In Vitro Studies of Megakaryocytopoiesis in Thrombocytotic Disorders of Man

By Alan M. Gewirtz, Edward Bruno, Joyce Elwell, and Ronald Hoffman

Increased numbers of bone marrow megakaryocytes and thrombocytosis are frequent accompaniments of the myeloproliferative disorders (MPD). This phenomenon is thought to be a direct consequence of an intrinsic stem cell defect that characterizes these diseases.1-4 There is little existent data, however, regarding the nature of this defect as it affects the megakaryocyte progenitor cells or colony-forming unit-megakaryocyte (CFU-M). Still less is known of the responsiveness of CFU-M from patients with these disorders to humoral regulators known to affect the in vitro cloning efficiency of colony-forming units-megakaryocyte (CFU-M). We have also examined the cloning efficiency of CFU-M from patients with these diseases. The results of our investigations suggest that: (1) increased production of Meg-CSA is not responsible for the megakaryocyte hyperplasia and thrombocytosis noted in these patients; (2) the intrinsic stem cell defect described in MPD appears to affect the CFU-M of these patients as well, resulting in an effective expansion of the CFU-M pool with consequent megakaryocyte hyperplasia and thrombocytosis; (3) the CFU-M of patients with MPD remain responsive to an exogenous source of Meg-CSA, suggesting that this megakaryocyte hyperplasia may not be entirely autonomous of its effects; and (4) the CFU-M pool in RT is normal both in size and responsiveness to Meg-CSA, suggesting that in these disorders, the stimulus leading to megakaryocyte hyperplasia and thrombocytosis is active at the post-CFU-M level of megakaryocyte differentiation.

MATERIALS AND METHODS

Patients

All patients studied attended the Yale-New Haven Hospital. Diagnostic assignments were made by accepted criteria after the appropriate diagnostic studies were completed. No patient was diagnosed as having a myeloproliferative disorder or reactive thrombocytosis solely on the basis of the platelet count. Platelet counts were performed in the Clinical Hematology Laboratories by electronic particle counting with a Clay-Adams Ultraflow 600 counter. The range of normal platelet counts is from 150,000 to 350,000; this range is established yearly by enumeration of platelet counts in the second-year members of the Yale Medical School. Platelet counts outside the normal range are routinely confirmed by examination of a Wright-stained peripheral blood smear.
Eleven patients with thrombocytosis and myeloproliferative disorders (three with chronic granulocytic leukemia (CGL); four with polycythemia vera (P. vera); and four with primary thrombocytosis (PT)) were studied. All but three had received some form of chemotherapy with either busulfan, chlorambucil, or hydroxyurea prior to study, but none had received any drugs within 2 mo prior to the time of study. The range of platelet counts in this group of patients was between 400,000 and 1,430,000.

Ten patients with reactive thrombocytosis were studied. Four were suffering from acute infectious processes, three had solid tumors, one had vasculitis, one had iron deficiency anemia, and one was recovering from an alcohol-induced marrow suppression and subsequently developed thrombocytosis. The range of platelet counts in this group of patients was between 580,000 and 1,800,000.

**Plasma Clot Megakaryocyte Cultures**

The culture system employed has been previously described.6,8 In brief, peripheral blood or marrow mononuclear cells were obtained by density centrifugation with Ficoll-Paque (specific gravity, 1.077 g/cu cm; Pharmacia Fine Chemicals, Piscataway, N.J.). The interface mononuclear cell layer was collected and washed with a medium minus nucleosides containing 2% fetal calf serum. Mononuclear cells (at a concentration of 5 x 10⁷ cells/ml for marrow or 10⁷ cells/ml for peripheral blood) were cultured in 1-ml volumes in 35-mm petri dishes. The plasma clot technique of McLeod et al. was utilized with modification as previously described.4 Culture dishes were incubated for 12 days at 37°C in a 100% humidified atmosphere of 5% carbon dioxide in air. Harvesting was performed by fixation in situ with methanol:acetone (at a ratio of 1:3) for 20 min, washing with 0.01 M phosphate-buffered saline (PBS) with a pH of 7.2, washing with distilled water, and then drying in air. Plasma clots were stored frozen at −80°C until immunofluorescence staining was performed.

Serum samples to be tested for colony-stimulating activity were sterilized by ultrafiltration. When the individual serum samples were added to the culture growth media, they replaced an equal volume of normal human AB serum. The serum samples from the different patients were tested simultaneously against the same marrow cells.

