Differences Among Myeloproliferative Disorders in the Behavior of Their Restricted Progenitor Cells in Culture

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We have studied the behavior in culture of circulating restricted hemopoietic progenitor cells from patients with idiopathic myelofibrosis (IMF), polycythemia vera (PV), and essential thrombocytopenia (ET). We have found differences in circulating granulocyte-macrophage, erythroid, and megakaryocytic progenitors that appear to be specific for these chronic myeloproliferative disorders. In IMF, most affected were granulocyte-macrophage progenitor cells (CFU-C), which circulated in increased numbers and were heterogeneous in their sensitivity to the regulatory factor(s) present in phytohemagglutinin (PHA) stimulated T-lymphocyte conditioned medium (CM). Most CFU-C were either highly sensitive to, or independent from, stimulatory factors, while others showed normal sensitivity. In some IMF patients, circulating megakaryocytic progenitors (CFU-M) were present that were capable of giving rise to colonies in the absence of added CM or erythropoietin (EPO). In PV, we confirmed the presence of circulating erythroid progenitor cells that give rise to colonies in culture without the addition of EPO. The number of circulating CFU-C was normal and they responded normally to CM. In ET, failure to detect 7-day circulating restricted progenitor cells was a common observation: the level of other circulating restricted progenitors was in the low normal range. Thus, despite certain common features, including a primary lesion at the level of the pluripotential hemopoietic stem cell, the myeloproliferative disorders differ with respect to the behavior in culture of their circulating restricted progenitor cells. These results have led us to postulate a second regulatory lesion in the pluripotential stem cell that differs in these disorders and is expressed at the level of the respective restricted progenitor cells.

THE TERM “myeloproliferative disorders” was introduced by Dameshek in 1951, with the intention of stressing an underlying unity among several apparently different hematologic diseases, including chronic granulocytic leukemia (CGL), polycythemia vera (PV), idiopathic myelofibrosis (IMF), essential thrombocytopenia (ET), megakaryocytic leukemia (Meg L), and erythroleukemia (EL). The concept was based on morphological and clinical evidence: the frequency with which involvement of more than one myeloid lineage occurs in each of these disorders, the frequency of transition from one disorder to another, and the occurrence of forms that appear to fall between the more clear-cut diagnostic entities. It was later established by isoenzyme studies that CGL, PV, IMF, and ET are all clonal diseases involving the pluripotential hemopoietic stem cell. This finding provided a cellular basis for the unity first conceived of by Dameshek.

In each of the myeloproliferative disorders, however, a different lineage appears to be preferentially involved. In 1971, Ward and Block, on the basis of

MATERIALS AND METHODS

Clinical Criteria Used for Patient Selection

Idiopathic myelofibrosis (IMF). All 8 patients with this diagnosis had a leuko-erythroblastic anemia, anisopoikilocytosis, hepatosplenomegaly, and bone marrow fibrosis proved by biopsy, with no evidence of an underlying disease. The patients had received no chemotherapy. Two patients had had splenectomy (I, VI); one (I) of them was studied both prior to and after operation. One patient (XII) received periodic blood transfusions.

Essential thrombocytopenia (ET). Six patients were studied; all had platelet counts of 1,000 × 10^9/liter or greater at the time of diagnosis. Splenomegaly was not present in any of the patients and all showed megakaryocytic bone marrow hyperplasia. In 3 patients, fibrotic changes in marrow were present. All 6 patients had been treated with busulfan, 1 yr or more prior to the study.

Polycythemia vera (PV). Each of the 4 patients in this group met the diagnostic criteria of the Polycythemia Vera Study Group and had been treated by phlebotomy alone; none had received myelosuppressive therapy.
The clinical data on the patients in these three groups are given in Table 1.

**Culture Conditions**

Mononuclear cells were separated from 30–40 ml of heparinized peripheral blood by 30-min centrifugation at 400 g in Ficoll-Hypaque (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The cells from the interface were resuspended in HMEM (Eagle’s minimal essential medium with Hanks’ balance salt solution, GIBCO Laboratories, Grand Island, NY), supplemented with 2% heat-inactivated fetal calf serum (FCS, GIBCO Laboratories), and centrifuged for 10 min at 400 g. The cells were resuspended in a small volume of the same medium, enumerated, and then diluted with HMEM to a final concentration of 6 x 10^6 cells/ml for plating in plasma culture.

The plasma culture system used for the study of 14-day erythroid, granulocyte-macrophage, and megakaryocytic colonies was modified from that described by McLeod et al. Briefly, 6 x 10^5 nucleated cells were plated in culture medium so that each milliliter contained a final concentration of 20% FCS, 10% of a solution of CaCl_2 containing 280 mg/liter in medium NCTC 109 (M.A. Bioproducts), 10% of a 10% BSA solution (bovine plasma citrated, GIBCO), and either 10% of EPO (4 U/ml; Erythropoietin, Step III, Connaught Labs), or 10% CM (PHA-stimulated human T-lymphocyte conditioned medium generously donated by the late Dr. A. M. Wu) or 10% of each. The final volume was brought to 1 ml with medium NCTC 109.

