Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 11310–11314, December 1991
Medical Sciences

Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: Influence of colony-stimulating factors

(hemopoietic colonies/differentiation commitment/granulocyte–macrophage/hemopoietic stem cells)

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Contributed by Donald Metcalf, August 26, 1991

ABSTRACT **In clonal cultures of normal mouse marrow cells, combination of granulocyte, granulocyte–macrophage, or multipotential colony-stimulating factor (G-CSF, GM-CSF, or multi-CSF, respectively) with stem cell factor (SCF) did not alter the number of blast colonies stimulated to develop compared with SCF alone but induced an up to 25-fold increase in their mean cell content and an up to 6-fold increase in their mean progenitor cell content. Costimulation of blast colony formation by SCF plus G-CSF did not change the relative frequency of progenitor cells of different types within the colonies compared with colonies stimulated by SCF alone. However, combination of GM-CSF or multi-CSF with SCF significantly increased the relative frequency of granulocytic progenitors and, for multi-CSF, also of eosinophil progenitor cells. These changes in the relative frequencies of progenitor cells committed to the various lineages support the hypothesis that hemopoietic regulators have some ability to induce selective lineage commitment in the progeny of multipotential cells.**

The immediate ancestors of morphologically recognizable immature hemopoietic cells are a population of committed progenitor cells that can be monitored by their capacity to generate hemopoietic colonies in semisolid cultures (1). These progenitor cells have a blast cell morphology and are heterogeneous in their proliferative potential but most appear to have undergone irreversible lineage commitment and to have no capacity for self generation (1). Murine progenitor cells committed to the granulocyte–macrophage lineage can form colonies of granulocytes and/or macrophages and are responsive in various degrees to proliferative stimulation by at least six defined hemopoietic regulators: granulocyte–macrophage, granulocyte, macrophage, and multipotential (or interleukin 3) colony-stimulating factors (GM-CSF, G-CSF, M-CSF, and multi-CSF, respectively), interleukin 6, and the stem cell factor (SCF) (also known as mast cell growth factor, kit ligand, or Steel factor) (2).

Progenitor cells are themselves generated by a heterogeneous population of more ancestral cells, termed collectively "hemopoietic stem cells." Although highly enriched stem cell populations can be obtained by fluorescence-activated cell sorting, the number of functionally distinct subsets within this class remains obscure. In mouse bone marrow, some of these stem cells can form colonies in semisolid culture that are characterized by their uniform content of undifferentiated blast cells (3–5). Reculture of the cells from blast colonies has revealed that many, perhaps all, are committed progenitor cells able to form typical hemopoietic colonies of one or other lineage (4, 5).

SCF (6, 7) is able, in cultures of normal mouse bone marrow cells, to stimulate the formation of multicentric blast cell colonies and granulocytic colonies, and the combination of SCF with G-CSF, GM-CSF, or multi-CSF results in a major enhancement of colony growth rates (5).

This system has permitted the present analysis of the content and nature of the committed progenitor cells in developing blast cell colonies to determine whether the combined use of SCF with a particular CSF results in the selective formation of particular subsets of committed progenitor cells. This model has been used to address the general question of whether the pattern of differentiation commitment in the progeny of multipotential cells can be influenced by the action of an extrinsic growth factor.

MATERIALS AND METHODS

Mice. Mice used were 2-month-old C57BL/6/J/Wehi mice of either sex reared under specific pathogen-free conditions.

Cultures. In each experiment, marrow cells were pooled from two donor animals and converted by pipetting to a dispersed cell suspension. All cultures were grown in 35-mm Petri dishes using 1 ml of Dulbecco's modified Eagle's medium initially containing 75,000 marrow cells and a final concentration of 20% (vol/vol) newborn calf serum and 0.3% agar (1). Stimuli were added to the culture dishes in 0.1 ml prior to the addition of the marrow suspension in agar medium. After mixing and gelling, cultures were incubated for 7 days at 37°C in a fully humidified atmosphere of 10% CO_2/90% air. Colonies (clones containing 50 or more cells) were counted at a magnification of ×35 using an Olympus dissection microscope and then the entire culture was fixed by the addition of 1 ml of 2.5% (vol/vol) glutaraldehyde in 0.9% NaCl. After 4 hr, the intact cultures were floated onto glass slides, allowed to dry, and then stained for acetylcholinesterase to detect megakaryocytes. The cultures were then stained in sequence with Luxol fast blue, to detect eosinophils, and with hematoxylin. The slides were mounted with cloverslips using D.P.X. neutral mounting medium and all colonies in at least two cultures were typed at a magnification of ×400 (1).