**Preparation of Antiserum Against PGPs**

Purified human PGPs were prepared by lithium diiodosalicylate-pheno1 extraction of pooled human platelet concentrates, as described by Marchesi and Chasis.11 New Zealand white rabbits were immunized with the purified human PGPs as previously described.8 Serum was harvested at 6 wk by cardiac puncture and stored in aliquots at −80°C.

**Immunofluorescence Staining and Scoring**

Whole rabbit anti-PGP antiserum was diluted to a concentration of 1:200 in PBS and layered over the fixed plasma clot cultures. These were incubated for 60 min at room temperature in 100% humidified air. After 3 washings with PBS, the specimens were reincubated with fluorescein-conjugated goat anti-rabbit IgG (Meloy, Springfield, Va.) diluted in PBS (final concentration, 0.36 mg protein/ml) for an additional 60 min. After another washing with PBS, the specimens were counterstained with 0.125% Evans blue for 7 min and washed with distilled water. Coverslips were then wet mounted with isotonic barbital buffer (pH 8.6) in glycerol at a concentration of 1:3.

In vitro plasma clot cultures were scored in situ. The 35-mm Petri dishes were inverted, the base area completely scanned, the fluorescein-positive colonies enumerated with fluorescence microscopy at a magnification of 100 through a Zeiss standard microscope 18 with an IV FL vertical fluorescent illuminator. A megakaryocyte colony was defined as a cluster of three or more intensely fluorescent cells.

**RESULTS**

In order to determine if increased serum levels of Meg-CSA might lead to the megakaryocytic hyperplasia in RT or MPD, normal bone marrow cells derived from a single donor were cloned in the presence of individual serum samples from seven patients with RT and seven patients with MPD. Final test serum concentrations of 5%, 10%, and 20% of the plated culture medium volume were analyzed. The mean number of colonies generated by the individual patient sera within each disease category at each of the final serum concentrations tested are shown in Fig. 1. Neither RT sera nor MPD sera were shown to contain levels of Meg-CSA over those that may be present in normal AB serum. Over a range of serum concentrations...
varying between 5% and 20%, no significant increase in the number of CFU-M-generated colonies over baseline could be demonstrated. In contrast, the mean number of colonies generated by two different sera known to contain Meg-CSA increased in a dose-dependent fashion over the entire concentration range tested, from a baseline of 60 colonies to 184 colonies at the 20% serum concentration.

The cloning efficacy and proliferative capacity of CFU-M as well as the responsiveness of CFU-M to a source of Meg-CSA was also assessed in the MPD and RT patients. This was accomplished by culturing either peripheral blood mononuclear cells in the case of MPD patients, or bone marrow or peripheral blood mononuclear cells in the case of RT patients in the presence of either normal human AB serum or a serum with known capability of increasing CFU-M cloning efficacy of normal human marrow cells. Six MPD patients and five RT patients were studied. As shown in Fig. 2, the number of CFU-M-derived colonies formed by MPD patient peripheral blood mononuclear cells in the presence of normal AB serum was strikingly increased over those obtained from six normal controls. The mean number of colonies generated by six normal controls in the presence of 30% AB serum was 11.3 compared to a mean number of colonies of 211.3 for the 6 MPD patients in 30% AB serum. This presents a mean increase of cloning efficiency of 1769% for the MPD patients compared with normal donors. Enumeration of the number of cells per megakaryocytic colony formed in the absence of exogenous Meg-CSA suggested that MPD CFU-M had a higher proliferative capacity as well when compared to normal CFU-M. Figure 3 illustrates that MPD megakaryocytic colonies had a mean of 13.5 (SD ± 10.0) cells/colony compared to 4.6 (SD ± 1.4) cells/colony for normal controls. This difference was highly significant (p < 0.001). As with the normal controls, however, addition of a source of Meg-CSA led to an increase in the number of CFU-M-derived colonies in the MPD patients. At the 10% Meg-CSA serum-containing concentration, the mean number of colonies generated by the MPD group was 449.5 compared to 27.4 for the normal controls. The relative percent increase in the number of colonies generated by the MPD patients and normals was similar however, being 113% for the former and 142% for the latter.

