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Expression of an activated erythropoietin or a colony-stimulating factor 1 receptor by pluripotent progenitors enhances colony formation but does not induce differentiation

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ABSTRACT **Whether the presence of specific receptors on the surface of developing cells is the cause or consequence of lineage restriction is not known. If activation of specific receptors is the driving event in differentiation, the premature expression of specific receptors would promote differentiation along that pathway. In this study pluripotent progenitors, obtained from blast cell colonies (pooled or individual) of 5-flurouracil-treated mice, were infected with retroviral vectors containing either an activated receptor for erythropoietin (EPO), an erythroid progenitor growth factor, or the receptor for colony-stimulating factor 1 (CSF-1), a macrophage growth factor. These receptors exhibit expression patterns restricted to committed progenitors. The developmental potential of infected pluripotent progenitors was not changed, although they expressed the exogenous genes, suggesting that in these cells activation of lineage-specific receptors does not induce differentiation. Acquisition of a constitutively activated EPO receptor allowed erythroid development in mixed colonies in the absence of EPO, as expected. Infection of progenitors with a virus containing the CSF-1 receptor promoted the development of granulocyte/macrophage (GM) colonies but did not alter the differentiation potential of either colony-forming unit (CFU)-GM or CFU-mix.**

Cells at different stages in development are thought to have different complements of receptors. Whether the appearance of specific receptors initiates a particular developmental sequence is not known. In this study we directly addressed the question: Does the acquisition of a lineage-specific receptor induce differentiation? Retroviral vectors were used to insert receptor genes into multilineage progenitors. Receptors for two lineage-restricted growth factors, erythropoietin (EPO), an erythroid (E) progenitor growth factor, and colony-stimulating factor 1 (CSF-1), a macrophage growth factor, were chosen for study. These receptors represent two different receptor families that are prominent in hemopoietic cells. The EPO receptor (EPO-R) is a member of the cytokine receptor family, which includes the receptors for interleukin 2 (IL-2), IL-3, IL-4, granulocyte/macrophage (GM)-CSF, G-CSF, IL-6, and others (1). The CSF-1 receptor (CSF-1R) is a member of the tyrosine kinase receptor family, which includes c-*kit*, flk-2, and others (2). Previous studies have demonstrated that EPO and CSF-1 act on committed progenitors and are not active on early progenitors (3–5). If the activation of these lineage-restricted receptors is a key inductive event in commitment, then expression of the receptor gene at early stages of development would promote differentiation in that particular lineage at the expense of other lineages. Blast cell colonies cultured from the spleens of mice injected with 5-fluorouracil (5-FU) 4 days earlier were chosen for this study because they are a good source of progenitors for mixed colonies, and the incidence of committed E progenitors in this population is quite low (6). Other considerations were that the cells are actively cycling and are readily infectable with retroviruses (7).

Our approach was to infect multilineage progenitors with a constitutively activated EPO-R [EPO-R(R129C)] that has been shown to confer growth factor-independent proliferation upon certain hemopoietic cell lines (8, 9). Infection of mice with a recombinant spleen focus-forming virus (SFFV) expressing EPO-R(R129C) (SFFVcEPO-R) resulted initially in increased platelet and reticulocyte counts and later in the development of leukemia (9, 10). Infection of committed E progenitors with SFFVcEPO-R abrogates their requirement for EPO but does not abrogate the requirement of progenitors for other cytokines (11). In the current study we infected uncommitted progenitors to test whether E development would be favored over differentiation into other lineages. We also carried out similar studies with wild-type [CSF-1R(wt)] and an activated mutant CSF-1R [CSF-1R(Y969F)]. The results of this study indicate the role of the EPO-R and the CSF-1R is primarily on cell proliferation rather than on the induction of differentiation.

MATERIALS AND METHODS

Viruses. Construction of the SFFVcEPO-R and MPSV-cEPO-R (MPSV: myeloproliferative sarcoma virus) retroviruses has been described (9–11). Both express the constitutively activated form of the EPO-R, EPO-R(R129C). Retroviruses expressing wild-type human CSF-1R (hCSF-1R) cDNA or hCSF-1R(Y969F) were supplied by M. Rousell and C. Sherr (St. Jude Children's Research Hospital, Memphis, TN) (12, 13).

