Synergism of Interferon-γ and Stem Cell Factor on the Development of Murine Hematopoietic Progenitors in Serum-Free Culture

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We examined the effects of interferon-γ (IFN-γ) on the growth of murine hematopoietic progenitors supported by interleukin-3 (IL-3) or stem cell factor (SCF) in a serum-free culture system. IFN-γ inhibited IL-3–dependent granulocyte-macrophage colony growth by normal bone marrow cells, but increased the number of pure and mixed megakaryocyte colonies by post–5-fluorouracil bone marrow cells. The addition of IFN-γ to the culture containing SCF resulted in a synergistic action on the development of primitive hematopoietic progenitors as well as on the development of mature populations. Primitive progenitors responding to SCF + IFN-γ were suggested to be supported by SCF in the early stage of development and require IFN-γ for subsequent growth. Replating experiments of blast cell colonies and comparison of total colony growth among SCF + IFN-γ, SCF + IL-3, and SCF + IFN-γ + IL-3 suggest that multipotential progenitors supported by SCF + IFN-γ are a part of those reactive to SCF + IL-3. These findings suggest that IFN-γ has bifunctional activity on murine hematopoiesis.

NORMAL HEMATOPOIESIS is regulated under a complex network consisting of stimulatory and inhibitory molecules. Interleukin-3 (IL-3) can directly act on the proliferation and differentiation of multipotential hematopoietic, as well as committed, progenitors.[1] Other cytokines, including IL-6 and granulocyte colony-stimulating factor (G-CSF), have been demonstrated to interact with IL-3 on the growth of primitive progenitors, whereas they fail to support colony growth by themselves.[2-5] Recently, stem cell factor (SCF), the ligand for the c-*kit* tyrosine kinase receptor, has been demonstrated to be constitutively produced by stromal cell layers.[6-8] This cytokine also acts synergistically with a variety of cytokines, including IL-3, IL-6, and G-CSF, on the development of early hematopoietic progenitors.[9-15]

Interferon-γ (IFN-γ) is a cytokine produced by activated T cells and natural killer cells in response to viral infection or other stimuli, and plays an important role in many different immune responses. Numerous investigators have demonstrated that it acts as a suppressor on hematopoietic progenitors.[16-20] Our previous studies[21] have provided preliminary evidence that IFN-γ inhibited G-CSF– and IL-3–dependent colony growth by granulocyte-macrophage progenitors derived from the bone marrow cells (BMC) of normal mice, but it significantly augmented the numbers of single and mixed megakaryocyte colonies supported by IL-3 derived from post–5-fluorouracil (post–5-FU) BMC in serum-containing culture.

Antagonistic effects are also seen in IL-4 and transforming growth factor-β (TGF-β). IL-4 enhances the formation of G-CSF–dependent granulocyte colonies and erythropoietin (Epo)-dependent erythroid and multilineage colonies,[22] and also acts synergistically with IL-6 or IL-11 on the development of primitive hematopoietic progenitors.[23,24] On the contrary, IL-4 inhibits the formation of GM colonies supported by IL-3,[25] and that of macrophage colonies supported by granulocyte-macrophage–CSF (GM-CSF) or macrophage-CSF (M-CSF).[26] Keller et al[27] have demonstrated that TGF-β suppresses the IL-3–dependent growth of multipotential hematopoietic progenitors, whereas a combination of TGF-β and GM-CSF increases the number of colonies formed by the BMC of normal and 5-FU–treated mice. In addition, TGF-β1 has been shown to markedly enhance the growth of macrophages in response to GM-CSF, but inhibits their responsiveness to M-CSF.[28] These observations suggest that potent negative regulators are not always inhibitors of hematopoietic progenitors.

In this study, we examined the effect of IFN-γ on the proliferation and differentiation of murine hematopoietic progenitors in response to IL-3 or SCF using serum-free culture system. The findings showed that IFN-γ inhibits IL-3–dependent GM colony growth by BMC of normal mice, but acts synergistically with SCF on the growth of primitive hematopoietic progenitors as well as mature progenitors, which suggests a bifunctional potential of IFN-γ on murine hematopoietic progenitors.

MATERIALS AND METHODS

Cell preparation. Female BDF_1 mice, 10 to 15 weeks old, were obtained from Shizuoka Experimental Animal Center (Shizuoka, Japan). BMC were prepared in α-medium (Flow Laboratories, Inc, Rockville, MD). 5-FU (Kyowa Hakko Kogyo Co, Tokyo, Japan) was administered through the tail vein of the mice at a dose of 150 mg/kg. BMC were harvested 2 days after 5-FU injection.

Purification of hematopoietic progenitors. To reduce the influence of accessory cells, we purified hematopoietic progenitors by negative selection. BMC were flushed from the femur of the mice with α-medium. Lineage marker-negative (lin^-) populations were obtained by depletion of lineage marker-positive cells with the cocktail of monoclonal antibodies (MoAbs) specific for L3T4, Lyt-2, B220, Gr-1, and Ly-6C (each purchased from PharMingen, San Diego, CA); Mac-1 (Serotec, Oxford, UK); and immunomagnetic beads (Dynabeads M-450 coated with sheep antirat Ig G; Dynal A.S., Oslo, Norway) by using a modification of the method of described previously.[29]

CSFs and antibody. Recombinant murine SCF was generously given to us by Dr Junji Nakao (Laboratory of Molecular Genetics, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan).

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Submitted July 31, 1992; accepted November 3, 1992.

Supported by a grant (3-24) from the Ministry of Health and Welfare, and grants from the Ministry of Education, Science, and Culture, Japan.