Colony Recloning. Seven-day multicentric blast colonies were identified by their characteristic shape and removed intact using a fine pipette. The sampling of blast colonies was strictly sequential unless a particular colony overlapped another type of colony, preventing its adequate removal. Each colony was added to 10 ml of agar medium and the colony cells were thoroughly redispersed. This suspension of colony cells was then added to duplicate cultures containing 100 ng of SCF, 1×10^3 units of CSF, 100 ng of SCF plus 1×10^3 units of CSF, or 0.1 ml of pokeweed mitogen-stimulated spleen conditioned medium (SCM) (1). After gelling, the cultures were incubated and then analyzed as above. Mean

Abbreviations: SCF, stem cell factor; CSF, colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; Multi-CSF, multipotential colony-stimulating factor; SCM, spleen conditioned medium.

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colony cell numbers were determined from pools of at least 50 sequentially harvested colonies by resuspending the cells in 1 ml of 5% calf serum/0.9% NaCl and then counting cells by using a hemocytometer.

Stimuli. All stimuli were recombinant factors purified to homogeneity after expression in bacterial expression systems. Rat SCF (kindly provided by K. Zsebo, Amgen Biologicals) was used as a final concentration of 100 ng/ml and human G-CSF (kindly provided by L. Souza, Amgen Biologicals), murine GM-CSF, and multi-CSF were used at a final concentration of 1×10^3 units/ml in the cultures. The specific activity of the CSFs were as follows: G-CSF, 1×10^8 units/mg; multi-CSF, 1×10^8 units/mg; GM-CSF, 3×10^8 units/mg.

Statistical Analysis. Analysis for statistically significant differences was performed using the Student's *t* test.

RESULTS

When cultures of 75,000 C57BL marrow cells were stimulated by SCF (100 ng/ml), between 10 and 20 multicentric blast cell colonies developed by day 7 of incubation in addition to larger numbers of mainly granulocyte colonies (Table 1). The mean cell content of these colonies was low, usually between 200 and 400 cells. Inclusion of G-CSF, GM-CSF, or multi-CSF at 1×10^3 units/ml increased colony numbers in a nonadditive manner and led to a 10- to 25-fold increase in mean colony size compared with colony size in cultures stimulated by SCF alone and, depending on the CSF used, a 3- to 20-fold increase in colony size compared with CSF-stimulated cultures (Table 1). As shown in the examples in Table 1, analysis of colony types developing in these cultures showed that combination of SCF with CSF resulted in no major perturbation of the pattern of colony formation expected from the use of the particular CSF and, in particular, did not significantly alter the absolute number of blast cell colonies developing compared with parallel cultures stimulated by SCF alone. However, as shown in Table 2, combination of each of the CSFs with SCF led to a 12- to 25-fold increase in cell numbers in such blast colonies, with multi-CSF and GM-CSF stimulating larger increases than G-CSF.

Examination showed that these enlarged blast colonies retained their characteristic multicentric morphology. Usually they were composed wholly of undifferentiated blast cells, although in a few colonies some immature granulocytes were also observed. Colonies retaining the general morphology of blast colonies but exhibiting an obvious corona of differentiating granulocytes and/or monocytes were excluded from the subsequent recloning studies.

