Cancer Chemother Pharmacol (1996) 38 (Suppl): S58–S63

ORIGINAL ARTICLE

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Differential effects of c-fms and c-kit ligands on the lineage development of the lymphohematopoietic cell line EML C1

Abstract The lymphohematopoietic progenitors represent $<0.01\%$ of nucleated marrow cells. We have shown that murine lymphohematopoietic progenitors can be immortalized by a recombinant retroviral vector harboring a dominant-negative retinoic acid (RA) receptor. The immortalized progenitors proliferate as a stem-cell factor-dependent clonal line designated EML C1. The EML C1 cell line spontaneously generates prepro-B-lymphocytes and erythroid and myeloid progenitors. Upon stimulation with interleukin 7 and marrow stromal cells, the prepro-B-lymphocytes express recombination-activating gene 1 (RAG-1) and undergo D-J rearrangements of the immunoglobulin heavy-chain genes. With erythropoietin, the erythroid progenitors proliferate and differentiate into red cells. Generation of the common progenitors for neutrophils and macrophages [colony-forming units-granulocyte-macrophage (CFU-GM)] is suppressed in EML C1 cells but is inducible by high concentrations of RA. An additional block in neutrophil differentiation occurs at the promyelocyte stage, but this can also be overcome by high concentrations of RA. Although c-fms is homologous to c-kit, which encodes the receptor for stem-cell factor (SCF), EML C1 cells neither express c-fms nor respond to macrophage colony-stimulating factor (M-CSF), the ligand for c-fms. Transduction and expression of c-fms cDNA in EML C1 cells confers responsiveness to M-CSF. This finding indicates that c-kit and c-fms share substantially overlapping signal-transduction pathways. However, c-fms-transduced EML C1 cells (EML C1/c-fms cells) exhibit different development patterns when stimulated by SCF alone or by M-CSF alone. When stimulated by SCF alone, EML C1/c-fms cells show mostly erythroid and B-lymphoid development. When stimulated by M-CSF alone, development switches to mostly myeloid (neutrophil and macrophage) development. This observation suggests that c-kit and c-fms must have unique signal-transduction pathways in addition to the common ones.

Key words C-kit · C-fms · Retinoic acid receptor · Lymphohematopoietic progenitors · Stem-cell factor

Work presented at the 11th Bristol-Myers Squibb Nagoya International Cancer Treatment Symposium "Cytokines and New Anitcancer Agents on the Horizon of Oncology", 24–25 November 1995, Nagoya, Japan

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Introduction

The retinoic acid receptors (RARs) are members of the steroid/thyroid hormone-receptor superfamily that function as ligand-inducible transcription factors [7]. In most cases of acute promyelocytic leukemia (APL), the RARα gene on chromosome 17 is translocated and fused with the PML gene on chromosome 15 [1, 3, 5]. Interestingly, leukemic promyelocytes from these patients can be induced by high concentrations of all-trans retinoic acid (ATRA) to differentiate into mature neutrophils [9].

The exact role of the PMLRARα fusion gene in the pathogenesis of APL remains unclear [6, 28]. However, we have shown that retroviral vector-mediated expression of a dominant-negative RARα (RARα403; Fig. 1) [25] in normal mouse bone-marrow cells produces a differentiation block at the promyelocyte stage; this block can be overcome with high concentrations of ATRA [24]. We subsequently demonstrated that murine lymphohematopoietic progenitors could be immortalized by the same retroviral vector containing the dominant-negative RARα403 cDNA [26]. The immortalized lymphohematopoietic progenitors proliferate as a stem-cell factor (SCF)-dependent cell line (designated EML C1) that spontaneously generates predominantly prepro-B-lymphocytes and erythroid progenitors [burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E); Fig. 2]. With erythropoietin and SCF, the erythroid progenitors proliferate and differ-

