Both Megakaryocytopoiesis and Erythropoiesis Are Induced in Mice Infected With a Retrovirus Expressing an Oncogenic Erythropoietin Receptor

By Gregory D. Longmore, Pamela Pharr, Drorit Neumann, and Harvey F. Lodish

Increasing direct and indirect evidence suggests that erythropoietin (Epo) promotes both erythropoiesis and megakaryocytopoiesis. Here we report that, in mice infected with a recombinant spleen focus-forming virus (SFFV) expressing an oncogenic erythropoietin receptor (EpoR), there was an increase in platelet count preceding the ensuing erythropoiesis. Concurrently, there was a substantial increase in splenic megakaryocytes. Culture of the bone marrow and spleen cells from infected mice showed enhanced numbers of multipotent megakaryocytic progenitors. DNA polymerase chain reaction analysis of individual megakaryocyte-containing colonies showed recombinant SFFV (SFFVeEpoR) proviral integration. Immunofluorescence of spleen sections showed overexpression of EpoR protein in the megakaryocytes. Mice infected with a strain of SFFV also developed splenic megakaryocytosis without activating overexpression of the EpoR in megakaryocytes. This in vivo system shows that a relationship between erythropoiesis and thrombopoiesis can exist at the level of the Epo-EpoR signaling pathway. Also, SFFV-based vectors may be excellent vehicles for the introduction of genes into multipotent, hematopoietic progenitors, in vitro. © 1993 by The American Society of Hematology.

MEGAKARYOCYTOPOIESIS and thrombopoiesis are regulated by a number of cytokines, including granulocyte-macrophage colony-stimulating factor, leukemia inhibitory factor, interleukin-3 (IL-3), IL-6, IL-11, and c-kit ligand, also called stem cell factor. In cell culture experiments all of these individually or together affect the proliferation and differentiation of megakaryocytic progenitors, yet the role of these growth factors on platelet production in humans or animals remains to be determined. Some studies indicate that erythropoietin (Epo), the primary humoral agent regulating erythropoiesis, promotes megakaryocytopoiesis and increases platelet counts, thereby suggesting a relationship between erythropoiesis and thrombopoiesis. Chronic administration of Epo to rats results in early, nonsustained increases in platelet counts and an increase in thymidine labeling of megakaryocytes. Transgenic mice expressing the human Epo gene did not show increases in their platelet counts. However, mice administered high doses of Epo subcutaneously did develop an increase in platelet levels. In some but not in all human Epo trials, there was a slight thrombocytosis in response to Epo. Whether these effects of Epo on platelet development are the result of direct or indirect activation of megakaryocyte-progenitor proliferation and differentiation is not clear. However, some recent findings, together with our present results, suggest that the effect of erythropoietin on megakaryocytopoiesis can be direct.

Human leukemic cell lines showing bipotential erythroid and megakaryocytic features have been isolated. These cell lines express receptors for Epo (EpoR) and can proliferate and/or differentiate in response to added Epo. When murine bone marrow (BM) cells were placed in serum-free liquid cultures, addition of Epo caused increases in megakaryocyte size, ploidy, and acetylcholinesterase activity, which are indicative of Epo stimulation of proliferation of early megakaryocytes. Consistent with expression of EpoR on megakaryocytes, radiolabeled Epo binds to rat and mouse marrow megakaryocytes, but not to platelets, with kinetic properties similar to those of erythroblast EpoR.