Table 1. Colony formation stimulated by SCF with or without added CSF

Stimulus	Total colonies, no.	Number of colonies: Bt	G	GM	M	Eo	Meg	Cells per colony, no.
SCF	57	12	38	6	0	0	1	230
G-CSF	50	2	35	10	3	0	0	240
SCF/G-CSF	93	12	60	15	4	0	2	4640
SCF	74	17	46	7	4	0	0	320
GM-CSF	83	0	25	19	33	6	0	860
SCF/GM-CSF	125	18	40	43	22	2	0	3350
SCF	63	13	42	5	1	0	2	280
Multi-CSF	118	4	41	20	37	5	11	1920
SCF/multi-CSF	117	15	40	19	28	7	8	5300

Three experiments in which cultures of 75,000 C57BL marrow cells were stimulated by 100 ng of SCF and/or CSF at 1×10^3 units/ml, as indicated. Cultures were scored after 7 days of incubation and figures represent mean data from duplicate cultures. Bt, blast; G, granulocyte; GM, granulocyte–macrophage; M, macrophage; Eo, eosinophil; Meg, megakaryocyte.

Table 2. Mean cell content of colonies and blast colonies stimulated by SCF with or without added CSF

Stimulus	Mean cell count: No. per blast colony	No. for all colonies
SCF	250	190
SCF/G-CSF	3130	1720
SCF/GM-CSF	5650	3430
SCF/multi-CSF	6500	5060

Cultures contained 75,000 C57BL bone marrow cells and SCF (100 ng/ml) and/or the CSF indicated at 1×10^3 units/ml. After 7 days of incubation, mean colony cell counts were determined from pools of 50 sequentially harvested colonies.

These observations indicated that the combination of a CSF with SCF merely enhanced the size attained by blast colonies that would have developed with SCF alone and was unlikely to have induced the formation of another subset of blast colonies.

As shown (5), reculture of cells from SCF-stimulated blast cell colonies revealed that many of the colony cells were progenitor cells able to generate typical granulocyte–macrophage colonies in secondary cultures stimulated by the "all purpose" stimulus SCM. In control cultures of normal bone marrow, the SCM used was capable of stimulating the formation of granulocyte, granulocyte–macrophage, macrophage, eosinophil, and megakaryocyte colonies in the culture medium used. Because no erythropoietin was added to recipient cultures, no erythroid colonies developed and the present analysis was not able to determine whether erythroid progenitor cells might have been present in such colonies.

With each SCF/CSF combination, four types of recipient culture were used for each resuspended colony cell population—SCF, the CSF used in the combination, a combination of SCF with that CSF, and SCM. For the SCF/CSF combinations used, SCM stimulated the greatest number of colonies to be formed by resuspended blast colony cells and the data to follow were derived from such SCM-stimulated recipient cultures.

The progenitor cell content of individual colonies varied widely but averaged 96 for SCF-stimulated colonies and averaged from 313 to 586 for blast colonies stimulated by the various SCF/CSF combinations (Table 3). It should be noted that similar progenitor cell numbers were present in blast cell colonies stimulated either by G-CSF plus SCF or multi-CSF plus SCF. Comparison of these figures with the data in Table 2 indicates that the CSF-stimulated enlargement of blast colonies, while substantially increasing the total number of progenitor cells in colonies, also resulted in a decrease in the relative frequency of such cells in these colonies. However, a wide variation was observed between individual colonies in the content of progenitor cells with all stimuli used in the primary cultures.

Because G-CSF stimulates predominantly granulocyte colony formation (Table 1), it was surprising to find in blast colonies stimulated by the combination of G-CSF with SCF that the relative frequency of granulocyte-committed progenitor cells was not elevated and was slightly but not significantly lowered (Fig. 1). Conversely, as shown in Fig. 2, the frequency of macrophage-committed progenitors was slightly elevated. In contrast, combination of either GM-CSF or multi-CSF with SCF resulted in a significant elevation in the relative frequency of granulocyte progenitors in SCF-stimulated colonies (Fig. 1), a significant decrease in the frequency of macrophage progenitors (Fig. 2 and Table 3) and, for the multi-CSF/SCF combination, a significant in-

Table 3. Progenitor cell content of blast colonies stimulated by SCF with or without CSF