Infection and Culture. BALB/c mice were obtained from Charles River Breeding Laboratories. 5-FU (Adria Laboratories) was administered i.v. through the tail vein of mice at a dosage of 150 mg/kg of body weight. Spleen cells were harvested 4 days after 5-FU injection. Methylcellulose culture was carried out as described (14). Culture medium (1 ml) contained 6×10^5 spleen cells from 5-FU-treated mice, α-medium (Flow Laboratories), 1.2% methylcellulose, 30% fetal bovine serum, 1% deionized bovine serum albumin, 0.1 mM 2-mercaptoethanol, 100 units (U) of IL-3 per ml, and 100 ng of IL-6 per ml. Blast cell colonies containing 20–200 cells were identified on day 6 or 7 of culture. Cells were resuspended in fresh or frozen virus supernatant or control medium containing 4 μg of Polybrene per ml and incubated at

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Abbreviations: CSF-1, colony-stimulating factor 1; CSF-1R, CSF-1 receptor; 5-FU, 5-flurouracil; EPO, erythropoietin; EPO-R, EPO receptor; GM, granulocyte/macrophage; SFFV, spleen focus-forming virus; IL, interleukin; U, unit(s); IU, international unit(s); h, human; m, murine; E, erythroid; SF, steel factor; MPSV, myeloproliferative sarcoma virus; CFU, colony-forming unit(s).

37°C for 3 hr. Following infection, samples of blast cells (50–100 per dish) were replated in α-medium containing 30% fetal bovine serum (Sterile Systems, Logan, UT), 1% crystallized bovine serum albumin (Sigma), 1.2% 1500 centipoise methylcellulose (1 poise = 0.1 Pa·sec; Fisher), 50 μM 2-mercaptoethanol (Sigma), and growth factors as indicated. Steel factor (SF) was provided by Steven Clark (Genetics Institute, Inc., Cambridge, MA) (15). Murine IL-3 (mIL-3) was a gift from Tetsuo Sudo (Biomaterial Research Institute, Yokohama, Japan). Recombinant hIL-6 was a gift from M. Naruto (Toray Industries, Yokohoma, Japan). Recombinant hCSF-1 [specific activity, 10^6 U/mg; 2×10^7 international units (IU)/mg] was purchased from Sigma. Partially purified urinary hEPO (specific activity, 250 U/mg of protein) was a generous gift from M. Kawakita (Kumamoto University, Kumamoto, Japan).

PCR. DNA was extracted as described (11). RNA was extracted by modification of the guanidine isothiocyanate/acid/phenol method described by Chomczynski and Sacchi (16). Cells were lysed in 4 M guanidine isothiocyanate/25 mM sodium citrate/0.5% sarcosyl/25 mM 2-mercaptoethanol (GuSCN solution) containing 5 μg of yeast tRNA. RNA was extracted with phenol/chloroform, pH 4.0, ethanol precipitated, dissolved in GuSCN solution, reprecipitated with ethanol, dissolved in 0.2 M sodium acetate (pH 4.0), ethanol precipitated, and washed in 70% ethanol. cDNA synthesis was carried out with random hexamer primers and Superscript reverse transcriptase (BRL). PCR was carried out with AmpliTaq (Perkin–Elmer/Cetus) using a DNA thermocycler (Perkin–Elmer/Cetus) under reaction conditions recommended by Cetus. Forty cycles were used for 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. PCR samples were transferred to Biotrans nylon membranes (ICN) and hybridized with ^{32}P-labeled oligonucleotides (oligos) as described (11). The PCR primer sets and hybridization oligos were as follows: (A) *MPSVcEPO-R*. Sense, from the long terminal repeat (LTR) TGGTCTCGCTGTTCCTTGGA; antisense, 371–392 of EPO-R CTCCAGCGGCACAAAACTCGA; oligo, 31–51 of EPO-R GACAAACTCAGGGTGCCCCTC. (B) *EPO-R*. Sense exon 8, 1252–1272 TTGGCCTCAAAGCCCAGGCCA; antisense exon 8, 1519–1539 CACATAGCCGGGATGCAGAGG; oligo exon 8, 1454–1475 ACGGGGACTCATCTGATGGCC. (C) *EPO-R*. Sense exon 1, 31–51 GACAAACTCAGGGTGCCCCTC; antisense exon 4, 368–387 CTCCAGCGGCACAAAACTCGA; oligo, 67–88 CCCCTCTGTCTCCTACTTGCT. (D) *Zipneo-hCSF-1R*. Sense, LTR TGGTCTCGCTGTTCCTTGGA; antisense, 361–381 of hCSF-1R ACACTGGGCTCTATCACTGG; oligos, 314–334 TTCTGCTGCTCCTGCTGGTG. (E) *mCSF-1R*. Sense, 1441–1462 CTGAGTCAGAAGCCCTTCGAC; antisense, 1862–1883 CCCAGACCAAAGGCTGTAGCC; oligo, 1464–1484 AGTGATCATTCAGAGCCAGCT. (F) *Actin*. Sense, 198–219 CTGAAGTACCCCATTGAACAT; antisense, 619–642 CTCTTTGATGTCACGCACGATTTC; oligo, 244–264 ATGGAGAAGATCTGGCAC.

