RECOMBINANT HUMAN MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF) REQUIRES SUBLIMINAL CONCENTRATIONS OF GRANULOCYTE/MACROPHAGE (GM)-CSF FOR OPTIMAL STIMULATION OF HUMAN MACROPHAGE COLONY FORMATION IN VITRO

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Proliferation and differentiation of hemopoietic progenitor cells in culture lie under the control of a number of different glycoprotein regulatory molecules including the colony-stimulating factors (CSFs) (1–6), erythropoietin (7), and many of the interleukins (8–10, Wong, G. G., J. S. Witek-Giannoti, P. A. Temple, R. Kriz, C. Ferenz, R. M. Hewick, S. C. Clark, K. Ikebuchi, and M. Ogawa, submitted for publication). At present, six of these factors, the genes of which have been molecularly cloned are, by themselves, capable of supporting hemopoietic colony formation in semisolid culture. Granulocytic-CSF (G-CSF)[1] (12–14), macrophage-CSF (M-CSF, also known as CSF-1) (15–17), and IL-5 (10) (also known as eosinophil differentiation factor) all appear to be selective for late committed progenitors, generating neutrophilic granulocytic, macrophagic, and eosinophilic granulocytic colonies, respectively (although IL-5 also interacts with both B and T lymphocytes) (18). The gene for each of these cytokines is highly conserved in evolution such that the respective human and mouse proteins react with target cells from either species. Human IL-6 (also known as B cell stimulatory factor 2/interferon β2) has also recently been found to directly support granulocyte/macrophage colony formation in the mouse system, an activity that has been more difficult to detect with human target cells (Wong, G. G., et al., submitted for publication). The multipotent colony stimulating factors granulocyte/macrophagic CSF (19) (GM-CSF) and IL-3 (20–25) support the formation of a broader range of hemopoietic colony types than the other cytokines, but the activities of the human and murine proteins are species specific.

Two of the human cytokines, M-CSF (15–17, 26–29) and IL-6 (Wong, G. G., et al., submitted for publication), have proved more effective in supporting colony formation by murine rather than human hemopoietic progenitors. Puri-

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[1] *Abbreviations used in this paper:* CAE, chloroacetate esterase; GM, granulocyte/macrophage; H-1, hemopoietin 1; IMDM, Iscove's modified Dulbecco's medium; NSE, nonspecific esterase.

fied human urinary M-CSF, as well as the recombinant factors derived from two different human cell lines will efficiently support the formation of pure murine macrophage colonies, but much higher concentrations are required to elicit even a minimal response with human target cells (26–29). Similarly, the CSF activity of human IL-6 has only been clearly demonstrated using murine target cells. In the mouse system, a factor designated hemopoietin-1 (H-1) has been reported to act synergistically with many of the CSFs, including M-CSF, to yield enhanced hemopoietic colony formation (30). This synergistic activity, designated H-1, has proved to be one of the many biologic activities of IL-1 (8), a factor that by itself has no CSF activity. Because of the many interesting interactions between the cytokines, we have investigated the possibility that human macrophage colony formation might be enhanced using combinations of hemopoietins. Our results demonstrate that subliminal concentrations of GM-CSF, but not of IL-1α, IL-3, nor G-CSF, render human marrow progenitors responsive to M-CSF in clonal culture systems and suggest that GM-CSF may prime these cells for the action of M-CSF.

Materials and Methods

Reagents. Recombinant human (rh) GM-CSF, G-CSF, and M-CSF were expressed from Chinese hamster ovary (CHO) cells engineered to express high levels of the individual factors. Homogeneous GM-CSF (3–4 × 10^6 U/ml) from these cells was kindly provided by the Genetics Institute Pilot Development Lab (Cambridge, MA). The G-CSF and M-CSF preparations were crude conditioned media from the respective cell lines. The human IL-3 preparation was made by transfecting *cos*-1 with an expression plasmid pXM containing the human IL-3 gene (kindly provided by Dr. Y.-C. Yang) as described elsewhere (24). Human rIL-1α is From Biogen, Geneva, Switzerland. A polyclonal antiserum (anti-M-CSF) was prepared at the Genetics Institute by immunizing rabbits with highly purified human recombinant M-CSF, and was kindly provided by Drs. K. Turner and E. Alderman. This antiserum was added at a final dilution of 1:300 to the cultures containing M-CSF or GM-CSF at the time of plating.

