Murine Pluripotent Hematopoietic Progenitors Constitutively Expressing a Normal Erythropoietin Receptor Proliferate in Response to Erythropoietin without Preferential Erythroid Cell Differentiation

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Erythropoietin (EPO) is a prime regulator of the growth and differentiation of erythroid blood cells. The EPO receptor (EPO-R) is expressed in late erythroid progenitors (mature BFU-E and CFU-E), and EPO induces proliferation and differentiation of these cells. By introducing, with a retroviral vector, a normal EPO-R cDNA into murine adult bone marrow cells, we showed that EPO is also able to induce proliferation in pluripotent progenitor cells. After 7 days of coculture with virus-producing cells, bone marrow cells were plated in methylcellulose culture in the presence of EPO, interleukin-3, or Steel factor alone or in combination. In the presence of EPO alone, EPO-R virus-infected bone marrow cells gave rise to mixed colonies comprising erythrocytes, granulocytes, macrophages, and megakaryocytes. The addition of interleukin-3 or Steel factor to methylcellulose cultures containing EPO did not significantly modify the number of mixed colonies. The cells which generate these mixed colonies have a high proliferative potential as shown by the size and the ability of the mixed colonies to give rise to secondary colonies. Thus, it appears that EPO has the same effect on EPO-R-expressing multipotent cell proliferation as would a combination of several growth factors. Finally, our results demonstrate that inducing pluripotent progenitor cells to proliferate via the EPO signaling pathway has no major influence on their commitment.

Erythropoietin (EPO) is the major regulator of erythroid cell lineage. The effects of this glycoprotein hormone on cells of this lineage include proliferation, maintenance of viability, and initiation of erythroid maturation. This biological activity is mediated by a cell surface receptor (EPO-R) (1, 28). EPO-R belongs to the hematopoietic growth factor receptor superfamily characterized by conserved cysteine residues, the WSXWS motif in the extracellular domain, and the absence of motifs evocative of a transduction signal mechanism in the cytoplasmic domain (7, 10, 15, 22). Nevertheless, binding of EPO to its receptor results in tyrosine phosphorylation of cellular proteins (19, 21). EPO-R activation is followed by both proliferation and differentiation of burst-forming unit-erythroid cell (BFU-E) and CFU-erythroid cell (CFU-E), which express 300 to 1,000 EPO-binding sites on their membranes (2). Pluripotent stem cells do not proliferate or differentiate in response to EPO. This can be due either to an insufficient level of EPO-R expression on these cells or to the absence of molecules necessary for the signal transduction induced by EPO. No data are available to directly answer this question. It has been shown, however, that ectopic expression of EPO-R in interleukin-3 (IL-3)-dependent murine hematopoietic cell lines (Ba/F3 [27], FDC-P1 [11], 32D [18], or DA-3 [19]) results in a proliferative response to EPO. The limited potentialities of these cell lines prevented studies of the role of EPO-R expression on their differentiation. EPO-R is expressed on UT7 (9) and TF1 (8) cells, two human multipotent hematopoietic cell lines, and addition of EPO or granulocyte-macrophage colony-stimulating factor (GM-CSF) to these cells

induces their differentiation toward erythropoiesis or granulopoiesis, respectively. The introduction of a normal EPO-R cDNA into bone marrow cells is a direct strategy to determine whether EPO can transduce a proliferative and/or differentiative signal in murine pluripotent hematopoietic cells. We showed that the constitutive expression of EPO-R in pluripotent cells allows their *in vitro* proliferation in the presence of EPO alone. This proliferative response to a single growth factor contrasts with the behavior of uninfected pluripotent cells, which need a cocktail of three to five factors to proliferate. Therefore, pluripotent cells express all the genes required for proliferation in response to EPO. This study also shows that EPO-R pluripotent progenitor cells are not systematically committed to erythroid lineage, since mixed colonies generated by these EPO-R pluripotent progenitors are composed of cells from all the hematopoietic lineages and especially of the granulomacrophagic lineage.

MATERIALS AND METHODS

Cell lines and cultures. FDC-P1 cells, a murine IL-3-dependent myeloid cell line (5), were cultured in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum and 5% WEHI-3B conditioned medium (WEHI-CM) (26). To determine the effects of growth factors on the proliferation of EPO-R transduced FDC-P1 cells, we washed the cells twice, resuspended them in the presence of IL-3 or EPO, and plated them at 10^5 cells per ml. Cell samples were harvested for counting every day during the 4 days of culture.

Constructions and retroviral titer. The retroviral vector encoding EPO-R was constructed by inserting 1,500 bp of the EPO-R cDNA (*SalI* fragment) in the *XhoI* site of the pBTZ-SVNeo vector (6). The murine EPO-R cDNA fragment starts 24 bases upstream of the ATG codon and ends at the stop

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codon. This construct or the NEO construct (see Fig. 2) was transfected into the packaging cell line Ψ 2 (14). Individual neomycin-resistant clones were derived, and their supernatant was tested for viral production on NIH 3T3 cells. The EPO-R virus-producing clone which produces the largest number of viruses was finally selected, and its viral integrated structure and expression were tested. The titer of the clones (Ψ 2NEO and Ψ 2EPO-R) used for bone marrow infection was 2×10^5 infectious viral particles per ml of supernatant.

Retroviral infection of FDC-P1 cells. FDC-P1 cells were cocultured for 48 h on subconfluent irradiated (10 Gy) Ψ 2NEO or Ψ 2EPO-R virus-producing cells. Nonadherent cells were then harvested, washed, and cloned into methylcellulose; 10^3 infected FDC-P1 cells were plated as described below in the presence or absence of G418 (500 μ g/ml). G418-resistant clones were picked up 10 days later and expanded in liquid medium in the presence of G418 (500 μ g/ml) and either EPO or IL-3.