Induction of erythroleukemia in mice by infection of spleen focus-forming virus (SFFV) occurs in several stages. Initiation of early polyclonal erythroblastosis results from activation of the EpoR by gp55, a membrane glycoprotein encoded by the env gene of SFFV, causing Epo-independent proliferation of erythroid progenitors. The cell-type specificity of the disease is thought to result from the restricted expression of the EpoR to early erythroid precursors. Mice injected with a recombinant spleen focus-forming virus (SFFVeEpoR) lacking gp55 but expressing the constitutively active (Epo-independent) mutant, R129C, of the murine EpoR, also develop polycythemia and erythroblastosis, which evolves into clonal erythroleukemia in a multistep fashion distinct from that induced by wild-type SFFV. Five weeks after SFFVeEpoR virus infection, mice show an increase in splenic megakaryocytes, temporally concurrent with the development of polycythemia and splenic erythroblastosis. However, no alterations in blood platelet levels were observed. We now show that, early in the course of SFFVeEpoR infection, there is an increase in platelet count that precedes the increase in hematocrit (Hct). Concurrently, there is an increase in splenic megakaryocytes. Infection with SFFVeEpoR conferred a growth factor-independent proliferative
antisera against the carboxy-terminal-
EPO AND MEGAKARYOCYTOPOIESIS
of the murine EpoR have been de~cribed.'~ For immunofluores-
dotype of SFFV.AP-L, a chimera of the polycythemic and anemic
poietic  progenitor^.^^

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erythropoiesis and thrombopoiesis at the level of the Epo-EpoR-
that gp55 is capable of activating this receptor for prolifera-
is capable of transducing a growth signal in megakaryocyte
infected with SFFV also suggests that a functional EpoR is
showed the expression of an EpoR in the splenic megakaryo-
the supematants. All viral stocks were filtered through 0.45-pm sterile
progenitors, in methylcellulose cultures. A polymerase
chain reaction (PCR) analysis of
Virus Week(s) After Spleen
Injection (no. of
mice studied) Hct
Reticulocyte No. (% of RBCs) Platelet Count (x10^12)/L Spleen Weight (mg) Spleen Megakaryocytes (x10^4)

None
1 (3) 47 5 450 ± 50 120 36 ± 12
3 (3) 46 5 470 ± 40 110 44 ± 11
5 (3) 47 5 455 ± 35 120 ND
SFFVAP-L
1 (3) 45 8 400 ± 70 130 52 ± 26
2 (3) 44 15 350 ± 50 1,250 650 ± 125
3 (4) 49 20 300 ± 80 1,300 500 ± 170
SFFVcEpoR
1 (3) 45 5 430 ± 80 120 48 ± 12
2 (4) 47 7 500 ± 80 190 76 ± 38
3 (8) 48 12 814 ± 90 400 1,190 ± 127
5 (4) 66 16 950 ± 180 975 2,602 ± 350

NIH/Swiss mice were injected intravenously with virus, and weekly blood samples were obtained. At weekly intervals mice were killed, and spleen weight and histology were determined. Values recorded are mean values ± SD. The methods for deriving estimates of reticulocyte count, platelet count, and spleen megakaryocyte number are detailed in Materials and Methods. The results are the combination of two separate experiments.

Abbreviation: ND, not determined.

advantage on BM and splenic multipotent megakaryocyte
progenitors, in methy1c cellulose cultures. A polymerase
chain reaction (PCR) analysis of DNA extracted from indi-
vidual megakaryocyte colonies showed that the SFFVcEpo-
provirus had integrated. Immunofluorescence studies
showed the expression of an EpoR in the splenic megakaryo-
cytes of mice infected with SFFVcEpoR, albeit to a lesser extent than in SFFVcEpoR-infected mice. This increase in
platelet count was not observed in mice infected with a strain of SFFV, despite an increase in total splenic mega-
karyocytes. Similarly, immunofluorescence did not detect
any increase in EpoR expression in splenic megakaryocytes of SFFV-infected mice.

These results suggest that a constitutively activated EpoR
is capable of transducing a growth signal in megakaryocyte
progenitors, resulting in megakaryocytosis and mild throm-
obycytosis. The increase in splenic megakaryocytes in mice infected with SFFV also suggests that a functional EpoR is
normally expressed in some megakaryocyte progenitors and
that gp55 is capable of activating this receptor for prolifera-
This in vivo system shows a relationship between eryth-
ropoiesis and thrombopoiesis at the level of the Epo-EpoR-
signaling pathway. Also, this study and others suggest that,
experimentally, SFFV-based vectors may be excellent vehi-
cles for introducing genes into murine multipotent-hematopoietic progenitors.23