Bone Marrow Colony Assays. Human bone marrow cells were obtained from healthy volunteers from the iliac bone with a heparin-containing syringe. Informed consent was obtained. Cells were separated on a Ficoll-Hypaque gradient (31), washed twice with PBS, and further depleted of adherent cells by double incubation in plastic flasks (Falcon Labware, Oxnard, CA) for 1 h each in RPMI medium (Gibco, Grand island, NY) containing 15% FCS (Hyclone, Logan UT). Phagocytic elements were removed with a sterile magnet after incubation of cells in RPMI/15% FCS containing 5 mg/ml carbonyl iron for 45 min. Cells were then T cell depleted by double rosetting with neuraminidase-treated sheep erythrocytes and centrifugation on Ficoll-Hypaque gradient (32). 8–12-wk-old female C3H/HEJ mice (The Jackson Laboratories, Bar Harbor, ME) were used. Animals were killed by cervical dislocation. Bone marrow cells were obtained from femora, cleared of surrounding tissue, and opened at both ends with a scalpel. The marrow plug was ejected into RPMI medium using a syringe connected to the femur with a 20-gauge needle. Cells were aspirated several times through the needle to obtain a single-cell suspension. Cells were then depleted of adherent and phagocytic cells as described for the human bone marrow cells. 2.5 × 10^4 human cells and 6 × 10^4 mouse cells, separated as described above, were plated in 35-mm Petri dishes (Corning Glass Works, Corning, NY) at a final volume of 1 ml/plate, in Iscove's modified Dulbecco's medium (Gibco) containing FCS at a final concentration of 20%, agar (Difco Laboratories, Detroit, MI) at a final concentration of 0.3%, rhGM-CSF at concentrations varying between 10 pg and 1 ng/ml, rhIL-3 and rhG-CSF at concentrations between 0.01 and 10 U/ml, rhM-CSF at concentrations between 0.1 and 50 U/ml, rhIL-1α at concentrations between 10 pg and 10 ng/ml, respectively. For the assays in chemically defined medium, cells were plated in 35-

TABLE I
Effect of rhM-CSF on Human and Mouse Bone Marrow Progenitor Cells

rhM-CSF	Mouse			Human		
	Colonies*	Colony size‡	NSE^+/CAE^-	Colonies*	Colony size‡	NSE^+/CAE^-
U/mL			%			%
0	0	—	—	4 ± 2	40–50	100
1	7 ± 3	50–150	100	7 ± 2	40–50	100
10	57 ± 8	100–250	97	9 ± 3	40–50	100
20	68 ± 9	100–300	95	9 ± 5	40–50	100
50	81 ± 11	100–300	95	6 ± 3	40–50	100

2.5×10^4 cells/ml and 6×10^4 cells/ml were plated in the human and murine assay, respectively.
* Colonies were scored at day 10 for the mouse assay and at day 14 for the human assay. Numbers are mean ± SD of triplicate plates. Values refer to one representative experiment of six cases tested.
‡ Number of cells per colony.