Retroviral infection of primary bone marrow hematopoietic cells. Hematopoietic cells were harvested from the tibias and femurs of mice 72 h after a 5-fluorouracil treatment (150 mg/kg). These cells were suspended in α MEM, washed, and counted. Nucleated cells were depleted of adherent cells by two successive 90-min attachments on 100-mm-diameter culture-treated petri dishes. Nonadherent cells were then collected and counted, and 9×10^6 cells were cocultured for 7 days at 33°C in 75-cm² flasks containing subconfluent irradiated Ψ 2NEO or Ψ 2EPO-R virus-producing cells in α MEM supplemented with 10% fetal calf serum and 5% WEHI-CM. On the last day of coculture, G418 was added to the culture (400 μ g/ml every 6 h repeated three times). At the end of the coculture, nonadherent cells were either plated in methylcellulose in the presence of different growth factors or cultured in suspension in α MEM.

Culture in liquid medium. To study the effect of exogenous EPO on EPO-R-infected bone marrow cells, nonadherent cells were harvested after the 7 days of coculture on Ψ 2NEO or Ψ 2EPO-R and cultured in α MEM complemented with 10% fetal calf serum and either 1 U of EPO per ml or 50 U of IL-3 per ml.

Kinetics of clonogenic cell production during the coculture and during liquid culture in EPO or IL-3. The kinetics of clonogenic cell production during the 7 days of infection on Ψ 2NEO or Ψ 2EPO-R and during the following 7 days in liquid culture in the presence of EPO or IL-3 were determined. One 75-cm² flask was used on each day of the coculture and of the liquid culture, and nonadherent bone marrow cells were harvested and plated in methylcellulose in the presence of different growth factors or in plasma clot in the presence of EPO.

For CFU-E kinetics, nonadherent cells were plated in plasma clot culture on days 1, 2, 3, and 5 after the beginning of the coculture.

Cultures in methylcellulose. Colony formation in semisolid medium was assessed in the presence of different growth factors (EPO, IL-3, EPO plus IL-3, or EPO plus IL-3 plus Steel factor) by the method of Worton et al. (25). Cells (from 10^5 to 5×10^5 without G418 and 10^5 to 10^6 in the presence of G418) were mixed in 1 ml of 0.8% methylcellulose in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, and growth factors were added as indicated in Results. Recombinant murine IL-3 was a gift from Amgen, Thousand Oaks, Calif., and was used at 100 ng/ml; and recombinant human EPO was a gift from Cilag, Paris, France, and was used at 1 U/ml. Recombinant murine Steel factor was purchased from Genzyme, Cambridge, Mass., and used at 50

ng/ml. Geneticin was used at a concentration of 750 μ g/ml of methylcellulose as noted. Colonies were scored on day 7 for granulomacrophagic colony-forming cells (GM-CFC) and mature BFU-E and on day 10 or 14 for mixed colonies.

Plasma clot cultures. A fraction of nonadherent cells (1/18 of the cells contained in one flask) was plated in the plasma culture system as described by McLeod et al. (16) in the presence of EPO and in the absence of G418. Cultures were harvested on days 1, 2, 3, and 4 and stained by the benzidine reaction. Clusters contains 8 to 16 benzidine-positive erythroblasts on day 2, 32 to 64 on day 3, and more than 64 on day 4.

DNA and RNA analysis. Total RNA was isolated by the guanidium thiocyanate lysis procedure followed by centrifugation over a CsCl cushion (3). RNAs were separated by electrophoresis and transferred to a nylon membrane (Hybond N; Amersham) which was hybridized with a labeled NEO probe (Amersham Multiprime DNA-labeling system and [α -³²P]dCTP). The NEO probe was the *Hind*III-*Bam*HI fragment of plasmid pSVNEO.

PCR was performed directly on cell lysate from colonies generated by EPO-R virus-infected cells in methylcellulose in the presence of EPO. Primers specific for the EPO-R construct and the β_2 -microglobulin gene were used. For the EPO-R construct, the 5' primer was 5'-GAAGATCTGGCCTGGCA TCC3' in the EPO-R gene and the 3' primer was 5'-CTGTTC CTATTCCAGCGGC3' in Moloney murine leukemia virus; the amplified fragment was 690 bp long. For the β_2 -microglobulin gene, the 5' primer was 5'-CAGTTCCACCCGCCTC AC3', the 3' primer was 5'-GTAAGGAAGAACTTGAGGC3', and the amplified fragment was 200 bp long.

Binding experiments. Binding experiments were performed with mixtures containing different proportions of infected and noninfected FDC-P1 cells (0, 25, 50, 75, and 100% of infected cells) and with hematopoietic cells from colonies grown in methylcellulose with G418 in the presence of IL-3 or IL-3 plus Steel factor. For binding experiments, 10^6 cells were incubated with 10^5 cpm of ¹²⁵I-EPO (specific activity, 35 mCi/mg), with or without a 100-fold excess of unlabeled recombinant human EPO, in a final volume of 100 μ l. Binding specificity was tested on EPO-R FDC-P1 cells with different amounts of unlabeled EPO (from 20 pM to 200 nM). For hematopoietic cells, only one dose of cold EPO (100 nM) was used in duplicate because of the small number of cells harvested in methylcellulose (4×10^6 to 5×10^6). Cells were incubated in the presence of NaN₃ (0.1%) either at 37°C for 3 h or at 4°C for 16 h, resuspended, centrifuged through a dibutyl-dinonylphthalate cushion ($d = 1.015$), and transferred into a tapered 0.5-ml flexible plastic centrifuge tube. Pellets were counted in a LKB gamma counter.