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0006-4971/93/8106-0028$3.00/0

It was prepared by expressing a cDNA for SCF in yeast cells, followed by further purification, according to a method described previously.[7] Murine recombinant IL-3 synthesized in yeast cells was a gift from Dr Ken-ichi Arai (DNAX, Research Institute of Molecular and Cellular Biology, Palo Alto, CA). Human recombinant G-CSF, with a specific activity of 1×10^8 U/mg protein, was provided by Chugai Pharmaceutical Co (Tokyo, Japan). Human recombinant IL-6, with a specific activity of 3.6×10^6 U/mg protein,[30] was generously provided by Dr Akira Okano and Dr Yukio Akiyama (Ajinomoto Co, Inc, Kawasaki, Japan). Murine recombinant IFN-γ was purchased from Genzyme Co (Boston, MA; 1 U of IFN-γ approximates the bioactivity contained in 1 U of the National Institutes of Health [NIH] standard IFN-γ preparation). Because the reagent contained 0.025% sodium azide as a preservative, it was reconstituted in 2 mL α-medium containing 0.1% crystallized globulin-free deionized bovine serum albumin (BSA; Sigma Chemical Co, St Louis, MO), dialyzed in 500 mL α-medium three times (8 hours each time) at 4°C, and filtered (0.22-μm filter; #SLGV025LS; Japan Millipore Ltd, Tokyo, Japan). Dialysis did not alter the inhibitory effect of IFN-γ (data not shown). Rat antimouse IFN-γ MoAb was purchased from Lee Biomolecular Research Laboratories Inc (San Diego, CA). Antimouse IFN-γ was quantified by a comparative assay with the NIAID antimouse IFN-γ reference reagent.

Clonal cell cultures. Serum-free culture was performed in 35-mm suspension culture dishes (#171099; Nunc Inc, Naperville, IL) using the technique described previously.[5] Unless otherwise specified, the culture consisted of 2×10^4 unseparated or 5×10^3 lin^- BMC of normal mice, and 5×10^4 unseparated or 2×10^3 lin^- BMC of 5-FU–treated mice, α-medium, 0.9% methylcellulose (Shinetsu Chemical Co, Tokyo, Japan), 1% deionized crystallized BSA, 10^{-4} mol/L 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, NY), 300 μg/mL of fully iron-saturated human transferrin (approximately 98% pure; Sigma), 160 μg/mL of soybean lecithin (Sigma), 96 μg/mL of cholesterol (Nacalai Tesque, Inc, Kyoto, Japan), IL-3 (200 U/mL), IL-6 (80 ng/mL), G-CSF (100 ng/mL), SCF (100 ng/mL), and IFN-γ (1,000 U/mL), alone or in combination. The concentrations of hematopoietic factors except IFN-γ were shown to be optimal for colony growth by titration studies.[3,31,32] The standard cultures contained 30% fetal bovine serum (FBS; Hyclone Laboratories, Inc, Logan, UT) instead of a combination of transferrin, lecithin, and cholesterol. Plates were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air.

Determination of colony types and sizes. Colony types were determined by in situ observation on an inverted microscope. We used "mapping studies" to determine the full potential of the colonies.[31] When a new colony appeared, its location on the plates was recorded, and subsequent growth and differentiation were observed every day. When it appeared to have matured, the colony type was classified according to the criteria described previously.[33] All colonies that had emerged until day 16 were enumerated. Abbreviations for the colony types are: GM, granulocyte-macrophage colonies; M, megakaryocyte colonies; GEM, granulocyte-erythrocyte-macrophage colonies; GMM, granulocyte-macrophage-megakaryocyte colonies; GEMM, granulocyte-erythrocyte-macrophage-megakaryocyte colonies; and BLAST, blast cell colonies. M colonies were scored when they had four or more megakaryocytes, and the other types of colonies were defined as aggregates of more than 50 cells.

M and GMM colonies were identified in situ with their typical features of containing large cells that had nonglanular, translucent cytoplasm and highly refractile cell membrane, as shown previously.[5,34,35] To assess the accuracy of in situ identification of M and GMM colonies, individual colonies were lifted with an Eppendorf micropipette under direct microscopic visualization, spread on glass slides by a cytocentrifuge (Cytospin II; Shandon Southern, Sewickley, PA), and stained with May-Grünwald-Giemsa and acetylcholine esterase (AChE) staining. Morphologic examination of GMM colonies showed the coexistance of megakaryocytes, granulocytes, and/or macrophages. Subsequently, M and GMM colonies were identified in situ using an inverted microscope and were stained only for confirmation and special purposes.

Replating experiments. To determine the full potential of blast cell colonies, we performed replating experiments of individual blast cell colonies grown in cultures of lin^- BMC from 5-FU–treated mice with SCF + IL-3 or SCF + IFN-γ. Individual blast cell colonies were picked with an Eppendorf micropipette and plated into secondary cultures containing 200 U/mL IL-3, 2 U/mL Epo, and 30% FBS. Replated cells were incubated, and secondary colonies were scored in the same manner as primary cultures.

Statistical analysis. All experiments were performed at least twice and were shown to be reproducible. The data are expressed as the mean ± SD of quadruplicate plates containing BMC of normal or 5-FU–treated mice. The probability of significant differences was determined according to the Student's *t*-test. Statistical analysis in colony size was performed using the Student's *t*-test on logarithms of the cell numbers per individual colonies.

