Apoptosis and Hematopoiesis in Murine Fetal Liver

By Hong Yu, Brad Bauer, Gretchen K. Lipke, Robert L. Phillips, and Gary Van Zant

The fetal mouse liver (FL) is an organ of intense, but transient, hematopoietic activity during mid-gestation, with erythropoiesis being predominant during days 11 through 16. It therefore seemed reasonable to expect that hematopoietic cytokines, such as erythropoietin (epo), interleukin-3 (IL-3), and stem cell factor (SCF), may play important roles in maintaining a homeostatic balance of erythropoiesis and apoptosis in liver during ontogeny. First, we determined the effects of these growth factors on hematopoiesis by measuring colony formation and hemoglobin synthesis of cultured FLs. Secondly, we determined the protection from apoptosis afforded by these cytokines, using electrophoretic analysis of DNA and by flow cytometry of FL cells deprived in culture of epo, IL-3, and SCF. Erythropoietin was necessary and alone sufficient for hemoglobin synthesis in colony-forming units-erythroid colonies, but IL-3 was a required cofactor to obtain maximal development of burst-forming units-erythroid colonies. SCF alone caused little colony formation in methylcellulose cultures of FLs, but when combined with epo and IL-3, it had dramatic effects both on the number of colonies and their size. Secondly, indices of apoptosis were determined by measuring DNA fragmentation caused by endogenous nuclease activity in apoptotic cells. Liver cells from cultures without cytokines showed the extensive degradation of DNA to low molecular weight nucleosomal oligomers, which is characteristic of apoptosis. Protection from apoptosis afforded by epo directly corresponded to the level of erythropoiesis in FLs of different gestational age. Erythropoietin was by far the most critical cytokine in sparing FL cells from apoptosis. Analyses of agarose gels showed that SCF and IL-3 alone had no apparent effect in reducing the amount of DNA in fragments, and when combined with epo they had no more protective effect than that provided by epo alone. However, using the more sensitive flow cytometric determination of cells with subdiploid amounts of DNA, SCF, and IL-3 alone had measurable protective effects that were less than those caused by epo. Thus, we show that normal, untransformed cells of the developing hematopoietic system not only require cytokines for proliferation and differentiation, but they have an initial and absolute requirement of them for protection from apoptosis.

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From the Department of Cell Biology and Anatomy, Texas Tech University Health Sciences Center, Lubbock, TX.

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Address reprint requests to Gary Van Zant, PhD, Department of Cell Biology and Anatomy, Texas Tech University Health Sciences Center, 3601 4th St. Lubbock, TX 79430.

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Apoptosis is heavily involved in the functions of the immune system: lymphocyte clones may be selectively eliminated by apoptosis,18 cytolytic T cells and natural killer cells destroy target cells by this mechanism,19 and corticosteroid-induced thymic regression occurs as a result of this mechanism.20 The granules of cytolytic T cells have been shown to contain a polyadenylate binding protein that causes apoptotic DNA fragmentation in their target cells.21 In cancer biology, it has recently been shown that the phorbol ester tetradecanoyl-phorbol acetate (TPA) may act as a leukemia promoter by interfering with apoptosis.22 Similarly, transfection of a normal p53 tumor suppressor gene into leukemic cells (whose indigenous p53 gene was mutated) restores apoptosis.23 Taken together, these two studies provide evidence that may link neoplasia with a loss of the capacity for apoptosis.

With respect to hematopoiesis, Williams et al24 have shown that hematopoietic cell lines derived from bone marrow that are dependent on IL-3 for survival and growth undergo apoptosis when deprived of IL-3. Koury and Bondurant25 and Spivak et al26 have shown that epo-dependent erythroleukemia cells proliferate and mature in its presence, but when deprived of epo, rapidly undergo DNA cleavage characteristic of apoptosis. Koury and Bondurant have proposed that circulating epo titers may control the normal homeostatic balance of erythropoiesis by modulating apoptosis and proliferation.2 Finally, mature human eosinophils have been shown to rapidly undergo apoptosis in the absence of IL-5.27

Thus, binding of epo, IL-3, and SCF to receptors on FL cells may serve two broad functions: the first, a prerequisite of the second, involves protection of cells from apoptosis, a normal consequence of cytokine deprivation. The second is the more familiar induction of proliferation and differentiation, activities collectively termed growth.28 We show here that these three cytokines have differential effects on apoptosis and erythropoiesis in FL cultures, and our results suggest that these mechanisms may be appropriate to normal and neoplastic hematopoiesis, and tumorigenesis in vivo.

MATERIALS AND METHODS

Mice. C57BL/6 mice were obtained from the Animal Resources Division of the National Cancer Institute or The Jackson Laboratory. Pregnancy was determined by the presence of a vaginal plug in spontaneously mated females; day 0 of gestation was the day of plug discovery.

