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Expression of a constitutively active erythropoietin receptor in primary hematopoietic progenitors abrogates erythropoietin dependence and enhances erythroid colony-forming unit, erythroid burst-forming unit, and granulocyte/macrophage progenitor growth

PAMELA N. PHARR*, DAVID HANKINS†, ANN HOFBAUER*, HARVEY F. LODISH‡§¶, AND GREGORY D. LONGMORE‡‖**

*Medical University of South Carolina and Veterans Affairs Medical Center, Charleston, SC 29403; †Johns Hopkins Medical School, Baltimore, MD 21205; ‡Whitehead Institute for Biomedical Research, Cambridge, MA 02142; §Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; and ‖Division of Hematology-Oncology, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA 02115

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ABSTRACT **We tested the ability of a constitutively activated erythropoietin receptor [EpoR(R129C)] to alter the growth requirements of primary hematopoietic precursors that terminally differentiate in culture. Two recombinant retroviruses expressing EpoR(R129C), spleen focus-forming virus (SFFVc-EpoR) and myeloproliferative sarcoma virus (MPSVcEpoR), were used to infect fetal liver cells that served as a source of hematopoietic progenitors. Methylcellulose cultures were incubated in the absence of any added growth factors or in combination with selected growth factors. EpoR(R129C) completely abrogated the Epo requirement of erythroid colony-forming units to form erythrocytes after 2–5 days in culture and did not interfere with the differentiation program of these cells. In the absence of added growth factors EpoR(R129C) did not enhance erythroid burst-forming unit development. In contrast to experiments in heterologous cell lines, EpoR(R129C) did not render progenitor cells independent of interleukin 3 or granulocyte/macrophage colony-stimulating factor (GM-CSF). However, when progenitors were cultured with added steel factor, but not with interleukin 3 or GM-CSF, EpoR(R129C) augmented the growth and differentiation of erythroid bursts, mixed erythroid/myeloid, and granulocyte/macrophage (GM) colonies. Furthermore, both viruses were capable of expressing EpoR(R129C) in erythroid, mixed erythroid/myeloid, and GM colonies. Thus an aberrantly expressed and constitutively activated EpoR can stimulate proliferation of some GM progenitors. The ability of EpoR(R129C) to abrogate the Epo requirement of primary hematopoietic cells, but not the requirement for other cytokines, is consistent with the induction of erythroblastosis *in vivo*.**

The precise role of hematopoietic growth factors in hematopoiesis has not been established, but they can affect cellular viability, differentiation, proliferation, and end stage function (1–3). These regulatory molecules influence their target cells through interaction with ligand-specific receptors on the cell surface. The receptors for erythropoietin (Epo), interleukins 2–7 (IL-2, IL-3, IL-4, IL-5, IL-6, IL-7), granulocyte/macrophage colony-stimulating factor (GM-CSF), and G-CSF define a new hematopoietin receptor superfamily (4, 5). These type 1 membrane proteins share common structural features within their extracellular domains yet have divergent cytoplasmic domains that lack any known signal transducing motifs; the mode(s) of signal transduction remains largely unknown. Expression in heterologous cell lines of cDNAs encoding members of the hematopoietin receptor superfamily has fostered the notion that some share aspects of common signaling pathways. For example, expression of the Epo receptor (EpoR) in IL-3-dependent cell lines (6, 7) allows these cells to proliferate in medium containing added Epo. Whether or not the EpoR can supplant the IL-3 receptor or other cytokine receptors during hematopoiesis is not known.

A point mutation in the extracellular domain (R129C) of the EpoR generates a receptor, EpoR(R129C), that confers growth factor-independent proliferation upon certain hematopoietic cell lines (8, 9). Mice infected with a recombinant spleen focus-forming virus (SFFV) expressing EpoR(R129C) (SFFVcEpoR) develop erythrocytosis, and growth factor-independent erythroleukemic cell lines can be isolated from the spleen of infected mice (9, 10). In these infected mice unregulated proliferation of erythroid progenitors predominates. This erythroid restriction may be the result of a transcriptional restriction imposed upon EpoR(R129C) by the SFFV long terminal repeats (LTRs) (11–13) or the inability of EpoR(R129C) to function in cells other than erythrocyte progenitors.