MATERIALS AND METHODS

Reagents. The generation of the SFFVcEpoR virus has been
described,20 this EpoR bears the activating R129C point mutation.
Rauscher virus was derived from an NIH 3T3 clone secreting
Rauscher MuLV.20 V-406 viral supernatant, a Friend MuLV pseu-
dotype of SFFV.AP-L, a chimera of the polychromic and anemic
strains of SFFV,24 was provided by Dr S. Ruscetti (National Cancer
Institute [NCI], Frederick, MD). The generation and properties of
antisera against the carboxy-terminal-14 amino acid oligopeptide
of the murine EpoR have been described.25 For immunofluores-
cence experiments, this antisera was affinity-purified over an affigel
column coupled to the carboxy-terminal peptide.26 Monoclonal an-
tibody (MoAb) 7C 10 specifically recognizes the gp55 envelope pro-
tein of SFFV and was provided by Dr S. Ruscetti.27 Rabbit antihu-
mann von Willebrand factor (vWF) sera was provided by Drs D.
Beecham and R. Handin (Brigham and Women's Hospital, Boston,
MA).28

Mice. Six-week-old female NIH/Swiss mice were obtained
from NCI-Frederick. They were injected intravenously, via the lat-
eral tail vein, with 0.5 mL of a 1:20 dilution of SFFVAP-L or with a
7.3 mixture of SFFVcEpoR and 3T3-Rauscher MuLV culture su-
pernants. All viral stocks were filtered through 0.45-μm sterile
filters before injection.

Cell counting. Mice were anesthetized, and orbital plexus blood
collected with EDTA used as an anticoagulant. The Hct was deter-
mimed by sedimentation in heparinized capillary tubes and calcu-
lated from red blood cell (RBC) indices obtained from a Seronom-
Baker 9000 Cell Counter. Platelet numbers were determined from the
automated cell counter and were clumping-excluded through concurrent examination of blood smears. Blood smears were
stained with Wright-Giemsa or May-Grünewald stains (Sigma, St
Louis, MO). Reticulocyte numbers were determined by staining
blood smears with new methylene blue, and were reported as a
percentage of RBC's. Spleens were fixed in 10% formalin-phos-
phate, sectioned, and stained with hematoxylin and eosin. Splenic
megakaryocyte cell counts were performed by viewing at 400X
magnification and were expressed as megakaryocytes × area^3 ×
spleen weight (mg).

Protein extraction and immunoblot analyses. Isolated spleens,
placed in ice-cold phosphate-buffered saline (PBS), were teased
apart with forceps; large debris was removed, and the remaining
splenic cells were washed twice in PBS. Total detergent-soluble protein was isolated by addition of lysis buffer (50 mmol/L Tris [pH
7.4], 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS] 0.5 TIU/mL Aprotinin, and 1 mmol/L
phenylmethylsulfonyl fluoride [PMSF]). The cell suspension was
mixed vigorously and allowed to sit in ice for 20 minutes. Cellular
debris was removed by centrifuging at 10,000 g for 10 minutes and
the supernatant stored at -70°C. Protein concentration was deter-
mmeled by the BCA Protein Assay (Pierce Chemical Corp, Rockford,
IL). Samples were subjected to 8% polyacrylamide gel electrophoresis in the presence of SDS and 2-mercaptoethanol and blotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Immunoblotting was performed in 4% skim milk, 10 mMol/L TRIS (pH 7.4), 0.15 mol/L sodium chloride, and 0.05% Tween 20 using rabbit antiserum against the carboxy-terminus of the murine EpoR, as described previously. Gp55 immunoblots were performed using mouse MoAb 7C10. In some experiments, before electrophoresis, samples were treated with endoglycosidase H (Genzyme, Boston, MA). Briefly, 200 to 300 μg of protein extract was suspended in 0.1 mol/L sodium citrate (pH 7.0) and 1% SDS and boiled for 5 minutes. After cooling to room temperature, 2 μl of endoglycosidase H was added, and the samples incubated overnight at 37°C.