RESULTS

Effects of IFN-γ on IL-3–dependent colony growth by BMC of normal and 5-FU–treated mice. A dose response study of IFN-γ showed that IL-3–dependent GM colony growth derived from BMC of normal mice was maximally inhibited by IFN-γ at higher than 1,000 U/mL in serum-containing

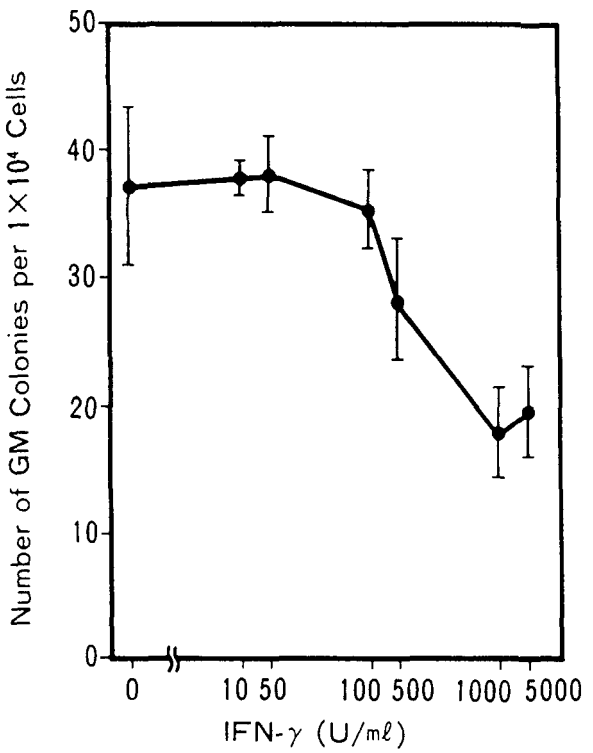

Fig 1. Dose response of IL-3–dependent GM colony growth to IFN-γ. BMC (1×10^4) of normal mice were plated in a culture dish containing 200 U/mL of IL-3 and IFN-γ, in concentrations ranging from 10 to 5,000 U/mL.

Table 1. Effect of IL-3 and/or IFN-γ on Colony Growth by Normal or Post–5-FU BMC

Colony Type	BMC of Normal Mice			BMC of 5-FU–Treated Mice		
	IL-3	IFN-γ	IL-3 + IFN-γ	IL-3	IFN-γ	IL-3 + IFN-γ
GM	63.5 ± 7.0	0	32.8 ± 6.4*	3.3 ± 1.3	0	3.5 ± 0.5
M	2.8 ± 1.3	0	3.5 ± 2.1	1.0 ± 0.7	0	4.3 ± 0.8*
GMM	5.5 ± 1.1	0	7.3 ± 3.4	4.0 ± 1.6	0	11.8 ± 1.9*
BLAST	2.8 ± 1.3	0	3.5 ± 1.1	10.5 ± 0.5	0	8.8 ± 1.5
Total	74.6 ± 7.2	0	47.1 ± 1.0*	18.8 ± 1.3	0	28.3 ± 1.3†

Data represent the mean ± SD of quadruplicate plates containing 2×10^4 and 5×10^4 BMC of normal and 5-FU–treated mice, respectively. IL-3 was used at 200 U/mL; IFN-γ was used at 1,000 U/mL.

* Significantly different from IL-3 alone ($P < .01$).

† Significantly different from IL-3 alone ($P < .001$).

culture (Fig 1). Thus, IFN-γ at 1,000 U/mL was used in the subsequent studies.

We investigated the effects of IFN-γ on the growth of different types of colonies supported by IL-3 at 200 U/mL derived from BMC of normal mice in a serum-free culture system, because FBS is potentially an endogenous source of various types of hematopoietic factors. Data presented in Table 1 are representative of four independent experiments. The addition of IFN-γ to the culture containing IL-3 reduced GM colony growth by approximately 50%, whereas the numbers of M, GMM, and BLAST colonies were not influenced. IFN-γ exerted inhibitory effects on the size, as well as the number, of GM colonies supported by IL-3 (data not shown). There was no colony growth in culture dishes containing IFN-γ alone.

To examine the effects of IFN-γ on the development of primitive hematopoietic progenitors in the dormant cell cycle state, we used BMC of 5-FU–treated mice. In contrast to the BMC of normal mice, the growth of the GM colonies was not inhibited by the addition of IFN-γ. It is of interest that the addition of IFN-γ to culture containing IL-3 significantly enhanced the growth of M and GMM colonies. These findings were assessed in four repeated experiments.

Synergistic effect of SCF and IFN-γ on the development of hematopoietic progenitors from normal and post–5-FU BMC. SCF has been demonstrated to interact with a variety of hematopoietic factors on the development of the early stages of hematopoietic progenitors as well as that of mature populations.[9-15] Therefore, we examined whether IFN-γ influenced colony growth supported by SCF using unseparated

Table 2. Synergistic Effect of SCF and IFN-γ on Colony Growth by Normal or Post–5-FU BMC