Here we tested the capabilities of EpoR(R129C) to alter the growth requirements of primary fetal liver hematopoietic precursors that terminally differentiate in culture. EpoR(R129C) completely abrogated the Epo requirement for erythroid colony-forming units (CFU-E) to form erythrocytes after 2–5 days in culture and did not interfere with the differentiation program of these cells. In the absence of added growth factors EpoR(R129C) did not enhance erythroid burst-forming unit (BFU-E) development or result in other progenitor cell growth. However, when progenitors were cultured with added steel factor (SF), but not with IL-3 or GM-CSF, EpoR(R129C) augmented the growth and differentiation of BFU-E, mixed erythroid/myeloid, and some granulocyte/macrophage (GM) progenitor cells. This demonstrates that an aberrantly expressed, constitutively activated EpoR can stimulate proliferation of at least some GM progenitors, indicating also that the transcriptional regulatory unit of SFFV is capable of functioning in nonerythroid cells.

MATERIALS AND METHODS

Retroviruses. Construction and generation of the SFFVEpoR and SFFVcEpoR viruses have been described (9) (Fig.

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Abbreviations: CFU-E, erythroid colony-forming unit(s); BFU-E, erythroid burst-forming unit(s); GM, granulocyte/macrophage; GM-CSF, GM colony-stimulating factor; SF, steel factor; SFFV, spleen focus-forming virus; MPSV, myeloproliferative sarcoma virus; Epo, erythropoietin; EpoR, Epo receptor; IL, interleukin; LTR, long terminal repeat.

¶To whom reprint requests should be addressed.
**Present address: Division of Hematology-Oncology, Washington University School of Medicine, St. Louis, MO 63110.

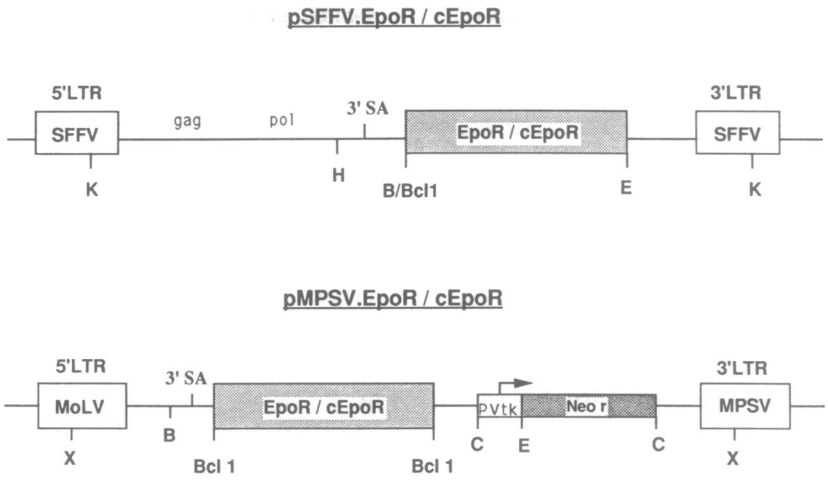

FIG. 1. Plasmid vectors used to generate retroviruses. The *Kpn* I–*Eco*RI fragments of wild-type EpoR and EpoR(R129C) cDNAs were modified and subcloned into plasmids pSFF and pGD′ as described in the text. MoLV, Moloney leukemia virus.

1). To generate a myeloproliferative sarcoma virus (MPSV) expressing the wild-type EpoR and EpoR(R129C), the *Kpn* I–*Eco*RI fragments encoding EpoR and EpoR(R129C) cDNAs were isolated from plasmids pXMEpoR and pXMc-EpoR, respectively (8). The ends were blunted, *Bcl* I linkers were ligated to the ends, and the fragments were ligated into the *Bcl* I site of plasmid pGD′ (14). The resultant plasmids pMPSVEpoR and pMPSVcEpoR (Fig. 1) were transfected into the retroviral packaging cell lines ψCRE and ψCRIP (15) and G418-resistant pools of each packaging cell line were isolated. High-titer viruses were generated by mixing the two G418-resistant pools. The resultant viruses were termed MPSVEpoR and MPSVcEpoR.

