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Research Article

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Distinct Roles of Erythropoietin, Insulin-like Growth Factor I, and Stem Cell Factor in the Development of Erythroid Progenitor Cells

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Abstract

Erythropoietin (EP), insulin-like growth factor I (IGF-I) and stem cell factor (SCF) each reduce apoptosis of human erythroid progenitor cells. To determine if these growth factors have additional roles in stimulating erythropoiesis, the proliferation, maturation, and survival of highly purified human erythroid colony-forming cells (ECFCs) were studied during the application of different combinations of these growth factors in a serum-free liquid culture. EP maintained cell viability and supported heme synthesis during erythroid maturation, with little increase in viable cell number or stimulation of DNA synthesis. The addition of SCF with EP resulted in a substantial increase in DNA synthesis, which was greater than that seen with the addition of EP and was associated with a large expansion in the number of ECFCs. Thus EP, by itself, produces little increase in cell proliferation, and expansion of the number of erythroid cells depends upon the presence of SCF with EP. The addition of IGF-I with EP led to enhanced heme synthesis and moderate cellular proliferation, but also greatly enhanced nuclear condensation and enucleation in the late erythroblasts. Thus EP, by itself, is not sufficient for complete end-terminal nuclear condensation/enucleation and the presence of IGF-I is necessary for this complete process. While EP greatly reduced apoptosis during 16 h of incubation at 37°C, the addition of SCF and IGF-I with EP had little additional effect, but these additions enhanced DNA synthesis > 3.4-fold. Thus SCF may have an additional role in directly stimulating proliferation through a process that is distinct from apoptosis. Our observations indicate that EP prevents apoptosis and maintains erythroid cell viability and development. IGF-I enhances erythroid maturation and proliferation, but the proliferation of erythroid progenitors is mainly controlled by the addition of SCF with EP, independent of an effect on apoptosis. (*J. Clin. Invest.* 1994. 94:34–43.) Key words: erythropoietin • insulin-like growth factor I • stem cell factor • apoptosis

Introduction

Investigation of the biology of hematopoietic progenitor cells and the molecular events associated with the binding of hematopoietic growth factors to the receptors of these cells have been limited by the use of heterogeneous populations with large numbers of accessory cells. Analysis of the effects of hematopoietic growth factors has also been hindered by the presence of undetermined growth and/or differentiation factors present in serum-supplemented culture media. The provision of highly purified erythroid colony-forming cells (ECFCs)[1] generated from partially purified peripheral blood burst-forming units-erythroid (BFU-E) (1) and a culture medium that supports the growth of these cells without the addition of serum (2) has enabled us to overcome these obstacles. Using this system we previously demonstrated that erythropoietin (EP) and insulin-like growth factor I (IGF-I) are necessary for the development of ECFC into erythroid colonies in semisolid culture (2), while highly purified peripheral blood BFU-E are also dependent initially on interleukin-3 (IL-3) for 48–72 h and on stem cell factor (SCF) for the first 7 d of erythroid development in vitro (3). However, since colony formation requires several elements of erythroid development, consisting of survival, proliferation, and maturation of the cells, the above observations based on colony assays did not determine which elements of erythroid development each of these hormones might support.

Although the precise chemical mechanism of action of these hematopoietic growth factors is not fully understood, the reduction of apoptosis, and the enhancement, thereby, of cell survival has been recognized as one biologic effect of growth factors that serves as a regulatory mechanism for enhanced hematopoietic cell production. Apoptosis is an active process of programmed cell death, which is distinguished from necrosis, a passive form of cell death, by distinctive morphologic hallmarks and chemical events such as internucleosomal cleavage of cellular DNA (4, 5). It has been demonstrated that hematopoietic growth factors such as IL-3, granulocyte (G) and granulocyte-macrophage (GM) colony-stimulating factor (CSF) suppress apoptosis of target cells (6, 7, 8). In the erythropoietic system, Koury and Bondurant (9, 10) demonstrated that EP protects murine erythroid progenitor cells from apoptosis and proposed the hypothesis that the principal action of EP is to maintain the viability of erythroid progenitor cells allowing more cells to proceed through various stages of proliferation and maturation, thereby expanding the erythroid component. Using human ECFC with a serum-free liquid culture system, we further demonstrated that EP, IGF-I, and SCF each reduced apoptosis of early erythroid progenitor cells (11). While this reveals a critical role for apoptosis in the regulation of human red cell production, it is possible that each of these growth factors has an additional role in directly stimulating erythroid proliferation