FL culture for DNA analysis and flow cytometry. Livers were surgically removed from fetuses under a dissecting microscope in I scove's Modified Dulbecco's Medium (IMDM) with 10% fetal calf serum (FCS; defined grade; Hyclone, Logan, UT). Livers were washed, minced, and passed via a syringe through a 2-gauge needle, then a 25-gauge needle to make a single cell suspension. Cells were either analyzed immediately or cultured (8 to 48 hours) with or without cytokines in IMDM containing 10% FCS in a humidified incubator gassed with 5% CO2 in air. Recombinant murine IL-3 was a gift of Biogen (Geneva, Switzerland) and was used at 25 U/ml; either tissue culture grade epo (Hyclone) or recombinant human epo (a gift of Amgen, Thousand Oaks, CA) was used at a concentration of 0.5 U/ml. Tissue culture grade and recombinant epo gave almost identical results when compared with respect to colony formation, hemoglobin (Hb) synthesis, or protection of cells from apoptosis. Recombinant rat SCF was a gift of Amgen and was used at 100 ng/ml.

**FL culture for determining Hb synthesis** Cells (100,000/0.5 mL culture, see Fig 2; 20,000/0.5 mL culture, see Fig 3) were grown as previously described29 in medium composed of IMDM containing (in final concentrations) 20% FCS, 1% bovine serum albumin (Boehringer-Mannheim, Indianapolis, IN), 1 × 10^{-4} mol/L 2-mercaptoethanol, and 1% methycellulose (4,000 cps; Sigma, St Louis, MO). Cells were plated in 0.5-mL aliquots in polypropylene 15-mL tubes and were pulsed with 3H-labeled (Amersham, Arlington Heights, IL) rat serum 24 hours before harvest. Cultures (quadruplicates) were washed with 10 mL of phosphate-buffered saline (PBS) and spun down; 1 mL of 33% Drabkins solution was added to the pellet, then 0.2 mL of 1 mol/L HCl, and finally 2 mL of cyclohexanone.30 The tube was vortexed and spun again, and 1 mL of the upper (cyclohexanone) phase was counted in a gamma counter. Because Hb was measured as a function of a 24-hour pulse of radiolabeled iron, an average daily rate of synthesis was measured. A set of cultures to which no cytokine additions were made was run with each experiment, in parallel with cultures containing epo and epo plus IL-3. The data are plotted in Fig 2 with the “no addition” curve subtracted from the time courses for cultures with added cytokines, thus giving Hb synthesis due to the added cytokines. In practice, day 1 was the only time point at which significant Hb synthesis was detected in the no addition group, presumably due to ongoing Hb synthesis in erythroblasts at the time of plating. The cpm of the no addition cultures on day 1, which was subtracted from the cytokine added groups, is given in the legend to Fig 2.

It should be emphasized that Hb synthesis measured by this method represents that occurring in erythroblasts generated during the course of the experiment from erythroid colony—and burst-forming cells, and not Hb-synthesizing cells that were present at the initiation of culture. Thus temporal waves of Hb synthesis during culture are directly attributable to the waxing and waning of Hb synthesis in erythroid colonies and bursts.29 Moreover, the amplitude of those waves is a direct reflection of the CFU-E and BFU-E content of FLs used to initiate the cultures, and bears no relationship to the number of Hb-synthesizing erythroblasts in the plating inoculum.

Colony growth in methylcellulose. The same methylcellulose-containing medium as described above for Hb determination was used, with the following changes. Culture genesis was performed in 35-mm suspension dishes (Sarstedt, Princeton, NJ) containing 2,000 to 50,000 FL cells. Epo was used at 0.5 U/mL concentrations for CFU-E and at 1 to 2 U/mL for BFU-E, CFU-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM), and CFU-granulocyte, macrophage, megakaryocyte (CFU-GMM) cultures. IL-3 was used at 50 U/mL. SCF was used at 25 to 250 ng/mL. CFU-E plates were counted on day 2 of culture after staining with dianisidinolfluorene (DAF) as described.30 BFU-E colonies were counted on day 6, 7, or 8 after DAF staining. CFU-GM and CFU-GEMM colonies were counted on days 10 through 12 after DAF staining; those colonies with an erythroid component (containing DAF-stained cells) were counted as CFU-GEMM, and those without any DAF-positive cells were counted as CFU-GMM.

DNA electrophoresis. A cell pellet of approximately 2 × 10^7 FL cells was gently mixed with 1 mL of lys buffer (10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 150 mmol/L NaCl, 0.2% sodium dodecyl sulfate, 250 μg/mL of proteinase K) overnight at 23°C or 3 hours at 37°C. DNA was successively extracted with 1 vol of phenol, a phenol–chloroform mixture (1:1), and finally with chloroform alone. The aqueous phase was brought to 150 mmol/L NaCl and precipitated with 2 vol of ethanol at −20°C overnight. Pellets were air-dried and resuspended in Tris–EDTA, pH 8.0 (10 mmol/L TrisCl, 0.1 mmol/L EDTA). The DNA concentration was determined by fluorometry after staining with Hoechst 33258 (bisbenzimide 33258; Calbiochem, San Diego, CA). DNA samples (5 μg per lane) were electrophoresed.
in 1.6% agarose gels for 17 to 18 hours at 30 V; Tris-acetate buffer (pH 8.0) was used for the electrophoretic separation. DNA was visualized by ultraviolet transillumination after staining with ethidium bromide (0.5 µg/mL) for 30 minutes.