Cell Lines. The Epo-dependent erythroleukemic cell line HCD57 (16) was maintained in Iscove's modified Dulbecco's essential medium supplemented with 20% heat-inactivated fetal calf serum and 0.5 unit of Epo per ml. Factor-independent HCD57cEpoR cells were generated by infecting HCD57 cells with the SFFVcEpoR virus (9). ψCRE and ψCRIP cells were maintained in Dulbecco's modified essential medium containing 10% heat-inactivated calf serum.

Infection and Culture of Hematopoietic Progenitor Cells. Single cell suspensions of fetal livers were prepared from day 13 pregnant BALB/c mice (Charles River Breeding Laboratories). Cells were washed three times in α-medium. Cells (10^6) were resuspended in medium containing fresh or frozen virus, and 4 μg of Polybrene per ml was added; cells were then kept on ice for 2.5 hr. Following infection, samples were washed in α-medium and replated in α-medium containing 30% fetal bovine serum (Sterile Systems, Logan, UT), 1% crystallized bovine serum albumin (Sigma), 1.2% 1500-cP (1 P = 0.1 Pa·sec) methylcellulose (Fisher Scientific), and 50 μM 2-mercaptoethanol (Sigma) at a cell concentration of 10^5 per ml unless otherwise specified. Mouse SF was provided by Steven Clark (Genetics Institute, Cambridge, MA) (17). Recombinant mouse IL-3 was a gift from Tetsuo Sudo (Biomaterial Research Institute, Yokohama, Japan). Partially purified human urinary Epo, specific activity = 250 units/mg, was a generous gift from M. Kawakita (Kumamoto University, Japan).

RNA PCR. RNA was extracted by a modification of the guanidine isothiocyanate/acid/phenol method described by Chomczynski and Sacchi (18). cDNA synthesis was carried out with random hexamer primers and Superscript reverse transcriptase (BRL). cDNA from individual colonies was prepared by a modification of the method described by Iscove and coworkers. (19). PCR was carried out with AmpliTaq (Perkin–Elmer/Cetus) using a DNA thermocycler (Perkin–Elmer/Cetus) under reaction conditions recommended by Cetus. Fifty cycles were used: 1 min at 94°C, 2 min at 55°C, and 4 min at 72°C. The primers were as follows (Fig. 3) (5′ → 3′): primer 1, 1421-CAGATTACAGT-TCGGGGGGCT-1441 of the EpoR cDNA (20); primer 2, 1015-CTGGAGGAGGAGGCTGAAGAG-995 from a remaining carboxyl-terminal piece of the *env* gene of SFFV-P (21); primer 3, 31-GACAAACTCAGGGTGCCCCTC-51 of the EpoR cDNA (20); primer 4, 387-CTCCAGCGGCA-CAAAACTCGA-371 of the EpoR cDNA (20); and primer 5, 5′-TGGTCTCGCTGTTCCTTGGGA-3′ from the MPSV LTR (22). The β-actin primer set was from Clontech. The concentration of each primer in the reaction mixture was 1 μM. PCR samples were blotted onto Biotrans nylon membranes (ICN). Filters were prehybridized in 50% formamide, 6× SSPE (0.4 M NaCl/0.06 M sodium phosphate, pH 7.4/6 mM EDTA), 5× Denhardt's solution, 0.5% SDS, and 200 μg of denatured, fragmented salmon sperm DNA per ml for 1 hr at 42°C. Overnight hybridization was carried out under the same conditions with an internal EpoR oligonucleotide probe, end labeled with [^{32}P]ATP (23). Blots were washed in 6× SSPE, three times for 15 in at room temperature and once for 15 min at 58°C, and then exposed by autoradiography.

