CONCISE REPORT

Nonclonal Hemopoietic Progenitor Cells Detected in Long-Term Marrow Cultures From a Turner Syndrome Mosaic With Chronic Myeloid Leukemia

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We have investigated the clonality of Ph[1]-negative hemopoietic progenitor cells appearing in long-term marrow cultures established with cells from a mosaic Turner syndrome patient (46,XX/45,X) with Ph[1]-positive chronic myeloid leukemia (CML). The Ph[1]-positive clone had been shown previously to have arisen from a cell of the 45,X lineage. At the time of the present study, the patient was five years postdiagnosis and had been off chemotherapy for two months following a year of treatment for lymphoid blast crisis. All analyzed unstimulated marrow metaphases and each of 23 individually analyzed erythroid and granulocyte colonies produced in assays of the starting marrow were 45,X,Ph[1]. Pooled granulocyte colonies from the same assays yielded four metaphases that were 45,X,Ph[1] and one that was 46,XX. Very few hemopoietic progenitors were detected in long-term cultures at any time; however, all of four individually analyzed large granulocyte colonies and a pooled granulocyte colony preparation obtained from assays of 4- to 6-week-old adherent layers yielded exclusively 46,XX metaphases. These results provide evidence that nonclonal progenitors can persist in patients with CML, even after the onset and treatment of blast crisis, and that the long-term marrow culture system provides a sensitive method for detecting such cells.

PHILADELPHIA (Ph[1])-positive chronic myeloid leukemia (CML) is a clonal neoplasm that appears to arise in a hemopoietic stem cell with both lymphoid and myeloid differentiative potential.[1,2] By the time of diagnosis, all dividing cells in the marrow are usually Ph[1]-positive and conventional therapy does not alter this.[3,4] Recent studies have shown, however, that Ph[1]-negative hemopoietic progenitors are also commonly present.[5-8] In most cases, these are diluted to undetectable levels by Ph[1]-positive progenitors from which they cannot be distinguished phenotypically.[9] The detection of Ph[1]-negative progenitors thus requires methods for reducing the proportion of Ph[1]-positive cells. This effect has been obtained clinically with intensive chemotherapy[5] or treatment with α-interferon.[6] Recently, we have shown that a similar result can be achieved in vitro with cells obtained from either untreated or conventionally treated patients. For reasons not yet clear, Ph[1]-positive cells are usually poorly maintained in long-term cultures initiated with CML marrow. As a result, Ph[1]-negative progenitors that are relatively well maintained under the same conditions become readily detectable within three to four weeks if sufficient numbers were present in the original inoculum.[7,8]

An important question is whether these Ph[1]-negative progenitors represent residual normal (nonclonal) hemopoietic cells, or whether they may belong to the neoplastic clone at a stage in its evolution that antedates the acquisition of the Ph[1] chromosome. Evidence both for[10,11] and against[12-14] such a multistage development of Ph[1]-positive malignancy has been described.

The availability of a mosaic Turner syndrome patient whose CML clone arose in a cell of the 45,X lineage allowed us to pursue the question of the clonality of Ph[1]-negative progenitors appearing in long-term CML marrow cultures. This report presents the results of that experiment.

MATERIALS AND METHODS

Case History

The patient studied was a 52-year-old female, known to have mosaic Turner syndrome (45,X/46,XX) and first diagnosed as having Ph[1]-positive CML in January 1979. Details of her history and treatment until January 1981 have been published previously.[14] Briefly, she presented with bruising, back pain, splenomegaly and hepatomegaly, a WBC count of 220,000 and 100% (11 of 11) Ph[1]-positive marrow metaphases. The Ph[1]-positive clone was found to have arisen in a cell of the minor 45,X lineage. Between January 1979 and January 1981 she was treated first with hydroxyurea and 6-mercaptopurine and then with the L-15 protocol.[5] On two occasions during this period, the proportion of Ph[1]-positive metaphases in her marrow was transiently reduced to 3% or less. Regeneration of hemopoiesis from persisting nonclonal progenitors was inferred from the demonstration of 46,XX and 45,X metaphases in the same 9:1 ratio characteristic of her phytohemagglutinin (PHA)-stimulated lymphocytes and skin fibroblasts. By November 1980, her marrow had reverted to 100% Ph[1]-positive metaphases. Subsequently, her disease was controlled with hydroxyurea or 6-mercaptopurine. In November 1982, she was diagnosed as having lymphoid blast crisis

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and was treated with a modification of the L-10 protocol.[15] This induced a rapid restoration of the chronic phase of her illness. The marrow used in the present study was obtained in January 1984, at which time her WBC count was stable at 50,000. She had not received any chemotherapy for two months and her marrow showed no evidence of blastic transformation. However, in March 1984, she presented with severe bone pain, a WBC count of 30,000 including 31% lymphocytes and 47% blasts, and a marrow consistent with a relapse of her lymphoid blast population.

Cultures

Bone marrow aspirate cells were collected in heparin in New York and kept on ice until receipt in Vancouver. Within 18 hours of aspiration, buffy coat cells were used to initiate long-term cultures as previously described[16] and an aliquot was plated in methylcellulose cultures[16] for assessment of erythroid and granulopoietic colony-forming cell numbers and karyotype[17] in the starting marrow sample. Long-term cultures were maintained by weekly removal of half of the nonadherent cells and replacement of half of the growth medium. After four and six weeks, long-term culture-adherent layers were suspended using collagenase,[16] and the cells were plated in methylcellulose for assessment of the number and types of progenitors present.

