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Analysis in serum-free culture of the targets of recombinant human hemopoietic growth factors: Interleukin 3 and granulocyte/macrophage-colony-stimulating factor are specific for early developmental stages

(hemopoietic stem cells/clonal cell culture)

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ABSTRACT **We have used a serum-free culture system for enriched human hemopoietic progenitors to analyze the developmental stages and lineage specificities of the human hemopoietic colony-stimulating factors. None of the individual factors alone efficiently supported hemopoietic colony formation. Neither interleukin 3 nor granulocyte/macrophage-colony-stimulating factor alone or in combination effectively supported proliferation of progenitor cells. However, when combined with granulocyte-colony-stimulating factor or erythropoietin, these factors yielded neutrophil colonies or erythroid bursts, respectively. Serial observations of interleukin 3-supported cultures revealed sequential emergence and subsequent degeneration of clusters of cells. These observations suggest that the primary targets of interleukin 3 and granulocyte/macrophage-colony-stimulating factor are multipotent progenitors at the early stages of development rather than cells in the terminal process of maturation.**

During the past two decades, the mechanisms of differentiation and proliferation of hemopoietic stem cells have been actively investigated by clonal cell culture methods. Hemopoietic colony formation in culture requires polypeptide hormones termed colony-stimulating factors (CSFs). Recent progress in the molecular biology of CSFs has not only made available the majority of human CSFs in biosynthetic forms but has also revealed complex interactions between CSFs (interleukins) and cells (1, 2). For example, interleukin 1 and tumor necrosis factor α can stimulate the production of granulocyte/macrophage (GM)-CSF and granulocyte (G)-CSF by a variety of cell types including fibroblasts and endothelial cells (1). In turn, GM-CSF has been shown to enhance the secretion of tumor necrosis factor by monocytes stimulated with endotoxin (3). Therefore, when crude populations of marrow cells are plated in culture for studies of hemopoiesis, endogenous production of CSFs by the various types of cells renders interpretation of the data difficult. In addition, fetal bovine serum (FBS), which is present in the standard clonal cell culture media, most likely contains CSFs and other substances capable of modulating the effects of CSFs. For these reasons, we have endeavored to analyze the effects of the different CSFs in serum-free cultures of highly enriched populations of target progenitor cells plated at low cell density. In this system, 1000 bone marrow null cells typically yielded 20–50 colonies when cultured in the presence of a combination of interleukin 3 (IL-3), GM-CSF, G-CSF, macrophage (M)-CSF, and erythropoietin (Epo) (4). We have used this system to analyze in detail the formation of hemopoietic colonies in the presence of the individual CSFs or multiple combinations of these factors. Under these conditions, neither IL-3 nor GM-CSF supported efficient hemopoietic colony formation. However, in combination with the lineage-restricted factor G-CSF or Epo, significant colony formation was observed. These results indicate that the primary function of IL-3 and GM-CSF is to support the proliferation of progenitors during the early stages of hemopoietic development but not of the cells in the terminal process of maturation.

MATERIALS AND METHODS

Recombinant CSFs. Recombinant human IL-3 (5), G-CSF, M-CSF, and Epo were produced by COS cells that had been transfected with cDNAs encoding each CSF. GM-CSF was purified from medium conditioned by Chinese hamster ovary cells engineered to express human GM-CSF (6). Purified, bacterially produced recombinant human IL-3 was a generous gift of J. Seehra, P. Schendel, and J. McCoy (Genetics Institute). The IL-3 preparation was homogeneous and had a specific activity of 2×10^7 units (u)/mg (5).

Clonal Cell Culture. Bone marrow samples were aspirated from the posterior iliac crest of healthy adult volunteers. Mononuclear cells were prepared with Ficoll-Paque (Pharmacia) and the nonadherent cells were isolated by overnight adherence to plastic dishes. The nonadherent, mononuclear cell fractions were further enriched for null cells by use of nylon-wool-fiber columns (Wako Chemicals, Dallas) and rosette formation with neuraminidase-treated sheep erythrocytes. The marrow null-cell fractions usually contained <3% T lymphocytes and <5% B lymphocytes plus monocytes (4).