RESULTS

Blast cell colonies from 5-FU-treated mice were assessed for susceptibility to infection with MPSVcEPO-R- or hCSF-1R-expressing viruses. Blast cell colonies were identified in culture, pooled, and divided into two portions. One was exposed to virus overnight and the other was not infected. Twelve hours after infection RNA was prepared from each sample and RNA PCR was performed with primers specific for retrovirally derived hCSF-1R or EPO-R transcripts. Uninfected blast cells expressed very little, if any, endogenous mCSF-1R or EPO-R transcript (Fig. 1*C*, columns I and III, respectively). Also, neither retrovirally derived transcript was evident in uninfected blast cells (Fig. 1 *B* and *C*, columns II and III). Following viral infection, hCSF-1R and EPO-R transcripts were present. Equivalent amounts of cDNA were included in all PCR reactions as shown by the actin control (Fig. 1*C*, column IV). These results indicated that blast cells do not express EPO-R or CSF-1R genes, that they could be infected with EPO-R(R129C) and CSF-1R retroviruses, and that both retrovirally encoded receptor genes are expressed at the RNA level.

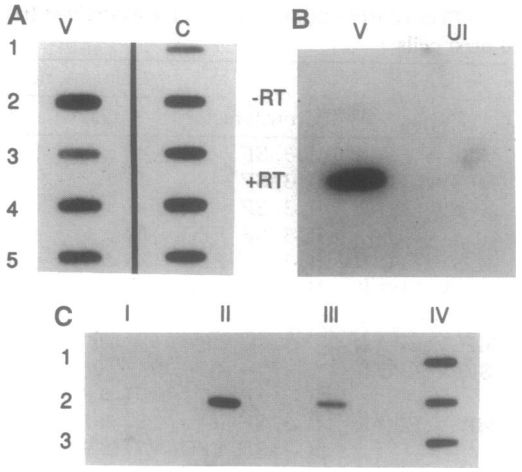

FIG. 1. Slot blot analysis of PCR samples. (*A*) Detection of MPSVcEPO-R provirus in mixed colonies derived from infected blast cell colonies. DNA was extracted from individual mixed colonies (see text). In column V, DNA PCR was carried out with primer set A; in column C, DNA PCR was carried out with primer set B. Sample 1 was from an uninfected culture; samples 2–5 were from colonies infected with MPSVcEPO-R. (*B*) Expression of EPO-R(R129C) by infected blast cells. V, MPSVcEPO-R-infected sample; UI, uninfected sample; RT, reverse transcriptase. RNA PCR was performed with primer set A. (*C*) Comparison of endogenous and exogenous receptor gene expression in blast cells. Column I, endogenous mCSF-1R expression was assessed by RNA PCR with primer set E. Line 1, uninfected sample; line 2, Zipneo-hCSF-1R-infected sample; line 3, no sample. Column II, expression of exogenous hCSF-1R was assessed by RNA PCR with primer set D. Line 1, uninfected sample; line 2, Zipneo-hCSF-1R-infected sample; line 3, no sample. Column III, expression of EPO-R was assessed by RNA PCR with primer set C. Line 1, uninfected sample; line 2, MPSV-cEPO-R-infected sample; line 3, no sample. Column IV, expression of actin was assessed by RNA PCR with actin-specific primers (set F). Line 1, uninfected sample; line 2, Zipneo-hCSF-1R-infected sample; line 3, MPSVcEPO-R infected sample. The same cDNA sample was used for all corresponding PCRs. Hybridization was carried out using an internal end-labeled oligonucleotide, described in the PCR primer sets.