Trypan blue exclusion. Trypan blue was added to cell suspensions to a final concentration of 0.2% for 1 minute. The fraction of cells unable to exclude the dye (nonviable) was calculated from a count of at least 200 total cells on a hemocytometer.

Propidium uptake. Propidium iodide (final concentration, 5 µg/mL) was added to an aliquot of approximately 2 × 10⁶ FL cells. After 2 to 3 minutes the cells were run on the flow cytometer and the fraction of cells stained (nonviable cells) was calculated from a total of 10,000 cells analyzed.

Flow cytometry. A FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) was used throughout these studies. A 5-W argon ion laser tuned to the 488-nm emission line and operated at 250 mW was used to determine either cell size (forward angle light scatter) or propidium iodide staining. Data analysis and display were performed either using the instrument software (LYSYS II) or after transfer to an Apple IIfx computer (Cupertino, CA).

Erythroid cell staining with TER-119. FL cell suspensions (approximately 2 × 10⁶ in 0.5 mL) were mixed gently for 20 minutes at 4°C with a 1:200 dilution of a purified preparation of TER-119 (rat IgG2b-kappa) antibody kindly supplied by Dr Tatsuo Kina (Kyoto University, Kyoto, Japan). Cells were then washed twice in PBS containing 0.1% bovine serum albumin, and gently mixed for another 20 minutes at 4°C with a 1:200 dilution of fluorescein isothiocyanate-labeled anti-rat IgG (Cappel, Durham, NC). After washing twice more, 10,000 cells were analyzed by flow cytometry. Control cell suspensions were incubated with the labeled secondary antibody only. Specific staining was determined as the fraction of cells with greater than control fluorescence.

Determination of cell size. Size distribution profiles were obtained for cultured FL cells by arbitrarily dividing the forward angle scatter profiles of 10,000 cells into three ranges. The fraction of cells in each of the ranges was calculated for each culture group by applying the same criteria. Medium and large cells were lumped together in Table 2.

Flow cytometric determination of cells with subdiploid DNA content. Numbers of apoptotic cells were determined using the method of Nicoletti et al.²¹ Briefly, 1.5 × 10⁶ FL cells were suspended in 1 mL of 0.1% sodium citrate containing 0.1% Triton X-100 (Sigma) and 50 µg/mL of propidium iodide. Resulting permeabilized cells were kept in the dark at 4°C overnight or for as long as 3 days before analysis by flow cytometry. We found, at least with FL cells, that storage for longer than 3 days before analysis resulted in degradation of the samples. At least 10,000 cells were analyzed for each sample. Cells with subdiploid amounts of DNA were apoptotic and viable cells with the typical DNA content reflecting their distribution in the cell cycle consisted of some in G₁ (diploid), some in S (DNA content between diploid and tetraploid), and some in G₂ + M (tetraploid).

RESULTS

We first determined the hematopoietic composition of FLs three times during gestation: days 12, 15, and 18. (The gestation period in the mouse is 21 days.) Erythroid cells were predominant during the entire period, and at days 12 and 15, erythroblasts comprised over 90% of all FL cells (Table 1). CFU-E, BFU-E, CFU-GMM, and CFU-GEEMM were most plentiful at day 12 of gestation and decreased later. CFU-E and BFU-E decreased in frequency by about 90% from day 12 to day 18. CFU-GMM frequency decreased less (60%) during the same period, probably reflecting increased myelopoiesis at later gestational ages.²² By day 18 and thereafter, the liver assumes adult function and is decreasingly a significant hematopoietic site.

To establish by another criterion that erythropoiesis was predominant in livers through day 16, we used an erythroid-specific antibody, TER-119, and flow cytometry to analyze FLs of different gestational ages. Figure 1 shows that day 13 and day 16 FLs contained 82% and 85% erythroid cells, respectively. TER-119 has been shown to identify all stages of erythroblasts, but not the erythroid progenitors, CFU-E and BFU-E, or stem cells in both fetal liver and bone marrow.²³,²⁴

Figure 2 depicts Hb synthesis in cultures of liver from fetuses of gestational days 11 through 14 using a method we developed to study dynamics of erythroid maturation in CFU-E and BFU-E colonies.²⁹ It should be noted that this assay (see Materials and Methods) measures the generation of new Hb-synthesizing cells by proliferation and differentiation of their precursors (CFU-E and BFU-E) and, except for the 24-hour time point, does not measure Hb synthesis in erythroblasts present in the FLs at the time of plating. The addition of epo stimulated Hb synthesis by CFU-E-derived erythroid cells, which peaked on day 2 of culture, a time when CFU-E colonies reach full maturity with respect to Hb synthesis; the amplitude of the peak reflects the CFU-E content of FLs of different gestational ages. Day 11 and 12 FLs had the highest frequency of CFU-E capable of this response, whereas by day 14 Hb synthesis was sharply diminished, thus reflecting the sharp drop in CFU-E and BFU-E with increasing gestational age shown in Table 1. Similarly, when both epo and IL-3 were added, cultures of day 11 and 12 FLs showed by far the greatest synthesis of Hb. A major difference in this set of cultures was the presence of two periods of maximum synthesis: the previously described peak at 2 days stimulated by epo alone, and a second wave at 4 to 6 days due to the maturation of BFU-E colonies or bursts, not present in cultures containing epo alone. Thus, BFU-E, the more primitive progenitor, but not CFU-E, had the dual requirement of epo and IL-3 for differentiation and maturation. Due to the far greater proliferative potential of BFU-E, when compared with CFU-E, the number of erythroblasts generated and Hb synthesized per burst colony was dramatically greater.

