Analysis of Myeloproliferative Disorders Using Cell Markers in Culture

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A. Introduction

We have used cell markers to study aspects of marrow regulation in selected patients with hematological neoplasms. While this report focuses on studies of normal individuals and of patients with polycythemia vera, the principles are widely applicable to any one of a number of disorders.

The analysis takes advantage of naturally-occurring cellular mosaicism in selected females. The marker used is that of the X chromosome-linked isoenzymes of glucose-6-phosphate dehydrogenase (G-6-PD) and has been detailed by Fialkow ([3] and see chapter in this text).

These studies have demonstrated that the peripheral blood elements in G-6-PD heterozygotes with chronic myelogenous leukemia [5], polycythemia vera [1], and agnogenic myeloid metaplasia with myelofibrosis [7] contain only a single isoenzyme type while extracts of skin fibroblasts demonstrate two isoenzyme types. This has led to the conclusion that these disorders arise at the level of a pluripotent stem cell, and, at least at the time of analysis, the peripheral blood elements probably represent the products of a single clone.

In an extension of these studies, we have cultured bone marrow cells from such patients to determine if progenitors could be detected which did not bear the isoenzyme type found in peripheral blood cells. Because such precursors must exist in the marrow in small numbers, we took advantage of the fact that these cells have marked proliferative potential when cultured under appropriate conditions, and we have employed methods for the growth of both granulocytic and erythroid colonies. Such an analysis, therefore, might detect the products of normal stem cells and, if so, provide an estimate of the ratio of abnormal to normal progenitors in the marrow and give insight concerning mechanisms of normal hematopoietic regulation.

B. Methods and Materials

I. Subjects

Hematologically normal G-6-PD heterozygotes were identified and served as controls. Heterozygosity was established by isoenzyme analysis of peripheral blood cells.

Two G-6-PD heterozygotes with polycythemia vera were studied. These patients, at the time of diagnosis, had pancytosis with splenomegaly. At the time of analysis, peripheral blood red cells, granulocytes, and platelets ex-

hibited only isoenzyme type A on electrophoresis [1]. Direct analysis of full thickness skin biopsies, in contrast, demonstrated approximately equal amounts of isoenzyme types A and B. Heterozygosity was repeatedly confirmed by analysis of cultured skin fibroblasts from both patients.

II. Marrow culture

To determine whether products of presumably normal stem cells were present in the marrow of the patients with polycythemia vera, we cultured cells from both individuals and exposed them to increasing concentrations of erythropoietin (ESF). Similar studies were carried out on marrow cells of the normal controls. Bone marrow cells were aspirated in a routine fashion into heparinized tissue culture medium and cultures established as previously published [10, 11]. Between days 6 and 12, colonies containing hemoglobinized cells appeared in the methylcellulose. These colonies were plucked from the medium using fine capillaries and placed on cellulose acetate strips soaked in buffer and subjected to electrophoresis [11]. In later studies, colonies derived from different classes of erythroid progenitors (CFU-E and BFU-E) were analyzed independently.

C. Results

I. Normal subjects

Erythroid colonies harvested from the normal marrow cultures exhibited either A or B type isoenzyme but rarely both [11]. The incidence of colonies containing a double isoenzyme phenotype was no greater than would be predicted on the basis of two spatially related progenitors. As shown in Table 1, the ratio of colonies containing one isoenzyme type versus the other reflected the ratio of isoenzyme types determined by direct analysis of peripheral blood elements. This was true irrespective of the concentration of ESF used and whether CFU-E- or BFU-E-derived colonies were analyzed. Recently, it has also been shown that CFU-C-derived colonies, as well, reflect the isoenzyme ratio observed in circulating granulocytes [12].