Autoradiography. Cell pellets from binding experiments were resuspended to a final concentration of 10^6 cells per ml, and 100 μ l of cell suspension was centrifuged onto microscope slides by using a Shandon-Elliott centrifuge. Slides were then air dried and fixed in methanol for 5 min. Dry slides were dipped in NTB2 photographic emulsion (Kodak, Marne-La-Vallée, France) at 42°C in the dark and dried for 5 h. Slides were placed in lightproof boxes containing Drierite and exposed for 4 weeks. The slides were then developed in Kodak D-14 developer for 2 min at 12°C, washed in distilled water, and fixed in an Agfa G334C fixer for 5 min. They were then washed and stained with May-Grünwald Giemsa stain.

RESULTS

FDC-P1 cell infection. To test whether our viral construct was functional, we first used the myeloid FDC-P1 cells as

FIG. 1. Proliferative response of two clones of EPO-R-infected FDC-P1 cells to either IL-3 or EPO. Two clones selected after infection with EPO-R retroviruses in methylcellulose in the presence of G418 were expanded and separated into two aliquots: one was cultured in the presence of EPO (◆), and the second was cultured in the presence of IL-3 (■). After 3 days of this treatment, the growth curve was established over the following 4 days. On day 1, 10^4 cells per ml was seeded; the number of viable cells was determined by a trypan blue exclusion every day for 3 days.

targets. After 48 h of coculture on Ψ 2NEO or on Ψ 2EPO-R, FDC-P1 cells were cloned in methylcellulose in the presence or absence of G418. Some of them (30%) were NEO resistant after either NEO or EPO-R coculture. Two clones from the NEO infection and two clones from EPO-R infection were picked up and, after expansion, tested for their response to EPO. A similar proliferation was observed in the presence of IL-3 or EPO with the EPO-R FDC-P1 clones, as shown in Fig. 1, whereas parental or NEO-infected FDC-P1 cells grew only in the presence of IL-3 (data not shown). Northern (RNA) blot analysis of mRNA of the two NEO and two EPO-R clones revealed, after hybridization with a NEO probe, three bands (3.6, 3.2, and 2 kb for NEO-infected cells, and 5.6, 5.2, and 2 kb for EPO-R infected cells) of the expected sizes (Fig. 2). To confirm the biological results of EPO-R expression, we performed binding and autoradiography experiments on infected FDC-P1 cells. Binding experiments showed that $1,829 \pm 227$ cpm was specifically bound on 10^6 EPO-R-infected FDC-P1 cells in the presence of an excess of cold EPO whereas only 26 ± 13 cpm was bound when NEO-infected cells were used.

Results of autoradiography experiments demonstrated that all the EPO-R-infected FDC-P1 cells were labeled and showed the same pattern of labeling. The number of grains per cell was 60.2 ± 4.5 . A low background (1.5 ± 0.5 grains per cell) was observed in NEO-infected FDC-P1 cells.

The response of EPO-R infected FDC-P1 to EPO and our results of autoradiography and binding experiments demon-

FIG. 2. (A) Retroviral constructs. The EPO-R cDNA coding sequence was cloned at the *Xho*I site of the pBTZen-SVNeo vector. Splice donor (D) and acceptor (A) sites and ATG and TGA codons are indicated. The sizes of the different expected mRNAs are given. (B) Northern blot analysis. RNAs were prepared from the cells of two clones (C11 and C12) of NEO-infected FDC-P1 cells and from two clones of EPO-R-infected FDC-P1 cells. A 4- μ g portion of RNA of the different samples was electrophoresed on a formaldehyde-agarose gel. After transfer, the membrane was hybridized with a NEO probe.

strate that EPO-R expressed from the viral gene is processed to the membrane of FDC-P1 cells and is functional, since EPO-R-infected FDC-P1 cells proliferate in response to EPO.

Infection of bone marrow hematopoietic cells. (i) Percentage of infected progenitors after 7 days of infection. After 7 days of coculture on either Ψ 2NEO or Ψ 2EPO-R, bone marrow cells were plated in methylcellulose in the presence of EPO alone, IL-3, IL-3 plus EPO, or IL-3 plus EPO plus Steel factor with or without the addition of 750 μ g of G418 per ml. The percentage of G418-resistant progenitors was calculated (Table 1). In the presence of EPO alone, almost all EPO-R-infected bone marrow cells which gave rise to granulocyte-macrophage and mixed colonies were G418 resistant. This demonstrated that only infected progenitors are able to grow in the presence of EPO alone.

The percentage of G418-resistant BFU-E growing in EPO alone (mature BFU-E) was the same after infection with NEO or EPO-R virus and was around 10%. When BFU-E are cultured in IL-3 plus EPO (immature BFU-E), the percentage of EPO-R-infected BFU-E is lower (4%) than that of NEO-infected BFU-E (11%).

TABLE 1. Percentage of G418-resistant progenitors after 7 days of coculture

Type of colony	Virus	% of resistant progenitors after coculture with:			
		EPO	IL-3	IL-3 + EPO	IL-3 + EPO + Steel factor
Mixed	NEO	— ^a	—	ND ^b	22
	EPO-R	88.5	—	61	53
GM-CFC	NEO	—	13	18	22
	EPO-R	100	25	16	37
BFU-E	NEO	11	—	14	ND
	EPO-R	13	—	4	ND

^a —, no colony of these types was obtained even in the absence of G418.^b ND, not done.