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**Fig 1.** TER-119 staining of FL cells. Day 13 and day 16 FL cells were stained as described in Materials and Methods with either FITC-labeled secondary antibody (light line) or TER 119 erythroid-specific antibody followed by secondary antibody (dark line). The fraction of TER 119-positive cells is designated as the percentage of the 10,000 cells analyzed that were to the right of the vertical marker used to distinguish between negative and positive cells.
added. Note that addition of both growth factors results in a peak associated with CFU-E colony growth. The lower panel depicts Hb synthesis in parallel cultures to which epo and IL-3 were added. Each point represents the mean of replicate cultures harvested 24 hours after a pulse of 55Fe-labeled rat serum. Note the peak of Hb synthesis at either 2 days or at the burst-related 4 to 6 days associated with erythroid burst colonies. As described in Materials and Methods, the day 1 cpm of the no addition groups, which was subtracted from Epo added (top panel) and Epo + IL-3 added (bottom panel) curves, is as follows: (□) day 11, 206; (◇) day 12, 329; (●) day 13, 162; (○) day 14, 191.

The disparity in responses between livers from day 11 to 12 and day 13 to 14 fetuses (Fig 2) was marked, and again illustrates the dynamics of erythroid precursor populations in FLs during gestation as shown in Table 1. What appears to be a more rapid decrease in colony- and burst-derived Hb synthesis between day 11 and 14 FLs (Fig 2) than the decrease in CFU-E and BFU-E colonies between days 12 and 15 (Table 1) can probably be accounted for by individual differences between experiments. The colony data in Table 1 represents a compilation of three separate experiments, and the large size of the standard deviations is indicative of the quantitative differences encountered between sets of pooled FLs of the same chronologic, but apparently not the same developmental, age. We underscore the fact that despite a dramatic decrease in CFU-E and BFU-E between gestational days 11 and 14, erythroblasts remained the predominant liver cell type through at least day 16 as evidenced by differential counts (Table 1) and TER 119 antibody staining (Fig 1).

We next determined the effect on Hb synthesis of delaying epo addition to day 12 FL cultures containing IL-3 (Fig 3). A delay of just 24 hours resulted in the complete disappearance of the 2-day peak (CFU-E) and significant reduction in the size of the later wave of Hb synthesis due to burst development from BFU-E. A 2-day delay in epo addition showed an even more dramatic reduction of BFU-E-derived Hb synthesis, and the small amount of Hb synthesis observed at that time was delayed by 2 to 3 days. These results show that epo presence was a continual and absolute necessity for CFU-E and BFU-E survival and/or differentiation.

Figures 2 and 3 suggested that erythroid progenitors were remarkably dependent on the presence of epo and IL-3 for progression of their developmental programs, and possibly for their survival and viability. We therefore examined the effects of epo and IL-3 deprivation on the integrity of DNA in cultured FL cells (Fig 4). Apoptotic cells, in all tissues studied, are characterized by activation of an internucleosomal endonuclease, which results in distinctive "ladders" of chromatin fragments upon separation by electrophoresis.12 The same three gestational times as depicted in Table 1 were chosen for study: days 12, 15, and 18. DNA was extracted from either freshly harvested liver (lane A) or liver cultured for 19 to 24 hours with no added cytokines (lane B), with epo (lane C), with IL-3 (lane D), or with both epo and IL-3 (lane E). Figure 4 shows that most of the DNA extracted from fresh FL cells, and cells cultured with epo or epo and IL-3, existed as high molecular weight species greater than 20 kb in size. However, in liver cultures with no added cytokines or IL-3 only, smaller DNA species were apparent, particularly fragments that were multiples of roughly 200-bp oligomers, representing the internucleosomal DNA cleavage characteristic of apoptosis. The addition of epo to cultures of day 12, but not day 18, livers reduced the amount of DNA in these smaller fragments. Therefore, epo protected FLs from apoptosis only during the ontogenic period when it was most highly erythropoietic. Apoptosis obviously occurred in other, nonerythropoietic cells, particularly in cultures of day 15 and 18 FLs, presumably because conditions necessary to prevent apoptosis were not met by the addition of epo and/or IL-3.

Figure 5 shows a temporal analysis of apoptosis in cultured day 14 FL cells. As little as 8 hours of epo deprivation was sufficient to result in detectable apoptosis by this method. The protective effect of epo was most striking after 24 hours in culture and was diminished after 48 hours, a time when significant amounts of apoptosis were evident in all groups. Most epo-responsive cells may have completed their developmental program by this time, leaving cell populations with requirements for additional factors or nutrients not present.