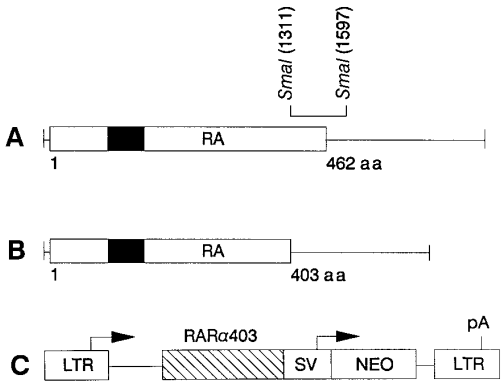

Fig. 1A–C Structure of RARα, RARα403, and the retroviral vector LRARα403SN. **A** Schematic representation of human RARα cDNA. The *bar* represents the open reading frame that encodes human RARα, containing 462 amino acids. The *solid bar* indicates the DNA-binding domain of RARα. *RA* indicates the retinoic acid-binding domain and the autonomous activation function-2 (*AF-2*) domain located in the carboxy terminus. **B** Structure of the truncated receptor RARα403. Nucleotides 1311–1596 of human RARα were deleted by SmaI digestion, and a stop codon was inserted using the NheI linker. The truncated receptor encodes 403 amino acids and is designated RARα403. **C** The structure of the retroviral vector LRARα403SN. The cDNA of RARα403 was cloned into the EcoRI-BamHI sites of LXSN [13] (*LTR* Moloney murine leukemia virus long terminal repeat, *SV* simian virus 40 promoter, *NEO* neomycin phosphotransferase gene, *pA* polyadenylation site). *Arrows* indicate transcription initiation sites

entiate into erythroblasts. Upon stimulation with interleukin 7 (IL-7) and stromal cells, the prepro-B-lymphocytes express RAG-1 and undergo D-J rearrangements of the immunoglobulin heavy-chain genes. Generation of myeloid progenitors [CFU-granulocyte-macrophage (CFU-GM)] is partially blocked in EML C1 cells but can be increased by a combination of high concentrations of ATRA and interleukin 3 (IL-3). An additional block is observed at the promyelocyte stage, but it can also be overcome using high concentrations of ATRA [26]. These findings are summarized in Fig. 3.

In the current study we examined the effects of ectopically expressed c-fms on the survival, proliferation, and differentiation of EML C1 cells. Both c-kit and c-fms are members of the type III tyrosine kinase growth-factor receptor family, characterized by the presence of five immunoglobulin-like motifs in the extracellular domain and two tyrosine kinase domains in the cytoplasmic portion, separated by a kinase insert domain [16, 27]. In the hematopoietic system, c-kit is expressed primarily by stem cells and early progenitors, whereas c-fms expression is largely restricted to the monocyte/macrophage lineage. Since c-kit and c-fms are structurally and functionally similar but developmentally (or temporally) separated in their expression, it would be interesting to determine whether and how premature expression of c-fms in lymphohematopoietic progenitors can affect their development.

Ectopic expression of c-fms in EML C1 cells confers a proliferative response to macrophage colony-stimulating factor

Parental EML C1 cells do not express c-fms as determined using Northern-blot analysis or flow cytometry, nor do they respond to recombinant human macrophage colony-stimulating factor (hM-CSF). To examine the effect of c-fms in EML C1, EML C1 cells were infected with the supernatant of a packaging cell line producing the retroviral vector pZENc-fms [18]. Infected cells were collected using a fluorescence-activated cell sorter (FACS) and anti-c-fms antiserum and were subsequently expanded in a medium supplemented with SCF.

Unlike parental EML C1 cells, pZENc-fms-infected EML C1 cells (EML C1/c-fms cells) expressed high levels of the retroviral message harboring the murine c-fms sequence when examined using Northern-blot analysis and survived and proliferated in response to hM-CSF alone. The optimal hM-CSF concentration for the survival