mm petri dishes in 1 ml of HB-102 chemically defined medium (DuPont, Westwood, MA) supplemented with L-glutamine 0.35 mg/ml (Flow Laboratories, McLean, VA), sodium pyruvate 1 mM (Flow Laboratories) and agar 0.3% final; cell number per plate and growth factor concentrations were exactly the same as for the experiments in the presence of FCS. Cultures were incubated at 37°C, in a humidified incubator at 5% CO_2. In some experiments GM-CSF was incubated with the cells in 1 ml of IMDM/20% FCS in Falcon plastic tubes at 37°C; after 24 h cells were washed twice in PBS and plated with or without M-CSF as described above. Experiments were performed on marrow samples from six different donors in triplicate plates, and treated in exactly the same way. Colonies were scored after 14 d of culture in the human assay, and after 10 d of culture in the murine assay. To determine the cellular composition of the resulting colonies, entire agar dishes were fixed in formal calcium (formalin, 3.7%; $CaCl_2$, 10 mg/ml) for 10 min, transferred to a slide, and stained with nonspecific esterase (NSE) and chloroacetate esterase (CAE) (33, 34).

Results

The recombinant human M-CSF expressed in CHO cells, as reported for other M-CSF preparations, proved to be much more effective in supporting murine than human macrophage colony formation (Table I). When adherence-depleted murine bone marrow cells were plated in the presence of different concentrations of M-CSF between 0.1 and 50 U/ml, an optimal concentration of 20 U/ml was observed, which yielded a mean of 68 colonies per plate, with an average size of 100–300 cells/colony. In contrast, <10 colonies/plate were observed at any concentration of M-CSF in cultures of human bone marrow cells that were depleted of both adherent cells and T lymphocytes. Bone marrow depleted of adherent cells but not of T cells gave similar scores (data not shown). All of the observed human macrophage colonies in these cultures consisted of aggregates of not more than 50 cells.

To test for enhancement of M-CSF-dependent human macrophage colony formation, human marrow cells were cultured in the presence of varying concentrations of M-CSF, and with several different doses of either GM-CSF, IL-3, or IL-1α. As shown in Fig. 1 *A*, subliminal concentrations of GM-CSF (in the range of picograms per milliliter) in combination with M-CSF at ≥10 U/ml

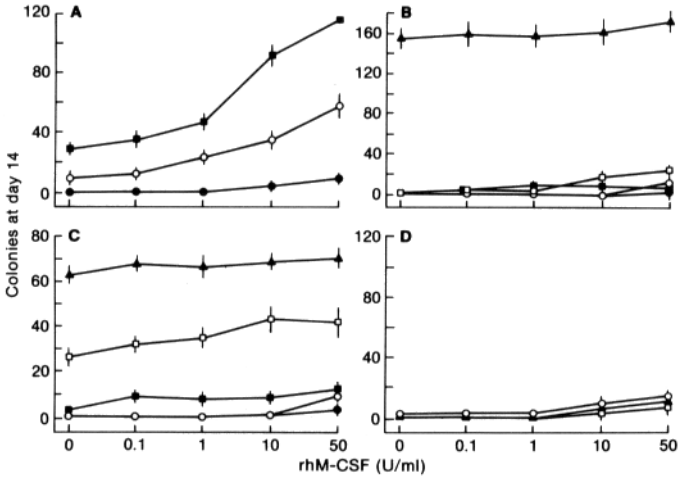

FIGURE 1. Effect of rhGM-CSF (*A*), rhG-CSF (*B*), rhIL-3 (*C*), and rhIL-1α (*D*) at various concentrations in combination with different concentrations of rhM-CSF on human bone marrow progenitor cells. *A*: (●), 0 pg/ml; (○), 10 pg/ml; (■), 100 pg/ml GM-CSF. *B*: (●), 0 U/ml; (○), 0.01 U/ml; (■), 0.1 U/ml; (□), 1 U/ml, (▲), 10 U/ml G-CSF. *C*: (●), 0 U/ml; (○), 0.01 U/ml; (■), 0.1 U/ml; (□), 1 U/ml; (▲), 10 U/ml IL-3. *D*: (●), 0 pg/ml; (○), 10 pg/ml; (■), 100 pg/ml; (□), 1 ng/ml; (▲), 10 ng/ml IL-1α. Data are given as mean of triplicate values. Bars indicate SD. Cells, separated as described in Materials and Methods, were plated at a final density of 2.5×10^4 cells/ml. Colonies (aggregates of ≥40 cells) were scored at day 14 of culture.