**Immunofluorescence.** Spleens were fixed by immersion in 4% paraformaldehyde in PBS for 6 hours at 4°C. For preparing 4-μm frozen sections, the spleens were equilibrated for 16 hours in 0.6 mol/L sucrose, subsequently embedded in O.C.T. compound (Miles, Elkhart, IN), frozen for 10 seconds in 2-methylbutane in liquid nitrogen, and kept at -70°C until sectioning in a Reichert Frigocut cryostat. Sections were placed on polylysine-coated glass slides, air dried, and kept at -70°C until staining. All antibodies were diluted in PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton X-100. Tissue sections were incubated for 30 minutes in PBS containing 0.1% Triton X-100 (Triton-PBS). Sections were then incubated in PBS containing 3% BSA for 30 minutes followed by 100 μg/mL normal goat IgG for 30 minutes. Sections were then incubated for 60 minutes with one of the following antibodies: 7 μg/ml affinity-purified sera raised against the carboxy-terminus of the Epo-R, rabbit antihuman vWF sera diluted 1:100, 10 μg/mL normal rabbit IgG. Thereafter sections were washed with Triton-PBS 3 times for 10 minutes each. Sections were then incubated with 100 μg/mL normal goat IgG for 10 minutes followed by 5 μg/mL TRITC-conjugated goat anti-rabbit IgG (Jackson Immuno Research Laboratories Inc., Avondale, PA) for 30 minutes. Sections were washed for 16 hours with Triton-PBS at 37°C, and mounted in glycerol, 2% n-propylgallate, and 0.2 mol/L Tris-HCl (pH 8.1). Sections were observed and photographed with Kodak TMAX film (Eastman Kodak, Rochester, NY) on a Zeiss Photomicroscope III (Zeiss, New York, NY).

**Methylcellulose culture of hematopoietic cell from infected mice.** Mice infected with SFFV-EpoR(R129C) were killed at various times, and BM and spleen cells were isolated. The BM suspension was sedimented through Ficoll-Paque (Pharmacia, Uppsala, Sweden) and the interface cells collected. Cells were washed 3 times in α-medium and replated in α-medium containing 30% fetal bovine serum (Sterile Systems, Inc., Logan, UT), 1% crystallized BSA (Sigma), 1.2% 1,500 centipose methylcellulose (Fischer Scientific Co, Norcross, GA), and 50 μmol/L 2-mercaptoethanol (Sigma) at a cell concentration of 10^5 cells/mL unless otherwise specified. Individual colonies were identified and picked for further analysis. Acetycholinesterase-positive staining confirmed the identity of all megakaryocyte containing colonies.

**DNA PCR.** Single colonies were placed directly in 100 μL of buffer B (10 mmol/L Tris, 1 mmol/L EDTA, 0.1% SDS, 100 μg/mL proteinase K, pH 8.0). Samples were digested overnight at 50°C. DNA was extracted with phenol/chloroform, precipitated with ethanol, and redissolved in 100 μL Tris, 1 mmol/L EDTA pH 8.0. PCR was performed with AmpliTag (Perkin Elmer-Cetus, Norwalk, CT) using a DNA thermocycler (Perkin Elmer-Cetus) under reaction conditions recommended by Cetus. Fifty cycles were used of 1 minute at 94°C, 2 minutes at 55°C, and 4 minutes at 72°C. The primers were as follows (from 5' to 3'): sense primer, 1421-CAGATTACAGTTCGGGGGTCC-1441 of the EpoR cDNA; and antisense primer, 1015-CTTGGAGGAAGAGGCGGTAAGAG-995 from a remaining carboxy-terminal piece of the env gene of polyclathemyc SFFV (SFFV-P). The concentration of each primer in the reaction mixture was 1 μmol/L. PCR samples were blotted onto Biotrans nylon membranes (ICN Pharmaceuticals, Inc., Plainview, NY). Filters were prehybridized in 50% formamide, 6X SSPE (0.4 mol/L NaCl, 0.06 mol/L sodium phosphate, pH 7.4, and 6 mmol/L EDTA), 5X Denhardt's solution, 0.5% SDS, and 200 μg/ml denatured, fragmented salmon sperm DNA for 1 hour at 42°C. Overnight hybridization was performed under the same conditions with an internal EpoR oligonucleotide probe, end-labeled with 32P adenosine triphosphate. Blots were washed in 6X SSPE three times for 15 minutes at room temperature, once for 15 minutes at 58°C, and then exposed by autoradiography.