RESULTS

To understand the role of signals transduced by the EpoR in hematopoiesis, a constitutively activated EpoR was introduced into hematopoietic progenitor cells by infecting fetal liver cells with two high-titer retroviruses expressing EpoR(R129C). In the absence of any added hematopoietic growth factor, only committed erythroid progenitors were stimulated to proliferate and differentiate (Table 1). Epo-independent erythroid colonies were observed between days 2 and 5 of culture, indicating that these resulted from CFU-E proliferation and differentiation, normally dependent on added Epo. The abrogation of Epo dependency for CFU-E growth following infection was complete and maximal, in that no significant increase in erythroid colony development occurred with the addition of Epo to EpoR(R129C)-infected cultures, as compared to uninfected or wild-type EpoR-infected cultures with added Epo (Table 1, experiment A). All colonies contained hemoglobinized cells, implying that there was no arrest in the erythroid differentiation program of these infected CFU-E (data not shown). SFFVcEpoR and MPSVc-EpoR had the same stimulatory effect (Table 1). Introduction of the wild-type EpoR gene into progenitor cells had no effect upon erythroid colony growth in the absence of added growth

Table 1. Abrogation of Epo requirement of erythroid colonies infected with EpoR(R129C)-expressing retroviruses

Exp.	Virus	Epo, unit/ml 0	Epo, unit/ml 0.3
A	None	152 ± 17	350 ± 127
	EpoR-MPSV	136 ± 11	1126 ± 8
	cEpoR-MPSV	934 ± 302	1306 ± 133
B	None	30 ± 4	ND
	EpoR-SFFV	20 ± 5	ND
	cEpoR-SFFV	172 ± 38	ND

Fetal liver cells, isolated at day 13 of gestation, were infected and cultured at 5×10^4 cells per ml. Data are expressed as the mean ± standard deviation of triplicate cultures. Cultures were scored on day 4. Experiments (Exp.) A and B were done with different fetal liver samples and on different days. ND, not determined.

factors (Table 1). Heat-treated virus was inactive as was the supernatant from untransfected retroviral packaging cell lines (data not shown). Colonies of other lineages, including megakaryocytes, macrophages, and granulocytes, were not seen. Furthermore late-appearing (day 7) erythroid colonies or bursts that normally require additional growth factors such as IL-3, GM-CSF, or SF were not observed following infections with EpoR(R129C)-containing viruses.

To determine if EpoR(R129C) could abrogate the Epo requirement of BFU-E, we cultured SFFVcEpoR virus-infected fetal liver cells with SF, IL-3, or GM-CSF. EpoR(R129C) was capable of augmenting BFU-E development only in the presence of SF (Fig. 2*A*) but not IL-3 (Fig. 2*D*) or GM-CSF (data not shown). The average size of day 7 erythroid burst colonies (BFU-E) in cultures containing SF was 3.5 times larger in infected than in uninfected cultures. In addition, EpoR(R129C) in the presence of SF, but not of IL-3 or GM-CSF, resulted in a slight but significant increase in the number of GM colonies (Fig. 2 *C* and *F*, and data not shown). The average size of GM colonies in infected cultures did not differ from those in uninfected cultures grown in SF. Also, the number of day 7 mixed erythroid/myeloid colonies was increased in SFFVcEpoR-infected cultures, relative to uninfected cells, in the presence of SF (Fig. 2*B*) but not in the presence of IL-3 (Fig. 2*E*) or GM-CSF (data not shown).

