Detection of a Human CFC With a High Proliferative Potential

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Colony forming cells (CFC) with high proliferative potential have been detected in nutrient agar cultures of human bone marrow cells containing recombinant human interleukin-3 (IL-3) and granulocyte macrophage colony stimulating factor (GM-CSF). These CFC were detected by the formation of large colonies with diameters greater than 0.5 mm and containing approximately 50,000 cells after 28 days incubation. The incidence of these CFC was only two in 100,000 normal bone marrow cells; however, bone marrow from patients treated with 5-fluorouracil contained up to sevenfold higher numbers of these CFC. The characteristics of these CFC, multifactor-responsive progenitors with high proliferative potential, requiring a prolonged growth period in culture and showing a relative preservation in marrow from individuals pretreated with 5-fluorouracil, are consistent with a human cell type equivalent to the primitive murine progenitor termed HPP-CFC.

PREVIOUS REPORTS have demonstrated multiple synergistic interactions between hematopoietic growth factors on murine bone marrow cells.[1,2] Combinations of factors have been shown to stimulate an early murine hematopoietic progenitor cell, termed high proliferative potential colony forming cell (HPP-CFC), which has been shown to have a highly significant correlation with cells capable of repopulating the bone marrow of lethally irradiated mice.[3-5] The HPP-CFC have been characterized by (1) their capacity to produce very large macrophage colonies in vitro (diameter >0.5 mm and an average of 50,000 cells per colony) and (2) their relative resistance to treatment with the cytotoxic drug 5-fluorouracil in vivo.[3] One population of HPP-CFC have been shown to generate progenitor cells for the megakaryocyte, granulocyte, and macrophage lineages, as well as cells with in vivo repopulating potential for reticulocytes, platelets, and bone marrow progenitors.[3-7] These data suggest that the murine HPP-CFC represent a primitive cell population whose properties are closely related to those of the true hematopoietic stem cell and therefore represent a critical cell type for our understanding of the hematopoietic system. In this study we have investigated the ability of combinations of human growth factors to synergistically stimulate human bone marrow cells and have identified a unique cell type that has a high proliferative potential in agar culture and that appears to be analogous to the murine HPP-CFC.

MATERIALS AND METHODS

Agar culture of bone marrow. The cultures were established in double-layer agar in 35-mm dishes as previously described.[8] Alpha medium supplemented with 20% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) was used for all cultures. Factors were incorporated in the underlays at a maximum of 13.2% of the culture volume per dish. Normal human bone marrow cells were collected in a 50-mL syringe containing 1,000 U of heparin after informed consent was obtained from all subjects. Bone marrow cells were centrifuged over Ficoll-paque (Pharmacia, Piscataway, NJ) and the low-density cells collected, washed with a balanced salt solution, and plated at 10^5 cells per dish in the upper layer and incubated for up to 28 days in a 7% O_2, 10% CO_2, 85% N_2 gas mixture.[8] Bone marrow cells were also sampled from three patients who were treated with 5-fluorouracil (FU) as part of the bone marrow transplant program at the University of Virginia. Each patient gave informed consent under guidelines approved by the Institutional Human Investigations Committee.

Patient 1 was a 54-year-old white male who was diagnosed as having esthesioneuroblastoma involving the nasal cavity. He had had previous treatment with cyclophosphamide, vincristine, and adriamycin and received 5,000 cGy locally to the nasal cavity. FU was administered by intravenous (IV) injection of 15 mg/kg on days 1, 2, and 3 and the bone marrow harvested on day 9.

Patient 2 was a 47-year-old white male who was diagnosed as having carcinoma of the descending colon. He had received no prior treatment and was given a single IV injection of FU at 15 mg/kg on day 1 and his bone marrow harvested on day 5.

Patient 3 was a 17-year-old white male who was diagnosed as having stage IIIA Hodgkin's disease. He had received prior treatment with BCNU, cyclophosphamide, vinblastine, procarbazine, and prednisone. FU was administered by IV injection of 15 mg/kg on days 1, 2, and 3 and the bone marrow harvested on day 8.