	GM	M	GMM	BLAST	Total
(A1) Unseparated normal BMC					
SCF	23.3 ± 2.5	0	0	3.3 ± 1.6	26.6 ± 1.2
IFN-γ	0	0	0	0	0
SCF + IFN-γ	31.5 ± 2.7*	0	0.3 ± 0.4	9.0 ± 1.6†	40.8 ± 3.6‡
IL-3	63.5 ± 7.0	2.8 ± 1.3	5.5 ± 1.1	2.8 ± 1.3	74.6 ± 7.5
(A2) Lin^- normal BMC					
SCF	29.3 ± 5.2	0	0	5.3 ± 1.6	34.6 ± 5.2
IFN-γ	0	0	0	0	0
SCF + IFN-γ	61.3 ± 3.3‡	0	0	21.8 ± 2.9‡	83.1 ± 4.3‡
IL-3	66.8 ± 7.2	2.8 ± 1.5	3.8 ± 0.8	9.3 ± 3.1	82.7 ± 7.0
(B1) Unseparated post–5-FU BMC					
SCF	0	0	0	1.3 ± 0.8	1.3 ± 0.8
IFN-γ	0	0	0	0	0
SCF + IFN-γ	0	0	0	10.0 ± 1.9‡	10.0 ± 1.9‡
IL-3	0	0	0.3 ± 0.4	0.5 ± 0.5	0.8 ± 0.4
SCF + IL-3	8.0 ± 1.6†	1.3 ± 1.1	9.5 ± 2.1†	1.5 ± 1.1	20.3 ± 2.9‡
(B2) Lin^- post–5-FU BMC					
SCF	0	0	0	4.9 ± 1.0	4.9 ± 1.0
IFN-γ	0	0	0	0	0
SCF + IFN-γ	0	0	0	14.0 ± 0.7‡	14.0 ± 0.7‡
IL-3	0.3 ± 0.5	0.7 ± 0.5	0	3.0 ± 1.6	4.0 ± 0.8
SCF + IL-3	3.8 ± 1.5†	2.8 ± 1.5	26.3 ± 3.4‡	2.0 ± 1.4	34.9 ± 1.5‡

Data represent the mean ± SD of quadruplicate plates containing 2×10^4 unseparated or 5×10^3 lin^- BMC of normal, and 5×10^4 unseparated or 2×10^3 lin^- BMC of 5-FU–treated mice. IL-3 was used at 200 U/mL; SCF was used at 100 ng/mL; IFN-γ was used at 1,000 U/mL.

* Significantly different from SCF or IL-3 ($P < .05$).

† Significantly different from SCF or IL-3 ($P < .01$).

‡ Significantly different from SCF or IL-3 ($P < .001$).

Table 3. Effect of Anti–IFN-γ Antibody on Colony Growth by BMC of 5-FU–Treated Mice

	GM	M	GMM	BLAST	Total
SCF	0	0	0	1.3 ± 0.4	1.3 ± 0.4
SCF + anti–IFN-γ	0	0	0	2.0 ± 1.1	2.0 ± 1.1
SCF + IL-3	3.0 ± 1.2	3.5 ± 0.9	4.8 ± 1.1	2.3 ± 1.3	14.0 ± 3.0
SCF + IL-3 + anti–IFN-γ	2.0 ± 1.2	2.5 ± 0.5	6.5 ± 1.7	1.8 ± 0.8	12.8 ± 2.2
SCF + IFN-γ	0	0	0	8.9 ± 1.8	8.9 ± 1.8
SCF + IFN-γ + anti–IFN-γ	0	0	0	2.8 ± 1.9*	2.8 ± 1.9*

Data represent the mean ± SD of quadruplicate plates containing 5×10^4 BMC of 5-FU–treated mice. IL-3 was used at 200 U/mL; SCF was used at 100 ng/mL; IFN-γ was used at 1,000 U/mL; anti–IFN-γ was used at 1,000 neutralizing U/mL.

* Significantly different from SCF + IFN-γ ($P < .01$).

and lin⁻ BMC of normal and 5-FU–treated mice. As presented in Table 2 (A1), SCF supported the growth of GM and BLAST colonies by BMC of normal mice. Addition of IFN-γ to a culture containing SCF caused a significant increase in numbers of GM and BLAST colonies compared with SCF alone ($P < .05$ and $P < .01$, respectively). Depletion of accessory cells from BMC of normal mice retained the synergistic action by two factors (A-2). The combination of SCF and IFN-γ was not capable of supporting the growth of megakaryocytic colonies. These findings were assessed in four repeated experiments.

As shown in Table 2 (B-1 and -2), the combination of SCF and IFN-γ also exhibited a synergism on the growth of primitive hematopoietic progenitors derived from both unseparated and lin⁻ BMC of 5-FU–treated mice. These findings were assessed in four repeated experiments. The number of total colonies grown by SCF + IFN-γ was approximately one-half of that supported by SCF + IL-3. Day-16 colonies formed by SCF + IFN-γ consisted mostly of blasts, whereas those grown by SCF + IL-3 were composed of neutrophils, macrophages, and megakaryocytes as well as a small percentage of blasts, determined by the preparation stained with May-Grünwald-Giemsa. Next, we examined whether the monoclonal rat anti–IFN-γ antibody neutralized the stimulatory effect of IFN-γ. Anti–IFN-γ antibody was added to culture dishes containing BMC of 5-FU–treated mice with IL-3, SCF + IL-3, or SCF + IFN-γ. The addition of anti–IFN-γ antibody to culture containing SCF + IFN-γ reduced BLAST colony formation to the level seen in culture with SCF alone. However, the colony growth supported by SCF or SCF + IL-3 was not influenced by the addition of the antibody (Table 3). These findings suggest that IFN-γ, rather than contaminants of the preparation of IFN-γ, is the effector molecule in stimulation of BLAST colony growth. When we estimated the number of constituent cells of colonies that did not change in size for 3 days, there was a significant difference between SCF + IFN-γ and SCF + IL-3 ($P < .001$). The mean number of cells per 25 individual colonies was 6,017 ± 4,504 cells per colony in SCF + IFN-γ, and 14,790 ± 5,233 cells per colony in SCF + IL-3.