In the presence of IL-3, EPO plus IL-3 plus EPO or IL-3 plus EPO plus Steel factor, the proportion of G418-resistant GM-CFC was between 10 and 30%, whatever the virus used. Interestingly, a large proportion (50 to 60%) of mixed colonies obtained with EPO-R-infected cells observed in the presence of IL-3 plus EPO or IL-3 plus EPO plus Steel factor were G418 resistant. In the next, we examined only the behavior of G418-resistant progenitors.

(ii) **Growth factor response of infected bone marrow cells after 7 days of infection.** In this set of experiments, we studied the response of NEO- and EPO-R-infected bone marrow cells to EPO, IL-3, IL-3 plus EPO, and IL-3 plus EPO plus Steel factor in methylcellulose after 7 days of coculture. EPO-R-infected bone marrow cells were able to generate macroscopic mixed colonies in the presence of EPO alone (Table 2). The number of mixed colonies was not significantly increased by the addition of IL-3 or IL-3 plus Steel factor. Mixed colonies were also obtained with NEO-infected bone marrow cells but only in the presence of EPO plus IL-3 or EPO plus IL-3 plus Steel factor, but the colonies were smaller and there were fewer colonies per flask. The mixed colonies obtained with EPO-R-infected marrow cells grown in methylcellulose in the presence of EPO alone were further characterized.

Colonies were individually picked up from methylcellulose cultures and were either stained for morphological characterization or studied for their secondary retransplantation capability. Mixed colonies were composed of blast cells and mature granulocytes, erythrocytes (characterized by benzidine), macrophages, and megakaryocytes (characterized by the acetylcholinesterase staining). A total of 48 colonies from three experiments were analyzed on retransplantation assays; 20 of them gave rise to secondary colonies (41%). Only 6% of primary colonies generated a single type of committed progenitor (GM-CFC or BFU-E); the other 94% gave rise to mixed colonies or to different types of committed progenitors. The

number of secondary colonies per primary colony was highly heterogeneous (from 1 to 241 colonies). Most (92%) of these secondary colonies were mixed, whereas 6 and 2% were granulomacrophagic and erythroid, respectively. No tertiary colonies were obtained after transplantation of these secondary colonies.

DNA from the cells which composed these colonies was used to look for the viral gene integration by PCR. PCR analyses were performed on 20 mixed colonies. The cell lysates were divided into two parts and amplified with primers specific to either the EPO-R construct or the β_2 -microglobulin gene. An integration of the construct was detected in all the colonies tested (Fig. 3).

After 7 days of coculture on Ψ 2EPO-R, GM-CFC colonies could be grown in the presence of EPO, IL-3, EPO plus IL-3, and EPO plus IL-3 plus Steel factor. The number of GM-CFC colonies able to grow in the presence of EPO alone was smaller than the number able to grow in IL-3 alone (10%). In contrast, bone marrow cells from the Ψ 2NEO infection did not give rise to GM-CFC in EPO alone. However, these NEO-infected cells did give rise to the same number of GM-CFC as that produced by EPO-R-infected cells in the presence of IL-3, IL-3 plus EPO, or IL-3 plus EPO plus Steel factor.

Two different types of GM-CFC were obtained with EPO-R-infected bone marrow cells in methylcellulose in the presence of EPO alone: typical GM-CFC colonies containing 100 to 1,000 cells, and very large colonies (more than 10,000 cells). These large colonies were composed of mature granulocytes and macrophages (characterized by May-Grunwald Giemsa staining on cytospin smears). However, when these large colonies were individually retransplanted in methylcellulose in the presence of EPO alone, 50% of them gave rise to secondary mixed colonies containing erythroid cells in addition to granulocytes and macrophages. These colonies, from their size and their potential, could be more easily compared to pluripotent progenitors than to true GM-CFC. For this reason, we subsequently scored these types of colonies growing in EPO alone as mixed colonies (Table 2).

Surprisingly, the number of BFU-E obtained with bone marrow cells cocultured for 7 days on Ψ 2EPOR was smaller than the number of BFU-E obtained with bone marrow cells from Ψ 2NEO coculture. This difference was greater when BFU-E were grown in methylcellulose in EPO alone (mature BFU-E) than in EPO plus IL-3 or EPO plus IL-3 plus Steel factor (immature BFU-E). EPO-R BFU-E are not more adherent on Ψ 2EPO-R than are NEO BFU-E on Ψ 2NEO. This was shown by culturing adherent cells in methylcellulose under the same conditions as nonadherent cells (data not shown). Only macrophage clusters were obtained in the presence of Steel factor alone. Whatever the culture conditions, no progenitor growth was observed in the absence of added

TABLE 2. Number of G418-resistant progenitors per flask obtained with methylcellulose after 7 days of coculture with virus-producing cells

Type of colony	Virus	No. of resistant progenitors ^a after coculture with:			
		EPO	IL3	IL3 + EPO	IL3 + EPO + Steel factor
Mixed	NEO	0	0	366 $\pm$ 151 (3)	565 $\pm$ 285 (3)
	EPO-R	770 $\pm$ 154 (10)	0	1,425 $\pm$ 303 (6)	1,265 $\pm$ 342 (5)
GM-CFC	NEO	0	3,109 $\pm$ 770 (4)	1,754 $\pm$ 595 (4)	3,090 $\pm$ 1,013 (4)
	EPO-R	480 $\pm$ 95 (3)	3,571 $\pm$ 900 (5)	1,747 $\pm$ 338 (5)	5,003 $\pm$ 2,119 (4)
BFU-E	NEO	338 $\pm$ 13 (3)	0	962 $\pm$ 349 (5)	1,332 $\pm$ 847 (3)
	EPO-R	47 $\pm$ 13 (5)	0	406 $\pm$ 234 (6)	448 $\pm$ 342 (3)

^a Number of progenitors $\pm$ standard deviation. The number of experiments performed is given in parentheses.

growth factor, showing that abnormal expression of a normal EPO-R did not induce factor independence.