yielded more macrophagic colonies than achieved with any concentrations of M-CSF alone. Neither G-CSF (Fig. 1 *B*), nor IL-3 (Fig. 1 *C*), nor IL-1α (Fig. 1 *D*) showed any significant interaction with M-CSF in enhancing the numbers of colonies observed, and neither factor had any effect on the observed colony morphology.

More detailed analysis of the colonies in the GM-CSF-containing cultures revealed that GM-CSF and M-CSF act synergistically to enhance the formation of macrophagic colonies that stain positively for NSE (Table II). GM-CSF at concentrations below 1 ng/ml combined with any concentration of M-CSF gave 100% NSE^+/CAE^- colonies. At 1 ng/ml of GM-CSF, the effect on M-CSF-dependent colony formation was also evident, but was partially masked by the emergence of conspicuous numbers of other colony types presumed to be due to stimulation of other GM-CSF-dependent progenitors. Although significant numbers of both granulocytic (CAE^+) and mixed granulocytic/macrophagic (CAE^+/NSE^+) colonies were obtained in the presence of 1 ng/ml GM-CSF, the percentage of macrophagic (NSE^+/CAE^-) colonies was increased in combination with concentrations of M-CSF of ≥10 U/ml. The enhancement of M-CSF-dependent colony formation was also evident in the colony size; M-CSF alone yielded small colonies of 40–50 cells, but in combination with GM-CSF, colonies containing up to 300 cells were commonly observed (Table II).

To determine if subliminal doses of GM-CSF were required throughout the culture period or if the factor might exert its effects through brief exposure to the progenitors, we pretreated bone marrow cells in suspension for 24 h with both M-CSF and GM-CSF, washed the cells, then replated them in semisolid

TABLE II
Synergism of GM-CSF and M-CSF on Macrophagic Colony Formation

GM-CSF	M-CSF	Colonies* (day 14)	Colony‡ size	NSE⁺/CAE⁻§	NSE⁻/CAE⁺§	NSE⁺/CAE⁺§
pg/ml	*U/ml*				%	
0	1	0	—	0	0	0
0	10	3 ± 2	40–50	100	0	0
0	50	8 ± 5	40–50	100	0	0
10	0	8 ± 4	40–50	100	0	0
10	1	23 ± 7	40–80	100	0	0
10	10	35 ± 9	50–150	100	0	0
10	50	58 ± 11	100–200	100	0	0
100	0	28 ± 11	40–80	100	0	0
100	1	47 ± 13	50–150	95	2	3
100	10	93 ± 19	50–200	98	1	1
100	50	116 ± 31	100–300	100	0	0
1,000	0	156 ± 34	50–150	27	41	32
1,000	1	163 ± 25	50–150	37	35	28
1,000	10	190 ± 43	50–250	48	26	26
1,000	50	228 ± 54	50–300	55	22	23

2.5×10^4 human bone marrow cells, separated as described in Materials and Methods, were plated.
* Colonies were scored after 14 d of culture; numbers are mean ± SD of triplicate plates. Values refer to a representative experiment of six different cases tested.
‡ Number of cells per colony.
§ Values expressed as percent positive colonies.