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1. *Abbreviations used in this paper:* BFU-E, burst-forming units-erythroid; ECFC, erythroid colony-forming cell; EP, erythropoietin; IMDM, Iscove's modified Dulbecco's medium; r, recombinant; SCF, stem cell factor.

and/or erythroid maturation which has not been recognized (12). To address this question we have made a detailed investigation of the effect of EP, IGF-I, and SCF on proliferation, maturation, and survival of ECFC in serum-free liquid suspension culture. The results indicate that while EP mainly prevents apoptosis and supports orderly erythroid maturation, IGF-I enhances terminal erythroid maturation plus proliferation and SCF substantially stimulates proliferation of erythroid progenitor cells without significant additional effects on apoptosis.

Methods

Blood. Blood was obtained from normal volunteers after informed consent. These studies were approved by the Vanderbilt University and Nashville Department of Veterans Affairs Medical Center Institutional Review Boards. Approximately 400 ml of peripheral blood was collected in sodium heparin (Upjohn Co., Kalamazoo, MI) at a final concentration of 20 U/ml.

Generation of ECFCs. ECFCs were prepared by a modified method described in detail by Sawada et al. (1). Light density mononuclear cells were obtained by density centrifugation using Ficoll-Hypaque (1.077 g/cm^3). Platelet depletion was accomplished by cell centrifugation through phosphate-buffered saline (PBS) containing 10% bovine serum albumin (BSA; Intergen Co., Purchase, NY). This was followed by T-lymphocyte depletion using sheep erythrocyte rosetting. Adherent cell depletion was then performed by overnight incubation in polystyrene tissue culture flasks at 37°C, followed by negative panning with CD11b/OKM*1, CD2/OKT*11, CD45/MY11, and CD16/MY23 antibodies to purify BFU-E to ~ 0.4%. The remaining cells were then cultured for 5–6 d (day 6–7 cells) to generate ECFCs in 0.9% methylcellulose containing 30% fetal calf serum, 0.5% deionized BSA, recombinant human EP (rEP; 2 U/ml; Amgen Inc., Thousand Oaks, CA), recombinant human IL-3 (50 U/ml; Amgen Inc.) 10^{-4} M 2-mercaptoethanol, penicillin 500 U/ml, streptomycin 40 μg/ml, and Iscove's Modified Dulbecco's Medium (IMDM; Sigma Chemical Co., St. Louis, MO) at 37°C, in a high humidity 5% CO_2/95% air incubator.

The cells were then collected and ECFCs were further enriched by centrifugation through 10% BSA and then over Ficoll-Hypaque, followed by incubation at 37°C for 1 h to remove adherent cells. This preparation generally resulted in 94% viable cells of which 30–90%, referred to here as ECFCs, gave rise to colonies of 2–500 hemoglobinized cells in the plasma clot system. In some experiments, ECFCs were further incubated at 2×10^5 cells/ml in liquid medium consisting of IMDM, 15% FCS, 15% human AB serum, 0.5% human serum albumin (HSA), 2 U/ml rEP, penicillin, and streptomycin to obtain more mature erythroid cells. At the indicated times (days 7–12), the cells were collected by centrifugation at 1,000 *g* for 5 min and were washed with IMDM. The cells were then resuspended in IMDM containing 0.1% BSA and incubated at 37°C for 1 h. After centrifugation over PBS containing 10% BSA, the cells were then washed with IMDM and used for further experiments.