**RESULTS**

To determine the effect of expression of the constitutively activated EpoR on megakaryocytopenis and erythropoiesis, mice were infected with the SFFV-EpoR virus and examined weekly for changes in reticulocyte counts, Hcts, platelet counts, spleen size, splenic histology, and the number of megakaryocytes present in the spleen (Table 1). These mice were compared with uninfected mice and with mice infected with a polyclathemyc strain of SFFV, SFFVAP-L. SFFV-EpoR-infected mice had a later onset of reticulocytosis and splenomegaly than did SFFV-infected mice, 3 to 4 weeks versus 1 to 2 weeks. The degree of splenomegaly was greater in the SFFV-infected mice, and these spleens showed a greater degree of tumor invasion than did spleens from mice infected with SFFV-EpoR (Fig 1; compare B and C). Approximately 3 weeks after infection with SFFV-EpoR, there was a twofold increase in the number of circulating platelets (Table 1). The increase in platelet count preceded the increase in Hct but paralleled the observed reticulocytosis. Concurrently, there was a 25-fold increase in the number of splenic megakaryocytes (Table 1 and Fig 1C). Despite a 10-fold increase in the number of splenic megakaryocytes, SFFV-infected mice did not show any change in the number of circulating platelets (Table 1 and Fig 1B). Grossly splenic tissue slices from SFFV-EpoR- or SFFV-infected mice did not show any change in size of megakaryocytes compared with those present in uninfected spleens. Similarly, we could detect no change in platelet size in blood smears.

Immunoblotting of splenic cell extracts from infected mice showed that the EpoR(R129C) and gp55 polypeptides were being expressed in SFFV-EpoR- and SFFV-infected spleens, respectively (Fig 2). Over the course of infection there was an increase in the amount of EpoR(R129C) (Fig 2A, lanes 1 through 6) and of gp55 protein expression (Fig 2B, lanes 3 through 6), respectively. No EpoR or gp55 proteins were detectable by immunoblotting in any SFFV- or SFFV-EpoR-infected splenic cell extracts, respectively (data not shown). This would exclude the possibility that SFFV activated expression of the EpoR gene.

To verify the identity of splenic megakaryocytes identified under phase contrast microscopy, we performed immunofluorescence analyses with an antibody against human vWF, a multimeric protein with expression restricted to megakaryocytes, platelets, and cytoplasmic granules (Weibel-Palade bodies) of endothelial cells. 29-31 Splenic mega-
karyocytes from uninfected mice, SFFV-infected mice, and SFFVcEpoR-infected mice all reacted positively with anti-vWF antibodies, in a pattern typical of the distribution of vWF in megakaryocytes (Fig 3 and data not shown).

To show that the megakaryocytosis observed in spleens after SFFVcEpoR infection was indeed due to the integration and expression of the SFFVcEpoR provirus in megakaryocytes or their progenitors, we cultured BM and spleen
cells from infected mice in the absence of any added growth factors or in the presence of added IL-3 and IL-6 (Table 2). Individual megakaryocyte-containing colonies were identified morphologically, confirmed with acetylcholinesterase staining, scored, and DNA isolated. DNA PCR analysis designed to amplify only an EpoR present in the SFFVcEpoR provirus were performed (Fig 4). Two weeks after infection, there was a proliferative advantage observed in megakaryocyte-progenitor cells, but only in the presence of IL-3 and IL-6. This effect preceded the increase in circulating platelet levels. The growth factor-dependent effect of SFFVcEpoR on megakaryocyte-progenitor growth was more pronounced at week 3. By week 5 growth factor-independent proliferation of megakaryocyte progenitors from infected mice was apparent. Of particular note was the dramatic effect on erythroid-megakaryocyte (E-Meg) progenitors. There also was a dramatic increase in day-7 erythroid and granulocyte-macrophage (GM) colonies in cultures of SFFVcEpoR-infected mice (data not shown), which is in agreement with our prior in vitro results. DNA PCR analysis of individual megakaryocyte-containing colonies from the infected mice BM and spleen cultures at week 2 (Fig 4), week 3, and week 5, in the presence and absence (data not shown) of growth factors, all showed integration of the SFFVcEpoR provirus. This was true for all megakaryocyte progenitors (day-5 Meg, day-7 E-Meg, and day-7 granulocyte-macrophage-megakaryocyte [GMM]), as well as day-7 erythroid and day-7 GM colonies. Thus, SFFVcEpoR infection with proviral integration appeared to impart a growth advantage on multipotent hematopoietic progenitors affecting erythroid, megakaryocytic, and GM colony proliferation.

