

Polycythemia Vera

THE IN VITRO RESPONSE OF NORMAL AND ABNORMAL STEM CELL LINES TO ERYTHROPOIETIN

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ABSTRACT Bone marrow cells from two glucose-6-phosphate dehydrogenase (G-6-PD) heterozygotes with polycythemia vera were cultured to determine whether progenitors which were not of the polycythemia vera clone were present, and, if present, which cell lines contributed to the increase in erythroid colonies observed in response to added erythropoietin (ESF). To accomplish this, the G-6-PD isoenzyme activity of individual erythroid colonies was determined. All of the erythroid colonies analyzed in cultures without added ESF, contained the G-6-PD isoenzyme type characteristic of the abnormal clone. With higher ESF concentrations in the culture, however, there was an increase in the colonies that were not of the polycythemia vera clone. Analysis of the ratio of the various types of colonies indicated that normal and polycythemia vera cells are capable of responding to ESF in vitro. In selected patients, this technique permits analysis of the ratios of normal to abnormal cells during the course of the disease, in response to therapy and during late complications, such as myelofibrosis or leukemic transformation.

INTRODUCTION

Polycythemia vera is a chronic myeloproliferative disorder characterized by generalized marrow hyper-

plasia with increased numbers of circulating erythrocytes, granulocytes, and platelets (1). The suggestion, based on the involvement of three hematopoietic lines, that this disease arises in a pluripotent stem cell, has been recently confirmed using a naturally occurring system of cellular mosaicism (2). Because of X-chromosome inactivation, somatic tissues of females heterozygous at the X-linked glucose-6-phosphate dehydrogenase (G-6-PD)¹ locus for a B and an A gene have two populations of cells—one synthesizing type A and the other, type B enzyme. In a study of two G-6-PD heterozygotes with polycythemia vera, normal tissues displayed both B and A enzymes, but peripheral erythrocytes, granulocytes, and platelets displayed only one enzyme, type A (2). The results demonstrate that polycythemia vera is a disease of a pluripotent stem cell and strongly suggest that the disorder is clonal in origin.

When marrow cells from polycythemia vera patients are cultured in semi-solid medium, a number of erythroid colonies form in the absence of exogenous erythropoietin (ESF) (3–5). However, the number of such colonies increases if ESF is added to culture. Because each colony arises from a single progenitor (6, 7), and can be typed individually for G-6-PD (7), the system can be used to study hematopoietic regulation in polycythemia vera.

This study was undertaken to formally determine whether descendants of the stem cells that did not arise from the polycythemia vera clone are present in the marrow, and if so, whether these presumed normal

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¹Abbreviations used in this paper: ESF, erythropoietin; G-6-PD, glucose-6-phosphate dehydrogenase; IRP, international reference preparation.

cells are solely responsible for the observed response to ESF in culture.

METHODS

Study subjects. Two G-6-PD heterozygotes (Gd^B/Gd^A) with polycythemia vera were the studied subjects. Clinical data and blood cell G-6-PD phenotypes have been reported (2). Peripheral blood values for the two subjects at the time of marrow culture were: patient 1: hematocrit, 41%; hemoglobin, 12.8 g/100 ml; leukocyte count, 4,200/mm³; platelet count, 225,000/mm³. patient 2: hematocrit, 44%; hemoglobin, 12.3 g/100 ml; leukocyte count, 24,800/mm³; platelet count, 590,000/mm³. Patient 1 had been intermittently treated with chlorambucil, but had not received any medication for 4 mo before the study; patient 2 had been treated only with phlebotomy.

Procedures

Erythroid colonies. Marrow cells were obtained by aspiration from the posterior iliac crest under local anesthesia. Patient 1 was studied in the Clinical Research Center at the University of Washington Hospital. Marrow cells from patient 2 were obtained in Philadelphia and shipped by air to Seattle at room temperature. The elapsed time between aspiration and culture was 8 h. Marrow cells were also aspirated from a hematologically normal male volunteer to provide a source of erythroid colonies of known G-6-PD isoenzyme type (B) for electrophoretic controls.

Approximately 1 ml of marrow aspirate was placed into 5 ml of α -medium (Flow Laboratories, Inc., Rockville, Md.) which was maintained at room temperature (20°C), and contained 2% fetal calf serum (Rehatein; Reheis Co., Inc., Kankakee, Ill.) and 500 U of preservative-free heparin. The cells were centrifuged (400 g; 10 min) at 4°C in a refrigerated centrifuge, the buffy coat removed and resuspended in ice-cold heparin-free medium, and then centrifuged again. A single-cell suspension of the buffy coat layer was prepared by repeated pipetting and then added to ice-cold α -medium containing 30% fetal calf serum, 1% bovine serum albumin (Pentex; Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) and 0.8% methylcellulose (Methocel; Dow Corning Corp., Midland, Mich.). The final concentration of nucleated cells was 2×10^5 per ml. Partially purified sheep plasma ESF (step III, lot 3006, Connaught Medical Research Laboratory, Willowdale, Ontario, Canada) was added in varying concentrations to appropriate cultures. The cell-containing medium was placed by syringe into 10 \times 35-mm petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) and incubated at 37°C in a high humidity, 5% CO₂-95% air, tissue culture incubator.