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Fig 2. Hemoglobin synthesis in FL cultures. The upper panel depicts Hb synthesis in cultures of 100,000 day 11, 12, 13, and 14 FLs to which epo (0.5 U/mL) was added. Each point represents the mean of replicate cultures harvested 24 hours after a pulse of 55Fe-labeled rat serum. Note the peak of Hb synthesis at 2 days associated with CFU-E colony growth. The lower panel depicts Hb synthesis in parallel cultures to which epo and IL-3 (25 U/mL) were added. Note that addition of both growth factors results in a peak of CFU-E-derived Hb synthesis at 2 days and a second peak at 4 to 6 days associated with erythroid burst colonies. As described in Materials and Methods, the day 1 cpm of the no addition groups, which was subtracted from Epo added (top panel) and Epo + IL-3 added (bottom panel) curves, is as follows: (□) day 11, 206; (◇) day 12, 329; (●) day 13, 162; (○) day 14, 191.
in our culture system. Because CFU-E, and especially BFU-E, represent such a small minority of the total erythroid tissue even in day 12 FLs where CFU-E comprised 14% of total cells (Table 1), our results suggest that epo exerted a protective effect on more plentiful, post-CFU-E erythroid populations. Exactly which populations these are is yet to be determined.

Other indices were examined to support the conclusion from DNA analysis that epo-deprived FL cells were lost through apoptosis. Failure to exclude trypan blue, uptake of propidium iodide, and decreased cell size have all been shown by others to be additional, but not necessarily diagnostic, hallmarks of apoptotic cells. Table 2 shows that measurement of these parameters supported our previous findings; namely, that epo, with or without IL-3, increased cell viability and decreased the number of small cells in FL cultures. Again, these effects were more pronounced in cultures of day 12 FLs than in cultured day 18 FLs.

Figure 6 shows the morphology of cells freshly prepared from day 14 FLs (A), and from the same FLs cultured 24 hours with no additions (B) and with epo added (C). Erythroblasts of all stages of development comprised 91% of all FLs in this experiment, and many mitotic figures were apparent in erythroblasts at harvest (A). Twenty-four hours in the absence of any added growth factors resulted in deterioration of erythroid cell morphology and an absence of erythroblasts undergoing mitosis (B). Numerous cells with pyknotic nuclei were present that resembled orthochromatophilic erythroblasts and that we believe were apoptotic cells.

Fig 3. Effect of delayed addition of epo on Hb synthesis in FL cultures. Day 12 FL cells (20,000) were cultured in methylcellulose-containing medium and Hb synthesis was measured as in Fig 2 in three sets of cultures: (○) those containing epo (1 U/mL) and IL-3 (50 U/mL) from inception; (●) a set started with IL-3 and receiving epo on day 1 of culture; and (□) a set started with IL-3 and receiving epo on day 2 of culture. Note that any delay in epo addition completely prevented the 2-day peak and greatly reduced the size of the burst-related peak.

Fig 4. Electrophoresis of DNA extracted from cultured FLs of different gestational ages. DNA was extracted from freshly harvested liver (A), or liver cells cultured 24 hours with nothing added (B), epo added (C), IL-3 added (D) or both epo and IL-3 added (E). Five micrograms of DNA was loaded per lane. The designated molecular weight markers are in kilobases. Note the retardation of DNA cleavage in epo-containing cultures of day 12 and day 15 FLs, but not of day 18 FLs.
then harvested, suspended in a hypotonic citrate buffer consisting of growth factor combinations, including SCF. Cells were cultured in the presence of added epo is surprising and without present explanation. This result has been obtained in three separate experiments with day 12 and 13 FL cells, but not in cultures of adult bone marrow (data not shown).

In sharp contrast, the addition of epo (C) resulted in a cell picture much like the freshly prepared cells, with erythroblasts of all stages of development, many of which were in mitosis. Stem cell factor, as its name implies, is thought to act at the stem/progenitor cell level, but not further down blood cell lineages.\(^3\) We therefore determined the effect of SCF on colony formation and apoptosis in day 12 FL cultures. Figure 7 shows the effects of SCF alone, or in combination with IL-3 and epo, on the growth of CFU-GMM and CFU-GEMM colonies. SCF alone resulted in very few colonies at concentrations of 25 to 250 ng/mL. In the presence of IL-3 and/or epo, however, it increased dramatically the number and size of colonies formed by epo, IL-3, or the two together. The largest number of CFU-GEMM colonies was produced by SCF plus IL-3, with or without epo. The fact that an erythroid component of these mixed colonies was generated in the absence of added epo is surprising and without present explanation. This result has been obtained in three separate experiments with day 12 and 13 FL cells, but not in cultures of adult bone marrow (data not shown).

FLs were cultured for 24 hours in the absence or presence of growth factor combinations, including SCF. Cells were then harvested, suspended in a hypotonic citrate buffer containing a detergent and propidium iodide (PI), and analyzed by flow cytometry for PI staining.\(^3\) This method has been shown to provide a quantitative measure of apoptotic cells in a population by virtue of the fact that subdiploid amounts of DNA are found in their nuclei, presumably due to endonuclease activity.\(^3\) Figure 8 compares PI staining of day 14 FLs in the “no addition” culture with cells cultured with epo plus SCF. The windows demarking apoptotic and viable populations are shown. The reduction caused by epo plus SCF in the number of apoptotic cells is dramatic (from 84% to 24%).