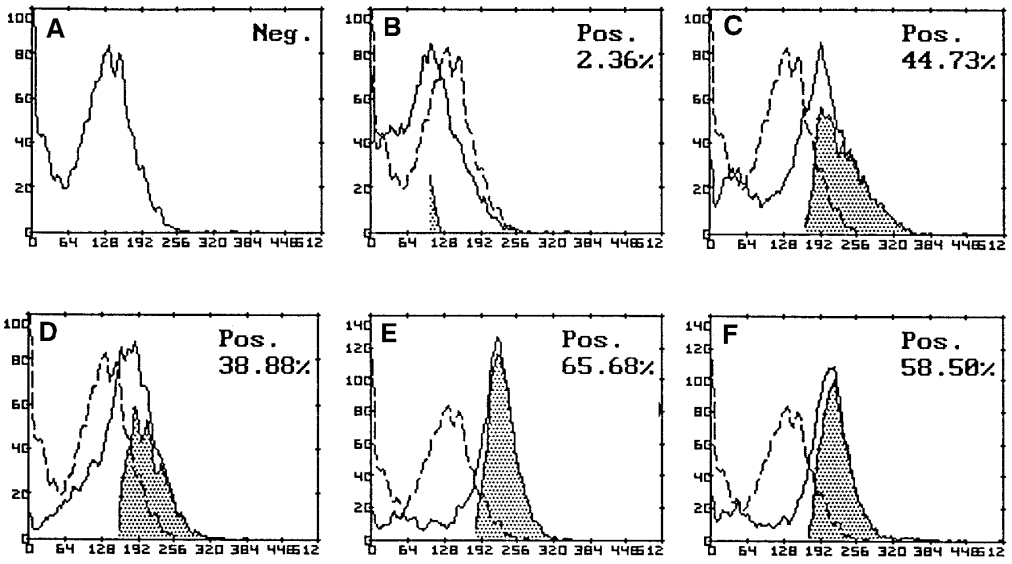

Fig. 2A–F Cell surface antigen profiles of the EML C1 cell line. The profile of isotype antibody (negative control) staining is shown in **A** and included in all other panels for direct comparison. The antibodies used were **B** Mac-1, **C** Sca-1, **D** Ack-2, **E** ter-119, and **F** B-220; positive fractions (*shaded areas* and *percentages*) are indicated. The specificities of the antibodies are as follows: stem cells and primitive progenitors (Sca-1 [23]), B-cell lineage (B220 [4]), macrophages and neutrophils (Mac-1), c-kit (Ack-2), and erythroid (Ter-119 [10])

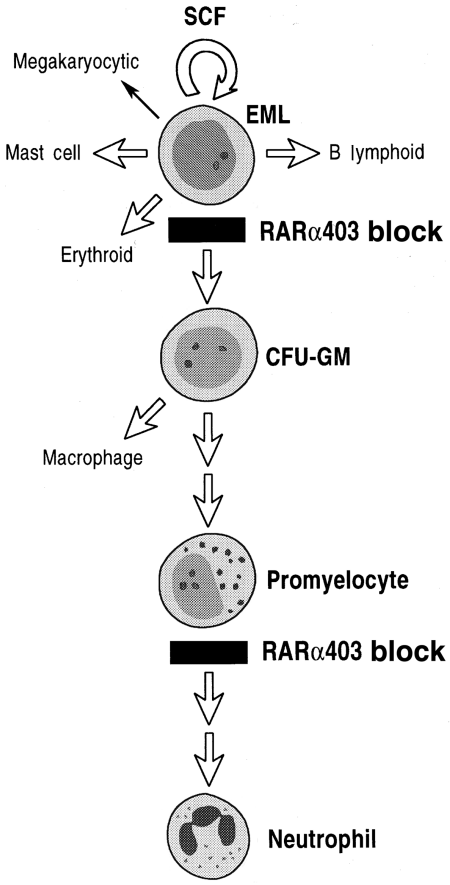

Fig. 3 Schematic summary of the lineage potentials of EML C1 cells, with emphasis on the neutrophil lineage. The SCF-dependent, self-renewing EML C1 lymphohematopoietic progenitor is shown at the *top*. The various lineages that have been observed in EML C1 populations are indicated. The 2 developmental blocks (at the pre-CFU-GM and the promyelocyte stages) caused by the dominant-negative RARα403 receptor are indicated by *solid bars*. Both blocks can be overcome by high concentrations of ATRA ($0.1-1.0\times10^{-5}$ M)

and proliferation of EML C1/c-fms cells was 80–100 ng/ml (Fig. 4). At this concentration, EML C1/c-fms cells proliferated exponentially upon addition of hM-CSF, without any apparent lag period.

