of infection in July, 1991, the patient’s WBC was 4,100/μL with 59% neutrophils, approximately 1 week after discontinuation of the cytokine therapy.

Case 2

A 78-year-old man was discovered to have a macrocytic anemia in 1982. A BM specimen at that time showed megaloblastoid erythroid hyperplasia. Serum B12 and folic acid levels were normal. In 1986, cytogenetic studies showed that 95% of the metaphase cells examined from a marrow specimen had trisomy 8; the remaining metaphase cells were normal (48,XY, +Y, +8 [19 cells, 95%]/46,XY [1 cell, 5%]). The patient received no therapy until January 1988, when a complete blood count (CBC) showed Hb 8.3 g/dL, mean corpuscular volume (MCV) 117 fl., WBC 2,700/μL, and platelets, 146,000/μL. A BM aspirate at that time showed 35% cellularity and multilineage dysplasia but, only 2% blasts. The patient was treated with transfusions until October 1988, when he was placed on 230 μg GM-CSF daily (3 μg/kg). This dose was reduced after 1 month because of marked neutrophilia and eosinophilic leukocytosis and was reduced again in January 1989, when he was placed on a maintenance dose of 58 μg/d for the first 14 days of each 4-week period for 32 months. During this time, the patient became increasingly thrombocytopenic, but maintained a neutrophilic response to the GM-CSF. Just before his death of infection in July, 1991, the patient's WBC was 4,100/μL with 59% neutrophils, approximately 1 week after discontinuation of the cytokine therapy.

In Situ Hybridization/Detection

The Wright-stained smears were used for correlative and noncorrelative interphase cytogenetic studies. For the studies correlating cytology with the in situ hybridization, coverslips were applied to the Wright-stained blood smears with a dilute xylene-mounding medium mixture (Pro-Texx; American Scientific, McGaw Park, IL), and the slides were analyzed by routine light microscopy. Photomicrographs of 37 to 111 cells from each specimen, including neutrophils, monocytes, eosinophils, basophils, and lymphocytes, were taken. We used a graduated microscope stage to note the location of each cell photographed. After the photomicroscopy, the coverslips were carefully removed and the slides were washed in xylene for 10 minutes and air-dried. The slides were hybridized directly without any pretreatment. We used a biotin-labeled DNA probe directed to the centromere of chromosome 8 (kindly provided by Imagegenetics Inc, Naperville, IL). The hybridization procedure was similar to that reported previously. The hybridized probe was detected with fluorescein-labeled avidin (Vector Laboratories, Burlingame, CA). An anti-fade solution was used, but no counterstain was employed. After the hybridization and detection steps, we used the same microscope to locate the cells previously photographed.

Enumeration

Hybridization signals were scored by two observers (J.A., J.F.) and averaged. The percent trisomy of each cell type (granulocytes, monocytes, eosinophils, and lymphocytes) was determined. In addition to the photographed cells, we scored 400 to 600 cells at random. In some specimens we did not directly correlate the cytogenetic data with cell type, and in these instances we only scored 400 to 600 cells at random.

Conventional Cytogenetics

Cytogenetic analyses were performed with a trypsin-Giemsa banding technique on BM cells from aspirates or biopsy specimens. Cells from 24- or 48-hour unstimulated cultures were examined. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature.

RESULTS

Control Specimens

On average, three hybridization signals were detected in less than 1% of PB cells in the three control cases. Two signals were seen in 93% of cells, and a single signal was seen in 6%. Although we did not detect small populations of cells with trisomy in our patient specimens, we would have considered a cell population to have a trisomic component if 2% or more exhibited three signals. This is in agreement with a similar value determined in a larger series of control specimens in which the same probe was used.

Patient Specimens

Lineage. In each of the specimens from the three patients, we found cells with three hybridization signals in various percentages of the granulocytes, monocytes, eosinophils, and basophils (Fig 1, A and B and Fig 2, a through c). The percentages ranged from 11% to 89% of the individual cell type. Monocytes consistently showed a higher percentage of trisomy than did granulocytes and eosinophils, and this trend was observed in each patient at each sampling in which a
Correlative study was undertaken. Eosinophils consistently had a lower percentage of cells with trisomy; however, the lower number may have been caused by the difficulty in identifying all signals in these cells that exhibit considerable autofluorescence (Fig 1, C and D). It was difficult to study basophils because of their low number; however, in each case we were able to identify basophils with three signals. Lymphocytes did not show trisomy in any of the nine specimens studied with correlative techniques (patient 1, 4 specimens, total number of lymphocytes studied = 52; patient 2, 1 specimen, total number of lymphocytes studied = 27; patient 3, 4 specimens, total number of lymphocytes studied = 109). This was true even when trisomy 8 approached 100% in the nonlymphoid cells of patient 1 (see below).