The post-FU bone marrows were collected and treated in an identical manner as the normal human marrow and were frozen in liquid nitrogen in 10% dimethyl sulphoxide for storage. The post-FU bone marrow was thawed prior to plating, washed three times with a balanced salt solution, plated at 100,000 cells per dish, and incubated for 14 and 28 days at 37°C in the same gas mixture described above.

Colonies were scored using a dissecting microscope with total colonies scored as all colonies containing 50 or more cells, while HPP-CFC–type colonies were scored as dense colonies with diameters greater than 0.5 mm. The number of cells per colony was determined by picking up ten large colonies using a drawn-out Pasteur pipette. The colonies were pooled and the cell number determined using a hemocytometer.

Growth factors. Recombinant growth factors were prepared as previously described; human granulocyte-macrophage colony stimulating factor (GM-CSF) was purified from inclusion bodies formed in *Escherichia coli* containing the GM-CSF expression plasmid[9] and human interleukin-3 (IL-3) was partially purified from medium conditioned by COS-1 cells containing the pSXIL-3 plasmid.[10] The number of units of each factor plated per 35-mm petri dish were 625U of GM-CSF (10^7 U/mg) and 20 U of IL-3.

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Morphology. The morphology of cells in the colonies was determined by fixing the culture plates in 10% formalin and preparing whole agar-slide preparations. Some large colonies were individually picked up using a drawn-out Pasteur pipette, dried onto a microscope slide, and fixed with formalin. Both types of slide preparations were stained with hematoxylin and colony morphology determined.

RESULTS

The combination of GM-CSF and IL-3 stimulated the formation of a few large colonies, with diameters between 0.5 and 1.0 mm, in agar cultures incubated for 28 days (Table 1). Figure 1 shows typical colonies formed in cultures containing IL-3 or GM-CSF alone (A) and cultures containing IL-3 plus GM-CSF (B). The large colonies were typically dense colonies and contained approximately 50,000 cells per colony. The morphology of the cells in the large colonies was pure macrophage.

One of the properties of the murine HPP-CFC is their relative insensitivity to the cytotoxic drug 5-FU.[3] We have examined the bone marrow from three patients who were treated with 5-FU. In cultures containing GM-CSF plus IL-3 there was up to a sevenfold increase in the incidence of large colonies compared with normal bone marrow (Table 1, Fig 2). There were also a few large colonies formed in cultures containing GM-CSF alone (Table 1, Fig 2); however, these colonies were smaller than those formed with GM-CSF plus IL-3 (Fig 2). The size of most of the large colonies formed with the combination of GM-CSF plus IL-3 was between 1.0 and 2.0 mm in diameter (Figs 2 and 3), compared with the large colonies from normal bone marrow, which were all less than or equal to 1.0 mm. These results demonstrate the presence in the bone marrow of cells with high proliferative potential with enrichment in marrow from patients treated with 5-FU.

Table 1. Colony Formation in Agar Culture of Normal and Post-FU Human Bone Marrow Cells

	GM-CSF	IL-3	GM-CSF + IL-3
Normal bone marrow			
Experiment 1			
Total colonies	18.3 ± 1.2	1.0 ± 0.5	21.7 ± 0.5
Large colonies	0	0	2.3 ± 0.3
Experiment 2			
Total colonies	8.0 ± 1.7	33.5 ± 1.1	37.7 ± 3.1
Large colonies	0	0.5 ± 0.4	1.7 ± 0.3
Experiment 3			
Total colonies	7.7 ± 1.0	6.0 ± 2.1	24.0 ± 2.9
Large colonies	0	0	0.7 ± 0.3
Post-FU bone marrow			
Patient 1			
Total colonies	60.0 ± 4.2	43.3 ± 1.2	81.3 ± 6.0
Large colonies	5.5 ± 0.3	0	10.3 ± 1.3
Patient 2			
Total colonies	47.5 ± 0.4	33.5 ± 3.2	44.0 ± 4.9
Large colonies	2.7 ± 0.7	0.3 ± 0.3	12.3 ± 0.3
Patient 3			
Total colonies	16.0 ± 0[1]	6.0 ± 0.7	25.3 ± 0.3
Large colonies	3.0 ± 0[1]	0	3.7 ± 1.2

The figures shown are the numbers of total colonies and large colonies (diameter >0.5 mm) formed in agar cultures of 100,000 human bone marrow cells after 28 days incubation. Each value is the mean of triplicate dishes ± SEM. The normal bone marrow shown are for three separate experiments. The three patients presented were treated as described in Materials and Methods. In these cultures two plates were contaminated, and the value presented is from a single dish.