EML C1/c-fms cells exhibit different development patterns in response to SCF and M-CSF

When the parental EML C1 cell line is stimulated by SCF, it generates mostly erythroid (BFU-E and CFU-E) and B-lymphoid progenitors and few myeloid progenitors (CFU-GM) [26]. Similarly, when EML C1/c-fms cells are stimulated by SCF, they produce mostly erythroid and B-lymphoid progenitors, but a moderate increase in the number of CFU-GM is detected in the presence of SCF alone (Table 1). Upon switching from SCF to M-CSF, EML C1/c-fms cells exhibit a different differentiation pattern. At 3 days after the SCF-M-CSF switch, large numbers of CFU-

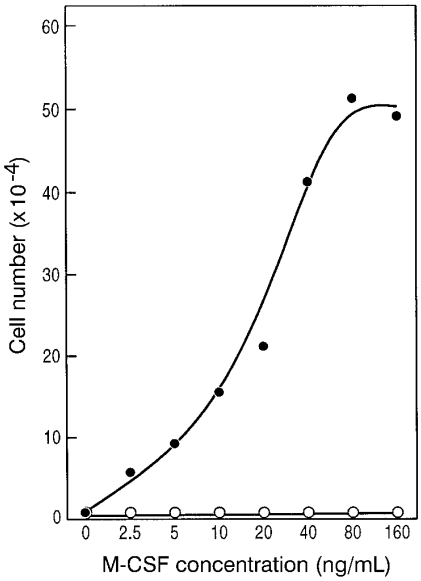

Fig. 4 M-CSF dose-response curves generated for EML C1 (*white circles*) and EML C1/c-fms cells (*black circles*). Washed cells (2×10^{-5}) were treated with hM-CSF at various concentrations, and surviving cells were counted after 24 h. Each data point represents the mean value for duplicate determinations. EML C1 cells cannot respond to hM-CSF at any concentration. The optimal concentration of hM-CSF for EML C1/c-fms is 80–100 ng/ml

GM can be found along with a concomitant decrease in the numbers of BFU-E as detected by colony assays (Table 1) and of B-lymphoid progenitors as determined by FACS (data not shown). This pattern is preserved after a longer, 6-day exposure to M-CSF (Table 2) and by clonal EML C1/c-fms lines (Table 3).

The identity of the cells in the CFU-GM colonies can be verified by examining Wright-stained cytospin preparations of individual colonies. All such colonies contain neutrophils, promyelocytes, and macrophages, with the majority being neutrophils and promyelocytes. Many of these promyelocytes can proliferate as GM-CSF-dependent promyelocyte cell lines similar to the MPRO (for mouse promyelocyte) cell line described previously [24]. This promyelocyte block is apparently caused by the dominant-negative RARα403, as is the case in MPRO cells. The finding of neutrophils and promyelocytes in EML C1/c-fms-derived CFU-GM colonies indicates that the premature expression and activation of c-fms do not commit the multipotent progenitors in the EML C1/c-fms cell line to the monocyte/macrophage lineage and do not preclude differentiation in the neutrophil lineage.

Two different mechanisms may account for the apparent activation of c-fms in EML C1/c-fms cells in the absence of exogenous M-CSF

The increased production of CFU-GM by EML C1/c-fms cells, even in the absence of exogenous M-CSF (Tables 1, 2), suggests that some of the ectopically expressed c-fms

Table 1 Effects of SCF and M-CSF on production of primitive erythroid and myeloid progenitors[a]

Cell	Preincubation growth factor (3 days)	BFU-E	CFU-GM	CFU-GM: BFU-E
EML C1	SCF	24.0 ± 1.7	0.03 ± 0.6	0.01
	M-CSF	(0)	(0)	(–)
EML/c-fms	SCF	23.7 ± 9.3	16.7 ± 6.8	0.7
	M-CSF	2.0 ± 1.7	52.0 ± 8.2	26