The question was raised that hematopoietic progenitors responding to SCF + IFN-γ were different from those grown by SCF + IL-3. Accordingly, we seeded 2×10^3 lin⁻ post–5-FU BMC in culture dishes containing SCF, SCF + IFN-γ, SCF + IL-3, or SCF + IFN-γ + IL-3. This experiment was performed twice, and similar results were obtained both times. As presented in Table 4, the total number of colonies grown by SCF + IL-3 was significantly greater than the total colony growth by SCF + IFN-γ. However, the combination of SCF, IFN-γ, and IL-3 did not increase the total number of colonies to exceed that supported by SCF + IL-3. This observation suggests that primitive hematopoietic progenitors supported by SCF + IFN-γ overlap with those responding to SCF + IL-3.

Effect of delayed addition of SCF or IFN-γ on colony growth by post–5-FU BMC. We examined whether the primitive progenitors required the two factors simultaneously at the beginning of culture. Lin⁻ post–5-FU BMC (2×10^3) were plated in a culture dish containing SCF or IFN-γ. The other factor was added on a subsequent day of incubation. Table 5 shows the results. This experiment was performed twice, and similar results were obtained both times. When IFN-γ was added on day 4 or day 7 to a culture initiated with SCF, the augmentation of total colony growth was preserved. Synergistic effect of IFN-γ was no longer seen after day 10. On the other hand, delayed addition of SCF to cultures initiated with IFN-γ on day 4 or day 7 allowed no colony

Table 4. Combined Effects of SCF, IFN-γ, and/or IL-3 on Colony Growth by Lin⁻ Post–5-FU BMC

Factor	GM	M	GMM	BLAST	Total
SCF	0	0	0	1.3 ± 0.8	1.3 ± 0.8
SCF + IFN-γ	0	0	0	7.8 ± 1.1	7.8 ± 1.1
SCF + IL-3	4.0 ± 1.2	3.3 ± 0.8	5.5 ± 1.8	2.0 ± 1.2	14.8 ± 3.0
SCF + IFN-γ + IL-3	2.8 ± 1.5	2.5 ± 0.5	6.5 ± 1.7	1.5 ± 0.5	13.3 ± 2.2

Data represent the mean ± SD of quadruplicate plates containing 2×10^3 lin⁻ BMC of 5-FU–treated mice. IL-3 was used at 200 U/mL; SCF was used at 100 ng/mL; IFN-γ was used at 1,000 U/mL. Total number of colonies supported by SCF + IL-3 is significantly greater than that supported by SCF + IFN-γ ($P < .01$), but there is no significant difference in total colony growth supported by SCF + IL-3 and SCF + IFN-γ + IL-3.

Table 5. Effect of Delayed Addition of SCF and/or IFN-γ on Colony Growth by Post–5-FU BMC

Addition of Factors						No. of Total Colonies per Dish
Day 0	Day 4	Day 7	Day 10	Day 13	Day 16	
SCF						6.9 ± 1.2
IFN-γ						0
SCF + IFN-γ						23.4 ± 1.9*
SCF	IFN-γ					18.6 ± 1.2*
SCF		IFN-γ				11.4 ± 1.1†
SCF			IFN-γ			9.4 ± 1.7
SCF				IFN-γ		6.6 ± 1.2
SCF					IFN-γ	7.0 ± 1.6
IFN-γ	SCF					0
IFN-γ		SCF				0
None	SCF + IFN-γ					0
None		SCF + IFN-γ				0

Data represent the mean ± SD of quadruplicate plates containing 2×10^3 lin^- BMC of 5-FU–treated mice. SCF was used at 100 ng/mL; IFN-γ was used at 1,000 U/mL.

* Significantly different from SCF + IFN-γ ($P < .001$).

† Significantly different from SCF + IFN-γ ($P < .01$).

growth. A delay in the addition of the two factors to an uninitiated culture resulted in no colony formation.

Synergism of IFN-γ with SCF, but not with IL-6 or G-CSF. Because both IL-6 and G-CSF have been reported to augment SCF-dependent colony growth by primitive hematopoietic progenitors,[6,10,11] we examined whether these factors interacted with IFN-γ. Two thousand lin^- BMC of 5-FU–treated mice were seeded in culture dishes containing SCF, IFN-γ, IL-6, and G-CSF, alone or in combination. Data presented in Table 6 are representative of four independent experiments. The combination of IL-6 or G-CSF with SCF gave rise to a significant increase in the number of total colonies compared with those supported by SCF alone. However, neither IL-6 + IFN-γ nor G-CSF + IFN-γ supported colony growth.

Replating experiments of blast cell colonies grown by SCF + IFN-γ. To determine the potential of blast cell colonies supported by SCF + IFN-γ, we performed replating experiments of individual blast cell colonies derived from lin^- BMC of 5-FU–treated mice, and compared them with the replating ability of blast cell colonies grown by SCF + IL-3. Blast cell colonies consisting of 152 ± 57 (range, 79 to 300) cells supported by SCF + IFN-γ and those consisting of 171 ± 51 (range, 103 to 253) cells supported by SCF + IL-3 were randomly selected on day 10 and day 7, respectively, because blast cell colonies emerged earlier in the culture containing SCF and IL-3 than in the culture containing SCF and IFN-γ. They were then individually replated into secondary culture containing 200 U/mL of IL-3, 2 U/mL of Epo, and 30% FBS (Table 7). Blast cell colonies formed by SCF + IFN-γ showed variable but high replating efficiencies comparable to those supported by SCF + IL-3 (44.6% ± 16.2% and 49.8% ± 18.6%, respectively). However, the percentages of GM and multilineage colonies (the sum of GEM, GMM, and GEMM colonies) in secondary colonies were statistically different between blast cell colonies formed by SCF + IFN-γ and those by SCF + IL-3. The percentage of secondary GM colonies was 72.5% ± 34.1% in SCF + IFN-γ and 44.3% ± 21.8% in SCF + IL-3 ($P < .05$), whereas the percentage of secondary, multilineage colonies was 22.8% ± 29.1% in SCF + IFN-γ and 50.8% ± 20.7% in SCF + IL-3 ($P < .05$).