Next the progeny of infected blast cells were examined for evidence of retroviral integration and expression of retroviral genes. Blast cells were infected, or not, and cultured in medium containing SF and IL-3. At day 8 mixed colonies were identified and DNA was extracted from individual colonies. Fig. 1*A* shows that the multipotential colony-forming cells in blast cell colonies are readily infectable with this virus (Fig. 1*A*). PCR was carried out with primers specific for the virus and slot blots of the PCR products were probed with an internal end-labeled oligonucleotide. Seven of eight colonies analyzed were infected. The uninfected control sample was negative as was the control sample lacking reverse transcriptase. A control using PCR primers for c-*kit* showed that the infected and uninfected samples had qualitatively equivalent amounts of cDNA (not shown). Both GM and mixed colonies expressed EPO-R(R129C) as determined by PCR of cDNA prepared from these colonies (not shown).

The effect of EPO-R(R129C) on the progeny of multipotent progenitors (blast cells) was analyzed in clonal cell culture. Infection did not make progenitors independent of growth

Table 1. Effect of EPO-R(R129C) on colonies derived from infected blast cells

Exp.	Virus	Growth factor	Colonies, no. per dish GM	E mixed
1	—	IL-3, SF	41 ± 1	0
	MPSVcEPO-R	IL-3, SF	51 ± 7	1
	SFFVcEPO-R	IL-3, SF	61 ± 6*	0.5
	—	IL-3, SF, EPO	37 ± 6	0.5
	MPSVcEPO-R	IL-3, SF, EPO	45 ± 14	6 ± 4
	SFFVcEPO-R	IL-3, SF, EPO	51 ± 8	7 ± 5*
	—	—	0	0
	MPSVcEPO-R	—	0	0
	SFFVcEPO-R	—	0	0
2	—	IL-3, SF	77 ± 8	0
	MPSVcEPO-R	IL-3, SF	87 ± 12*	2 ± 1*
	—	IL-3, SF, EPO	77 ± 9	7 ± 3
	MPSVcEPO-R	IL-3, SF, EPO	83 ± 11	9 ± 2
3	—	IL-3, SF	75 ± 7	0
	MPSVcEPO-R	IL-3, SF	96 ± 5**	0
	—	IL-3, SF, EPO	59 ± 5	0.25
	MPSVcEPO-R	IL-3, SF, EPO	90 ± 5**	3 ± 1**
	—	—	0	0
	MPSVcEPO-R	—	0	0

Data are the mean of four dishes ± SD of the mean for experiments 1 and 3 and the mean of nine dishes for experiment 2. IL-3, 100 U/ml; SF, 5 U/ml; EPO, 1 U/ml. In experiment 1, 70 cells were plated per dish; in experiments 2 and 3, 100 cells were plated per dish. *, $P < 0.05$; **, $P < 0.01$ when compared to the corresponding uninfected control by the Student's t test.

factors since no colonies developed in the absence of added growth factors (Table 1, experiments 1 and 3). Infection of pooled blast cell colonies with EPO-R(R129C) allowed the development of mixed E colonies in the absence of added EPO, in cultures containing SF and IL-3. No mixed E colonies were observed in the absence of EPO in cultures of uninfected cells (Table 1, experiments 1 and 2). Similar results were obtained with SFFVcEPO-R and MPSVcEPO-R viruses. The addition of EPO increased the number of mixed E colonies observed in infected samples. These results are consistent with the observation that a mutant receptor retains the ability to bind EPO and that some infected cells respond to EPO.

We then asked if EPO-R(R129C) enlarged the E component of multilineage colonies. The composition of the mixed colonies from experiment 2, Table 1, is shown in Table 2. The E fraction of mixed colonies obtained from infected cultures was not larger than that of uninfected cultures containing EPO. The average percentage of E cells in mixed colonies from MPSVcEPO-R-infected cultures lacking EPO was 11% as compared to 45% in the uninfected sample cultured with EPO, and 43% in the infected sample cultured with EPO. These results are representative of several similar experiments.