Response to growth-factor therapy. Although we did not analyze a pretreatment specimen from patient 1 or 2, it is clear from the PB counts and a comparison with the subsequently analyzed specimens that the GM-CSF stimulated both the trisomic and disomic clones in these patients and in patient 3 (Fig 2, d through f). The effect of the therapy was most marked in patient 1 at the first sampling, in which the GM-CSF increased the absolute number of neutrophils, eosinophils, and monocytes more than 10-fold (see Fig 2d "pre" v "on" bars), and in which the stimulated cells ("on"-therapy) were a mixture of trisomic and disomic cells. The effect of GM-CSF on the disomic population diminished as the disease progressed in this patient. In patient 2, GM-CSF had a similar effect on neutrophils and eosinophils, as observed in patient 1, but there was little effect on monocytes (see Fig 2e). We could not identify an effect of GM-CSF therapy in any cell line except for the eosinophils in patient 3 (Fig 2f). This may have been caused by the fact that patient 3 exhibited only mild to moderate cytopenia while off treatment.

Clonal expansion. We used data from the correlative and noncorrelative analyses to study clonal expansion. Because trisomy was not detected in lymphocytes, we expressed the data as "percent trisomy in nonlymphoid cells." To derive this value, we used the differential count and the percent trisomy identified in individual cell types from the correlative analysis. As a check, we used the percent trisomy from the random counts of 400 to 600 cells and divided by the fraction of PB cells that were nonlymphoid. The two values agreed within 10% in 7 of 9 samples on which both analyses were performed and within 20% in the remaining 2 (Fig 2, g through i).

The percentage of nonlymphoid cells with trisomy 8 increased rapidly over a 5-month period in the first patient until nearly 100% of the circulating nonlymphoid cells were trisomic for chromosome 8 (Fig 2g). This increase occurred 2 months before the patient showed evidence of evolution of her disease to RAEB-T. This patient received chemotherapy because she appeared to be evolving toward acute leukemia and her clinical condition was deteriorating. She died of infection before her PB counts recovered and before her BM showed signs of regeneration. In patient 2, the percentage of nonlymphoid PB cells with trisomy 8 was constant over a 10-month follow-up time (Fig 2h). This occurred throughout a period of time when the patient remained stable with regard to blood counts and percentage of BM blasts. This patient died of infection despite a normal granulocyte count. The third patient exhibited a slow increase in the percentage of trisomic cells (Fig 2i), but she remains stable hematologically.
Fig 2. FISH analysis in three patients with MDS associated with trisomy 8: Lineage, response to growth factors, and clone expansion. In a, b, and c (cases 1, 2, and 3, respectively) the percentages of PB granulocytes, eosinophils, monocytes, and lymphocytes showing trisomy 8 are presented. Data are from the initial specimen studied with correlative FISH analysis for each patient. In d, e, and f (cases 1, 2, and 3, respectively) the absolute number of PB cells showing trisomy 8 in "pre" or "off" therapy specimens and in "on" therapy specimens are presented. Data are from the initial specimen studied with correlative FISH analysis in each patient. In cases 1 and 2, only the absolute number of cells is noted for the "pre" or "off" therapy specimens, as these were not analyzed by FISH. In g, h, and i (cases 1, 2, and 3, respectively) the percent of PB nonlymphoid cells showing trisomy 8 versus time is presented. Data are derived from correlative study (circles) or from random counts (triangles) (see Results). The mode of the GM-CSF therapy is noted by the solid bar (continuous treatment) and broken bar (cycled therapy). The percentages of BM metaphase cells showing trisomy 8 is also given, with the time of study noted with a small line on the abscissa.

**DISCUSSION**

Trisomy 8 is one of the most frequent chromosomal abnormalities reported in MDS. In this study of three patients with MDS associated with trisomy 8, we have used FISH on previously stained PB smears to identify trisomic cells and to correlate the trisomic genotype with the morphologic phenotype. This study has permitted us to determine the lineage of the trisomic cells, as well as to observe the response of the trisomic clone to growth-factor therapy. It has also allowed us to study the apparent expansion of the cytogenetically abnormal clone over a period of time.