In contrast to the results obtained with MPD CFU-M, Fig. 3 also demonstrates that there was no significant difference in proliferative capacity (p > 0.05) of RT CFU-M (5.4 ± 3.0 cells/megakaryocytic colony) compared to normal CFU-M (4.6 ± 1.4 cells/megakaryocytic colony). Examination of Fig. 4 reveals that RT is also not characterized by an increase in the number of endogenous CFU-M-derived colonies. In fact, the numbers of colonies generated in normal AB serum by RT cells was approximately equal to those
generated by normal peripheral blood mononuclear cells and appeared to be slightly less than those generated by normal bone marrow mononuclear cells. In four of the five cases studied, however, the response of RT CFU-M paralleled the response of normal cells to stimulation by a source of Meg-CSA. These responses suggest that the CFU-M pool of patients with RT is qualitatively normal, as assessed by proliferative capacity and Meg-CSA responsiveness. These data also suggest that the RT CFU-M pool is probably qualitatively normal as well.

DISCUSSION

Current in vitro culture data derived from several groups suggest that there are at least two humoral regulators important in the control of normal human megakaryocytopoiesis. One regulator, Meg-CSA, is felt to exert its effects on CFU-M, causing them to proliferate and undergo partial maturation. The second regulator, thrombopoietin, does not by itself support CFU-M growth in vitro but is thought to finalize the maturation process of young megakaryocytes by influencing nuclear endoreduplication and completing cytoplasmic maturation.

Previous attempts to explain the increased megakaryocyte mass and platelet numbers often observed in patients with MPD or RT were formulated in the absence of this data and were based on either painstaking morphological observations of terminally differentiated marrow megakaryocytes in these patients or on the demonstration of thrombopoietic substances in their blood. Harker and Finch, for example, have shown that in normal individuals there is an inverse relationship between megakaryocyte volume and platelet numbers. They postulated that because megakaryocyte volume was inappropriately increased in relation to the elevated platelet count noted in primary thrombocytosis and polycythemia vera, that this was evidence for autonomous megakaryocyte proliferation. In contradistinction, McDonald has demonstrated, as has Schreiner and his colleagues, that some patients with MPD produce a substance with thrombopoietin-like properties and that this may be the putative agent for increased platelet production in these patients. Harker has also speculated that because megakaryocyte volume was appropriately diminished in patients with RT but the number of megakaryocytes was inappropriately increased, a factor causing increased megakaryocyte proliferation might be elaborated in these disorders. The nature of this putative factor is unknown, but if patients with RT do synthesize a factor resulting in an increased megakaryocyte mass, it would of necessity bear some biologic resemblance to Meg-CSA if the current schema are correct. In this regard, thrombopoietin-like substances have been demonstrated in the serum of RT patients, but no data exist regarding Meg-CSA activity in this population. We therefore studied various aspects of in vitro megakaryocytopoiesis in patients with RT and MPD in order to further define the role of Meg-CSA and CFU-M in generating the increased megakaryocyte mass noted in these disorders. Our investigations have revealed a number of interesting findings.

When mononuclear cells from MPD patients were assayed for CFU-M in the presence of normal human AB serum, they gave rise to many more megakaryocyte colonies that did normal controls that were studied under identical conditions. The number of cells conspiring each colony was also increased. The tremendous increase in cloning efficiency and proliferative capacity of MPD CFU-M in the absence of exogenous Meg-CSA could be interpreted in several ways. One explanation might be that the growth and proliferation of CFU-M from MPD patients is autonomous of Meg-CSA, as originally implied by Harker and Finch. Another equally tenable explanation could be that some CFU-M of MPD patients are exquisitely sensitive to the low levels of Meg-CSA that are presumably present in normal serum and that they clone with greatly increased efficiency in its presence. Such a situation would be analogous to the proposed regulatory role of Meg-CSA in MPD. Alternatively, since we have reported a high proportion of megakaryocyte colonies that did not respond to low levels of Meg-CSA, it is possible that some CFU-M in MPD patients are also not autonomous of Meg-CSA, as originally implied by Harker and Finch.

As shown in Fig. 4, serum from patients with RT stimulated megakaryocytopoiesis in vitro but was not as efficacious in its presence as AB serum. In order to further define the role of AB serum, we conducted experiments to examine the stimulatory activity of this serum in mitogenic responses. The results of these experiments are shown in Fig. 4. As noted in the legend, open circles represent the mean number of colonies formed by the bone marrow mononuclear cells of 11 normal controls. Open circles in the left-hand part of the figure represent the mean number of colonies formed by peripheral blood mononuclear cells of six normal controls.