Table 3 summarizes four experiments determining by flow cytometry the fraction of apoptotic cells in cultures of day 12 and 14 FLs with single and combinatorial additions of epo, IL-3, and SCF. Although the variation in overall apoptosis varied considerably between experiments, the effects of growth factors in protecting cells from apoptotic death was consistent. In contrast to our DNA electrophoretic results (Figs 4 and 5), in cultures of both day 12 and 14 FLs IL-3 alone reduced the size of the apoptotic population in comparison with the no addition group by about one third, with the protective effect being apparently greater on day 14 FLs. Similarly, SCF alone had a comparable protective effect as that found with IL-3; namely, a reduction in apoptosis by about one third. In corroboration of our DNA analyses (Figs 4 and 5), epo alone had a dramatic protective effect on FLs, causing a reduction in apoptosis of 45% to 60% in the four experiments. Admixture of IL-3 and/or SCF with epo did not appreciably alter the protective effect of epo added alone to either day 12 or day 14 FL cultures, demonstrating again that of the cytokines tested, epo was the single most important for FL survival.

The same cultured day 12 FL cells analyzed by flow cytometry for subdiploid amounts of DNA (Table 3, experi-

| Table 2. Cell Viability and Cell Size in Cultured FLs |
|---------------------------------|---------------------------------|
| Nonviable Cells                  | Cell Size                       |
| Staining (%)                    | Staining (%)                   |
| Trypan Blue                     | PI                              |
| Small                           | Medium                         | Large                          |
| Day 12                          |                                 |
| No addition                     | 27                              | 30                             | 48 | 52 |
| Epo                             | 11                              | 16                             | 35 | 65 |
| IL-3                            | 25                              | 28                             | 46 | 54 |
| Epo + IL-3                      | 9                               | 18                             | 33 | 67 |
| Day 18                          |                                 |
| No addition                     | 23                              | 48                             | 50 | 50 |
| Epo                             | 26                              | 41                             | 46 | 54 |
| IL-3                            | 21                              | 44                             | 51 | 49 |
| Epo + IL-3                      | 18                              | 43                             | 44 | 56 |

FL cells were cultured with nothing, epo (0.5 U/mL), IL-3 (50 U/mL), or epo and IL-3. Twenty-four hours later cells were harvested and viability was assessed by trypan blue exclusion or PI exclusion. Nonviable cells stained with trypan blue were determined in counts of at least 200 cells; nonviable cells whose DNA was stained with PI were determined by an analysis of 10,000 cells on the flow cytometer. Cell size was determined on the flow cytometer by arbitrarily dividing the forward angle light scatter distribution of 10,000 cells into (1) small and (2) medium and large categories. The same criteria were then applied to all eight groups analyzed in this table.
Fig 6. Morphology of freshly isolated and cultured FL cells. Day 14 FL cells freshly prepared (A), or cultured 24 hours with no added cytokines (B) or with added epo (0.5 U/mL) (C), were cytopspun onto slides and Wright’s stained. Note the mitotic figures in A and C, and the presence of erythroblasts in all stages of development. In contrast, erythroblasts in B with no added cytokines were small with pyknotic nuclei (at arrows) and no evidence of cell division, a morphology consistent with apoptotic cells. The normal morphology of myelocytes in (B) indicates that granulopoiesis was apparently unaffected under these conditions.

Treatment 1) were also analyzed for apoptosis by DNA electrophoresis. Figure 9 shows, as before, that in any culture containing a cytokine combination that included epo, a reduction in the amount of low molecular weight fragments was clearly evident. Compare lanes E, F, G, and I with lane B (no additions). Reduction in low molecular weight fragments, however, was not apparent in cultures to which IL-3 alone (lane C) or SCF alone (lane D) was added. Lane H shows that the addition of SCF and IL-3 reduced the fragmentary DNA, but not to the same extent as those cultures
containing epo, thus agreeing with the flow cytometric analysis in Table 3. Although the two methods of determining DNA fragmentation qualitatively agree with respect to cultures containing epo, the electrophoretic method apparently is not sensitive enough to detect the protection afforded by IL-3 and SCF alone that we detected using the flow cytometric method.

**DISCUSSION**

Occupation of cellular receptors by the appropriate growth factor has a number of coordinated effects on cell function. The first, requisite effect, preceding any effect associated with proliferation or differentiation, may be to ensure viability by sparing cells from apoptosis. This facet of homeostasis in blood-forming tissues has, until recently, been largely ignored. The concept of ineffective erythropoiesis has been a part of the blood literature for many years and implies a failure of optimal red cell production. Incomplete maturation of erythroblasts and their premature death in marrow is a part of this concept, although the mechanisms remain obscure. The work of Koury and Bondurant, Spivak et al, and the data presented here suggest that one mechanism may be deprivation of cytokines, most notably SCF containing epo, thus agreeing with the flow cytometric analysis in Table 3. Although the two methods of determining DNA fragmentation qualitatively agree with respect to cultures containing epo, the electrophoretic method apparently is not sensitive enough to detect the protection afforded by IL-3 and SCF alone that we detected using the flow cytometric method.