Cell culture. Purified ECFCs were incubated at 37°C in serum-free liquid medium consisting of 0.5% deionized, delipidated, dialyzed crystalline BSA (C-BSA-3D), iron-saturated transferrin (Sigma Chemical Co.; 300 μg/ml), lipid suspension (oleic acid, 2.8 μg/ml; L-α-phosphatidylcholine, 4.0 μg/ml; cholesterol, 3.9 μg/ml; Sigma Chemical Co.), penicillin, streptomycin, and 50% IMDM/50% F-12 (HAM) (Sigma Chemical Co.), which were prepared as previously described (2). When the rate of ^{59}Fe incorporation into heme was measured, the concentration of transferrin was reduced to 100 μg/ml. In some cultures, rEP, recombinant human IGF-I (rIGF-I; Intergen Co.), and/or recombinant human SCF (rSCF; Amgen Inc.) were added. When ECFCs were cultured further than day 11, the cells were collected by centrifugation at 1,000 *g* and recultured in fresh media every 3–4 d. The cell concentration was reduced to no more than 10^6/ml at each medium change.

The number of ECFCs after 40 h of incubation in serum-free liquid culture was determined by adding 200–800 cells to 0.2 ml of serum medium for plasma clot assay after 7 d of incubation as previously described (1, 2). Colonies of two or more hemoglobinized cells were scored as ECFC. The number of erythroid colonies per microliter at the onset of the 40-h liquid culture was designated as 100%. The erythroid colony-forming capacity of day 14 cells was similarly determined and was expressed as the percentage of erythroid colonies formed per cell number plated. The purity of the ECFCs in each experiment was determined by inoculating 200 cells in plasma clots prepared in the same manner and counting four replicates.

Determination of cell viability, benzidine positivity, and morphology. The viable cell number was determined by trypan blue exclusion using a hemacytometer. Aliquots of the cells were removed for cytocentrifugation onto glass slides at the indicated times on days 7–14. The slides were stained with 3,3′ dimethoxybenzidine and hematoxylin, and were examined by light microscopy. All the mononuclear cells that stained with benzidine were identified using a magnification of 1,000, and the percentage of benzidine positive cells was determined by counting 400 cells. The net benzidine positive cell number was calculated from the total cell number and the percentage of benzidine positive cells.

Immunofluorescence. To determine the expression of spectrin as a marker for the erythroid lineage, immunofluorescence using anti-erythroid spectrin antibody (Sigma Chemical Co.) was performed. The day 14 cells were washed with IMDM and allowed to adhere to an alcian blue-coated cover slip for 15 min at 37°C. The coverslips were dipped in 2.0% formaldehyde in PBS containing 0.1% DMSO for 8 min followed by washing in PBS for 1 h and the cells were then permeabilized by dipping in 0.5% Triton X-100 in PBS for 5 min. Incubation with rabbit anti–human erythrocyte spectrin (Sigma Chemical Co.) was performed at room temperature for 45 min, followed by incubation with a goat anti–rabbit IgG secondary antibody conjugated to FITC (Sigma Chemical Co.). Photomicrographs were taken using an Olympus BH-2 microscope equipped with epifluorescence optics. For a negative control, peripheral blood BFU-E were purified as previously described (3) and were cultured in serum-free media containing rIL-3 (100 U/ml), rSCF (50 ng/ml), and rEP (1U/ml) for 2 d to generate day 3 BFU-E.

^{59}Fe incorporation into heme. The effect of growth factors on heme synthesis were assessed by measuring ^{59}Fe incorporation into heme as previously described (13). The ECFCs (3×10^5 day 6 or 7 cells) were incubated in 0.5 ml of serum-free media with various combinations of growth factors at 37°C. At the indicated times, 50 μl of a solution of human transferrin (2.4 mg/ml; Calbiochem-Boehring, La Jolla, CA) containing 0.5 μCi of $^{59}FeCl_3$ (54 mCi/mg; New England Nuclear Corp., Boston, MA) was added directly to the culture. After 4 h the cells were collected by centrifugation at 1,000 *g* for 5 min and were washed with IMDM. The cells were lysed in Drabkin's solution which was then acidified with HCl, and this was followed by extraction of heme using cyclohexanone. The radioactivity of ^{59}Fe in the cyclohexanone fraction was measured in a gamma counter and expressed as cpm/ml culture or as a percentage of radioactivity in the cultures with rEP alone.