Serum-free culture was carried out in 35-mm Lux suspension-culture dishes (Miles) by a modification of the technique of Iscove *et al.* (7, 8). Details of the method are presented elsewhere (4). Bone marrow null cells (1000 per dish) were plated in 35-mm dishes containing attenuated α-medium (4), 1.2% (wt/vol) 15-poise (1.5 Pa·sec) methylcellulose (Fisher), 1% (wt/vol) fatty acid- and globulin-free bovine serum albumin (Sigma) that had been crystallized and deionized, 50 μM 2-mercaptoethanol (Eastman), 600 μg of fully iron-saturated human transferrin (≈98% pure, Sigma) per ml, 50 nM sodium selenite (Sigma), 10 μg of lecithin (Sigma) per ml, 6 μg of cholesterol (Sigma) per ml, and single or combinations of CSFs. Final concentrations of each CSF were as follows:

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Abbreviations: CSF, colony-stimulating factor; GM-CSF, granulocyte/macrophage-CSF; G-CSF, granulocyte-CSF; M-CSF, macrophage-CSF; IL-3, interleukin 3; Epo, erythropoietin; FBS, fetal bovine serum; u, unit(s).

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GM-CSF, 40 u/ml (9); IL-3, 1:5000 dilution of COS-cell supernatant or specified concentrations of IL-3; G-CSF, 1:1000 dilution of COS-cell supernatant; M-CSF, 1:1000 dilution of COS-cell supernatant; and Epo, 2 u/ml. The optimal dilutions of IL-3, G-CSF, and M-CSF had been determined by colony formation in methylcellulose culture by bone marrow mononuclear (nonadherent) cells (data not shown). Serum-containing culture was carried out also in methylcellulose by a modification (9) of the technique of Iscove *et al.* (10). Dishes were incubated at 37°C in a humidified atmosphere flushed with a mixture of 5% CO_2, 5% O_2, and 90% N_2.

All colonies were scored on day 14 of culture by *in situ* observation with an inverted microscope. In Tables 1–4, the colony types identified are abbreviated as follows: G, granulocyte colonies; M, macrophage colonies; GM, granulocyte/macrophage colonies; B, erythroid bursts; Eo, eosinophil colonies; EMeg, erythrocyte/megakaryocyte colonies; EEo, erythrocyte/eosinophil colonies (11); GEM, granulocyte/macrophage/erythrocyte colonies; Meg, megakaryocyte colonies. All colonies in experimental groups except for the CSFs groups were individually aspirated with a micropipette and analyzed for cell composition by May–Grünwald/Giemsa staining.

RESULTS

Colony Formation Supported by Human IL-3 or a Combination of CSFs. First, we plated 1000 bone marrow null cells per dish in the presence of IL-3 (1:5000 dilution of COS-cell supernatant) or a combination of CSFs including IL-3, GM-CSF, G-CSF, M-CSF, and Epo. On day 14, we analyzed colony formation (Table 1). The total number of colonies supported by a combination of CSFs in serum-free culture was ≈85% of that in serum-containing culture. In both serum-free and serum-containing cultures, colonies revealed a variety of lineages as shown previously (4). IL-3 alone in serum-free culture, however, failed to support colony formation except for one small granulocyte/macrophage colony and one eosinophil colony. In the same culture, however, there were several clusters containing fewer than 50 cells per colony. To characterize the nature of these clusters in more detail, we carried out serial observations of cluster formation in culture. In this experiment, dishes were examined on days 5, 7, 10, and 12 with an inverted microscope. When small clusters of cells were first identified, the areas of the clusters in the dish were recorded and the subsequent growth of the clusters was recorded. The results (Fig. 1) clearly depict a continuous turnover of the cluster populations. All clusters contained fewer than 50 cells. None of the clusters seen on day 5 were present on day 12. There was one cluster identified on day 7 that was still present on day 12. On day 12, only five clusters were present. When clusters of cells were picked and stained with May–Grünwald and Giemsa reagents, the majority of cells were immature, blast-like cells with open nuclear chromatin, nucleoli, and basophilic cytoplasm. Only a few neutrophilic promyelocytes were identified. We also replated 50 clusters identified on day 6 or 7 in culture into secondary cultures containing 30% serum, IL-3, and Epo. A total of 14 colonies developed, including 3 granulocyte colonies, 8 erythroid bursts, 2 eosinophil colonies, and 1 erythrocyte-containing mixed colony. This replating experiment demonstrated the viability of the cells in the clusters and indicated that the lack of colony formation in the presence of IL-3 alone was due to the absence of factors that are necessary for the terminal stages of differentiation.