The cells were plated in wells (Flow Laboratories) 6 mm in diameter in a final concentration of 3 x 10^5 cells/0.5 ml plasma culture. The plates were incubated for a period of 11–14 days at 37°C in 5% CO_2 in air at 100% humidity. After harvesting and fixing, the clots were stained and examined by light microscopy for granulocytic, erythrocytic, and megakaryocytic colonies.

Mononuclear cells from the interface were assayed for 7-day erythrocytic progenitors in modified plasma cultures in microwells (Flow Laboratories), as described previously. The nucleated cells were plated at 5 x 10^4 cells in 0.1 ml plasma culture medium with EPO at 0.2 U/0.1 ml.

**RESULTS**

**Circulating Granulocyte-Macrophage (GM) Progenitor Cell (CFU-C) Levels and Their Responses to Regulatory Factors in Culture**

In the absence of CM, few or no GM colonies were produced from peripheral blood cells of 5 normal individuals (Fig. 1A). In the presence of CM, the number of GM colonies ranged from 5 to 16/3 x 10^5 nucleated cells. From 6/7 IMF patients (Fig. 1B), GM colonies were generated in culture without the addition of CM. Upon the addition of CM, there was substantial increase in the number of GM colonies in most cultures. These results show that the numbers of circulating CFU-C highly sensitive to (or independent from) the regulatory factors in CM are greater in IMF patients than in normal.

The number of GM colonies produced in culture by peripheral blood cells of 6 ET patients was at the low end of the normal range (Fig. 1C), and colonies were generated only in the presence of CM.

In patients with PV (without extensive myelofibrosis), there was no obvious difference from normal in the frequency of GM colonies in the absence or presence of added CM (Fig. 1D).

**Circulating 7- and 14-Day Erythroid Progenitor Cell Levels and Their Responses to Regulatory Factors in Culture**

As shown in Table 2, when nucleated blood cells from normal individuals were plated in plasma culture with no EPO or CM added, no 7- or 14-day erythroid colonies were produced. With EPO added to the cultures, the number of 7-day colonies ranged from 0.1 to 8.6/5 x 10^4 cells (Table 2) and the number of 14-day
colonies, from 8 to 41.3 /3 \times 10^3 cells (Table 2). In patients with IMF, the distribution of numbers of circulating erythroid progenitors appeared more heterogeneous than normal. Half of the patients had no detectable circulating 7- or 14-day progenitors; the remainder had 7- and/or 14-day erythroid progenitors, which were detected only in the presence of EPO (Table 3).

The ET patients had no detectable circulating 7-day erythroid progenitors in the presence or absence of EPO; this finding is significant (0.01 > p > 0.001) as compared to normals (compare Table 4 with Table 2). Fourteen-day progenitors were seen in small numbers and in the presence of EPO only.

As shown in Table 5, 7- and 14-day erythroid colony formation by circulating progenitors of PV patients...
Nucleated cells ($5 \times 10^5$) were plated in 0.1 ml plasma culture, and the erythroid colonies were scored at day 7; 3 $\times$ $10^5$ nucleated cells were plated in 0.5 ml plasma culture, and the erythroid colonies were scored at day 14. Each value represents the mean of 4 samples. (ND, not done.)
differed from those of normal individuals and from those of IMF and ET patients in the following ways: (1) erythroid colonies grew in the absence of added EPO; and (2) in all but one instance, the number of erythroid colonies was increased 2–15 times in the presence of EPO.

**Circulating Megakaryocytic Progenitor Cell (CFU-M) Levels and Their Responses to Regulatory Factors in Culture**

We were unable to detect circulating CFU-M in the peripheral blood from either normal individuals or ET patients, and the cells of 1 patient with PV produced only low numbers of megakaryocytic colonies (3/3 x 10⁶ nucleated cells). In contrast, 4/7 patients with IMF had megakaryocytic progenitors present in peripheral blood. As shown in Fig. 2 (left panel), in these cultures, a variable number of megakaryocytic colonies could be generated in the absence of any added regulatory factor (CM or EPO). The addition of CM substantially increased the number of colonies obtained in 4 of the patients (I, V, XII, XIII), as demonstrated in Fig. 2 (right panel). In 2 patients, the number of megakaryocytic colonies produced in the absence of CM was too high to be counted accurately.