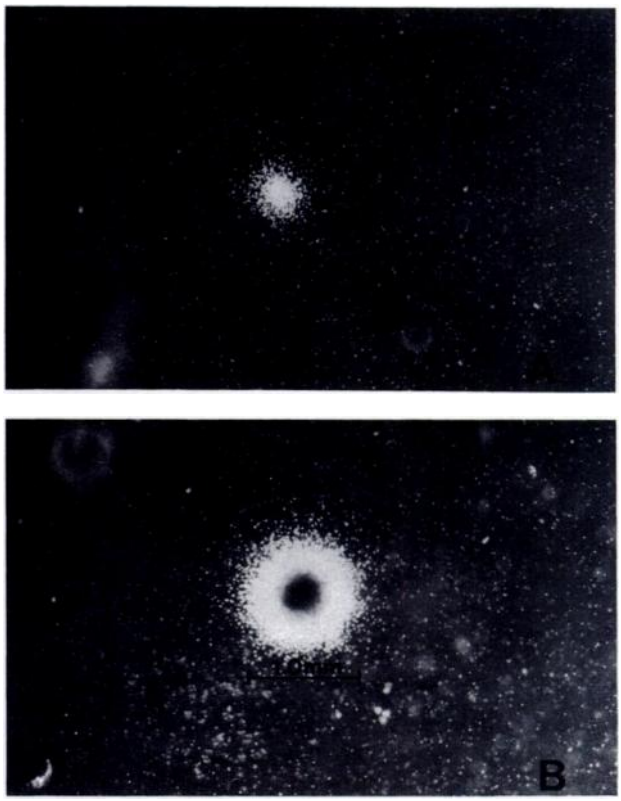

Fig 1. The relative sizes of colonies formed from normal human bone marrow cultures. In general, colonies were formed with diameters less than 0.5 mm; a typical colony is shown in panel A. Panel B shows a typical HPP-CFC–type colony that was stimulated by the combination of GM-CSF plus IL-3 (B). Colonies were photographed in situ in agar cultures after 28 days of incubation at 25 × original magnification using an inverted microscope.

The morphology of the cells in the large colonies formed in the cultures of post-FU marrow was predominantly granulocytic with a small admixture of macrophages and other nonidentifiable cells. This was in contrast to the large colonies formed in cultures of normal human bone marrow that consisted entirely of macrophages, suggesting the possibility of two distinct populations of large colony forming cells analogous to the murine system.[7]

DISCUSSION

We have described a new class of multifactor-responsive human hematopoietic progenitor cells characterized by a high proliferative potential, a prolonged growth period in culture, and a relative preservation in marrow from individu-