To demonstrate that integrated SFFVcEpoR proviruses expressed their gene products in megakaryocytes, we performed immunofluorescence analyses on spleen sections from infected mice and contrasted them with spleen sections from mice infected with SFFV. For such experiments we used an affinity-purified antibody, specific to the carboxy-terminal peptide of the murine EpoR. In cell lines expressing endogenous EpoR, neither immunofluorescence nor immunoblotting with this antisera detects an EpoR protein (Longmore and Lodish data not shown). However, overexpression of the EpoR, after infection of cells with SFFVcEpoR, allows for easy detection of EpoR protein by immunofluorescence and immunoblotting (data not shown). Presumably, this reflects the low level of EpoR protein expression normally present and also reflects the sensitivity limits of our particular antisera. Megakaryocytes in splenic sections from mice infected with SFFVcEpoR reacted positively (Fig 5C), whereas splenic megakaryocytes from mice infected with SFFV (Fig 5B) or those from uninfected mice (data not shown) did not. Approximately 80% of the megakaryocytes in spleens from mice infected with SFFVcEpoR showed EpoR(R129C) expression. Figure 5C also shows abundant expression the Epo-R in erythroblasts interspersed throughout the diseased spleens of mice infected with SFFVcEpoR; this immunofluorescence pattern was not observed in the erythroblasts present in the spleens of uninfected mice or in those infected with SFFV (Fig 5A and B). No immunofluorescence was seen in any of the experimental spleen sections when control, nonimmune antisera was used (data not shown).

**DISCUSSION**

It is not clear whether Epo normally plays a role in regulating megakaryocytopenia and, if so, whether the effect is direct or indirect. However, we showed here that infection of mice with SFFVcEpoR resulted in mild thrombocytosis,

**Fig 2.** Immunoblotting of detergent extracts of isolated splenic cells. (A) Mice were infected with SFFVcEpoR, and immunoblotting of splenic detergent-extracted proteins was performed with antisera against the C-terminus of the murine EpoR. A total of 200 μg of protein was applied per lane. Lane 1, spleen from a mouse 1 week after infection; lane 2, spleen from a mouse 2 weeks after infection; lanes 3 and 4, spleens from mice 3 weeks after infection; lane 5, spleen from a mouse 4 weeks after infection; lane 6, spleen from a mouse 5 weeks after infection; lane 7, detergent extract from 1 x 10⁶ cells of the A1.5 cell line, derived from a mouse infected with SFFVcEpoR²⁰; lane 8, uninfected spleen. (B) Mice were infected with SFFVAP-L, and immunoblotting of splenic detergent-extracted proteins was performed with a MoAb to gp55. A total of 200 μg of protein was applied per lane. Before electrophoresis, samples were treated with endoglycosidase H. Lane 1, uninfected spleen; lane 2, detergent extract of 1 x 10⁶ MEL cells, a line derived from a mouse infected with SFFV-P; lane 3, spleen from a mouse 1 week after infection; lane 4, spleen from a mouse 2 weeks after infection; lanes 5 and 6, spleens from mice 3 weeks after infection.
prominent erythrocytosis, splenic megakaryocytosis, and erythroblastosis. SFFV infections also induced splenic megakaryocytosis and erythroblastosis but did not affect the number of circulating platelets or mature RBC number. The erythroid effect of SFFV was much earlier and more prominent than that observed in mice infected with SFFVcEpoR. SFFVcEpoR was capable of proviral integration in early Meg, E-Meg, and GMM progenitors and enhanced their proliferation. Megakaryocytes present in the spleens of mice infected with SFFVcEpoR expressed high levels of EpoR protein whereas splenic megakaryocytes of SFFV infected spleens had no change in the level of EpoR protein expression. These results suggest that in normal animals EpoR-mediated signaling pathway(s) may affect both megakaryocytopoiesis and erythropoiesis; they also indicate that megakaryocyte, erythroid, and multipotent progenitors contain the intracellular signalling proteins that enable them to proliferate in response to ectopic expression of a constitutively active EpoR, EpoR(R129C). Furthermore, it appears that SFFVcEpoR, in contrast to SFFV, is capable of infecting and affecting much earlier hematopoietic progenitors. This may explain the delay in erythrocytosis observed in SFFVcEpoR-infected mice. Presumably, the inability of SFFV to affect early progenitors relates to the restricted expression of the EpoR, a necessary protein for SFFV pathogenicity.