Recognizable erythroid colonies, identifiable by the orange-red color of the cells, appeared by day 7. The number of erythroid colonies was determined and the colonies were harvested between days 10 and 13. Erythroid colonies or possible erythroid bursts were not formally distinguished other than the fact that closely associated clusters of erythroid colonies were counted as single entities.

Individual colonies were harvested under direct vision with a dissecting microscope. Intact colonies were randomly harvested, and care was taken to avoid closely adjacent colonies which might have arisen from two spatially paired progenitors. Whenever colonies were arranged in groups (possibly erythroid bursts) only one colony of the group was taken. Each colony was lifted from the methylcellulose medium into a glass capillary and placed in a drop of saline containing

0.006 M final concentration NADP on a glass slide. The bulk of supporting medium with surrounding cells was teased away from the colony by two needles. Individual colonies were then placed on cellulose acetate strips presoaked in electrophoretic buffer. Three colonies were placed onto each strip: two from experimental cultures, and one from the normal control marrow cultured under identical conditions, and lysis of the colonies was achieved by adding saponin to the buffer. The strips were immediately subjected to electrophoresis and then stained for G-6-PD activity (8).

Granulocytic colonies. Granulocytic colonies were harvested from the same culture dishes on day 14. No special effort to achieve optimal conditions for growth of granulocytic colonies was made, e.g., no colony-stimulating activity was added. Granulocytic colony growth was apparently facilitated by the colony-stimulating activity produced by macrophages present in the cultures. The methods of harvesting and electrophoresis of granulocytic colonies were identical to those described above for erythroid colonies.

ESF bioassay. Three consecutive 24-h urine collections were obtained from patient 1. Each specimen was concentrated and assayed for ESF activity in ex-hypoxic polycythemic mice by previously published methods (9). Concentrations of ESF International Reference Preparation (IRP) were simultaneously assayed to permit quantitative expression of the results. Saline-injected animals served as controls and each experimental and control group consisted of a minimum of six mice.

RESULTS

Erythroid colony formation. In cultures of cells from both subjects, erythroid colonies formed in the absence of added ESF (Table I). Addition of ESF, however, resulted in a stepwise increase in colony numbers beginning with 0.25 IRP U/ml. The colonies which formed in cultures without added ESF, averaged 100 cells, whereas colonies in cultures which added ESF, contained up to 10^3 cells. Over 90% of the colonies could be scored for G-6-PD type and each colony was characterized by a single isoenzyme type. All colonies examined from cultures without added ESF were of G-6-PD type A, the same enzyme type characteristic of the patients' circulating blood cells. However, with increasing concentrations of ESF, colonies containing

TABLE I
Erythroid Colonies: G-6-PD Isoenzyme Analysis

ESF	Patient 1		Patient 2	
	Colonies*	A/B†	Colonies*	A/B†
IRP U/ml				
0	15	19/0	36	27/0
0.25	32	21/1	75	12/0
1.0	47	28/3	115	22/1
5.0	68	26/10	156	44/2
10.0	—	—	161	30/8

* Per 10^5 cells.

† Number of individual colonies analyzed of specific G-6-PD isoenzyme type.

G-6-PD type B appeared. These were found in greatest number in cultures with the highest concentration of ESF (Table I).

Statistical analysis. To determine whether the colonies that were stimulated by the added ESF arose from the polycythemia vera clone or presumed normal progenitors, the ratio of colonies of different G-6-PD types was analyzed statistically.² This analysis assumes that equal numbers of type A and type B colonies should arise from normal cells. This assumption is based on finding virtually equal proportions of isoenzyme activities in the skin (2) in the two patients, and that the ratio of G-6-PD isoenzyme activities does not differ significantly among mesenchymal tissues (10). When the numbers of A- and B-type colonies were analyzed with respect to the increase in total colony number, it was determined that the increase due to added ESF could not be solely explained on the basis of the growth of cells from the non-polycythemia vera clones. Thus, the null hypothesis, that cells of the polycythemia vera clone were not responding to ESF, was rejected ($P < 0.001$).

Granulocytic colonies. 10–20 granulocytic colonies formed per 2×10^5 nucleated cells plated and grew in cultures with and without ESF. Colony size ranged from 50 to 200 cells. For patient 1, 20 A- and 9 B-type colonies were found; for patient 2, 22 A type and 1 B type were found. Thus, although only small numbers were analyzed for G-6-PD type, colonies containing type A or type B enzyme were detected.

ESF bioassay. Mean radioactive iron incorporation in mice receiving urine concentrates of patient 1 ranged from 2.82 to 4.11% of the injected tracer; saline-injected control animals incorporated 0.71% ($\text{SEM} \pm 0.13\%$). Quantitation of ESF excretion yielded a mean value of 2.3 IRP U/day and a range of 1.8–2.6 U/day. These values fall within the normal range (4.2 ± 1.3 IRP U/day) established for this laboratory (9) and are higher than those found in untreated polycythemia vera (11).