(iii) Time course of the number of progenitors during the 7 days of infection. To understand the behavior of the progenitors during the 7 days of the coculture, one flask was taken every day during the coculture and nonadherent cells were grown either in methylcellulose in the presence of different growth factors (Fig. 4) or in plasma clot in the presence of EPO but in the absence of G418 (Fig. 5). The number of mixed colonies obtained with bone marrow cells cocultured on Ψ 2EPO-R in methylcellulose in the presence of EPO and G418 increased slowly during the coculture (from 0 to 500 per flask [Fig. 4A]). No mixed colony was observed in methylcellulose with EPO alone when bone marrow cells cocultured on Ψ 2NEO were plated.

The time course of the number of GM-CFC grown in the presence of IL-3 (Fig. 4B) was quite similar in bone marrow cells cocultured on Ψ 2NEO or Ψ 2EPO-R. The number of G418-resistant BFU-E obtained from bone marrow cells cocultured on Ψ 2NEO increased during the 7 days of coculture (Fig. 4C). In contrast, during the 7 days of the coculture on Ψ 2EPO-R, the number of G418-resistant BFU-E remained very low.

As in the case of BFU-E, fewer CFU-E are produced during the first 5 days of the coculture on Ψ 2EPO-R than on Ψ 2NEO (Fig. 5A). In addition, the kinetics of the development of NEO and EPO-R CFU-E are identical: CFU-E appear in the plasma clot on day 2 and reach a maximum on day 3 (Fig. 5B), and the clusters of benzidine-positive erythroblasts have the same size (8 to 16 erythroblasts on day 2 and 32 to 64 on day 3). CFU-E were never observed in plasma clot before day 2. These results clearly show that EPO-R-infected BFU-E do not mature faster than NEO-infected BFU-E.

Time course of the number of infected progenitors during 7 days in liquid culture in the presence of EPO or IL-3. To precisely evaluate the effects of exogenous EPO on EPO-R- or NEO-infected cells, marrow cells were cultured in liquid medium after the 7 days of infection, in the presence of G418 and either EPO or IL-3. One flask was taken every day of the liquid culture, and cells were cloned in methylcellulose in the presence of G418 and either EPO or IL-3.

Whatever the conditions, no progenitor growth was observed with bone marrow cells cocultured on Ψ 2NEO and incubated with EPO (data not shown). The number of mixed colonies obtained with EPO-R-infected cells in methylcellulose in the presence of EPO alone increased rapidly after the beginning of the culture in the presence of EPO (Fig. 4A), demonstrating an important effect of exogenous EPO on the pluripotent cells which express the transduced EPO-R gene. In the presence of IL-3, the number of mixed colonies was maintained at the same level during the 7 days of the liquid

culture. The number of EPO-R BFU-E stayed very small even in the presence of exogenous EPO (Fig. 4C). The number of NEO-infected committed progenitors (BFU-E and GM-CFC) and the number of EPO-R-infected GM-CFC decreased rapidly after the beginning of the liquid culture in the presence of EPO as well as in the presence of IL-3 (Fig. 4B).

Binding experiments and autoradiography. Binding experiments and autoradiography were performed on infected bone marrow cells to quantify the bound EPO and to determine the type of the cells which express EPO-R on their membrane. Binding with radiolabeled EPO was performed on cells from hematopoietic colonies harvested after culture of infected cells in methylcellulose in the presence of IL-3 or IL-3 plus Steel factor in the presence of G418. The specific binding (counts per minute bound in the absence of unlabeled competitor minus counts per minute bound in the presence of a 100-fold excess of unlabeled competitor), was 642 (1,219 - 577) cpm. This specific binding was compared with the specific binding of different proportions of infected FDC-P1 cells. Under the assumption that the number of receptors expressed at the surface of bone marrow cells is equal to that of FDC-P1 cells, one would expect that around 45% of the total G418-resistant hematopoietic cells were labeled (Fig. 6).

The same hematopoietic cell populations were used to perform autoradiography (Fig. 7). Only 10% of the bone marrow cells were labeled. The morphology of the labeled cells indicated that they are large cells with an elevated nucleus/cytoplasm ratio. The mean grain count for the labeled cells was 55 ± 8 grains per cell.

DISCUSSION

In this study, a normal EPO-R cDNA was introduced into bone marrow cells by using a retrovirus vector. The most striking result was the induction of proliferation of EPO-R-virus infected pluripotent progenitors in response to EPO. This was demonstrated by the presence of mixed colonies when EPO-R-infected bone marrow cells were plated in methylcellulose in the presence of EPO alone and by the rapid increase in the number of EPO-dependent pluripotent cells during the 7 days in liquid culture in the presence of EPO. The cells which give rise to these mixed colonies have a high proliferative potential since the colonies are macroscopic, and a significant proportion of primary mixed colonies generated by these EPO-R pluripotent progenitors give rise to secondary colonies. The EPO-R pluripotent progenitor properties seem to be similar to those of high-proliferative-potential colony-forming cells (HPP-CFC) described by McNiece et al. (17) but different from those of classical CFU-GEMM, which give rise to smaller and untransplantable mixed colonies. However, there is an important difference between normal HPP-CFC and EPO-R

FIG. 3. PCR analyses of mixed colonies. Mixed colonies were picked up with a capillary tube from cultures of EPO-R-infected cells in methylcellulose in the presence of EPO. From the same cell lysate, an EPO-R fragment specific for the construct and a β_2 -microglobulin fragment were amplified. The negative control was a cell lysate of 10^4 NIH 3T3 cells.