**Lineage**

We have demonstrated trisomy 8 in PB neutrophils, monocytes, eosinophils, and basophils, but not in the lymphocytes of each of the three patients with MDS whom we studied. Our results are similar to those recently reported by Kibbelaar et al. who studied clonality of myeloid and lymphoid cell fractions from patients with MDS by a combined immunophenotyping and DNA in situ hybridization technique. In that study, none of the cells bearing T- or B-cell antigens from any of the five patients reported on demonstrated trisomy for the probes used, whereas cells bearing antigens for myeloid cells, monocytes, and progenitor cells did show trisomy. Such findings of cytogenetic clonality restricted to the granulocytic and monocytic cells are not surprising, given the common development of acute myeloid leukemia, the absence of lymphoid leukemias, and the only rare occurrence of biphenotypic leukemias in patients with MDS. However, one cannot necessarily use the findings to conclude that the origin of the neoplastic clone in MDS is a myeloid-restricted progenitor. If MDS results from a multi-step process in which cytogenetic change occurs late, the finding, by cytogenetics-related techniques, of trisomy restricted to nonlymphoid cells may simply be an indication that the acquisition of a cytogenetic abnormality, such as +8 or abnormalities of chromosome 5 or 7, occurs only in an unstable myeloid-derived subclone. A number of molecular studies have suggested that lymphoid cells are clonal in MDS; however, if so, they apparently have a lesser ten-
Clonal Expansion

The ability to follow the cytogenetic clone by FISH analysis offers a simple means of gauging the response of the clone to growth-factor therapy in a patient with MDS associated with a known numerical chromosomal aberration, but this approach may also offer a means of assessing the progression of the disease. The rapid increase in the percentage of trisomic PB cells in patient 1 preceded both the left shift in granulocytes in the PB and BM and the eventual increase in circulating and marrow blasts. It seems that the increasing percentage of trisomic cells was an indication of clonal expansion. This conclusion is supported by the concurrent increase in the number of dividing BM cells with trisomy 8, as detected by conventional cytogenetic analysis (see Fig 2g). Whether the increased percentage of trisomic cells detected by FISH analysis was due to a growth or proliferative advantage of the trisomic clone, or due to its facilitated release into the PB; and whether these were caused by factors such as oncogene activation or karyotypic evolution, cannot be determined by this method of analysis. However, FISH analysis allowed the monitoring of a dramatic change in the PB that otherwise would have gone unnoticed. Determining whether there is a relationship between the kinetics of the cytogenetically abnormal clone detected in the PB and the risk of transformation to acute leukemia will require an evaluation of a larger series of patients. Further studies will also be needed for determining whether similar FISH analyses may be useful in monitoring of the effect of growth-factor therapy administered in conjunction with chemotherapy. Clearly, this rapid and easy to use technique offers the unique ability to follow both clone size and maturation. It is difficult, if not impossible, to study these using conventional cytogenetic techniques, in which technical factors, such as differential growth in cell culture and the analysis of a relatively small number of dividing cells, limit the conclusions that can be drawn.

Response to GM-CSF

Most studies on the response of patients with MDS to growth-factor therapy have demonstrated that the growth factor augments the production of neutrophils without increasing the likelihood for transformation to acute leukemia. In a single case reported previously, growth factor was believed to result in a complete remission by preferentially stimulating the nonclonal population and by suppressing the clonal cells. However, in few studies have clonal analyses been undertaken to elucidate the clonal response to therapy. In a study by Ganser et al, restriction fragment length polymorphism (RFLP) analysis was used to assess the neoplastic clone during cytokine therapy in patients with clonal hematopoietic disorders. Although there were different responses among the four patients examined, the investigators concluded that GM-CSF could stimulate both normal and abnormal hematopoiesis in MDS. Given the limitations of using trisomy 8 as a marker for the malignant clone as discussed above, we would draw a similar conclusion from the cases reported here. We saw no preferential stimulation of trisomic or disomic cells in any of the three patients analyzed in this study, and we clearly observed no elimination of the abnormal clone.

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NOTE ADDED IN PROOF

Patient 3 died 4 months after the discontinuation of growth factor therapy. She had no evidence of acute leukemia.

REFERENCES


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