DISCUSSION

Several investigators have demonstrated the inhibitory activity of IFN-γ on the growth of GM progenitors.[16-20] The present study showed that IFN-γ suppressed IL-3–dependent GM colony growth, but failed to inhibit the formation of M and GMM colonies by BMC of normal mice. On the other hand, in post–5-FU BMC, GM colony number was not influenced and numbers of M and GMM colonies were significantly enhanced by the addition of IFN-γ to culture with IL-3. These findings suggest that the different responses to IFN-γ depend on the type and maturation stage of the hematopoietic progenitors. Because we used a crude population, it is possible that factors, including IL-6, shown to act synergistically with IL-3 on the development of the primitive progenitors capable of megakaryocytic lineage expression,[5] are endogenously produced by coexisting nonhematopoietic cells in response to IFN-γ. Accordingly, we used polyclonal rabbit anti–IL-6 antibody to test this possibility.[5] The addition

Table 6. Combined Effect of IFN-γ With IL-6 or G-CSF on Colony Growth by Lin^- Post–5-FU BMC

Factor	GM	M	GMM	BLAST	Total
SCF	0	0	0	1.0 ± 0	1.0 ± 0
IFN-γ	0	0	0	0	0
SCF + IFN-γ	0	0	0	11.3 ± 2.3*	11.3 ± 2.3*
IL-6	0	0	0	0	0
IL-6 + SCF	2.8 ± 1.5†	4.0 ± 1.7†	5.3 ± 0.8*	36.8 ± 2.8*	48.8 ± 2.2‡
IL-6 + IFN-γ	0	0	0	0	0
G-CSF	0	0	0	0	0
G-CSF + SCF	4.5 ± 1.5§	0	0	41.0 ± 3.3‡	45.5 ± 2.7‡
G-CSF + IFN-γ	0	0	0	0	0

Data represent the mean ± SD of quadruplicate plates containing 2×10^3 lin^- BMC of 5-FU–treated mice. SCF was used at 100 ng/mL; IFN-γ was used at 1,000 U/mL; IL-6 was used at 80 ng/mL; G-CSF was used at 100 ng/mL.

* Significantly different from SCF alone ($P < .01$).

† Significantly different from SCF alone ($P < .05$).

‡ Significantly different from SCF alone ($P < .001$).

§ Significantly different from SCF alone ($P < .02$).

Table 7. Replating Experiment of Blast Cell Colonies Supported by SCF + IFN-γ or SCF + IL-3

No. of Cells per Colony	Total Replating Efficiency (%)	Secondary GM Colonies per Total Secondary Colonies (%)	Secondary Multilineage Colonies per Total Secondary Colonies (%)
SCF + IFN-γ			
152 ± 57	44.6 ± 16.2	72.5 ± 34.1	22.8 ± 29.1
SCF + IL-3			
171 ± 51	49.8 ± 18.6	44.3 ± 21.8	50.8 ± 20.7

Twelve blast cell colonies supported by SCF + IFN-γ or SCF + IL-3 were selected at random on day 10 and day 7, respectively, and individually replated into secondary cultures containing 200 U/mL of IL-3, 2 U/mL of Epo, and 30% FBS. The percentages of GM and multilineage colonies in secondary colonies exhibited a statistical difference between SCF + IFN-γ and SCF + IL-3 ($P < .05$).

of anti–IL-6 antibody to culture containing IL-3 + IL-6 significantly reduced the number of M and GMM colonies to the level obtained by IL-3 alone. However, neither M and GMM colony growth by IL-3 + IFN-γ– nor IL-3–dependent colony growth was influenced (data not shown). These findings suggest that enhancement of M and GMM colony growth by the addition of IFN-γ to culture containing IL-3 does not result from endogenous production of IL-6. Monte et al[36] reported the presence of high-affinity receptors for IFN-γ on the surface of a human megakaryocytic cell line. Taken together, it is possible that IFN-γ directly stimulates the development of megakaryocytic progenitors.

The addition of IFN-γ to a culture containing SCF enhanced the development of primitive hematopoietic progenitors. However, neither IL-6 nor G-CSF cooperated with IFN-γ, implying that IFN-γ does not always act synergistically with factors capable of affecting the development of multipotential progenitors. Delayed addition of IFN-γ to a culture containing SCF resulted in a significant enhancement of total colony growth by post–5-FU BMC, while a delay of SCF addition to the culture initiated with IFN-γ allowed no colony formation, which suggested that primitive progenitors responding to SCF + IFN-γ are supported by SCF in the early stage of development and require IFN-γ for subsequent growth. These results are consistent with the report by Metcalf[37] that transfer of G-CSF–initiated clones derived from BMC of normal mice to SCF-containing cultures does not result in any significant enhancement of proliferation, but that SCF-initiated clones transferred to G-CSF–containing cultures are strongly stimulated to proliferate.