**Fig. 4.** Effect of the addition of serum with known megakaryocyte colony stimulatory activity on the number of CFU-M-derived colonies cultured from five patients with RT utilizing either bone marrow mononuclear cells from two patients (left) or peripheral blood mononuclear cells from three patients (right). Each solid circle represents the mean ± SEM of a single experiment performed in duplicate. Open circles in the left-hand part of figure represent the mean number of colonies formed by the bone marrow mononuclear cells of 11 normal controls. Open circles in the right-hand part of the figure represent the mean number of colonies formed by peripheral blood mononuclear cells of six normal controls.
hypersensitivity of erythroid stem cells in polycythemia vera to erythropoietin, which has been recently demonstrated by a number of laboratories.\textsuperscript{18, 20} Unfortunately, our data do not allow us to distinguish between these two possibilities. Enhanced cloning efficiency of CFU-M from MPD patients in the absence of an exogenous stimulator has been reported by two other laboratories as well.\textsuperscript{21, 22}

Our data also suggest that the CFU-M of MPD patients remain responsive to the effect of exogenous Meg-CSA. In fact, except for the higher starting point attributable to the enhanced cloning efficiency of MPD CFU-M in normal AB serum, the slope of the dose-response curve to a source of Meg-CSA closely approximated that seen in normal controls. The preservation of Meg-CSA responsiveness suggests that the proliferation of CFU-M in these patients and the consequent megakaryocyte hyperplasia may not be entirely autonomous of Meg-CSA. Rather, these data imply that MPD CFU-M are heterogeneous with respect to their sensitivity to Meg-CSA. Some CFU-M may be hypersensitive to or autonomous of its effect, while others manifest normal sensitivity. The relative percentage of normal versus malignant CFU-M in either of these proposed populations is unknown.

We have also clearly shown that the apparent expansion of the assayable CFU-M pool of MPD patients is not due to elevated levels of Meg-CSA in these patients over those that may be present in normal serum. This finding is consistent with previous work reported from our laboratory demonstrating an inverse relationship between megakaryocyte number and Meg-CSA activity in the serum.\textsuperscript{8} Accordingly, while the thrombopoietic substances reported in MPD patients may play some role in the generation of thrombocytosis, their role in generating megakaryocyte hyperplasia remains to be clarified, since we were unable to demonstrate any serum factor in these patients capable of increasing normal CFU-M cloning efficiency.

Neither cloning efficiency nor proliferative capacity of CFU-M from RT patients was significantly different from that seen in normal controls. Further, the dose responsiveness of RT CFU-M to exogenous Meg-CSA did not deviate from that seen in a normal population. In addition, Meg-CSA levels in these patients were not elevated as measured in our assay. These data suggest that the size, proliferative ability, and dependence on Meg-CSA of the RT CFU-M pool is similar to what we have observed in normals. These data also necessarily imply that the phenomena leading to reactive thrombocytosis must be active at a point beyond the CFU-M level of megakaryocyte differentiation as it is currently conceived. It is possible that a humoral factor distinct from Meg-CSA is responsible for the megakaryocyte hyperplasia observed in RT. An alternate hypothesis to explain the etiology of RT would be to implicate a local marrow environmental factor that may influence megakaryocyte mass. In this regard, macrophages have recently been shown to be able to enhance murine CFU-M cloning efficacy.\textsuperscript{23}

Whether activated macrophages in the stress states commonly associated with RT might elaborate a factor that influences megakaryocyte development is yet to be defined but is the subject of future investigation in our laboratory.

The normal proliferative capacity of RT CFU-M as compared to MPD CFU-M may have diagnostic implications as well. Heretofore, only tedious morphological examination of bone marrow megakaryocytes were felt to be helpful in this regard.\textsuperscript{14} Examination of large populations of patients will have to be studied in order to determine whether the in vitro proliferative capacity of CFU-M will be useful in differentiation between RT and thrombocytosis associated with MPD.

By employing the above-mentioned methodology then, new information on the pathogenesis of the bone marrow megakaryocyte hyperplasia and elevated platelet counts commonly observed in MPD and RT has been gathered. In MPD, the intrinsic stem cell defect described in these disorders appears to affect the CFU-M, resulting in an expanded stem cell pool with an increased proliferative capacity. Megakaryocyte hyperplasia eventually results. In RT, the pathogenesis of the megakaryocyte hyperplasia appears to be different. The CFU-M pool is qualitatively and qualitatively normal, and levels of Meg-CSA are not elevated. We hypothesize that the megakaryocytic hyperplasia in RT is due to some cellular or humoral factor that exerts its effect beyond the level of the CFU-M. The elaboration of these as yet undefined factors appears to result from a variety of stimuli, including inflammation, stress, infection, and malignancy. The data is this article clearly show that these factors are not operational at the CFU-M level and emphasize the need for further investigations to elucidate their nature.

REFERENCES

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