[a] EML C1 or EML/c-fms (bulk) cells (1×10^5) were treated with either murine (m) SCF at 80 ng/ml or human (h) M-CSF at 80 ng/ml for 3 days and were then washed 3 times with phosphate-buffered saline (PBS). The total respective cell number after 3 days was: EML C1 + SCF, 13.2×10^5; EML C1 + M-CSF, 0; EML/c-fms + SCF, 11.7×10^5; and EML/c-fms + M-CSF, 12.0×10^5. An aliquot of each culture (1/400 v/v) was plated in methylcellulose culture medium in a 35-mm dish and then stimulated using either mSCF at 80 ng/ml + EPO at 10 U/ml for BFU-E detection or mGM-CSF at 20 ng/ml for CFU-GM detection. Colonies were scored on day 8. Data represent mean values ± SEM for 3 cultures

Table 2 Effects of SCF and M-CSF on production of primitive erythroid and myeloid progenitors[a]

Cell	Preincubation growth factor (3 days)	BFU-E	CFU-GM	CFU-GM: BFU-E
EML C1	SCF	28.0 ± 1.0	2.7 ± 1.5	0.1
	M-CSF	(0)	(0)	(–)
EML/c-fms	SCF	49.7 ± 1.5	19.7 ± 6.4	0.4
	M-CSF	1.7 ± 1.5	49.7 ± 4.5	29.2

[a] EML C1 or EML/c-fms (bulk) cells (1×10^5) were treated with either mSCF at 80 ng/ml or hM-CSF at 80 ng/ml for 6 days and then washed. The total respective cell number after 6 days was: EML C1 + SCF, 3.3×10^7; EML C1 + M-CSF, 0; EML/c-fms + SCF, 3.1×10^7; and EML/c-fms + M-CSF, 5.5×10^7. An aliquot of each culture (1/10,000 v/v) was plated in methylcellulose culture medium in a 35-mm dish and then stimulated using either mSCF at 80 ng/ml + EPO at 10 U/ml for BFU-E detection or mGM-CSF at 20 ng/ml for CFU-GM detection. Colonies were scored on day 8. Data represent mean values ± SEM for 3 cultures

Table 3 Effects of SCF and M-CSF on production of primitive erythroid and myeloid progenitors by clonal EML/c-fms cell lines[a]

Cell	Preincubation growth factor (3 days)	BFU-E	CFU-GM	CFU-GM: BFU-E
EML/c-fms C2.1	SCF	119	1.5	0.01
	M-CSF	41.9	34.4	0.82
EML/c-fms C10	SCF	16	0	< 0.06
	M-CSF	1.5	20	13.3

[a] Cells (2×10^5) were treated with either mSCF at 80 ng/ml or hM-CSF at 80 ng/ml for 3 days and then washed 3 times with PBS. An aliquot of each culture (1/200 v/v) was plated in methylcellulose culture medium in a 35-mm dish and then stimulated using either mSCF at 80 ng/ml + EPO at 10 U/ml for BFU-E detection or mGM-CSF at 20 ng/ml for CFU-GM detection. Data represent mean values for 2 cultures

may be activated. Two plausible mechanisms may explain this apparent activation of c-fms without exogenous M-CSF. It has been shown that high-level expression of c-fms on the cell surface may lead to activation of c-fms [22]. In view of the high-level expression of exogenous c-fms in EML C1/c-fms cells, at least some of the spontaneous activation of c-fms in this cell line could be attributed to this mechanism. Another possibility is that c-fms is activated by an autocrine or juxtacrine mechanism. To determine whether EML C1 cells were capable of autocrine/juxtacrine production of murine M-CSF, reverse transcription-polymerase chain reaction (RT-PCR) was performed on total RNA from EML C1 cells. The results indicate that EML C1 cells express M-CSF mRNA (Fig. 5). Thus, it seems that both mechanisms (i.e., activation at high-level expression and autocrine production of M-CSF) may account for the c-fms effects observed in the absence of exogenous M-CSF.