Replating experiments showed multipotentiality of blast cell colonies supported by SCF + IFN-γ, and no difference in replating efficiencies of blast cell colonies between SCF + IFN-γ and SCF + IL-3. However, SCF + IFN-γ generated blast cell colonies with a higher frequency of secondary GM colonies and with lower frequency of secondary multilineage colonies than did SCF + IL-3. This may be related to insufficient proliferation and differentiation of hematopoietic progenitors in the presence of SCF and IFN-γ, in which the mean colony size was smaller and day 16 colonies consisted largely of blasts. The total number of colonies formed by SCF + IFN-γ was significantly lower than that of the colonies grown by SCF + IL-3 in culture containing post–5-FU BMC. In addition, the combination of SCF, IFN-γ, and SCF did not support total colony growth over the level seen in SCF + IL-3. These findings suggest that multipotential progenitors responding to SCF + IFN-γ are a subpopulation of multipotential progenitors supported by SCF + IL-3.

We report here that IFN-γ inhibited the proliferative response to IL-3 of mature GM progenitors, but markedly enhanced the proliferation of primitive megakaryocytic progenitors in post–5-FU BMC, and that IFN-γ had a stimulatory effect on SCF-dependent colony growth by mature and primitive hematopoietic progenitors. Taken together, these lines of evidence indicate that potent negative regulators are not always inhibitors of hematopoietic progenitors, but that they may act bifunctionally.

ACKNOWLEDGMENT

We are deeply indebted to Prof A. Komiyama (Shinshu University School of Medicine) for helpful comments.

REFERENCES

1. Suda T, Suda J, Ogawa M, Ihle JN: Permissive role of interleukin-3 (IL-3) in proliferation and differentiation of multipotential hemopoietic progenitors in culture. J Cell Physiol 124:182, 1985

2. Ikebuchi K, Wong GG, Clark SC, Ihle JN, Hirai Y, Ogawa M: Interleukin-6 enhancement of interleukin-3–dependent proliferation of multipotential hemopoietic progenitors. Proc Natl Acad Sci USA 84:9035, 1987

3. Koike K, Nakahata T, Takagi M, Kobayashi T, Ishiguro A, Tsuji K, Naganuma K, Okano A, Akiyama Y, Akabane T: Synergism of BSF-2/interleukin 6 and interleukin 3 on development of multipotential hemopoietic progenitors in serum-free culture. J Exp Med 168:879, 1988

4. Ikebuchi K, Clark SC, Ihle JN, Souza LM, Ogawa M: Granulocyte colony-stimulating factor enhances interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. Proc Natl Acad Sci USA 85:3445, 1988

5. Koike K, Nakahata T, Kubo T, Kikuchi T, Takagi M, Ishiguro A, Tsuji K, Naganuma K, Okano A, Akiyama Y, Akabane T: Interleukin-6 enhances murine megakaryocytopoiesis in serum-free culture. Blood 75:2286, 1990

6. Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Karkare SB, Sachdev RK, Yushenkoff VN, Birkett NC, Williams LR, Satyagal VN, Tung W, Bosselman RA, Mendiaz EA, Langley KE: Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. Cell 63: 195, 1990

7. Williams DE, Eisenman J, Baird A, Rauch C, Ness KV, March CJ, Park LS, Martin U, Mochizuki DY, Boswell HS, Burgess GS, Cosman D, Lyman SD: Identification of a ligand for the c-kit proto-oncogene. Cell 63:167, 1990

8. Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, Wellner D, Leder P, Besmer P: The hematopoietic growth factor KL is encoded by the S1 locus and is the ligand of the c-*kit* receptor, the gene product of the W locus. Cell 63:225, 1990

9. Martin FH, Suggs SV, Langley KE, Lu HS, Ting J, Okino KH, Morris CF, McNiece IK, Jacobsen FW, Mendiaz EA, Birkett NC, Smith KA, Johnson MJ, Parker VP, Flores JC, Patel AC, Fisher EF, Erjavec HO, Herrera CJ, Wypych J, Sachdev RK, Pope JA, Leslie I, Wen D, Lin CH, Cupples RL, Zsebo KM: Primary structure and

functional expression of rat and human stem cell factor DNAs. Cell 63:203, 1990

10. Tsuji K, Zsebo KM, Ogawa M: Enhancement of murine blast cell colony formation in culture by recombinant rat stem cell factor, ligand for c-*kit*. Blood 78:1223, 1991

11. Metcalf D, Nicola NA: Direct proliferative actions of stem cell factor on murine bone marrow cells in vitro: Effects of combination with colony-stimulating factors. Proc Natl Acad Sci USA 88: 6239, 1991

12. Migliaccio G, Migliaccio AR, Valinsky J, Langlet K, Zsebo K, Visser JWM, Adamson JW: Stem cell factor induces proliferation and differentiation of highly enriched murine hematopoietic cells. Proc Natl Acad Sci USA 88:7420, 1991

13. Broxmeyer HE, Hangoc G, Cooper S, Anderson D, Cosman D, Lyman SD, Williams DE: Influence of murine mast cell growth factor (c-*kit* ligand) on colony formation by mouse marrow hematopoietic progenitor cells. Exp Hematol 19:143, 1991

14. Lowry PA, Zsebo KM, Deacon DH, Eichman CE, Quesenberry PJ: Effect of rrSCF on multiple cytokine responsive HPP-CFC generated from SCA^+ Lin^- murine hematopoietic progenitors. Exp Hematol 19:994, 1991