FIG. 4. Time course of G418-resistant progenitors over 7 days of coculture and over the following 7 days in liquid culture in the presence of IL-3 or EPO. Every day after the initiation of the coculture and every day of the liquid culture, nonadherent bone marrow cells were harvested from one flask and plated into methylcellulose in the presence of different growth factors. Bone marrow cells from coculture on Ψ 2NEO (■) or on Ψ 2EPO-R (▲) and bone marrow cells from liquid culture in the presence of IL-3 (Ψ 2NEO [■] or Ψ 2EPO-R [●]) or in the presence of EPO (Ψ 2EPO-R [◆]) were plated in methylcellulose in the presence of EPO to determine the number of mature BFU-E and mixed colonies and in the presence of IL-3 to determine the number of GM-CFC. (A) Number of mixed colonies per flask. (B) Number of GM-CFC per flask. (C) Number of mature BFU-E per flask.

pluripotent progenitors: the growth of HPP-CFC requires a combination of five to seven growth factors such as GM-CSF, IL-1, IL-3, G-CSF, Steel factor, IL-6, and M-CSF, whereas EPO alone appears to be sufficient to induce EPO-R pluripotent progenitor proliferation. It is noteworthy that the EPO-R pluripotent progenitor proliferative potential remains limited since, first, in the presence of EPO, we did not obtain tertiary colonies and, second, although the number of EPO-R pluripotent progenitors increased during the first 7 days in liquid culture in the presence of EPO, it decreased during the

FIG. 5. CFU-E production during coculture on Ψ 2NEO or Ψ 2EPO-R. (A) Time course of CFU-E production during the first 5 days in coculture on Ψ 2NEO or Ψ 2EPO-R. Nonadherent cells were plated in plasma clot in the presence of EPO and in the absence of G418 after 1, 2, 3, and 5 days of coculture on Ψ 2NEO (□) or on Ψ 2EPO-R (■). Plasma clot cultures were harvested and stained with benzidine on day 3. (B) Kinetics of CFU-E development in plasma clot culture after 2 days of coculture on Ψ 2NEO or Ψ 2EPO-R. After 2 days in coculture on Ψ 2NEO (□) or on Ψ 2EPO-R (■), nonadherent cells were plated in plasma clot culture and stained with benzidine on days 1, 2, 3, and 4. On day 2, clusters contained 8 to 16 benzidine-positive erythroblasts; on day 3, they contained 32 to 64; and on day 4, they contained more than 64.

following days. Two hypotheses may explain these results: either EPO-R pluripotent progenitors are not true stem cells and have only intrinsically limited self-renewal, or their long-term self-renewal requires other growth factors. The ability of EPO-R pluripotent progenitors to grow in the presence of EPO alone suggests that the virally transduced EPO-R is expressed at the surface of these cells and that proteins essential for the proliferative signal transduction induced by binding of EPO on its receptor are present in these cells. In contrast to EPO-R pluripotent progenitors, EPO-R GM-CFC respond poorly to EPO. Only a small fraction (10%) of IL-3-responsive EPO-R GM-CFC found at the end of the coculture can grow in the presence of EPO. In addition, at the end of the liquid culture in the presence of EPO, no GM-CFC can proliferate in methylcellulose in response to EPO. These results suggest that only EPO-R-expressing immature stem cells, probably including immature GM-CFC, proliferate in response to EPO.

Another goal of this study was to evaluate the effects of the overexpression of EPO-R on the commitment of EPO-R pluripotent progenitors. The mechanisms of hematopoietic lineage commitment remain largely unknown. It has been propounded that pluripotent stem cells exhibit a low level of most cytokine receptors and that binding of a lineage-restricted growth factor to its receptor may induce an irreversible

FIG. 6. ^{125}I -EPO binding experiment on EPO-R-infected FDC-P1 and hematopoietic cells (BM). Noninfected and EPO-R-infected FDC-P1 cells were mixed in different proportions (0, 25, 50, 75, and 100% of infected cells), and binding was performed on these mixed-cell populations with ^{125}I -EPO. Infected bone marrow cells from colonies obtained after plating of bone marrow cells cocultured on $\Psi 2\text{EPO-R}$ in methylcellulose in the presence of IL-3, Steel factor, or IL-3 plus Steel factor were used for binding experiments. Cells from these colonies were washed and incubated with ^{125}I -EPO. Specific binding obtained with bone marrow cells was compared with the binding obtained with the different populations of FDC-P1.

commitment. If EPO was involved in lineage commitment, a forced expression of its receptor in pluripotent stem cells could shift their differentiation toward erythropoiesis. However, our results suggest that EPO is not a major regulator of this process. First, EPO-R mixed colonies obtained in the presence of EPO have qualitatively the same cellular composition as do normal HPP-CFC colonies with a majority of granulomonocytic cells and give rise to GM-CFC after retransplantation in methylcellulose in the presence of EPO. Second, the number of GM-CFC, a progenitor which arises from a pluripotent cell, increases in the same manner during the 7 days of coculture on $\Psi 2\text{NEO}$ or $\Psi 2\text{EPO-R}$ cells and is not affected by addition of EPO. Third, preliminary experiments indicate that spleen colonies from EPO-R CFU-S have the same cellular composition as do spleen colonies from NEO CFU-S.