Table 1. The ratio of A and B isoenzymes of G-6-PD in peripheral blood cells and marrow-derived erythroid colonies from hematologically normal G-6-PD heterozygotes

	Peripheral blood	Colonies	
	A:B	A	B
Normal 1	50:50[a]	38[b]	37
Normal 2	60:40	51	25

[a] Ratio of isoenzyme types determinated by electrophoresis

[b] Actual number of colonies typed as A or B

II. Polycythemia vera subjects

When marrow from patients with polycythemia vera is cultured in semisolid medium in the absence of ESF, so-called endogenous erythroid colonies appear [6,9,13]. However, as higher concentrations of ESF are added to culture, an increased number of erythroid colonies is seen [9,13]. When these colonies were analyzed for G-6-PD isoenzyme type, those appearing in cultures having low concentrations of ESF contained only the isoenzyme type found in the peripheral blood, type A (Fig. 1). However, at higher concentrations of ESF, increasing numbers of erythroid colonies were detected which contained isoenzyme type B. At the highest concentrations of ESF, up to 25% of the colonies typed for isoenzyme B and thus, presumably, arose from normal stem cells. Although the initial study did not distinguish between colonies derived from CFU-E and BFU-E, a repeat study of one of the patients has demonstrated that normal (isoenzyme type B) colonies arise primarily from BFU-E. Essentially no normal colonies arose from CFU-E [2].

Also appearing in the cultures were limited numbers of granulocyte-macrophage colonies arising from granulocyte colony forming units (CFU-C). Their appearance was apparently stimulated by the release by marrow cells of

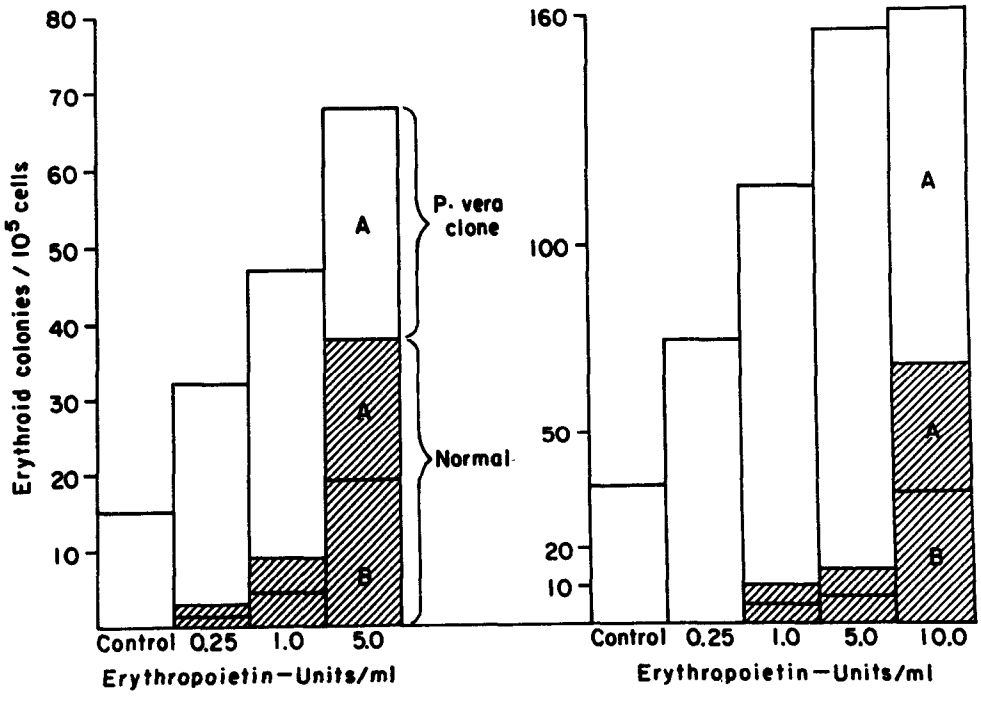

Fig. 1. The proportions of erythroid colonies of isoenzyme type A and B determined from marrow cultures of two G-6-PD heterozygotes with polycythemia vera. In cultures containing no added erythropoietin, only colonies bearing the isoenzyme type found in the peripheral blood were observed. As erythropoietin concentrations increased, an increasing percentage of colonies with isoenzyme type B was found. The shaded area denotes the calculated percentage of expected normal colony forming units based on the number of type B colonies detected and the assumption that an equal number of normal type A colonies exist in heterozygotes with balanced expression of the two G-6-PD isoenzyme types.

endogenous colony stimulating factor (CSF). A restricted number of these colonies were also harvested and analyzed. As shown in Table 2, CFU-C-derived colonies containing isoenzyme type B were also detected in the cultures of these patients, although a detailed CSF dose/response curve was not done and culture conditions had not been adjusted to optimize granulocyte/macrophage colony growth.