Stimulus	Colonies assayed, no.	Mean no. progenitors per colony	% total colonies				
			G	GM	M	Eo	G/Eo
SCF	63	96 ± 66	39 ± 22	13 ± 14	47 ± 32	0.5 ± 1.5	0.3 ± 1.5
SCF/G-CSF	40	544 ± 383	30 ± 28	10 ± 10	59 ± 31	1 ± 4	0.2 ± 0.7
SCF/GM-CSF	30	313 ± 221	55 ± 29*	6 ± 7	38 ± 30	1 ± 2	0
SCF/multi-CSF	36	586 ± 439	62 ± 27*	6 ± 6	25 ± 27*	7 ± 17*	0.6 ± 1.3

Sequentially harvested individual 7-day blast cell colonies were recultured in dishes containing 0.1 ml of SCM. Data shown are the calculated mean absolute progenitor cell content of the colonies assayed (±SD). The percent frequencies of the various colony types were determined from an analysis of all colonies in duplicate cultures stimulated by SCM (mean ± SD from the total number of blast colonies assayed). G, granulocyte; GM, granulocyte–macrophage; M, macrophage; Eo, eosinophil; G/Eo, mixed colonies of mature granulocytes and eosinophils.
*Significantly different from values from SCF-stimulated blast cell colonies ($P < 0.01$).

crease in the percentage of eosinophil progenitor cells (Table 3).

Calculation of the absolute number of progenitor cells in individual blast cell colonies showed that, although costimulation by each CSF significantly increased the total number of granulocyte progenitor cells, the combination of SCF with multi-CSF was the strongest stimulus for committed granulocyte and eosinophil progenitor cell formation. Conversely, the combination of SCF with G-CSF was the strongest stimulus for the formation of committed macrophage progenitor cells (Table 4).

There were several additional features of the recloning data worthy of note. Regardless of the stimulus used in the recloning cultures, no blast colonies or megakaryocyte colonies were detected in any culture of blast colony cells despite the facts that SCF and SCF/CSF combinations were used to stimulate secondary cultures of blast colony cells and that the multi-CSF and SCM used in the recloning of cultures were able to stimulate the formation of megakaryocyte colonies in control cultures of normal mouse marrow cells. Furthermore, most colonies developing in secondary cultures were only of medium or small size and, when a combined SCF/CSF stimulus was used, this did not cause the marked enhancement of colony size seen with such combinations in primary cultures. The secondary colonies developing were in almost all cases composed wholly of well-differentiated and readily recognizable cells. Finally, no abnormal combination of lineages was observed in secondary colonies, such as macrophage plus eosinophil cells. The only unusual colony type was composed of a mixture of mature granulocytes and eosinophils. This type was observed somewhat more commonly after the recloning of blast cell colonies stimulated by the SCF/multi-CSF combination. Colonies of this type do develop in low numbers in cultures of normal marrow cells and the progenitor cell concerned may be a distinct subset of hemopoietic progenitor cells.

DISCUSSION

Use of SCF (kit ligand or Steel factor) to stimulate cultures of normal mouse bone marrow cells has provided an opportunity to examine, in a reproducible manner, the formation and progenitor cell content of blast colonies. The recloning studies, even when using stimuli in recipient cultures capable of inducing blast colony formation, failed to detect blast-colony-forming cells in the blast colonies indicating that, at least under the culture conditions used, the type of blast-colony-forming cell studied had no capacity for self generation. This confirms the view that this type of blast-colony-forming cell is a relatively mature type of stem cell, with multipotentiality but little self-renewal capacity. The failure to detect progeny other than in the granulocyte–macrophage