EPO-R pluripotent progenitors were able to differentiate toward a terminal granulomonocytic lineage in methylcellulose by using only EPO as growth factor. Because no colonies are observed without EPO, EPO seems to be responsible for the induction of differentiation. In contrast, the role of EPO in the maturation of granulomacrophagic progenitors is not clear. ^{125}I -EPO labeling of cells from colonies generated by EPO-R-infected cells showed that EPO-R is not detectable on mature granulocytes but is present on blast cells. The absence of EPO-R expression on mature granulomacrophagic cells could be related to the regulation of the EPO-R expression on the cell membrane. The efficiency of EPO-R protein translocation to the cell surface is a major regulatory step in the erythroid cell-specific response to EPO. The regulation of this step appears to differ according to the cells. For example, Migliaccio et al. (18) have shown that EPO-R protein is present in the majority of 32D-derived cell lines but is expressed only at the surface of cells with the more immature phenotype. Therefore, EPO does not seem to be involved in granulomacrophagic maturation, which could be explained by one of the two

FIG. 7. Autoradiography of EPO-R-infected bone marrow cells labeled with ^{125}I -EPO. Hematopoietic cells from colonies obtained after culture of EPO-R-infected bone marrow cells in methylcellulose in the presence of IL-3 or IL-3 plus Steel factor and G418 were harvested, washed, and incubated at 4°C for 16 h with ^{125}I -EPO. After being washed, 10^5 cells were centrifuged onto slides. The slides were dipped in photographic emulsion, developed after 4 weeks of exposure, and stained with May-Grünwald Giemsa stain.

following mechanisms: an irreversible induction of a differentiation program occurring directly at the level of GM-CFC progenitors, or the presence of some autocrine or paracrine stimulation by GM-CSF, G-CSF or M-CSF inside these large colonies, allowing granulocyte-macrophage maturation to occur.

Finally, EPO-R expression has a major impact on the number of BFU-E. This was particularly apparent during the time course of the infection. Indeed, while the number of NEO-BFU-E increased during the coculture similarly to that of NEO- or EPO-R CFU-GM, the number of EPO-R BFU-E stayed very small. Because EPO-R BFU-E are not more adherent to virus-producing cells than are NEO BFU-E, and because the erythroid differentiation is not accelerated after EPO-R infection, the low rate of erythroid differentiation might result from a block in the erythroid differentiation. Therefore, the most likely hypothesis is that EPO induces proliferation of pluripotent progenitors but not their erythroid commitment.

In a recent study, other investigators transferred a mutated EPO-R, which is constitutively activated, into fetal liver cells (20). The expression of this constitutively active EPO-R completely abrogated the EPO requirement of CFU-E. This is not surprising since one could assume that the expression of a constitutively active EPO-R has the same behavior as a normal EPO-R after stimulation with EPO. The expression of a constitutively activated EPO-R did not enhance either BFU-E or mixed-colony development. On the other hand, the addition of Steel factor to the culture augmented the growth and differentiation of mutated EPO-R-expressing progenitors (BFU-E, GM-CFC and mixed erythroid-myeloid colonies). Our results and those of Pharr et al. differ with respect to the behavior of infected pluripotent progenitor cells: our work showed that pluripotent progenitors expressing a normal EPO-R proliferate in response to EPO, whereas their work showed that pluripotent progenitor cells expressing a constitutively active EPO-R are still dependent on growth factors for growth. Our results also differ in the response of infected progenitor cells to Steel factor. These discrepancies may be explained by differences in the target cells used for retroviral infection. Pharr et al. infected fetal liver cells, whereas we used adult bone marrow cells from 5-fluorouracil-treated mice. It has been shown that there are striking differences between primitive hematopoietic cells from adult bone marrow and fetal liver (12). Moreover, recently, the same group (13) injected mice with a mixture of recombinant spleen focus-forming retrovirus expressing the same constitutively active EPO-R gene and Rauscher murine leukemia virus. At 5 weeks after the injection, they observed growth factor-independent colonies (Meg, E-Meg, and GMM) from the bone marrow and the spleens of the infected animals. So, with the same virus, in their two different studies, they obtained growth factor independence in different infected progenitors. This reinforces the idea that the infected cells are different in the three studies. Furthermore, it is likely that the mutated EPO-R and wild-type EPO-R have different biochemical behaviors. Mutated EPO-R constitutively makes homodimers which induce a transduction signal leading to cell proliferation and differentiation (23). In contrast, there is only preliminary evidence that the normal EPO-R may also dimerize in the presence of EPO. Other investigators have suggested that EPO-R is associated with other membrane constituents to form an active EPO-R (4, 24).

In conclusion, in this study we have shown that a forced expression of the EPO-R gene in hematopoietic pluripotent progenitors enable them to proliferate in response to EPO and

that EPO has no major effect on the commitment of pluripotent progenitor cells.