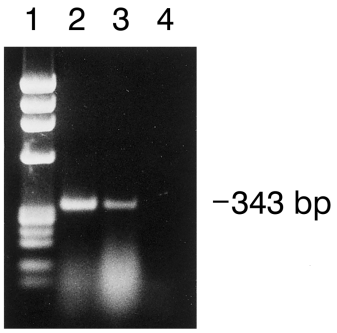

Fig. 5 Detection of M-CSF mRNA in EML C1 by RT-PCR performed using 1 μg of total RNA from the stromal cell line W20 F1 (positive control) and from EML C1 cells. The murine M-CSF primers used were 5′-TTTCCCCCTCCACGCCTGTAG and 5′-CTCCCCAAGGGGCAGCACAATGCCCCA. These primers span the intron-exon junctions of the murine M-CSF gene and prevent amplification of genomic DNAs. The PCR products were resolved on a 2% agarose gel and were stained with ethidium bromide (*Lane 1* HaeIII-digested ϕX174 DNA, *lane 2* W20 F1 mRNA, *lane 3* EML C1 mRNA, *lane 4* buffer)

M-CSF + erythropoietin is capable of supporting proliferation and differentiation of preformed BFU-E derived from EML C1/c-fms cells

The rapid disappearance of primitive erythroid progenitors (BFU-E) from EML C1/c-fms cell cultures stimulated with M-CSF could result from a decrease in commitment along the erythroid lineage, from selective killing of BFU-E by c-fms stimulated with M-CSF, or from suboptimal survival/proliferative support of BFU-E by c-fms stimulated with M-CSF. To rule out the second and third possibilities, colony assays of EML C1/c-fms cell (previously maintained in SCF, not M-CSF)-derived BFU-E were performed in methylcellulose culture medium supplemented with either SCF at 80 ng/ml + erythropoietin (EPO; 10 U/ml) or hM-CSF at 80 ng/ml + EPO at 10 U/ml. The results are summarized in Table 4. The combination of M-CSF and EPO can support proliferation and differentiation of EML C1/c-fms-derived BFU-E, although the number of BFU-E colonies supported by M-CSF + EPO appears to be slightly

Table 4 M-CSF + EPO supports development of BFU-E colony development[a]

Growth factor in colony assay	BFU-E	Mixed BFU-E + undifferentiated	Undifferentiated
SCF + EPO	30.0 ± 5.0	3.3 ± 2.5	16.3 ± 0.6
M-CSF + EPO	25.7 ± 6.7	2.0 ± 1.7	6.7 ± 0.6

[a] EML/c-fms cells maintained in mSCF at 80 ng/ml were washed 3 times with PBS and counted. Washed cells (3000) were plated in a methylcellulose culture medium in a 35-mm dish and then stimulated using either mSCF at 80 ng/ml + EPO at 10 U/ml or hM-CSF at 80 ng/ml + EPO at 10 U/ml. Colonies were counted on day 8. Data represent mean values ± SEM for 3 cultures

lower than that supported by SCF + EPO and the size of the average colony seems smaller. This result indicates that c-fms is not toxic to EML C1/c-fms-derived BFU-E but does not rule out the possibility of suboptimal survival/proliferative support of EML C1/c-fms-derived BFU-E by M-CSF as the basis for the decreased BFU-E numbers observed in EML C1/c-fms cell populations after a switch to M-CSF for 3–6 days.