To determine the growth factor requirements for the late stages of erythroid maturation, day 9–12 cells were harvested from serum-supplemented liquid cultures and 3×10^5 cells were further incubated with 0.125 μCi of ^{59}Fe-transferrin in 0.5 ml of serum-free liquid medium with various combinations of growth factors for 24 h at 37°C. Measurements of accumulated ^{59}Fe-heme were performed as described above.

Measurement of DNA synthesis. The effect of various growth factors on proliferation of ECFCs was determined by measuring DNA synthesis using [^{3}H]thymidine as previously described (9). Briefly, 0.5 ml serum-free cultures with 10^5 ECFCs were established with various combinations of growth factors and were incubated with 1.25 μCi of [^{3}H]-thymidine (6.7 Ci/mmol; New England Nuclear Corp.) for 1 h at the indicated times. The cells were then collected by centrifugation at 1,000 *g* for 5 min, washed with IMDM, and placed in 10% ice-cold trichloroacetic acid (TCA). The cell precipitates were collected on glass filter discs (type GF/A; Whatman, Maidstone, England), washed with 5% ice-cold TCA, followed by 95% ethanol, air dried, placed in a scintillant and counted in a scintillation counter. [^{3}H]thymidine incorporation was

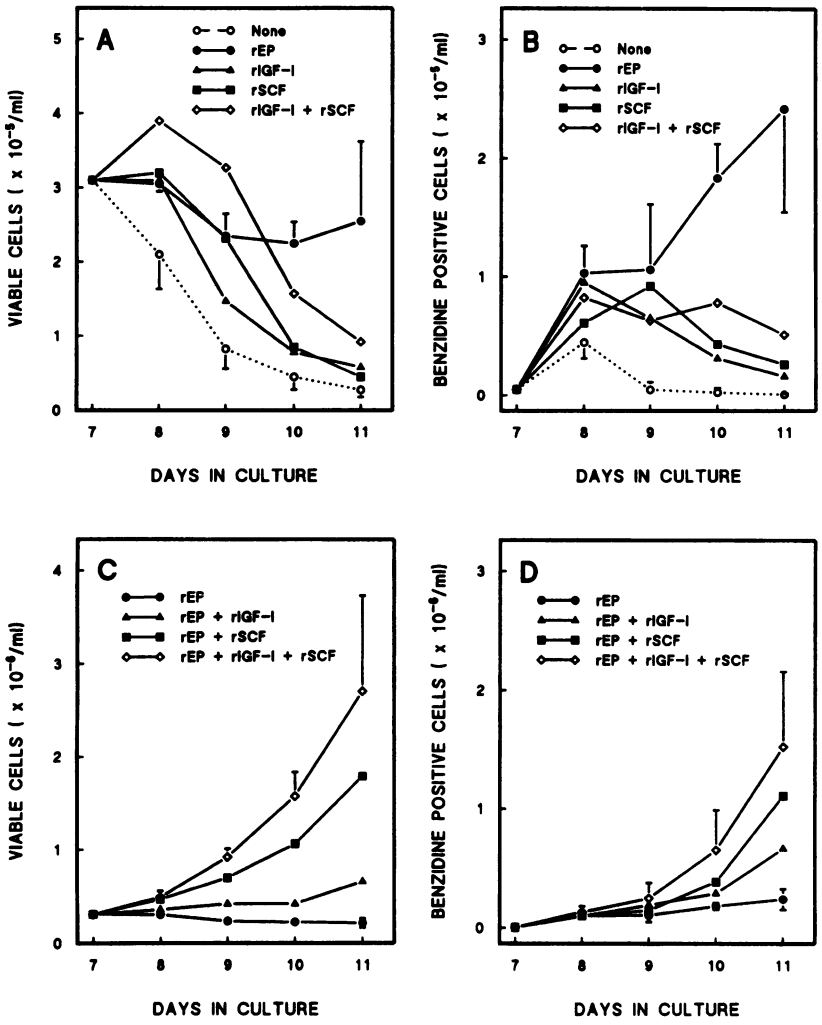

Figure 1. Effect of rEP, rIGF-I, and rSCF on growth of ECFCs. ECFCs (day 7 cells) were incubated in a serum-free liquid medium with rEP (1 U/ml), rIGF (100 ng/ml), and/or rSCF (100 ng/ml) (*A* and *B*), or with rEP plus rIGF-I and/or rSCF (*C* and *D*). Viable cells (*A* and *C*) and benzidine positive cells (*B* and *D*) were enumerated. The data are the mean (±SD) of four replicates from two experiments. The purity of the ECFC was 74±10%, and 53±10%.

expressed as cpm/ml of culture or as a percentage of cellular radioactivity in the cultures with rEP, rIGF-I, and rSCF.

Analysis of ECFC apoptosis. To quantitate growth factor protection of ECFCs from DNA fragmentation, the amount of uncleaved DNA was measured as described by Koury and Bondurant (10). The ECFCs were preincubated in IMDM containing 30% FCS and 0.5% HSA, at 37°C, for 30 min. [^{3}H]Thymidine (0.5 μCi/ml) was added to 10^6 cells/ml and incubation was carried out for a further 30 min. The cells were collected, centrifuged through PBS containing 10% BSA at 1,000 *g*, 4°C, for 5 min, and washed with