15. Papayannopoulou T, Brice M, Broudy VC, Zsebo KM: Isolation of c-kit receptor-expressing cells from bone marrow, peripheral blood, and fetal liver: Functional properties and composite antigenic profile. Blood 78:1403, 1991

16. Klimpel GR, Fleishmann WR, Klimpel KD: Gamma interferon (IFN-γ) and IFN-α/β suppress murine myeloid colony formation (CFU-C): Magnitude of suppression is dependent upon level of colony-stimulating factor (CSF). J Immunol 129:76, 1982

17. Broxmeyer HE, Lu L, Platzer E, Feit C, Juliano L, Rubin BY: Comparative analysis of the influences of human gamma, alpha and beta interferons of human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells. J Immunol 131:1300, 1983

18. Raefsky EL, Platanias LC, Zoumbos NC, Young NS: Studies of interferon as a regulator of hematopoietic cell proliferation. J Immunol 135:2507, 1985

19. Griffin JD, Sabbath KD, Herrmann F, Larcom P, Nichols K, Kornacki M, Levine H, Cannistra SA: Differential expression of HLA-DR antigens in subsets of human CFU-GM. Blood 66:788, 1985

20. Takagi M, Koike K, Nakahata T: Antiproliferative effect of IFN-γ on proliferation of mouse connective tissue-type mast cell. J Immunol 145:1880, 1990

21. Koike K, Ma F, Shiohara M, Kubo T, Takagi M, Amano Y, Nakahata T, Komiyama A: Interferon-gamma inhibits proliferation, but not commitment, of murine granulocyte-macrophage progenitors. J Cell Physiol 153:528, 1992

22. Peschel C, Paul WE, Ohara J, Green I: Effects of B cell stimulatory factor-1/interleukin 4 on hematopoietic progenitor cells. Blood 70:254, 1987

23. Rennick D, Jackson J, Yang G, Wideman J, Lee F, Hudak S: Interleukin-6 interacts with interleukin-4 and other hematopoietic growth factors to selectively enhance the growth of megakaryocytic, erythroid, myeloid, and multipotential progenitor cells. Blood 73: 1828, 1989

24. Musashi M, Clark SC, Sudo T, Urdal DL, Ogawa M: Synergistic interactions between interleukin-11 and interleukin-4 in support of proliferation of primitive hematopoietic progenitors of mice. Blood 78:1448, 1991

25. Rennick D, Yang G, Muller-Sieburg C, Smith C, Arai N, Takabe Y, Gemmell L: Interleukin 4 (B-cell stimulatory factor 1) can enhance or antagonize the factor-dependent growth of hematopoietic progenitor cells. Proc Natl Acad Sci USA 84:6889, 1987

26. Jansen JH, Wientjens GHM, Fibbe WE, Willemze R, Kluin-Nelemans HC: Inhibition of human macrophage colony formation by interleukin 4. J Exp Med 170:577, 1989

27. Keller JR, Jacobsen SEW, Still KT, Ellingsworth LR, Ruscetti FW: Stimulation of granulopoiesis by transforming growth factor β: Synergy with granulocyte/macrophage-colony-stimulating factor. Proc Natl Acad Sci USA 88:7190, 1991

28. Fan K, Ruan Q, Sensenbrenner L, Chen B: Transforming growth factor-β1 bifunctionally regulates murine macrophage proliferation. Blood 79:1679, 1992

29. Okada S, Suda T, Suda J, Tokuyama N, Nagayoshi K, Miura Y, Nakauchi H: Effect of interleukin 3, interleukin 6, and granulocyte colony-stimulating factor on sorted murine splenic progenitor cells. Exp Hematol 19:42, 1991

30. Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashiwamura S, Nakajima K, Koyama K, Iwamatsu A, Tsunasawa S, Sakiyama F, Matsui H, Takahara Y, Taniguchi T, Kishimoto T: Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature 324:73, 1986

31. Koike K, Stanley ER, Ihle JN, Ogawa M: Macrophage colony formation supported by purified CSF-1 and/or interleukin 3 in serum-free culture: Evidence for hierarchical difference in macrophage colony-forming cells. Blood 67:859, 1986

32. Takagi M, Nakahata T, Kubo T, Shiohara M, Koike K, Miyajima A, Arai K, Nishikawa S, Zsebo KM, Komiyama A: Stimulation of mouse connective tissue-type mast cells by hematopoietic stem cell factor, a ligand for the c-*kit* receptor. J Immunol 148:3446, 1992

33. Nakahata T, Ogawa M: Identification in culture of a hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. Proc Natl Acad Sci USA 79:3843, 1982

34. Nakahata T, Ogawa M: Clonal origin of murine hemopoietic colonies with apparent restriction to granulocyte-macrophage-megakaryocyte (GMM) differentiation. J Cell Physiol 111:239, 1982

35. Koike K, Shimizu T, Miyake T, Ihle JN, Ogawa M: Hemopoietic colony formation by mouse spleen cells in serum-free culture supported by purified erythropoietin and/or interleukin-3, in Levine RF, Williams N, Levine J, Evatt BL (eds): Megakaryocyte Development and Function. New York, NY, Liss, 1986, p 33

36. Monte D, Wietzerbin J, Pancre V, Merlin G, Greenberg SM, Kusnierz JP, Capron A, Auriault C: Identification and characterization of a functional receptor for interferon-γ on a megakaryocytic cell line. Blood 78:2062, 1991

37. Metcalf D: Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: Influence of colony-stimulating factors. Proc Natl Acad Sci USA 88:11310, 1991