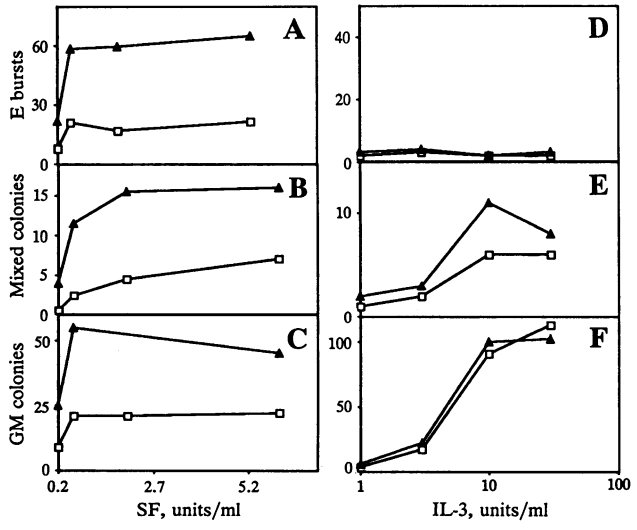

FIG. 2. Infected progenitor cell cultures in the presence of SF or IL-3. Fetal liver cells were infected with SFFVcEpoR (▲) or uninfected (□) and plated at 5×10^4 cells per ml in methylcellulose containing differing concentrations of SF (*A–C*) or IL-3 (*D–F*). On day 7 BFU-E (*A* and *D*), mixed erythroid/myeloid (*B* and *E*), and GM (*C* and *F*) colonies were scored. All points are the mean of triplicate cultures.

Significant enhancement in the number of erythroid bursts, mixed erythroid/myeloid, and GM colonies was also observed when MPSVcEpoR-infected progenitor cells were cultured in the presence of SF (data not shown). Importantly, the magnitude of the effect on GM colonies was the same regardless of which EpoR(R129C)-expressing retrovirus was used.

To demonstrate that the observed effect of EpoR(R129C) upon GM, mixed erythroid/myeloid, and BFU-E colony growth was due to a functional EpoR, fetal liver cells were infected with wild-type EpoR-expressing viruses. When cultured in the presence of SF along there was no effect upon progenitor cell growth as compared with uninfected cultures (data not shown). Addition of Epo (1 unit/ml) together with SF to cultures infected with wild-type SFFVEpoR or MPSVEpoR resulted in enhanced BFU-E and mixed erythroid/myeloid colony formation similar to that observed in EpoR(R129C)-infected cultures grown in SF alone (data not shown). However, addition of Epo plus SF to EpoR-infected cultures had little effect upon GM progenitor growth.

To determine if the BFU-E and GM differentiation following infection of progenitors with EpoR(R129C)-expressing viruses was indeed due to the presence of EpoR(R129C) in the respective progenitor populations, we developed an RNA PCR assay to detect EpoR mRNA expressed from integrated SFFVcEpoR proviruses. Specific primers (1 and 2) were designed to amplify only EpoR transcripts expressed from SFFV-LTR driven viral transcripts (see Fig. 3). To demonstrate that only SFFV-derived EpoR transcripts indeed were amplified we performed control PCRs on RNA isolated from either Epo-dependent HCD57 cells or factor-independent HCD57 cells derived from infection with SFFVcEpoR (HCD57cEpoR). As shown in Fig. 3, PCR amplification with primers 1 and 2 detects EpoR transcripts only in cells infected with SFFVcEpoR (lane 2 vs. lane 3), whereas primers 3 and 4 amplify the endogenous EpoR transcript present in HCD57 and the endogenous and SFFVcEpoR-derived EpoR transcript in HCD57cEpoR cell lines (lanes 4 and 5). As a control, actin mRNA was detected in HCD57 and HCD57cEpoR cells (lanes 6 and 7). Next, GM, mixed erythroid/myeloid, and day 7 erythroid BFU-E colonies from SFFVcEpoR-infected cultures, generated in the presence of SF, were picked and cDNA PCR amplification was performed with primers 1 and 2. PCR products were blotted onto nylon membranes and probed with an EpoR oligonucleotide internal to the primer set used (Fig. 4). Only colonies (lanes A2, A3, and A4) derived from cultures infected with SFFVcEpoR expressed an EpoR transcript, as did HCD57cEpoR cells (lane A1). Mixed erythroid/myeloid and GM colonies from uninfected cultures were, as expected, negative (lanes B2 and B3). Similar experiments done with colonies picked from MPSVcEpoR-infected cultures, generated in the presence of SF, also showed expression of retrovirally derived EpoR transcripts in GM, mixed erythroid/myeloid, and day 7 erythroid BFU-E colonies (data not shown). The reduced effect of EpoR(R129C) on GM colony growth, as compared to erythroid colony growth, could result from reduced efficiency of retroviral infection or gene expression in GM colonies relative to erythroid colonies. Table 2 shows the percentage of single colonies expressing EpoR(R129C) following infection with the two retroviruses. In both viral-infected cultures equal fractions of GM and erythroid colonies express EpoR(R129C).