In another group of experiments, individual blast cell colonies were divided into two portions—one was infected and the other was not exposed to EPO-R(R129C). Infection

Table 3. Infection of individual blast cell colonies with MPSVcEPO-R or hCSF-1R virus

Virus	Colony no.	Blast cell colony size	No. of secondary GM and E mixed colonies Uninfected	Infected
MPSVcEPO-R*	1	60	1	32
	2	88	0	16
	3	120	2	22
	4	65	1	31
	5	95	8	10
	6	74	0	2
	7	103	2	16
	8	55	0	11
hCSF-1R(Y969F)†	1	218	39	102
	2	162	55	107
	3	89	23	32
	4	75	14	38
	5	51	2	37
	6	177	37	63
	7	121	8	16
	8	163	19	37
	9	96	2	10
	10	174	10	63

Colonies were scored on day 8.
*Individual blast cell colonies were infected, or not, and replated into medium containing IL-3 (100 U/ml) and SF (5 U/ml).
†Postinfection cultures contained IL-3 (100 U/ml).

increased colony size and enhanced the development of GM and E mixed colonies (Table 3). The percentage of E cells was not increased when compared to an uninfected sample cultured with EPO (not shown). Average colony size was increased 2-fold.

A similar analysis of the effect of the CSF-1R on the differentiation of uncommitted progenitors was carried out. When bone marrow cells were infected with viruses containing either wild-type hCSF-1R or hCSF-1R(Y969F), enhanced development of GM colonies was observed (Table 4). Infection with either wild-type hCSF-1R or hCSF-1R(Y969F) increased the number of GM colonies from 8 to 20 ($P < 0.01$ by the Student's t test) in bone marrow cultures containing 2×10^4 cells per ml and 100 U of CSF-1 per ml. We then studied the effect of exogenous expression of CSF-1R on blast cells and, as expected for a late-acting lineage-specific factor, CSF-1 alone was unable to support the development of GM colonies from blast cells (Table 4). Infection with either the mutant or the wild-type receptor resulted in a significant increase in colony development when IL-3 was included in the medium, indicating that infection gives progenitors a growth advantage. Since the cultures contained serum, and monocytes produce CSF-1 (17), a small amount of CSF-1 was probably present in the cultures. Optimal development of GM colonies required both IL-3 and CSF-1. The effect of the CSF-1R on proliferation and differentiation was further analyzed by infecting individual blast cell colonies. Ten individual colonies were divided in two portions—one was

Table 2. Effect of EPO-R(R129C) on the composition of mixed colonies derived from infected blast cells

Virus*	EPO	Cells/colony†	Average % m	Mast	N	E	M	e	Bl
+	−	74,792 ± 54,731	54 ± 28	15 ± 16	19 ± 19	11 ± 14	0.2 ± 0.35	0.2 ± 0.4	0.3 ± 1.0
−	+	43,556 ± 34,205	31 ± 28	11 ± 14	10 ± 14	45 ± 31	0.6 ± 0.6	0.2 ± 0.45	3.1 ± 6.9
+	+	101,754 ± 115,510	28 ± 25	15 ± 24	13 ± 22	43 ± 35	0.8 ± 0.9	0.03 ± 0.11	0.6 ± 1.6

Cultures contained SF (3 U/ml) and IL-3 (100 U/ml) and were scored on day 9. m, Macrophages; Mast, mast cells; N, neutrophils; E, E cells; M, megakaryocytes; e, eosinophils; Bl, blast cells.
*MPSVcEPO-R.
†Mean number (±SD) of cells per colony.

Table 4. Infection of bone marrow and blast cells with hCSF-1R viruses

Virus	Growth factor	GM colonies, no. per dish
	Bone marrow cells†	
—	—	0
hCSF-1R(Y969F)	—	0
hCSF-1R(wt)	—	0
—	CSF-1	8 ± 3
hCSF-1R(Y969F)	CSF-1	21 ± 3*
hCSF-1R(wt)	CSF-1	20 ± 3*
	Blast cells‡	
hCSF-1R(Y969F)	—	0
hCSF-1R(wt)	—	0
—	CSF-1	0
hCSF-1R(Y969F)	CSF-1	0
hCSF-1R(wt)	CSF-1	0
—	IL-3	14 ± 5
hCSF-1R(Y969F)	IL-3	52 ± 5*
hCSF-1R(wt)	IL-3	49 ± 9*
—	CSF-1, IL-3	32 ± 10
hCSF-1R(Y969F)	CSF-1, IL-3	60 ± 7*
hCSF-1R(wt)	CSF-1, IL-3	58 ± 6*

wt, Wild type.
*$P < 0.01$.
†Bone marrow cells were plated at a concentration of 2×10^4 cells per ml. Data are the mean ± SD of quadruplicate cultures. CSF-1 was added to a concentration of 100 U/ml.
‡Cultures contained 70 blast cells per dish. Data represent the mean ± SD of quadruplicate cultures. CSF-1 and IL-3 were added to a concentration of 100 U/ml.