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REFERENCES

1. Barber, D. L., and A. D. D'Andrea. 1992. The erythropoietin receptor and the molecular basis of signal transduction. *Semin. Hematol.* **29**:293-304.
2. Broudy, V. C., N. Lin, M. Brice, B. Nakamoto, and T. Papayanopoulou. 1991. Erythropoietin receptor characteristics on primary human erythroid cells. *Blood* **77**:2583-2590.
3. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
4. Damen, J. E., L. Liu, R. L. Cutler, and G. Krystal. 1993. Erythropoietin stimulates the tyrosine phosphorylation of Shc and its association with Grb2 and a 145-Kd tyrosine phosphorylated protein. *Blood* **82**:2296-2303.
5. Dexter, T. M., J. Garland, D. Scott, E. Scolnick, and D. Metcalf. 1980. Growth of factor-dependent hemopoietic precursor cell lines. *J. Exp. Med.* **152**:1036-1047.
6. Johnson, G. R., T. J. Gonda, D. Metcalf, I. K. Hariharan, and S. Cory. 1989. A lethal myeloproliferative syndrome in mice transplanted with bone marrow cells infected with a retrovirus expressing granulocyte-macrophage colony stimulating factor. *EMBO J.* **8**:441-447.
7. Jones, S. S., A. D. D'Andrea, L. L. Haines, and G. G. Wong. 1990. Human erythropoietin receptor: cloning, expression and biologic characterization. *Blood* **76**:31-35.
8. Kitamura, T., T. Tange, T. Terasawa, S. Chiba, T. Kuwaki, K. Miyagawa, Y. F. Piao, K. Miyazono, A. Urabe, and F. Takaku. 1989. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J. Cell. Physiol.* **140**:323-334.
9. Komatsu, N., H. Nakauchi, A. Miwa, T. Ishihara, M. Eguchi, M. Moroi, M. Okada, Y. Sato, H. Wada, Y. Yamata, T. Suda, and Y. Miura. 1991. Establishment and characterization of a human leukemic cell line with megakaryocytic features: dependency on granulocyte-macrophage colony stimulating factor, interleukin 3, or erythropoietin for growth and survival. *Cancer Res.* **51**:341-345.
10. Kuromachi, S., T. Chiba, H. Amanuma, A. Tojo, and K. Todokoro. 1991. Growth signal erythropoietin activates the same tyrosine kinases as interleukin 3, but activates only one tyrosine kinase as differentiation signal. *Biochem. Biophys. Res. Commun.* **181**:1103-1109.
11. Kuromachi, S., Y. Ikawa, and K. Todokoro. 1990. Characterization of murine erythropoietin receptor genes. *J. Mol. Biol.* **216**:567-575.
12. Lansdorp, P. M., W. Dragowska, and H. Mayani. 1993. Ontogeny-related changes in proliferative potential of human hematopoietic cells. *J. Exp. Med.* **178**:787-791.
13. Longmore, G. D., P. Pharr, D. Neumann, and H. F. Lodish. 1993. Both megakaryocytopoiesis and erythropoiesis are induced in mice infected with a retrovirus expressing an oncogenic erythropoietin receptor. *Blood* **82**:2386-2395.
14. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**:153-159.
15. Maouche, L., C. Tournamille, C. Hattab, G. Boffa, J.-P. Cartron, and S. Chretien. 1991. Cloning of the gene encoding the human erythropoietin receptor. *Blood* **78**:2557-2563.
16. McLeod, D. L., M. M. Shreeve, and A. A. Axelrad. 1978. Culture system in vitro for the assay of erythrocytic and megakaryocytic progenitors, p. 31-37. In M. J. Murphy (ed.), *In vitro aspects of*

- erythropoiesis, Springer-Verlag, New York.
17. McNiece, I. K., T. R. Bradley, A. B. Krieglner, and G. S. Hodgson. 1986. Subpopulations of mouse bone marrow high-proliferative-potential colony-forming cells. *Exp. Hematol.* **14**:856–860.
 18. Migliaccio, A. R., G. Migliaccio, A. D'Andrea, M. Baiocchi, S. Crotta, S. Nicolis, S. Ottolenghi, and J. W. Adamson. 1991. Response to erythropoietin in erythroid subclones of the factor-dependent cell line 32D is determined by translocation of the erythropoietin receptor to the cell surface. *Proc. Natl. Acad. Sci. USA* **88**:11086–11090.
 19. Miura, O., A. D'Andrea, D. Kabat, and J. N. Ihle. 1991. Induction of tyrosine phosphorylation by the erythropoietin receptor correlates with mitogenesis. *Mol. Cell. Biol.* **11**:4895–4902.
 20. Pharr, P. N., D. Hankins, A. Hofbauer, H. F. Lodish, and G. D. Longmore. 1993. Expression of 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. *Proc. Natl. Acad. Sci. USA* **90**:938–942.
 21. Quelle, D. E., and D. M. Wojchowski. 1991. Localized cytosolic domains of the erythropoietin receptor regulate growth signaling and down-modulate responsiveness to granulocyte-macrophage colony-stimulating factor. *Proc. Natl. Acad. Sci. USA* **88**:4801–4805.
 22. Shoemaker, C. B., and L. D. Mitsock. 1986. Murine erythropoietin gene: cloning, expression and human gene homology. *Mol. Cell. Biol.* **6**:849–858.
 23. Watowich, S. S., A. Yoshimura, G. D. Longmore, D. J. Hilton, Y. Yoshimura, and H. F. Lodish. 1992. Homodimerization and constitutive activation of the erythropoietin receptor. *Proc. Natl. Acad. Sci. USA* **89**:2140–2144.
 24. Witthuhn, B. A., F. W. Quelle, O. Silvennoinen, T. Yi, B. Tang, O. Miura, and J. N. Ihle. 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* **74**:227–236.
 25. Worton, R. G., E. A. McCulloch, and J. E. Till. 1969. Physical separation of hematopoietic stem cell forming colonies in culture. *J. Cell. Physiol.* **74**:171–178.
 26. Ymer, S., W. Q. J. Tucker, C. J. Sanderson, A. J. Hapel, H. D. Campbell, and I. J. Young. 1985. Constitutive synthesis of interleukin-3 by leukaemia cell line WEHI-3B is due to retroviral insertion near the gene. *Nature (London)* **317**:255–258.
 27. Yoshimura, A., A. D. D'Andrea, and H. F. Lodish. 1990. Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. *Proc. Natl. Acad. Sci. USA* **87**:4139–4143.
 28. Youssoufian, H., G. Longmore, D. Neumann, A. Yoshimura, and H. F. Lodish. 1993. Structure, function and activation of the erythropoietin receptor. *Blood* **81**:2223–2236.