Thus, EpoR(R129C) expression in GM progenitors confers a small but significant growth advantage when these cells are cultured in the presence of SF, suggesting that EpoR(R129C), in the presence of SF, is capable of functionally transducing a signal in at least some myeloid progenitor cells.

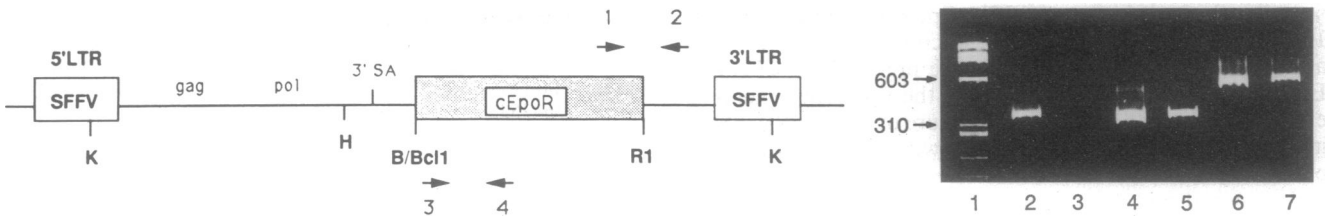

FIG. 3. RNA PCR analysis of EpoR expression in HCD57 and HCD57cEpoR cells. cDNA was generated from total RNA isolated from HCD57 cells (lanes 3, 5, and 7) or HCD57cEpoR cells (lanes 2, 4, and 6). PCR was performed using primers 1 and 2 (lanes 2 and 3), primers 3 and 4 (lanes 4 and 5), or β-actin primers (lanes 6 and 7). An aliquot of the PCR was separated on an 8% polyacrylamide gel stained with ethidium bromide. Lane 1 is a molecular size standard (BRL). The arrows identify 603- and 310-base-pair fragments in the standards lane.

DISCUSSION

The hematopoietic system is a dynamic one. Mature blood cells are continuously replaced with new cells thought to derive from pluripotent stem cells residing in the bone marrow of adult animals and the liver of fetal mice. Multipotent hematopoietic progenitors respond to various growth factors, including IL-3, IL-6, IL-11, and SF (24–29). After these progenitors become committed to a particular lineage, they require specific growth factors to complete the maturation process; for example, BFU-E and CFU-E require Epo to complete erythroid differentiation (30, 31). In the absence of Epo, proerythroblasts undergo apoptosis (2). The effects of Epo are restricted to the early erythroid, and possibly megakaryocytic cell lineages, and occur relatively late in the development of these cell types (30–32). This restricted cellular activity of Epo is thought to be, in part, modulated by the regulated expression of the gene encoding the cell-surface Epo receptor (33).

Here hematopoietic progenitors in fetal liver were infected with retroviruses expressing a constitutively activated EpoR. Epo-independent erythroid colonies were observed between days 2 and 5, consistent with differentiation of a population of cells, CFU-E, normally maximally sensitive to Epo (34, 35). The fact that wild-type EpoR-infected cultures showed no enhanced erythroid colony growth in the absence of Epo would argue against the role of small amounts of Epo, present in the fetal calf serum used in the cultures, in contributing to autonomous growth of erythroid colonies in EpoR(R129C)-infected cultures. The expression of EpoR(R129C) in this CFU-E population completely abrogated the need for exogenous Epo. In the absence of any added growth factor EpoR(R129C) did not affect BFU-E, GM, or megakaryocyte colony proliferation and differentiation. However addition of SF, but not IL-3 or GM-CSF, to EpoR(R129C)-infected progenitor cells led to the development of Epo-independent BFU-E colonies at day 7 as well as GM and mixed erythroid/myeloid colonies. The effects were due to expression of EpoR(R129C) since viruses expressing the normal EpoR were inactive unless Epo was added. RNA PCR analysis of colonies from SFFVcEpoR- and MPSVcEpoR-infected cultures containing added SF demonstrated that EpoR(R129C) was expressed in erythroid bursts, GM colonies, and mixed erythroid/myeloid colonies.