The data presented here show that, of the cytokines tested, epo was the most important in protecting FL cells from apoptosis, yet the addition of IL-3 and SCF to methylcellulose cultures was obligatory for maximal Hb synthesis and colony formation. This apparent discrepancy is resolved if one accepts two things: the first is that epo acts on more differentiated erythroid cells, whereas SCF and IL-3 affect more primitive progenitors of erythropoiesis; the second is that cell proliferation occurring between the level of progenitors responsive to SCF and IL-3 and those cells requiring epo amplify the latter with respect to the former. As a consequence, epo-responsive cells in unfractionated FLs far outnumber those responsive to SCF or IL-3. Thus, epo deprivation causes apoptosis in a significantly larger FL population than that responsive to SCF and IL-3, and it may therefore be more...
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Table 3. Percent Apoptotic Cells and Cell Cycle Analysis of Viable Cells in Cultured FLs

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<tr>
<th>Treatment (cultured for 24 h)</th>
<th>Day 12 FLs</th>
<th>Day 14 FLs</th>
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<tr>
<td></td>
<td>Apoptotic Cells (%)</td>
<td>Reduction in Apoptosis (%)</td>
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<td>Exp 1 Exp 2</td>
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<tr>
<td>No addition</td>
<td>22 56</td>
<td>—</td>
</tr>
<tr>
<td>IL-3</td>
<td>16 44</td>
<td>27 21</td>
</tr>
<tr>
<td>SCF</td>
<td>14 39</td>
<td>36 30</td>
</tr>
<tr>
<td>Epo</td>
<td>12 23</td>
<td>45 59</td>
</tr>
<tr>
<td>SCF + IL-3</td>
<td>15 ND</td>
<td>32 —</td>
</tr>
<tr>
<td>Epo + IL-3</td>
<td>12 26</td>
<td>45 54</td>
</tr>
<tr>
<td>Epo + SCF</td>
<td>13 20</td>
<td>41 64</td>
</tr>
<tr>
<td>Epo + SCF + IL-3</td>
<td>13 26</td>
<td>41 54</td>
</tr>
</tbody>
</table>

Abbreviations: Exp, experiment; ND, not determined.

FL cells, from either 12- or 14-day fetuses, were cultured as described for 24 hours. IL-3 was used at a concentration of 50 U/mL, epo at 0.5 U/mL, and SCF at 100 ng/mL. Cells were then harvested, lysed in hypotonic citrate buffer containing PI, and stored at 4°C for 1 to 3 days before analysis on the flow cytometer. The fraction of apoptotic cells was determined as shown in Fig 8.

easily detected by the methods used. The SCF/IL-3 target cell population may be so small as to be beyond the limits of detection, particularly using electrophoresis to assay DNA fragmentation (Fig 9). Whether SCF and IL-3 protect their target cells from apoptosis is conjectural at this point, but the precedence of such an effect on cell lines dependent on IL-3 has suggested that such may be the case. The results in Table 3 using flow cytometry to detect apoptotic cells demonstrate that SCF and IL-3, in the absence of epo, afford protection to a measurable subpopulation. It should also be noted that IL-3 and SCF addition (without epo) to methylcellulose cultures stimulated multilineage colonies with an erythroid component. Studies are underway on sorted populations of FLs enriched in primitive progenitors to better understand their cytokine requirements for viability and function.

We have demonstrated that epo spares FL cells from cell death and effects a differentiation program measurable by Hb synthesis and colony formation. Erhthropoietin has these coordinated effects during ontogeny only when the liver is a significant contributor to fetal erythropoiesis and contains significant numbers of epo-responsive cells (Figs 1 and 2). Temporal studies of Hb synthesis and colony formation in FL cultures (Figs 2 and Table 1) showed that days 11 and 12 of fetal development were the times of maximum concentration of CFU-E and BFU-E in the liver, a finding in agreement with previous reports by Rich and Kubanek and Johnson and Barker. How then does epo protect day 11 and later FLs from apoptosis? The observation that epo retarded DNA cleavage in day 14 and 15 FL cultures (Figs 4 and 5; Table 3) well after CFU-E and BFU-E had begun to decrease (Table 1) is understandable in light of the fact that their progeny (morphologically identifiable erythroid cells including early erythroblasts) are epo-dependent. Large numbers of these more differentiated cells were present in FLs at least through day 15 (92%, Table 1) or day 16 (85%, Fig 1), and perhaps required epo at least for their survival, if not for completion of their erythroid developmental program. In this regard, early erythroblasts from spleens of mice infected with Friend virus (anemia strain) have clearly been shown to require epo for their survival and for progression of erythroid differentiation. Apoptosis is a rapid consequence of epo deprivation in these cells. Similarly, Spivak et al have shown that an erythroid cell line derived from spleen cells of Friend virus–infected mice required epo both for survival and as a mitogen; apoptosis was a rapid consequence of epo deprivation. However, it should be emphasized that an epo requirement among erythroblasts may apply only to stages preceding and including the onset of Hb synthesis, not the period of maximal Hb synthesis. Koury and Bondurant found that their relatively homogeneous population of early erythroblasts from virus-infected mice, mostly proerythroblasts and basophilic erythroblasts, had the most stringent epo requirements during the first 24 hours of culture, a period preceding maximal Hb synthesis.