DISCUSSION

Like chronic granulocytic leukemia (12, 13), polycythemia vera appears to be a clonal disease arising in a pluripotent stem cell. Thus in G-6-PD heterozygotes, only one isoenzyme type is found in peripheral erythrocytes, granulocytes, and platelets, whereas two isoenzymes are found in skin. These findings suggest strategies to investigate aspects of hematopoietic regulation in these diseases. This study takes advantage of the naturally occurring system of cellular mosaicism, based on G-6-PD isoenzymes, and the fact that colonies of recognizable differentiated hematopoietic elements can be grown in culture. Such colonies arise from single

cells (7) and thus reflect properties inherent in their progenitors.

The first portion of the study was carried out to determine if there were cells present in the marrow which were not of the polycythemia vera clone and, if there were, to determine whether they alone were capable of responding in vitro to the humoral regulator, erythropoietin. This approach was taken because of in vitro (3–5, 14, 15) and in vivo evidence (16, 17) demonstrating an erythropoietic response of polycythemia vera marrow cells to ESF. It has not been possible to formally demonstrate the relative contribution to the response by abnormal or presumably normal progenitors in such studies. When colonies cultured from the marrow of our two patients were analyzed for G-6-PD type, all of the erythroid colonies which formed in the absence of exogenous ESF were of one isoenzyme type—that of the peripheral blood elements. Thus, they appear to have arisen from the abnormal clone, a finding consistent with the interpretations of others (5). With added ESF, increasing numbers of colonies were found, however, which did not belong to the polycythemia vera clone. The cells giving rise to these colonies presumably arose from normal stem cells. Similarly, small numbers of granulocytic colonies were found which were not of the polycythemia vera clone.

These experiments demonstrate that presumably normal committed erythroid progenitors respond in vitro to ESF. To determine whether erythroid progenitors of the polycythemia vera clone were also responding to increasing concentrations of ESF, the ratio of type A to B colonies was statistically analyzed. Previous studies of G-6-PD heterozygotes show that the proportions of isoenzymes do not significantly differ between mesenchymal tissues in a given normal subject (10). Therefore, because the ratios of A and B isoenzymes were nearly equal in skin fibroblasts from our patients, we presumed that non-polycythemia vera colony-forming units of G-6-PD type A and B were also nearly equal in number. The analysis is also based on the fact that ESF-stimulated marrow cultures do not select for colonies of a given isoenzyme type in hematologically normal G-6-PD heterozygotes (7). Based on these considerations, if the non-polycythemia vera population was solely responsible for the increase in colony number, $\approx 50\%$ of the ESF-dependent increment would be of G-6-PD type B and the other 50% “normal” type A. When analyzed statistically, this was not the case. The differences between the proportions of A- and B-type colonies, growing in response to added ESF, differ significantly from the null hypothesis at individual concentrations of ESF and when all data are combined. We conclude, from this analysis, that the increments in colony numbers are due, in part, to the increased numbers of type A colonies which have presumably arisen from the polycythemia vera clone.

² Details of the statistical analysis may be obtained by writing to the authors.

Relatively greater numbers of type B erythroid and granulocytic colonies were observed with low concentrations of ESF and colony-stimulating activity in cultures from patient 1 who was treated with chlorambucil, when compared to patient 2. We believe this reflects the differences in therapy employed. Thus, phlebotomy would not be expected to reduce the pool size of committed polycythemia vera progenitors, whereas myelosuppressive agents might alter the ratio of abnormal to presumed normal precursors.

The results are also relevant to mechanisms of regulation of hematopoiesis. The products of a normal clone were not found in the circulation of either subject, when a technique sensitive enough to detect as little as 5% of a minor enzyme component was used. This was observed despite the fact that the hematocrit and hemoglobin concentrations were normal in both subjects, all peripheral counts had been normalized in patient 1 by chlorambucil therapy, and this patient excreted normal amounts of ESF. Thus, despite what would be considered a satisfactory clinical remission induced by chemotherapy, only the products of the polycythemia vera clone were detected. This suggests that a part of hematopoietic expression in this disorder results from suppression of normal clones, a control process possibly operating over very short distances and perhaps through cellular communication, as has been postulated (18, 19). As a result, evidence of the growth of normal cells was only obtained in stimulated cultures of marrow cells.

Finally, these studies provide an example by which analyses of hematopoietic regulation may be performed in selected subjects with other clonal disorders. By these means it should be possible to determine whether progeny of normal stem cells are present in other myeloproliferative diseases, such as chronic granulocytic leukemia and agnogenic myeloid metaplasia with myelofibrosis. Perhaps of more importance, patients, such as the ones described here, may be studied over a period of time to determine the balance of cell lines during response to various therapies and at such times when the underlying disease undergoes transition to myelofibrosis or leukemia.

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