The reason that we did not observe an increase in blood platelet levels in mice infected with the strain of SFFV tested, despite a 10-fold increase in splenic megakaryocytes, may be the result of some immune-mediated destruction of platelets, as has been reported with other murine retroviral infections. Because the platelet counts did not decrease, one would need invoke a mild or compensated form of immune destruction. Although we have not excluded this possibility, it seems unlikely. Also, there may be a threshold megakaryocytosis necessary for manifestation of peripheral thrombocytosis. Two types of SFFV isolates exist, the polycythemic (SFFV-P) and anemic (SFFV-A) strains.

Table 2. Culture of BM and Spleen Cells from SFFVcEpoR-Infected Mice

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>BM Colonies/2 \times 10^6 Cells</th>
<th>Spleen Colonies/2 \times 10^6 Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Meg</td>
<td>E-Meg</td>
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<td>Week 2</td>
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<tr>
<td>UI</td>
<td>—</td>
<td>0</td>
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<tr>
<td>INF</td>
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<td>0</td>
</tr>
<tr>
<td>UI</td>
<td>IL-3/IL-6</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>INF</td>
<td>IL-3/IL-6</td>
<td>13 ± 4</td>
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<tr>
<td>Week 3</td>
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</tr>
<tr>
<td>UI</td>
<td>—</td>
<td>0</td>
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<tr>
<td>INF</td>
<td>—</td>
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<tr>
<td>UI</td>
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<td>25 ± 4</td>
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<tr>
<td>Week 5</td>
<td></td>
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</tr>
<tr>
<td>INF</td>
<td>—</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>INF</td>
<td>IL-3/IL-6</td>
<td>19 ± 3</td>
</tr>
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Uninfected or mice infected with SFFV EpoR(R129C) were killed at various time after infection. BM and spleen cells were isolated as described in Materials and Methods. BM cells were cultured at 1 \times 10^6 cells/dish, spleen cells were cultured at 2 \times 10^6 cells/dish. Cultures contained no additional growth factors or 100 U/mL IL-3 and 100 ng/mL IL-6. Meg colonies were scored on day 5; E-Meg and GMM were scored on day 7 of culture. All megakaryocyte-containing colonies were confirmed by staining with acetylcholinesterase. Data are the mean of four replicate cultures ± SD.

Abbreviations: UI, uninfected; INF, infected.

cause an overall increase in erythrocyte production, but SFFV-A-infected mice have a lower-than-normal Hct as a result of a significant increase in plasma volume, an increase not observed in SFFV-P-infected mice. The SFFV-A genetic information responsible for the increase in plasma volume is not known. Our study used a strain of SFFV, SFFVAP-L, which is a recombinant containing the 1.6-kb EcoRI-Kpn I env-3’LTR fragment of SFFV-P on a SFFV-A background. SFFVAP-L expresses a chimeric gp55 in which only the membrane-spanning domain is from the SFFV-P strain; the balance is from SFFV-A. Nonetheless, SFFVAP-L induces erythroblastosis in a manner similar to that of SFFV-P. A more likely explanation for the lack of thrombocytosis after SFFVAP-L infection is that this virus, like SFFV-A, induces an increase in plasma volume that masks any true increase in the platelet count. In support of this hypothesis is the lack of change in Hct after infection by SFFVAP-L, despite a massive increase in splenic erythroblasts and blood reticulocytes. Because we did not determine the plasma volume of mice infected with SFFV, this remains speculative.