Table 1. Colony formation by bone marrow null cells supported by IL-3 or combinations of CSFs

FBS, %	Growth factor(s)	No. of colonies								
		G	M	GM	B	Eo	EEo	GEM	Meg	Total
0	IL-3	0	0	1	0	1	0	0	0	2
	CSFs	9	3	15	13	3	0	1	0	44
30	CSFs	7	4	10	20	3	3	3	1	51

Data represent colony formation from 2000 marrow null cells. See *Materials and Methods* for colony-type abbreviations. CSFs consisted of IL-3, 1:5000 dilution; GM-CSF, 40 u/ml; G-CSF, 1:1000 dilution; M-CSF, 1:1000 dilution; and Epo, 2 u/ml.

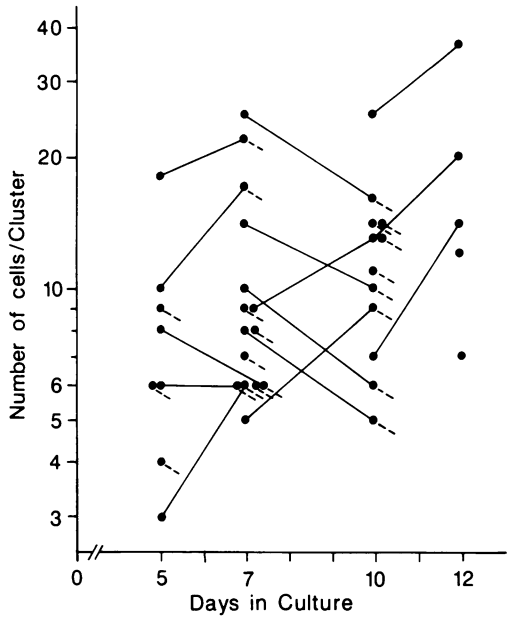

FIG. 1. Changes in the number of cells in clusters. Two dishes were examined, each containing 1000 bone marrow null cells and IL-3 in serum-free culture medium.

Colony Formation at Various IL-3 Concentrations. Although we used a plateau-range dilution of the IL-3-containing COS-cell supernatant, inhibitors may have been present in the conditioned medium and may have affected the colony formation. We therefore tested various concentrations of IL-3 for colony formation in serum-free cultures (Table 2). For this purpose, we used highly purified, bacterially derived, recombinant human IL-3. Concentrations of IL-3 between 1 u/ml and 100,000 u/ml supported formation of 1–6 colonies expressing only granulocyte/macrophage and eosinophil lineages from a total of 4000 null cells. In contrast, 102 colonies were supported by a combination of CSFs.

Colony Formation Supported by Individual CSFs Alone or in Combination with IL-3. Because the combination of CSFs yielded the highest plating efficiency of the target progenitor cells, we tested various factors alone and in combination with IL-3 to determine the relative contribution of each to the

Table 2. Colony formation in serum-free culture supported by various concentrations of purified IL-3

Growth factor(s)	Conc., u/ml	No. of colonies						
		G	M	GM	B	Eo	EEo	Total
IL-3	0.1	0	0	0	0	0	0	0
	1	0	0	0	0	1*	0	1
	10	0	0	0	0	0	0	0
	100	0	0	3	0	1	0	4
	1,000	0	0	0	0	2	0	2
	10,000	0	0	5	0	1	0	6
	100,000	0	0	4	0	0	0	4
CSFs	—	13	7	37	31	11	3	102

Data represent colony formation from 4000 bone marrow null cells. CSFs consisted of IL-3, GM-CSF, G-CSF, M-CSF, and Epo as described for Table 1.