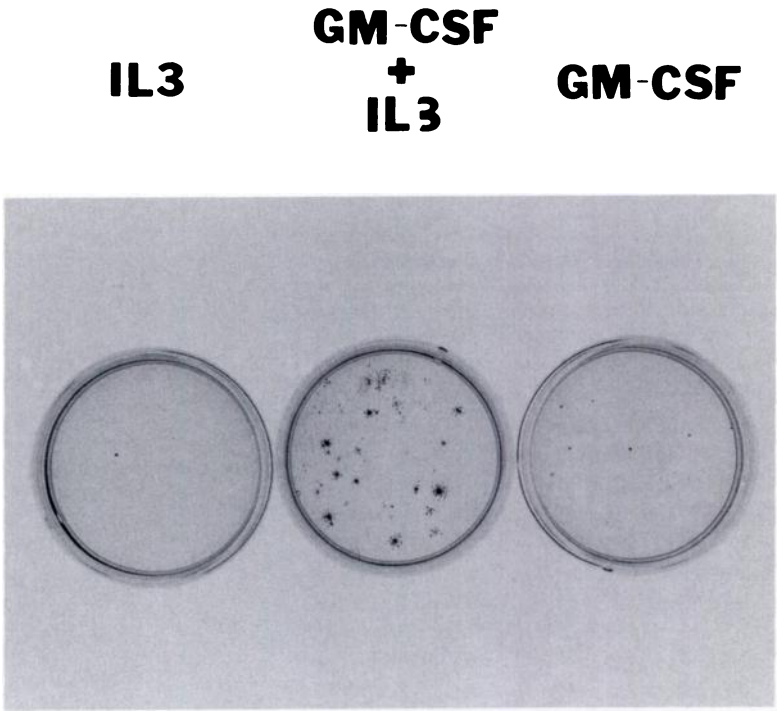

Fig 2. Culture plates (35-mm petri dishes) showing the colony formation in day 28 cultures of post-FU human (patient 2) bone marrow cells (100,000 cells per dish) in the presence of IL-3 or GM-CSF alone or in combination.

als pretreated with 5-FU. These characteristics are quite similar to those of the murine HPP-CFC, although the latter class of progenitor cells has been defined as responsive to CSF-1 plus either IL-3 or a synergistic factor from human spleen or placental-conditioned medium.[11,12] Further work in the murine species has shown that multiple 2-factor combinations, including GM-CSF plus IL-3, G-CSF plus CSF-1, GM-CSF plus G-CSF, and G-CSF plus IL-3, will stimulate macrophage HPP-CFC–like colonies.[2] The morphology of the cells in large colonies formed in cultures of normal human bone marrow was identical to reports for murine marrow, consisting entirely of macrophages. In contrast, the large colonies formed in cultures of post-FU marrow consisted of granulocytes and macrophages. This result is consistent with previous reports on the potential for murine HPP-CFC, stimulated by CSF-1 plus SF-1, to generate progenitor cells for both the granulocyte and macrophage lineages.[7]

The ability of GM-CSF alone to stimulate the formation of large colonies, although to a lesser degree than GM-CSF plus IL-3, is also of particular interest. We have data in the murine system that murine GM-CSF alone can also stimulate the formation of large colonies with diameters between 0.5 and 2.0 mm.[13] The present data suggest that GM-CSF may act on progenitor cells with high proliferative potentials; however, it is possible that GM-CSF may act in part by inducing the production of other hematopoietic growth factors (eg, IL-3) from accessory cells or from the target stem cells.

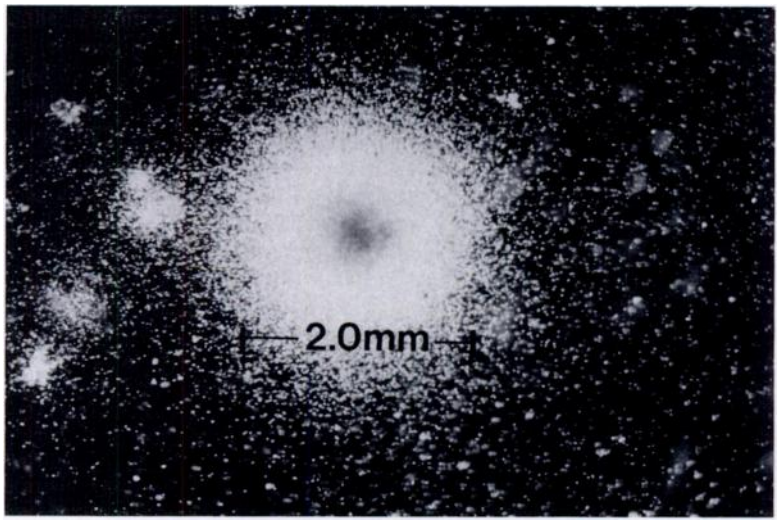

Fig 3. A typical large colony formed from post-FU human bone marrow in the presence of IL-3 plus GM-CSF after 28 days incubation. The colony shown was photographed in situ at 25 × original magnification using an inverted microscope.

Murine HPP-CFC have shown a better correlation with marrow renewal in vivo than other classes of progenitor cells assayed in vitro. In general, most classes of human progenitor cells assayed in vitro have not correlated closely with marrow renewal in transplant settings and do not appear to provide adequate models for human marrow renewal cells. These multifactor-responsive HPP-CFC may represent early human progenitor cells, and their assay in vitro may provide a better predictor for marrow renewal in the transplant setting.

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