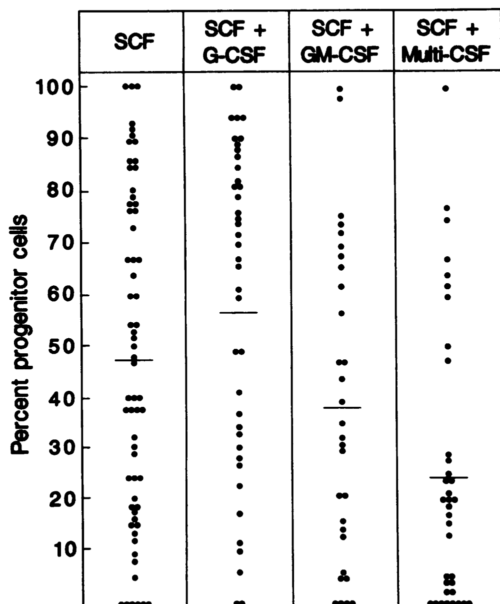

FIG. 1. Percent of granulocyte progenitor cells in progenitor cell populations present in 7-day blast colonies stimulated by SCF alone or a combination of SCF with G-CSF, GM-CSF, or multi-CSF. Each point represents data from a single colony in secondary cultures stimulated by SCM. Bars indicate mean values.

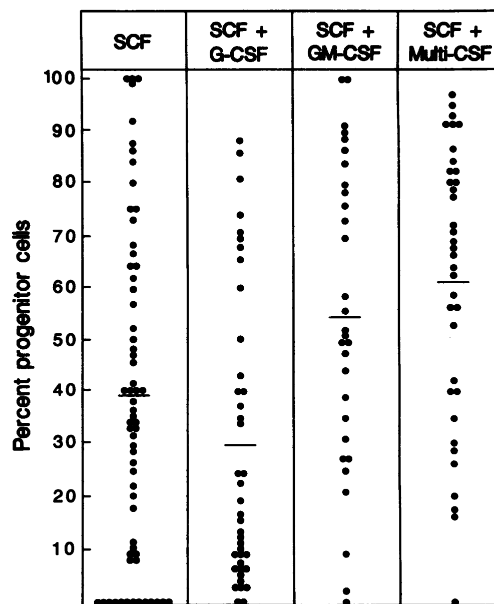

FIG. 2. Percent of macrophage progenitor cells in progenitor cell populations present in 7-day blast colonies stimulated by SCF alone or a combination of SCF with G-CSF, GM-CSF, or multi-CSF. Each point represents data from a single colony in secondary cultures stimulated by SCM. Bars indicate mean values.

Table 4. Absolute numbers of progenitor cells in blast colonies stimulated by SCF with or without CSF

Stimulus	Colonies assayed, no.	No. total progenitor cells per colony			
		G	GM	M	Eo
SCF	63	36 ± 39	12 ± 17	45 ± 45	0.6 ± 1.8
SCF/G-CSF	40	126 ± 127	65 ± 88	350 ± 313	2.3 ± 4.8
SCF/GM-CSF	30	149 ± 111	25 ± 42	137 ± 179	1.6 ± 4.6
SCF/multi-CSF	36	350 ± 332	37 ± 47	173 ± 260	12.0 ± 18.1

Total progenitor cell numbers in 7-day blast colonies were calculated (mean ± SD). G, granulocyte; GM, granulocyte–macrophage; M, macrophage; Eo, eosinophil.

and eosinophil lineages further suggests that these cells are not ancestors of megakaryocytes, and these latter cells may originate from distinct stem-cell subpopulations. This conclusion was supported by allowing blast colonies to remain in culture for 14 days, at which time again no megakaryocytes were detected in such colonies. No comment is possible from the present studies regarding the potential of these blast-colony-forming cells to generate erythroid, lymphoid, or mast cell progenitors. The cells forming the present blast colonies, therefore, probably need to be distinguished from those that form blast colonies developing megakaryocytes and erythroid cells and exhibiting self renewal (4, 8). These latter blast-colony-forming cells are less numerous and likely to be more ancestral than those analyzed in the present study.

Combination of SCF with each of the three CSFs clearly increased the size and progenitor cell content of the blast colonies developing but the data suggested that these colonies were substantially the same type of colonies that were stimulated to a lesser degree by SCF, when acting alone. As a similar CSF-induced size increase in SCF-stimulated colonies has been observed in cultures of enriched populations of stem cells (5, 9), the CSF enhancement appears to represent a direct action of the CSF on these cells. The system, therefore, offers the opportunity to explore the consequences of amplification of blast-colony populations by using CSFs with differing lineage specificity, at least as assessed by their actions in stimulating the proliferation of committed progenitor cells. Attempts were made also to use the selective macrophage stimulus, M-CSF, but difficulty in distinguishing M-CSF-induced multicentric macrophage colonies from blast colonies in unstained cultures prevented inclusion of this combination in the study.