Spleens from mice infected with SFFVcEpoR had a 25-fold increase in the number of megakaryocytes, yet the animals showed only a twofold increase in platelet count. By 8 weeks after infection with SFFVcEpoR, the spleens in mice surviving this long have increased in weight approximately 10-fold and were almost entirely replaced with erythroblasts or leukemic blasts. Very few megakaryocytes were present (data not shown). At this time after infection, all blood cells except RBCs are decreased, presumably due to the predominance of the leukemic process. Alternatively, there may be unknown negative regulatory mechanisms controlling increases in platelet numbers.

The present study did not determine which maturational stage in megakaryocyte development is affected by ectopic
expression of EpoR(R129C) or gp55. Neither the ploidy of splenic megakaryocytes nor the quantitative expression of acetylcholinesterase, all markers helpful in distinguishing megakaryocyte maturation, were determined. The ex vivo culture of BM and spleen cells from SFFVcEpoR-infected mice indicates that EpoR(R129C) is capable of transducing a proliferative signal in early, multipotent megakaryocyte progenitors (E-Meg and GMM). EpoR(R129C)-protein expression persists in the majority of megakaryocytes and erythroblasts examined in SFFVcEpoR-infected mice. This along with the observed effect of SFFVcEpoR on GM colony growth suggests that SFFVcEpoR is capable of infecting and affecting the growth of early multipotent, hematopoietic progenitors and is associated with continued protein expression in megakaryocyte and erythroid lineages. Our immunofluorescence data suggest that
EpoR(R129C) directly induces megakaryocytosis by its expression in megakaryocyte precursors. We cannot exclude the possibility of an indirect activation of megakaryocytosis, for instance, because of release of other cytokines as a result of SFFV-EpoR infection.

Ligand-binding experiments showed the presence of EpoRs on the surface of mouse and rat BM megakaryocytes. However, immunofluorescence of spleens from uninfected or SFFV-infected animals, using an antipeptide sera specific for the EpoR, did not detect any EpoR expression. This analysis does not exclude expression of an EpoR at a low level. Using the same anti-EpoR sera used in this study, we could not show the presence of an EpoR in HCD57 cells, an erythroleukemic cell line known to express the EpoR, by immunoblotting, immunoprecipitation, or immunofluorescence (Longmore and Lodish data not shown). However, HCD57 clones transformed with SFFV-EpoR express abundant EpoR, as ascertained by these three techniques (Longmore and Lodish data not shown). Thus, the antisera used in the present experiments was incapable of detecting the low level of endogenously expressed EpoR expressed in normal splenic erythroid progenitors as well as that which was present in normal splenic megakaryocytes. Therefore, the megakaryocytosis noted in spleens of SFFV-infected mice could result from gp55 activation of EpoRs normally expressed in megakaryocyte precursors. SFFV and SFFV-EpoR viruses have equivalent titers (data not shown), yet overexpression of EpoR(R129C) in megakaryocytes has a 2.5-fold greater proliferative effect than does overexpression of gp55 by the same SFFV vector. This contrasts with the delay in onset of erythrocytosis in mice infected with SFFV-EpoR compared with that in mice infected by wild-type SFFV. This, together with the differing genetic events required for leukemogenesis after virus infection, further highlights the biologic differences between gp55 and EpoR(R129C) as stimulators of cell proliferation and as early acting oncogenes.

In summary, mice infected with a recombinant SFFV expressing either a constitutively activated EpoR or gp55 develop substantial splenic megakaryocytosis. This is the result of either the proliferative signals transduced by EpoR(R129C) expressed in megakaryocytes or the activation of the endogenous megakaryocyte EpoR by the env gene, gp55, of SFFV, respectively. These data support the hypothesis of a direct effect of Epo in normal megakaryocytosis and erythropoiesis.

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REFERENCES


Both megakaryocytopoiesis and erythropoiesis are induced in mice infected with a retrovirus expressing an oncogenic erythropoietin receptor [see comments]

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