exposed to virus and the other served as an uninfected control. As shown in Table 3, more secondary colonies developed in the infected portion of each blast cell colony, clearly showing an effect of CSF-1R on colony development. All of the secondary colonies from blast cell colony 3 were analyzed individually. Infection increased the size of the secondary colonies, but the composition of the colonies was not altered. The mean number of neutrophils and macrophages per colony were increased (Table 5). The average percentage of macrophages ranged from 2% to 100% with an average of 67% in uninfected cultures, and ranged from 8% to 99%, with an average of 57% in the infected cultures. Analysis of other colonies gave equivalent results—an effect on size but not on composition. Furthermore, infection with CSF-1R did not block the expression of other lineages. A comparison of mixed E colonies obtained from infected and uninfected cultures is shown in Table 6. The percentage of macrophages was 37% in cultures of uninfected cells and 39% in cultures of infected cells. Similarly, there was no change in the fraction of other lineage types. There was, however, a significant effect on colony size (60,250 vs. 125,650, $P < 0.02$ by Student's t test). These results are representative of four similar experiments. Thus, the effect of CSF-1R activation on progenitors appeared to be primarily on their proliferation rather than differentiation.

Table 5. Analysis of the composition of GM colonies derived from infection of an individual blast cell colony with hCSF-1R(Y969F) virus

		Per colony	
Virus	Average size	Macrophages	Neutrophils
Uninfected	1970 ± 1480	1151 ± 1000	836 ± 1102
Infected	4150 ± 2408*	2284 ± 1870**	1911 ± 1607**

Analysis of the secondary colonies ($n = 20$) derived from blast cell colony 3, Table 3. Cultures contained IL-3 (100 U/ml). *, $P < 0.01$; **, $P < 0.02$.

DISCUSSION

The first part of our study asks if the expression of an activated EPO-R in an uncommitted cell could induce E differentiation. We demonstrated that pluripotent progenitors could be infected with retroviral vectors expressing EPO-R(R129C) and that blast cells and their progeny, colony-forming unit (CFU)-GM and CFU-mix, express retrovirally derived EPO-R(R129C) as determined by PCR of cDNA prepared from these colonies. We saw no evidence that EPO-R(R129C) could induce E differentiation when inserted into pluripotent progenitors. These results are consistent with classic *in vivo* experiments in which EPO has been shown to regulate the rate at which committed erythrocytic progenitors become erythroblasts (3, 4) and with other studies that demonstrate that EPO had no effect on pluripotential stem cells (18–20).

Conflicting results have been obtained regarding the role of another lineage-restricted receptor c-*fms*, the CSF-1R, in macrophage differentiation. When the CSF-1R genes, either wild-type or an activated form, were introduced by retroviral infection into long-term mouse cultures, pre-B-cell lines underwent spontaneous and irreversible differentiation to macrophages when transferred from RPMI 1640 medium to Iscove's modified Dulbecco's medium (21). However, when the hCSF-1R gene was expressed in the stem cell line LYD9, the cells did not differentiate in response to hCSF-1, although they had the capacity to differentiate since they could be induced to differentiate by stromal layers. Transfectants of the myeloid clone L-G3 differentiated into neutrophils in response to hCSF-1 (22). Retrovirus-mediated transfer of the CSF-1R in NFS-60 cells, an IL-3-dependent multipotent hematopoietic cell line, enabled the cells to proliferate in response to CSF-1. However, the phenotype of the NFS-60 cells did not significantly differ from the original NFS-60 cells and these cells retained their E potential. In contrast, a CSF-1-dependent variant of NFS-60 differentiated into monocyte/macrophages upon CSF-1 stimulation and almost totally lost its E potential (23). When a mutated CSF-1R (L301S, Y969F) was transfected into IL-3-dependent 32D cells, CSF triggered proliferation in association with monocyte differentiation. Monocyte differentiation was reversible upon removal of CSF-1, suggesting that CSF-1 was required for the maintenance of the monocyte phenotype but was not sufficient to induce an irreversible commitment to differentiation (24).