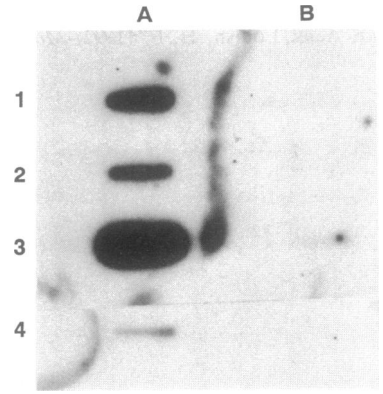

FIG. 4. Slot blot analysis of RNA PCR fractions prepared from the hematopoietic colonies isolated from SFFVcEpoR-infected fetal liver cells cultured in the presence of SF. Twenty-five colonies of each type were identified, picked, and pooled. Total RNA was isolated and cDNA was synthesized. PCR was performed using primers 1 and 2 and the product was blotted on nylon membranes. An EpoR probe, internal to the primers used, was end labeled with ^{32}P and hybridization carried out. Lanes: A1, HCD57cEpoR cells; A2, mixed erythroid/myeloid colonies; A3, GM colonies; A4, BFU-E colonies; B1, HCD57 cells; B2, uninfected mixed erythroid/myeloid colonies; and B3, uninfected GM colonies.

The effect of EpoR(R129C) expression on proliferation of GM progenitors and BFU-E was much less than on CFU-E proliferation: a 2.5- to 3-fold vs. a 10-fold stimulation, respectively. The reduced proliferative effect on EpoR(R129C) on BFU-E and CFU-GM was not the result of differing rates of infection or expression in the different colonies. Perhaps BFU-E and CFU-GM are relatively less responsive to the EpoR(R129C) than are CFU-E cells.

SF plus insulin and insulin-like growth factor I are capable of supporting the growth of highly purified human BFU-E in serum-free cultures, whereas IL-3 alone is not (36). Similarly, we observed a synergistic effect on SF and EpoR(R129C), but not IL-3 or GM-CSF, upon BFU-E proliferation and differentiation. IL-3 was, however, capable of supporting GM colonies in fetal liver cultures. One explanation for the observed cooperativity between the SF-c-kit and Epo-EpoR signal transducing pathways is that SF may expand an early Epo-unresponsive population of cells (37, 38). Alternatively, the interaction of SF with its receptor, c-kit, may directly or indirectly activate the EpoR, resulting in "priming" of the cells to Epo responsiveness (38).

The present results indicate that EpoR(R129C) is capable of transducing a growth signal in some nonerythroid, hematopoietic, cells. That the proliferative effects of EpoR(R129C) on nonerythroid cells are less than on erythroid progenitors may explain the predominant erythrocytosis ob-

Table 2. RNA PCR analysis of EpoR(R129C) transcript expression in single erythroid colonies and single GM colonies from viral-infected cultures

	Positive colonies			
	Erythroid		GM	
Virus	No.	%	No.	%
cEpoR-SFFV	6/8	75	6/11	55
cEpoR-MPSV	7/8	88	10/11	90

Single colonies were picked from virally infected cultures grown in the presence of 5 units of SF per ml. cDNA were generated, and PCR was performed using primers 1 and 2 for cEpoR-SFFV-infected colonies, and primers 5 and 4 were used for cEpoR-MPSV-infected colonies. The PCR product was blotted onto nylon membranes and probed with an EpoR oligonucleotide.

served in the early stages of SFFVcEpoR infection of mice (9, 10). The results obtained from the culture of primary cells agree with *in vivo* studies in that the predominant effect of EpoR(R129C) was the abrogation of the Epo requirement of erythroid progenitors (9, 10). The nature of the EpoR(R129C) intracellular signal is unknown. However, protein-tryosine phosphorylation is increased after Epo addition to erythroid and nonerythroid hematopoietic cell lines containing an EpoR (7, 39, 40). Similarly EpoR(R129C) might activate a protein kinase common to some myeloid and erythroid progenitors.

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