**DISCUSSION**

The results reported here indicate that the members of the myeloproliferative syndrome—IMF, PV, and ET—differ from one another with respect to their circulating restricted progenitor cells, despite the fact that each of these diseases apparently arises from the selective amplification of a single abnormal pluripotential stem cell.²⁻⁵

Chervenick¹¹ and others¹²⁻¹⁵ have reported increased numbers of circulating CFU-C in patients with IMF. We have confirmed this observation and now report, in addition, that, in IMF, the circulating CFU-C population is heterogeneous with respect to their response to the colony-stimulating activity (CSA) present in CM. Thus, most were apparently independent of CSA, i.e., they did not require addition of the factor to support their proliferation and differentiation; the rest of the CFU-C population showed a normal sensitivity to CSA, as suggested by the increase in the total number of GM colonies produced when CM was added to the cultures. Further work will be necessary to resolve the question as to whether the population that exhibited apparent independence from CSA is composed of progenitor cells that (1) have already been influenced by a stimulating factor in vivo prior to culture, or (2) are influenced in culture by microenvironmental cells producing the factor, or (3) are hypersensitive to minute amounts of CSA present in the culture medium, e.g., in fetal calf serum, or (4) are truly independent of CSA.

It is of interest that Klein et al.¹⁶ recently reported that in the spleens of mice infected with the myeloproliferative sarcoma virus, CFU-C exist that are capable of producing GM colonies in the absence of added CSA. They postulate the existence, in this disease, of one CFU-C subpopulation that is CSA independent and another that requires normal concentration of CSA for colony formation.

Our results confirmed that the PV patients with normal WBC count have a normal frequency of circulating CFU-C.

The number of circulating CFU-C in ET patients was within normal range, and CFU-C colonies could be generated only in the presence of CM.

Megakaryocytic progenitors were found in the peripheral blood of patients with IMF, but these could not be detected in the blood of patients with PV or ET or of normal individuals. In some of these IMF
patients, megakaryocyte progenitors were present in very high numbers and were able to give rise to megakaryocyte colonies in culture without the addition of CM or EPO. High numbers of CFU-M have also been found in patients with chronic granulocytic leukemia; however, it is not clear whether these cells showed a normal sensitivity to regulatory factors.

It is well known that narrow megakaryocytosis with morphological abnormalities in megakaryocytes is a constant finding in IMF. Recently, it was suggested that the intramedullary death of megakaryocytes and subsequent deposits of their components in intercellular spaces is a necessary condition for the fibroblast proliferation and secretion of collagen characteristic of IMF. It is possible that the defective megakaryocytes arise from the abnormal clone. The fact that we observed changes in the circulating CFU-M population would support this hypothesis.

Although circulating CFU-M were present in most IMF patients studied, there was a large variation in their number from one case to another. It may be that these variations reflect different stages of the disease, since the duration of the disease varied in the patients investigated. It is interesting to note that in one patient, we were able to perform cultures on 3 different occasions; we found an increase in the number of circulating CFU-M as the disease progressed and the patient deteriorated. At the terminal stage of his disease, the cultures were overgrown by megakaryocytes and we were unable to count the megakaryocytic colonies accurately. It is possible that this patient developed a terminal megakaryocytic leukemia.

Most of our IMF patients had no detectable circulating erythroid progenitors, and in the remainder, the number of such progenitors was normal. In contrast to the data of Lutton and Levere, the peripheral blood cells of our IMF patients produced colonies in culture only in the presence of normal concentrations of EPO. We have confirmed the appearance of subpopulations of erythroid progenitors able to produce erythroid colonies in the absence of EPO in PV only. The failure to detect circulating 7-day erythroid progenitors in ET patients may be significant. The possibility exists that this may represent the expression of regulatory defects specific to this condition. Further work will be necessary to throw light on this finding.

This study shows that patients with IMF, PV, and ET exhibit differences in the behavior of their circulating restricted progenitors. It will be interesting to determine the number and sensitivities to regulatory factors of the various restricted progenitors in the bone marrow of these patients. Such experiments are presently under way in our laboratory.

These observations have led us to postulate a second regulatory lesion at the level of the stem cell, which is different in these diseases and which is expressed at the level of circulating restricted progenitors. Thus, the essential requirement for these diseases would be a primary regulatory defect in the pluripotential stem cell, which confers on these cells a selective growth advantage, permitting emergence of the pathologic clone. A second lesion within this clone of pluripotential stem cells, differing in each of the disorders, would then lead to the emergence of the phenotypically abnormal population of circulating CFU-C and CFU-M in IMF patients, the abnormal population of circulating early and late erythroid progenitors in PV patients, and perhaps the lack of circulating late erythroid progenitors in ET patients. This model seems to fit well with the concept of multistep neoplastic development, recently proposed by Fialkow et al. It might also help to clarify the unity-within-diversity characteristic of the chronic myeloproliferative disorders.

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