Table 6. Effect of hCSF-1R(Y969F) on the composition of mixed colonies derived from infected blast cells

		Average %						
Virus†	Cells/colony‡	m	Mast	N	E	M	Bl	e
−	64,736 ± 87,256	37 ± 32	3 ± 4	33 ± 28	17 ± 27	0.4 ± 0.8	6 ± 9	2 ± 7
+	125,645 ± 67,814*	39 ± 26	5 ± 8	37 ± 24	16 ± 15	0.3 ± 0.5	3 ± 5	0.04 ± 0.2

Cultures contained SF (3 U/ml), IL-3 (100 U/ml), CSF-1 (100 U/ml), and EPO (1 U/ml) and were scored on day 9. Abbreviations as in Table 2.
*$P < 0.02$.
†hCSF-1R(Y969F).
‡Mean number (±SD) of cells per colony.

In this study the CSF-1R was inserted into primary progenitors rather than cell lines, since the differentiation of cells lines is often incomplete and does not necessarily reflect normal differentiation. RNA PCR of blast cells infected with hCSF-1R viruses demonstrated expression of the retroviral receptor gene in blast cells. The effect of the CSF-1R on GM progenitors was on their proliferative ability rather than on their differentiation. An increase in colony number and size was not due exclusively to an increase in the size of the macrophage component of mixed colonies, as might have been expected, as there was no change in the relative number of macrophages per mixed colony. Similarly, when inserted into pluripotent progenitors, the CSF-1R gene did not induce macrophage differentiation at the expense of other lineages. Thus, premature expression of the CSF-1R does not automatically result in macrophage differentiation. Support for this interpretation comes from *op/op* mice, which have a defect in the CSF-1 gene. Despite this defect these mice have macrophage progenitors and some tissue macrophages (25). Thus, it appears that activation of the CSF-1R is not absolutely required for the induction of macrophage differentiation.

As multilineage progenitors differentiate, EPO-R and CSF-1R could be down-regulated (26) or up-regulated (27) posttranscriptionally. Despite the presence of mRNA, we have no evidence that the respective receptor proteins are expressed appropriately. However, since all cell types in infected CFU-mix respond to EPO (Table 2) and both neutrophil and macrophage cells of infected CFU-GM respond to CSF-1 (Table 5), it is likely that the receptors are functionally expressed in early multipotent progenitors. Studies carried out in cell lines indicate that receptors are capable of stimulating proliferation in cell types in which they are not normally expressed. Evidence is accumulating that different cytokine receptor signals can converge intracellularly through interactions with the Jak-Tyk family of cytosolic tyrosine kinases (28, 29). Interestingly, Jak2 expression was detected by RNA PCR analysis of blast cell colonies (not shown). It is possible that other specific cytosolic proteins, essential for EPO-R and CSF-1R differentiative signals, are only expressed at a distinct stage of E and macrophage commitment and not in pluripotent blast cells.

The initiation of differentiation could be induced by an exogenous stimulus or could be a spontaneous random event with survival of committed cells dependent on the availability of a supportive milieu. Experimentally it is difficult to distinguish between these two possibilities. Metcalf (30) analyzed the progenitor content of developing blast cell colonies to address the question of whether extrinsic growth factors influenced the pattern of differentiation of multipotential cells. He found that costimulation of blast colony formation by SF plus G-CSF did not change the relative frequency of progenitor cells of different types within the colonies compared with colonies stimulated by SF alone. However, combination of GM-CSF or IL-3 with SF significantly increased the relative frequency of granulocytic progenitors. These results were interpreted to mean that hemopoietic regulators have some ability to induce selective lineage commitment in the progeny of multipotential cells. Mayani *et al.* (31) examined individually sorted human cord blood-derived primitive progenitors and demonstrated asymmetric cell divisions that apparently were not skewed by different cytokine combinations. Evidence reported in this study is more consistent with a supportive role for EPO and CSF-1 in the differentiation of red cells and macrophages. Thus, expression of lineage-specific receptors for EPO and CSF-1 may be a consequence rather than a cause of differentiation. These studies leave open the possibility that the induction of differentiation could be due to the activation of other genes.

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