The surprising result of the analysis of progenitor cells generated within blast colonies by combined SCF/CSF action was that the pattern of lineage commitment of the progenitor cells was not in agreement with expectations from the known actions of these CSFs on progenitor cells and their progeny. Specifically, G-CSF is virtually a granulocyte-specific proliferative stimulus (10) and was expected to lead to the generation of an unusually high proportion of granulocytic progenitors. An absolute increase in granulocyte progenitor cells was observed but not in the frequency of such cells relative to other progenitor cells. Similarly, GM-CSF is an active eosinophil proliferative stimulus (11) and might have been anticipated to have had some selective action on the formation of eosinophil-committed progenitor cells but this was not observed. From the present data, the categorization of these CSFs based only on their action on progenitor cells provides a misleading impression of their lineage-restricted activities. From their actions on slightly less mature populations, the CSFs exhibit somewhat different patterns of lineage activity with, notably, G-CSF being the best stimulus for the formation of macrophage-committed progenitors. This latter action may seem bizarre but has some support from two other known actions of G-CSF. G-CSF was shown to have the capacity to initiate the proliferation of both macrophage and granulocyte–macrophage progenitors but is unable to sustain their proliferation with the consequence that, by default, after 7 days of incubation the cultures characteristically contained only granulocytic colonies (10). Similarly, injection of G-CSF *in vivo* has been shown to greatly elevate blood levels of progenitor cells but, surprisingly, these progenitor cells were of all lineages (12). Although this might be based on an induced unselective release of preexisting progenitor cells from the marrow, it could again indicate a capacity of G-CSF when acting on stem cells to stimulate the generation of progenitor cells in a broader range of lineages.

If the assumption in these experiments is correct that the progeny of an equivalent set of blast-colony-forming cells was being analyzed, do the observed changes in the relative frequency of various progenitor cells indicate that the CSFs can influence differentiation commitment? Here, the comparison between G-CSF and multi-CSF is particularly revealing since the increase in absolute progenitor cell numbers was similar yet the relative frequencies of the various progenitor cells were significantly different. The data permit three formal alternative interpretations (*i*) selective differentiation commitment, (*ii*) unselective amplification of progenitor cell numbers with concomitant selective death of certain progenitors, or (*iii*) selective amplification of certain progenitors generated initially according to a fixed pattern of differentiation commitment.

Although the data do not allow an unequivocal choice among these three alternatives, there are two arguments against the latter two alternatives. (*i*) No cell death was observed in blast colonies yet cell death is easy to observe when occurring in hemopoietic colonies growing *in vitro* and such dying cells require 1–2 days to disappear. (*ii*) If selective amplification of certain progenitor cells had occurred, this should have resulted in a detectable reduction in the size of the colonies they generated during the secondary recloning cultures. In fact, no such size difference was apparent between secondary colonies initiated by SCF and G-CSF versus SCF and multi-CSF.

These observations favor the occurrence of induced differentiation commitment although the obvious heterogeneity in colony progenitor cell content in all groups would certainly indicate that directed commitment was not possible for all blast-colony-forming cells. Although the data fall short of unambiguous proof of induced differentiation commitment, they support data from the culture of multipotential cloned cell lines (13) that again are most simply explained by a process of regulator-induced differentiation commitment.

I am indebted to Dr. K. Zsebo and Dr. L. Souza of Amgen Biologicals (Thousand Oaks, CA) for generously supplying respectively the SCF and G-CSF used in these studies, to Dr. N. A. Nicola of this Institute for supplying the GM-CSF and Multi-CSF, and to Mrs. L. Di Rago and Miss S. Mifsud for careful technical assistance. This work was supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria, the National Health and Medical Research Council, Canberra, Australia and the National Institutes of Health, Bethesda, MD, Grants CA-22556 and CA-25972.

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