TABLE III
Requirement for Continuous Presence of Subliminal Concentration of GM-CSF in M-CSF-Induced Macrophagic Colonies

CSF added	Continuous presence of GM-CSF	GM-CSF removed after 24 h
GM-CSF, 10 pg	8 ± 4	8 ± 3
GM-CSF, 100 pg	28 ± 11	8 ± 5
GM-CSF, 10 pg + M-CSF, 50 U	58 ± 11	9 ± 2
GM-CSF, 100 pg + M-CSF, 50 U	116 ± 31	7 ± 4
M-CSF, 50 U (control)	8 ± 5	8 ± 5

Cells were incubated at 37°C in the presence of GM-CSF at the given concentrations. After 24 h cells were washed twice in PBS and plated in the presence of M-CSF at the given concentration with or without GM-CSF. Colonies were scored after 14 d of culture. Values are mean ± SD of triplicate plates. Values refer to one representative experiment of six cases tested. 100% of colonies in every condition stained NSE⁺/CAE⁻.

medium either in M-CSF alone or in the presence of both factors. As summarized in Table III, 24 h exposure of progenitors to GM-CSF had little or no effect on M-CSF-induced colony formation except at the highest concentration tested, suggesting that the M-CSF-responsive cells may require GM-CSF throughout the culture period. The same qualitative effect could be obtained also in serum-free conditions, although higher doses of GM-CSF (1–10 ng/ml) were required (data not shown).

Because GM-CSF has been shown to induce M-CSF production by human monocytic cells (35), we tested the possibility that pure macrophagic colonies in a colony assay supported by relatively high concentration (1–10 ng/ml) of GM-CSF arise as a secondary effect of M-CSF production in the culture. A neutralizing polyclonal antiserum against M-CSF proved to neutralize M-CSF colony formation in a murine colony assay had no effect on the growth of macrophagic colonies in cultures of human marrow progenitors grown in the presence of GM-CSF (data not shown). Thus, GM-CSF by itself at the relatively high concentrations that are usually used in clonogenic assays seems to be capable of supporting the proliferation of at least some of the late progenitors per se of the macrophage lineage.

Discussion

Human M-CSF, either the recombinant protein derived from two different sources or the natural molecule isolated from human urine, has proved to be ineffective in supporting human macrophage colony formation, although it is very efficient in generating murine macrophage colonies (26–29). We have found that human macrophagic colonies can be generated effectively if subliminal concentrations of GM-CSF are included in the M-CSF-supported cultures; in this system the human macrophagic colony formation was as efficient as observed previously in cultures of murine bone marrow cells. This synergistic activity between M-CSF and GM-CSF in support of human macrophage colony formation was specific for GM-CSF and was not observed with IL-3, G-CSF, or IL-1α. These results suggest that in the human marrow there is a population of hemopoietic progenitors that must be primed by exposure to GM-CSF before they become competent to respond to M-CSF. Our data indicate that concentrations of GM-CSF 100-fold lower than the threshold concentration required to support colony formation are sufficient to prime the macrophage precursors for responsiveness to M-CSF. This priming of macrophage precursors requires exposure to GM-CSF for longer than 24 h (or perhaps continuous exposure), because washing cells pretreated with GM-CSF before culture in the presence of M-CSF largely prevented subsequent macrophage colony formation.

The effects of subliminal concentrations of GM-CSF in the human bone marrow cultures suggest two possible explanations for the different responses of macrophage progenitors to M-CSF in the human and murine systems. The first possibility is that in the mouse marrow a large fraction of progenitor cells are already preprimed by GM-CSF or by IL-1α/H1 to respond to M-CSF. Alternatively, because murine bone marrow samples are usually plated without T cell depletion (at present no satisfactory techniques have been developed for T cell depletion in the mouse), it is possible that T cells or even other accessory cells in the murine cultures elaborate sufficient GM-CSF to prime the macrophage progenitors for responsiveness to M-CSF. This possibility should be testable with a neutralizing antibody to murine GM-CSF. Similarly, the demonstration that monocytes activated with either phorbol myristate acetate or IFN-γ express M-CSF (36) and that GM-CSF itself can induce the expression of M-CSF transcripts by monocytes (35) led us to speculate that the macrophagic component of the GM-CSF activity might be an indirect consequence of the expression of M-CSF