Cytogenetic Studies

A direct metaphase preparation[18] was obtained from part of the same marrow sample used to initiate long-term cultures and initial methylcellulose assays. Cytogenetic analysis of hemopoietic colonies generated in methylcellulose assays was performed as previously described using a minimum criterion of two analyzable metaphases for individually analyzed colonies.[17] Karyotypes were established after Giemsa-banding.

RESULTS

Cytogenetic analysis of unstimulated direct marrow preparations showed only cells of the 45,X,Ph^1 clone to be proliferating (Table 1). Methylcellulose assays revealed the presence of 62 colony-forming units (CFU)-Es, 14 burst-forming units (BFU)-Es, and 0.5 CFU-Cs per 10^5 marrow buffy coat cells. CFU-E and BFU-E values were within normal limits.[19,20] The CFU-C value was significantly reduced ($P < .05$). Twenty-three of these BFU-Es and CFU-Cs were individually karyotyped. All proved to be 45,X,Ph^1. Two pools of granulocyte colonies from the same methylcellulose cultures yielded five metaphases, four of which were 45,X,Ph^1 but one of which was 46,XX (Table 1).

Table 1. Cytogenetic Data From Analyses of Marrow Metaphases and Marrow Progenitors Detected Initially and After Four and Six Weeks in Long-Term Culture

	Initial Marrow				Progenitors From Long-Term Cultures*	
Karyotype	Unstimulated Metaphases	BFU-E†	CFU-C‡	Pooled CFU-C§	Four Weeks	Six Weeks
45, X, Ph^1	11	16	7	4	0	0
46, XX	0	0	0	1	2‖	3**
45, X	0	0	0	0	0	0
Total	11	16	7	5	2	3

*Progenitors of large granulocyte colonies were analyzed individually. No erythroid colonies were obtained.

†Progenitors of very large erythroid colonies (containing more than eight clusters of erythroblasts).

‡Progenitors of large granulocyte colonies suitable for individual analysis.

§Two pools of several granulocyte colonies each.

‖One individual large granulocyte colony and one metaphase from a pool of several smaller granulocyte colonies.

**Three individual large granulocyte colonies.

The number of progenitors detected in both nonadherent and adherent fractions of the long-term marrow cultures was consistently low. Progenitors of large erythroid colonies (BFU-Es) were not detected and only an occasional small granulocyte colony was observed in weekly assays of the nonadherent fraction. Therefore, cytogenetic data were obtained only for CFU-Cs present in the adherent layer. One large granulocyte colony from the week 4 assay and three such colonies from the week 6 assay yielded two to three analyzable metaphases each. A pool of smaller granulocyte colonies from the week 6 assay also yielded one metaphase. All were 46,XX (Table 1).

At week 6, the total number of primitive CFU-Cs (ie, those yielding large granulocyte colonies) per long-term culture dish was approximately ten. The number of such progenitors in the aliquot of marrow used to initiate each culture was calculated to be approximately 125 (with an estimated upper limit of 250). Since all seven large granulocyte colonies analyzed in initial marrow assays were 45,X,Ph^1, but one metaphase in a pooled granulocyte colony preparation was 46,XX (Table 1), it is possible that 13% of the primitive CFU-Cs initially added (ie, 16 with an upper limit of 33) were 46,XX. Thus, ten 46,XX primitive CFU-Cs per culture present after six weeks, although a relatively low number, is equivalent to at least 30% of the corresponding progenitor input value. This is consistent with the kinetics typical of CFU-Cs in normal human long-term marrow cultures.[16]

DISCUSSION

Previous studies have shown that in most cases, Ph^1-positive hemopoiesis is not maintained in long-term cultures initiated with marrow cells from patients with Ph^1-positive CML.[7,8] It is likely that this is due at least in part to a difference in the properties of Ph^1-positive progenitors, rather than a qualitative change in the environment established in the culture, since co-existing Ph^1-negative progenitors, when present in sufficient numbers in the original marrow sample, are relatively well maintained in the same cultures. In fact, the differential behavior of Ph^1-positive and Ph^1-negative progenitors in long-term culture makes this system a very sensitive method for revealing the

presence of Ph[1]-negative progenitors in patients in whom they cannot otherwise be detected. This was shown to apply to some CML patients with established treated disease, as well as to patients studied at diagnosis.

The present studies extend this observation to a patient studied five years postdiagnosis, having previously undergone two major chemotherapeutic regimens, one that transiently reestablished a partial Ph[1]-negative remission in the first year after diagnosis,[14] and the second that induced a one-year Ph[1]-positive "remission" after the emergence of a Ph[1]-positive, terminal deoxynucleotidyl transferase positive lymphoid blastic transformation.

All granulocyte colonies produced in assays of 4- to 6-week-old adherent layers that were analyzed cytogenetically were found to have been derived from cells belonging to the originally predominant 46,XX lineage and were therefore unlikely to be related to the 45,X,Ph[1] clone. This finding is significant because it provides evidence that nonclonal hemopoietic stem cells were still present in the marrow of this patient at a relatively late stage in her disease.

The present study further demonstrates that, at least in this instance, conditions established in long-term cultures did not select for a Ph[1]-negative clonal population. Since initial studies indicated that the 45,X lineage in this individual comprised only 10% of her total constitution,[14] a predominance of 45,X hemopoietic progenitors would have provided strong evidence for the existence of a Ph[1]-negative step in the evolution of Ph[1]-positive CML. Failure to demonstrate such a population indicates either that a significant number of such cells did not exist in this patient's marrow at the time of study or that the long-term culture system does not favor their persistence. Experiments with additional mosaic CML patients should help to resolve this question. Nevertheless, the present studies suggest that long-term marrow culture can be used to select for residual nonclonal Ph[1]-negative hemopoietic progenitors and may be useful for future development of appropriate methodology for reactivating such cells in patients with CML.

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