*This colony was a mixed eosinophil/basophil colony (12).

observed colony formation (Table 3). Again, IL-3 alone supported only 7 small colonies in serum-free culture, whereas a combination of CSFs supported a total of 154 colonies of diverse types, including some mixed colonies. In serum-containing medium, IL-3 supported a total of 68 colonies, including many macrophage and eosinophil colonies. Similar to IL-3, GM-CSF alone supported only 5 eosinophil colonies in serum-free medium, compared to a total of 66 colonies of neutrophil, macrophage, and eosinophil lineages in serum-containing medium. Concentrations of GM-CSF up to 100,000 u/ml exhibited similar weak colony-forming ability (data not shown). The combination of IL-3 and GM-CSF also failed to support formation of many colonies. Only 8 small eosinophil colonies were seen in serum-free culture, in contrast to 73 colonies seen in serum-containing culture. These data suggest that neither IL-3 nor GM-CSF effectively supports the terminal process of progenitor maturation.

G-CSF in serum-free culture supported formation of 7 neutrophil colonies. The combination of G-CSF and IL-3 supported formation of 20 neutrophil colonies. In contrast to serum-free culture, there were 57 neutrophil colonies supported by G-CSF and 56 neutrophil colonies supported by the combination of IL-3 and G-CSF in serum-containing culture. These results are in accord with G-CSF being a terminally acting CSF that works synergistically with IL-3 to support formation of neutrophil colonies. In the absence of other factors, Epo alone failed to support erythroid burst formation in serum-free culture. We observed only a few erythroid colonies during days 5–7 because our method of deleting adherent cells and B cells also eliminates the majority of erythroid colony-forming units. The combination of Epo and IL-3 supported 19 erythroid bursts in serum-free culture compared to 63 erythroid bursts in serum-containing culture. In serum-free culture, M-CSF, either singly or in combination with IL-3, did not support macrophage colonies. In serum-containing culture, macrophage colonies were supported by M-CSF in the absence or presence of IL-3.

Colony Formation Supported by Combinations of CSFs. Using a different bone marrow sample, we again tested the effects of various combinations of factors on colony formation (Table 4). Similar to the data presented in Table 3, IL-3 or GM-CSF as single agents supported only eosinophil colonies in serum-free culture. Again, M-CSF was a poor stimulator of macrophage colony formation both singly and in combination with other factors. The combination of IL-3 and GM-CSF was not effective in supporting granulocyte/macrophage colony formation. In contrast, the number of neutrophil colonies supported by the combination of IL-3 and G-CSF was similar to that supported by combinations of CSFs. The combination of GM-CSF and G-CSF was also effective in support of neutrophil colonies in serum-free culture. The combination of IL-3, GM-CSF, and G-CSF did not show higher neutrophil colony-stimulating activity than that of GM-CSF plus G-CSF or IL-3 plus G-CSF. These data indicate that there is significant overlap between the neutrophil progenitors that are responsive to IL-3 and those responsive to GM-CSF. Combinations of terminally active, lineage-specific factors such as G-CSF and M-CSF, G-CSF and Epo, or M-CSF and Epo did not reveal synergy.

In agreement with the data presented in Table 3, IL-3 consistently exhibited burst-promoting activity for erythroid progenitors. GM-CSF plus Epo also supported erythroid bursts, but the number of bursts was less than half of that supported by IL-3 plus Epo. The number of bursts supported by the combination of IL-3, GM-CSF, and Epo was similar to that seen in cultures with IL-3 and Epo. These data are consistent with the idea that IL-3 is a more potent source of burst-promoting activity than GM-CSF. When combinations of four factors were tested, a combination of CSFs without M-CSF produced the highest number of colonies. Epo or G-CSF was required for formation of erythroid bursts or

Table 3. Colony formation supported by individual CSFs singly or in combination with IL-3