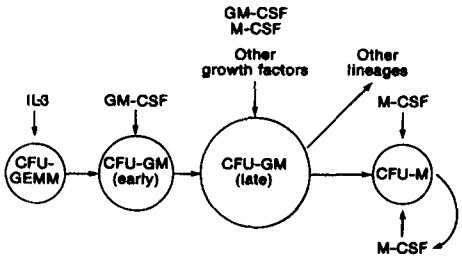

FIGURE 2. Model of sequential stimulation of different growth factors on human bone marrow progenitor cells. A very immature progenitor (CFU-GEMM, pluripotent stem cell) becomes committed to the myeloid lineage by an ancestral factor such as IL-3. This early CFU-GM acquires the ability to respond to GM-CSF, an interaction that might prime this cell to become a late CFU-GM, responsive not only to GM-CSF, but also to M-CSF and/or other growth factors. Either high (nanogram range) or low (picogram range) concentrations of GM-CSF in the presence of M-CSF could determine the final macrophagic morphology of this cell; the interaction with GM-CSF might also induce this terminal element to produce its own lineage-specific factor.

in the culture. Our experiments with an M-CSF-blocking antiserum in the human cultures demonstrated that the production of extracellular M-CSF by cells in the culture was not necessary for the formation of GM-CSF-supported macrophage colonies (although we cannot eliminate the possibility that M-CSF produced within a macrophage precursor that never leaves the cell might be necessary). These results suggest that GM-CSF exerts two separate effects on macrophage progenitors (or on different populations of progenitors). At subliminal concentrations for direct colony formation, GM-CSF primes at least some macrophage progenitors to become M-CSF responsive, while at higher concentrations it will directly support colony formation by at least some of the macrophage progenitors.

We have placed these experimental observations into a general model of sequential stimulation of the hemopoietic progenitors by different regulatory molecules in accordance with the stochastic model proposed by Metcalf for the murine system (3, 4). As shown in Fig. 2, a very ancestral factor, such as IL-3, could induce a multipotent cell to differentiate to a pluripotent cell capable of responding to GM-CSF. At this point, the progenitor becomes committed to the myeloid lineage, but within this lineage still retains the capacity to progress towards several subclasses such as the neutrophilic granulocytic, the monocytic, or the eosinophilic pathways. Contact with GM-CSF would make the cell competent for the lineage-restricted factors such as M-CSF or G-CSF. That is, the GM-CSF exposure would push the individual progenitor into a more mature state, as evidenced by acquiring the ability to respond to the more specific growth and differentiation factors. At this point, the progenitor (or perhaps progenitor subsets) might be induced to mature into macrophages by either of two different mechanisms: (*a*) direct induction by very high concentrations of GM-CSF; or (*b*) indirect induction by low concentrations of GM-CSF in the presence of M-CSF. The terminal macrophage in turn might amplify these signals by producing more M-CSF under the stimulus of GM-CSF. It will certainly be of interest to test this model and to determine if similar growth factor interactions operate in the murine system to support murine macrophage colony formation.

Summary

Human macrophage colony-stimulating factor (M-CSF or CSF-1), either in purified or in recombinant form, is able to generate macrophagic colonies in a murine bone marrow colony assay, but only stimulates small macrophagic colonies of 40–50 cells in a human bone marrow colony assay. We report here that recombinant human granulocytic/macrophage colony-stimulating factor (rhGM-CSF) at concentrations in the range of picograms enhances the responsiveness of bone marrow progenitors to M-CSF activity, resulting in an increased number of macrophagic colonies of up to 300 cells. Polyclonal antiserum against M-CSF did not alter colony formation of bone marrow progenitors incubated with GM-CSF at optimal concentration (1–10 ng/ml) for these in vitro assays. Thus, GM-CSF at higher concentrations (nanogram range) can by itself, elicit macrophagic colonies, and at lower concentrations (picogram range) acts to enhance the responsiveness of these progenitors to M-CSF.

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