Table 2. The isoenzyme types of granulocyte/macrophage colonies arising in marrow cultures from two G-6-PD heterozygotes with polycythemia vera

	Isoenzyme type	
	A	B
Subject 1	20[a]	9
Subject 2	22	1

[a] Number of colonies typed as A or B

D. Discussion

The demonstration of the origin of certain hematologic disorders at the level of the pluripotent stem cell and the observation that peripheral blood elements are predominantly the products of a single clone suggest that in the course of the disease there is clonal expansion. What is uncertain, however, is whether such expansion is associated with the elimination of normal stem cells and their progeny or the suppression of growth of such progenitors. In order to approach this question, we cultured bone marrow cells from patients with polycythemia vera who are also heterozygotes for a naturally-occurring marker of cellular mosaicism, the X-linked isoenzymes of G-6-PD. The method used employed the growth of marrow cells in culture. These assays detect the presence of progenitors capable of marked proliferation and differentiation. The advantages of such a colony forming assay are several, but perhaps the most relevant to this analysis are the facts that recognizable differentiated cells can be detected in culture and that the progeny reflect an inherent property of the parent cell – that of the G-6-PD being expressed by the remaining active X chromosome. Thus, through in vitro amplification, it is possible to detect progenitors which may have arisen from stem cells not of the abnormal clone, some of which, by definition, should not contain the dominant G-6-PD isoenzyme type.

For this analysis to be valid, however, there are two requirements. First, the various colony types must be able to be analyzed by available techniques and, second, culture conditions should not favor the growth of progenitors having one isoenzyme type over the other. Studies in hematologically normal G-6-PD heterozygotes confirmed the validity of the approach. The results of such studies reveal that erythroid and granulocytic colonies can be individually recovered from methylcellulose and their G-6-PD isoenzyme type determined with a high degree of fidelity. Since virtually all colonies expressed only one isoenzyme type, either A or B, it could be concluded that such colo-

nies arose from single cells. In addition, the ratio of isoenzyme types obtained from the analysis of individual colonies reflects the ratio determined directly from the peripheral blood cells of the normal heterozygotes. This was true whether such erythroid colonies were derived from CFU-E or BFU-E and has recently been shown to be true for granulocyte/macrophage colonies. Finally, increasing concentrations of ESF failed to alter the isoenzyme ratios, suggesting that, at least in cultures from hematologically normal subjects, the conditions did not favor the growth of colonies of one isoenzyme type over the other.

The analysis of the cultures from the polycythemia vera patients provided several insights. First, only one isoenzyme type, type A, was detected in the peripheral blood of such individuals. Endogenous erythroid colonies also contained this same isoenzyme. With increasing concentrations of ESF, however, up to 25% of colonies were detected which contained type B isoenzyme. This implies that up to half of all colonies formed at high concentrations of ESF arose from presumably normal stem cells. This estimate of the incidence of normal colonies is derived from the fact that in a G-6-PD heterozygote with a balanced (approx. 50:50) expression of isoenzymes, there should be a normal A type colony for every type B colony which is found. Such an estimate is allowed by the observation in normals of concordance of ratios of isoenzyme activities in tissues of various types, including skin and blood [4]. If one makes such an estimate, as indicated in Fig. 1, it can be seen that half of the erythroid colonies detected were presumably normal.

This estimate of the percent of normal progenitors is far greater than the level of sensitivity of the electrophoresis technique used to determine isoenzyme activity. This implies that if such progenitors underwent terminal differentiation and maturation in vivo, it should have been possible to detect their products in circulation. The fact that normal cells were not detected suggests that the progenitors are somehow suppressed, perhaps by the abnormal clone, in their final maturation steps. Whatever the nature of this suppression, it appears to influence not only erythropoiesis but granulopoiesis as well (Table 2).

The mechanism by which suppression of normal progenitors is carried out is unknown. Although speculative, it is possible that such suppression is achieved by regulatory substances acting over short distances [8] rather than an abnormal product of the neoplastic clone. Such regulatory factors may be normal products; however, only the normal progenitors are capable of recognizing and responding appropriately to such growth controllers. Thus, the proliferative advantage of the abnormal clone would involve a lack of responsiveness to growth controllers rather than increased sensitivity to stimulating factors.

Further studies in culture of such patients may permit definition of the factors regulating marrow growth and also provide the opportunity to examine the influence of different forms of therapy and time on the relative numbers of normal and abnormal progenitors.

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