Discussion

Both c-kit and c-fms are members of the type III tyrosine kinase growth-factor receptor family, which also includes the platelet-derived growth-factor receptor-α (PDGF-Rα) and PDGF-Rβ, flt1, flt3/flk2, flt4, and kdr/flk1. Murine c-fms is expressed primarily in cells committed to the monocyte/macrophage lineage, but it can also be detected in osteoclasts, trophoblasts, and, to a lesser degree, in B-cells [2]. Murine c-kit is expressed in a spatially and developmentally regulated manner in a variety of tissues [15], including pluripotent hematopoietic stem cells and progenitors, melanocytes, mast cells, germ cells, specific neurons and glial cells, and lung and gut epithelia [14]. Binding of their respective ligands (M-CSF and SCF) to c-fms and c-kit induces dimerization of the receptor and activation of the tyrosine kinase catalytic domain. The activated receptor dimers then transphosphorylate each other and phosphorylate a number of SH_2-containing proteins directly or indirectly, including the 85-kDa subunit of phosphatidylinositol 3′ kinase (PI3 K), Grb2, and p150 [12]. Whereas activated c-kit interacts with both GTPase-activating protein (GAP) and phospholipase Cγ (PLCγ), activated c-fms exhibits relatively weak associations with GAP and does not bind PLCγ [8, 11, 17, 19–21]. Thus, c-kit and c-fms appear to utilize overlapping as well as distinct signal-transduction pathways.

The current study compares the effects of c-kit and ectopically/prematurely expressed c-fms on the differentiation pattern of the unique, SCF-dependent murine lympho-hematopoietic cell line EML C1. The multipotentiality and the expression of c-kit but not c-fms in EML C1 cells make them an attractive model for comparative studies of the biochemical and cell-biological effects of c-kit and c-fms on primitive hematopoietic progenitor cells. It is surprising that ectopically expressed c-fms is capable of supporting proliferation of infected EML C1 cells. The growth rate of EML C1/c-fms cells in response to M-CSF (at the optimal concentration of 80 ng/ml) is equal to or greater than that of EML C1/c-fms cells stimulated with SCF at the optimal concentration (80–100 ng/ml) during the 1st week after the growth-factor switch. This finding suggests that these two receptors must utilize largely overlapping signal-transduction pathways to convey growth-promoting signals.

In contrast, the types of committed progenitors in EML C1/c-fms cell populations stimulated with SCF alone or M-CSF alone appear to be markedly different. Whereas SCF-stimulated EML C1/c-fms cell cultures contain mostly erythroid and B-lymphoid progenitors, as do parental EML C1 cells, M-CSF-stimulated EML C1/c-fms cultures contain mostly myeloid (CFU-GM) progenitors. This apparent erythroid-myeloid switch may be the result of different commitment processes, different survival rates of EML C1/c-fms-derived erythroid (BFU-E) and myeloid (CFU-GM) progenitors in response to SCF and M-CSF, or the selective toxicity of M-CSF for EML C1/c-fms-derived erythroid progenitors. The last possibility is ruled out by the observation that a combination of M-CSF and EPO can support the proliferation and differentiation of BFU-E derived from EML C1/c-fms cells (Table 4). In the same experiment we also observed that the number and the size of BFU-E supported by M-CSF + EPO are somewhat smaller than those of BFU-E supported by SCF + EPO. This finding suggests that the different survival rates of BFU-E and CFU-GM may account for at least some of the observed difference. However, it does not rule out the possibility that the commitment pattern of the multipotent progenitors in EML c1/c-fms populations may be different in the presence of SCF or M-CSF alone. Further experiments are required to make these distinctions.

Although it remains to be determined whether c-fms can influence the choice of erythroid (and B-lymphoid) versus myeloid lineages by the multipotent progenitors in EML C1 populations, it is clear that c-fms expression and activation do not commit all progenitors to the monocyte/macrophage lineage because most mature cells in CFU-GM-derived colonies are neutrophils and promyelocytes. In any case, different progenitors are found in EML C1/c-fms populations stimulated with SCF alone versus M-CSF alone, indicating that c-kit and c-fms must also utilize unique in addition to common signal-transduction pathways. The EML C1 cell line may serve as a useful system for identification of these unique (and common) pathways.

Acknowledgements The author wishes to thank Drs. Roland Bourette and Steve Bartelmez for technical assistance and Grant McArthur for useful discussions. This work was supported by a grant (DK48622) from the National Institutes of Health (Bethesda, Md., USA).

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