In important early work, Paul et al and Cole and Paul examined the ontogeny of erythropoiesis in mouse FLs and found that the number of hemoglobinized cells and the rate of Hb synthesis in livers peaked on fetal day 14 or 15. Their assay was designed to measure ongoing Hb synthesis in explanted livers and therefore directly reflected the content of Hb-synthesizing erythroblasts. Moreover, cultured FLs through about day 15 of development responded to epo addition with increased Hb synthesis, indicating that at least some erythroblast subpopulations were capable of an epo response. The assay of Hb synthesis used in our studies (Figs 2 and 3) was designed differently to measure the generation of new Hb-synthesizing erythroblasts from their clonogenic progenitors, CFU-E and BFU-E. Our results show that day 11 and 12 FLs gave the highest levels of Hb synthesis in response to epo, thus accurately reflecting the maximal CFU-E and BFU-E content of day 11 and 12 FLs (Table 1).

Masuda et al have recently reported epo receptor mRNA levels in mouse FLs during ontogeny. Our results are in agreement with their observations showing that epo receptor expression is highest on about day 13, remains high in FLs through day 16, and subsequently decreases dramatically by day 19. In further corroboration of this erythroid pattern, we have used an erythroid-specific antibody, TER 119, to stain FLs. The cognate antigen is present on erythroblasts of all
Fig 9. Effects of erythropoietin, IL-3, and stem cell factor on DNA fragmentation in cultured FL cells. The same cultures of day 12 FLs analyzed by flow cytometry for apoptosis and reported in Table 4 (experiment 1) were also analyzed by gel electrophoresis. DNA was extracted from freshly harvested livers (lane A), or liver cells cultured 24 hours with nothing added (B), IL-3 added (C), SCF added (D), epo added (E), epo and IL-3 added (F), epo and SCF added (G), IL-3 and SCF added (H), or epo, IL-3, and SCF added (I). Five micrograms of DNA was loaded per lane. The designated molecular weight markers are in kilobases. Note the retardation of DNA cleavage in all epo-containing cultures (lanes E, F, G, I) and a lack of effect in cultures given only IL-3 (lane C) or SCF (lane D).

The data in Fig 5 show that as little as 8 hours of epo deprivation is enough to cause measurable apoptosis in FLs. Similar results have been reported for lymphocytes deprived of IL-249 and eosinophils deprived of IL-5.27 Figure 3 shows that a 24-hour delay in adding epo to methylcellulose cultures of FLs completely eliminated CFU-E colonies, a finding in agreement with that of Iscove using bone marrow. However, in contrast with Iscove, we found that BFU-E in FLs was also dependent on epo and a 1- or 2-day epo deprivation, even in the continual presence of IL-3, caused a dramatic decrease in bursts (Fig 3). There may be a fundamental difference in cytokine requirements between BFU-E in marrow and in FLs.

Our results demonstrating an IL-3 requirement, in addition to epo, for BFU-E development (Fig 2) stands in contrast to the recent report of Valtieri et al,55 who found that human BFU-E obtained from FL required only epo for colony formation. We have no explanation for this discrepancy at present, except to note the species difference.

The predominant cell type in FL cultures lacking exogenous epo were what appeared to be orthochromatophilic erythroblasts (Fig 6), suggesting that maturation of erythroblasts may proceed without epo. Although this may be possible to a limited extent, we feel that what appeared to be orthochromatophilic erythroblasts were in fact apoptotic erythroblasts (Fig 6). The decreased size and highly pyknotic nucleus are characteristic of apoptotic cells from a variety of tissues.1

The exact mechanisms by which hematopoiesis is initiated in the FL on day 10 of gestation, becomes robust on days 12 through 16, and then decreases after day 16 are largely unknown. It is clear from studies by Toles et al52 that yolk sac-derived stem cells initially seed the FL to set the stage for active hematopoiesis at this site. Alterations in the liver stroma presumably precede stem cell seeding to create a microenvironment conducive to hematopoiesis. In this regard, stromal cells with a potent capacity to support hematopoiesis in vitro have recently been derived from FLS.56 Finally, the liver has been shown to be the major, if not sole, site of epo production in the fetus.54 55 Thus, stem cells, supporting stromal cells and epo-producing cells, are all resident to initiate and sustain erythropoiesis.

Eclipse of hematopoiesis in the liver may involve changes in the microenvironment that make it unsuitable for further hematopoiesis. Such a change may simply be the consequence of proliferation of hepatocytes and other cells normally associated with adult liver function. An alternate possibility involves the epo-producing cells of FLs that may create microenvironmental niches supportive of erythropoiesis.54 56 Temporal loss of these niches during ontogeny may lead to local epo deprivation, followed by apoptosis in associated erythroid foci. The concept of lineage-specific niches is supported by the observation that hepatic erythropoiesis decreases well before myelopoiesis; at day 17, when erythropoiesis is minimal, myelopoiesis remains substantial.32
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