Growth factor(s)	No. of colonies								
	G	M	GM	B	Eo	EMeg	EEo	GEM	Total
Serum-free medium									
IL-3	2	0	1	0	4	0	0	0	7
GM-CSF	0	0	0	0	5	0	0	0	5
G-CSF	7	0	0	0	0	0	0	0	7
M-CSF	0	0	0	0	0	0	0	0	0
Epo	0	0	0	0	0	0	0	0	0
Mock CM	0	0	0	0	0	0	0	0	0
IL-3 + GM-CSF	0	0	0	0	8	0	0	0	8
IL-3 + G-CSF	20	0	1	0	2	0	0	0	23
IL-3 + M-CSF	0	0	0	0	3	0	0	0	3
IL-3 + Epo	0	0	0	19	0	0	0	0	19
CSFs	20	11	57	30	30	0	4	2	154
Medium with 30% FBS									
IL-3	2	28	12	0	26*	0	0	0	68
GM-CSF	13	26	10	0	17	0	0	0	66
G-CSF	57	21	5	0	0	0	0	0	83
M-CSF	0	13	0	0	0	0	0	0	13
Epo	0	4	0	11	0	0	0	0	15
Mock CM	0	4	0	0	0	0	0	0	4
IL-3 + GM-CSF	19	27	6	0	21	0	0	0	73
IL-3 + G-CSF	56	44	18	0	28*	0	0	0	146
IL-3 + M-CSF	2	48	7	0	13	0	0	0	70
IL-3 + Epo	1	42	7	63	25	0	3	2	143
CSFs	50	45	64	82	32	2	0	6	281

Data represent colony formation from 4000 bone marrow null cells. CSFs consisted of IL-3, GM-CSF, G-CSF, M-CSF, and Epo. Mock CM, conditioned medium from COS cells not transfected by a plasmid.

*Including eosinophil/basophil colonies or pure basophil colonies (12).

Table 4. Colony formation supported by individual CSFs or combinations of CSFs

Growth factors					No. of colonies								
IL-3	GM-CSF	G-CSF	M-CSF	Epo	G	M	GM	B	Eo	EMeg	EEo	GEM	Total
Serum-free medium													
+	−	−	−	−	0	0	0	0	5	0	0	0	5
−	+	−	−	−	0	0	0	0	4	0	0	0	4
−	−	+	−	−	11	0	0	0	0	0	0	0	11
−	−	−	+	−	0	0	0	0	0	0	0	0	0
−	−	−	−	+	0	0	0	0	0	0	0	0	0
−	−	−	−	−*	0	0	0	0	0	0	0	0	0
+	+	−	−	−	0	0	0	0	16	0	0	0	16
+	−	+	−	−	26	0	0	0	9	0	0	0	35
+	−	−	+	−	0	0	0	0	6	0	0	0	6
+	−	−	−	+	0	0	0	27	9†	0	2	0	38
−	+	+	−	−	19	1	14	0	4	0	0	0	38
−	+	−	+	−	0	2	1	0	5	0	0	0	8
−	+	−	−	+	0	0	0	12	3	0	0	0	15
−	−	+	+	−	6	0	0	0	0	0	0	0	6
−	−	+	−	+	9	0	0	0	0	0	0	0	9
−	−	−	+	+	0	0	0	1	0	0	0	0	1
+	+	+	−	−	27	1	20	0	15	0	0	0	63
+	+	−	+	−	0	0	0	0	11	0	0	0	11
+	+	−	−	+	0	0	0	31	11	0	4	0	46
+	−	+	−	+	26	0	0	23	7	0	1	0	57
+	−	−	+	+	0	0	0	26	5	0	1	0	32
−	+	+	+	+	22	0	18	12	10	0	0	0	62
+	−	+	+	+	26	0	2	30	13	0	4	0	75
+	+	−	+	+	0	0	1	46	13	0	3	0	63
+	+	+	−	+	23	1	17	42	15	0	4	0	102
+	+	+	+	−	24	2	26	0	16	0	0	0	68
+	+	+	+	+	25	4	28	41	15	0	3	1	117
Medium with 30% FBS													
+	+	+	+	+	28	48	41	66	24	1	2	1	211

Data represent colony formation from 4000 bone marrow null cells.
*Mock CM (see Table 3 legend).
†Including eosinophil/basophil colonies.

neutrophil colonies, again demonstrating the lineage-specific nature of these factors.

DISCUSSION

Many investigators have demonstrated that murine and human IL-3 support multilineage colony formation in serum-containing culture (9, 13–16). Based on these studies, it has been proposed that the spectrum of biological activities of IL-3 includes the terminal processes of maturation of all of the hemopoietic lineages, with the exception of the Epo-dependent maturation of erythroid progenitors (2). Here we have shown that IL-3 in serum-free culture without other growth factors results in a plating efficiency much less than 10% of that achieved with a combination of all of the factors. Our observations of these IL-3-supported cultures on days 5–12 demonstrated serial emergence and degeneration of small clusters of cells; IL-3 under these conditions apparently supported only a few cell divisions and could not maintain the viability of the later generations of progeny cells. However, when combined with the lineage-restricted factor G-CSF or Epo, IL-3 augmented the formation of neutrophil colonies or erythroid bursts, respectively. These results are compatible with a model in which IL-3 supports the proliferation of progenitors in the early stages of development but not the later stages of maturation. This interpretation is consistent with previous data from serum-containing cultures, which showed that multipotent progenitors found in blast-cell colonies become less sensitive to IL-3 as the colonies mature into multilineage colonies (17).

GM-CSF also proved to be ineffective in supporting colony formation and appeared to be incapable, by itself, of supporting the terminal maturation of granulocyte/macrophage progenitors. However, GM-CSF worked synergistically with G-CSF and Epo in supporting neutrophil and erythroid progenitor proliferation in culture. Similar effects of GM-CSF on erythroid progenitors have been observed in serum-containing cultures (9, 18, 19). Previous work (9, 20) showed that the targets of both human and murine GM-CSF include a population of multipotent progenitors capable of forming blast-cell colonies in culture, but the frequency of such GM-CSF-responsive cells is substantially less than the frequency of IL-3-responsive blast-cell-colony progenitors. Metcalf *et al.* (21) have also suggested that murine GM-CSF is capable of supporting several divisions of multipotent progenitors. Taken together, these observations indicate that GM-CSF serves primarily as a growth factor for multipotent progenitors that are intermediate in the hemopoietic pathway between the early cellular targets of IL-3 and those of the terminally acting CSFs such as G-CSF and Epo.

M-CSF, either as a single agent or in combination with other factors, proved ineffective in supporting macrophage proliferation and differentiation. In our serum-free culture system, M-CSF had little if any effect on colony formation in the presence of any combination of the other factors. This is consistent with the observations from many laboratories that indicate that human M-CSF is far more effective in generating murine macrophage colonies than it is in generating human macrophage colonies in serum-containing culture (1). Regulation of the proliferation of human monocyte/macrophage progenitors by M-CSF may require additional factors.

In our serum-free culture system, G-CSF and Epo acted synergistically with IL-3 or GM-CSF in support of neutrophil colony and erythroid burst formation, respectively. These observations are consistent with a model in which G-CSF is a neutrophil lineage-specific regulator that acts primarily on the terminal stages of neutrophil development and the well-established model that Epo is a physiological regulator of erythropoiesis that acts primarily at the late stages of erythroid maturation (22–24). The lineage specificity of both G-CSF and Epo was further demonstrated in serum-free culture studies with four factor combinations; omission of G-CSF or Epo eliminated the neutrophil or erythroid colonies from these cultures.

The demonstration that the individual CSFs are more restricted in action than originally thought has important implications for the potential clinical uses of these molecules. If each of the different factors acts differently with different target cells, it seems likely that the most potent stimulation of hemopoiesis *in vivo* would be provided by a combination of all of the CSFs. While this prediction provides a major challenge by requiring the determination of the optimal relative amounts of each factor to be used in particular clinical indications, it also suggests that by appropriate manipulations it may be possible to selectively regulate the levels of the different blood-cell types for particular medical problems.

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