IMDM. Replicate [^{3}H]thymidine-labeled cells (5×10^5) were then incubated at 37°C in 1 ml of serum-free liquid medium containing thymidine and deoxycytidine (20 μM; Sigma Chemical Co.) with various combinations of growth factors. After incubation for 16 h, the cells were collected and the DNA was extracted and analyzed by electrophoresis on alkaline 0.6% agarose gels as previously described (10, 11). Each lane was cut into 16 5-mm fractions and the radioactivity of each fraction was determined. The sum of the radioactivity in fractions 1–4 was considered as the amount of uncleaved DNA and was expressed as a percentage of the total radioactivity (11). Data are presented as mean±SD and significance was calculated using the *t* test.

Results

Effect of rEP, rIGF-I, and rSCF on growth of ECFCs in serum-free liquid culture. Fig. 1 *A* shows the effect of rEP, rIGF-I, and rSCF on the number of viable cells when day 7 ECFCs were incubated in serum-free liquid culture. The majority of the cells cultured with rEP alone remained viable through day 11, but no increase in the number of viable cells was evident. rIGF-I, and/or rSCF, increased the number of viable cells above that seen in the cultures without rEP for 2 d ($P < 0.05$), but beyond that time, rEP alone maintained cell viability significantly more than rIGF-I and/or rSCF ($P < 0.01$). When the number of benzidine positive cells was determined (Fig. 1 *B*), an increasing number of ECFC incubated with rEP alone turned positive due to the accumulation of hemoglobin during erythroid maturation, even though the number of viable cells did not increase. By day 11, the number of benzidine positive cells was approximately equal to the number of viable cells. Increases in the number of benzidine positive cells were also seen with the addition of rIGF-I ($P < 0.05$ on days 8–11), rSCF ($P < 0.05$ on day 9), or rIGF-I plus rSCF ($P < 0.05$ on days 8–11). On day 9 the number of benzidine positive cells seen in the cultures with rEP was similar to those seen in the cultures with rIGF-I and/or rSCF, but the former increased after day 9, and was significantly greater ($P < 0.01$), while the latter declined.

Fig. 1 further shows the number of viable cells (*C*) and benzidine positive cells (*D*) in cultures incubated with rIGF-I and/or rSCF together with rEP. While rEP alone promoted little

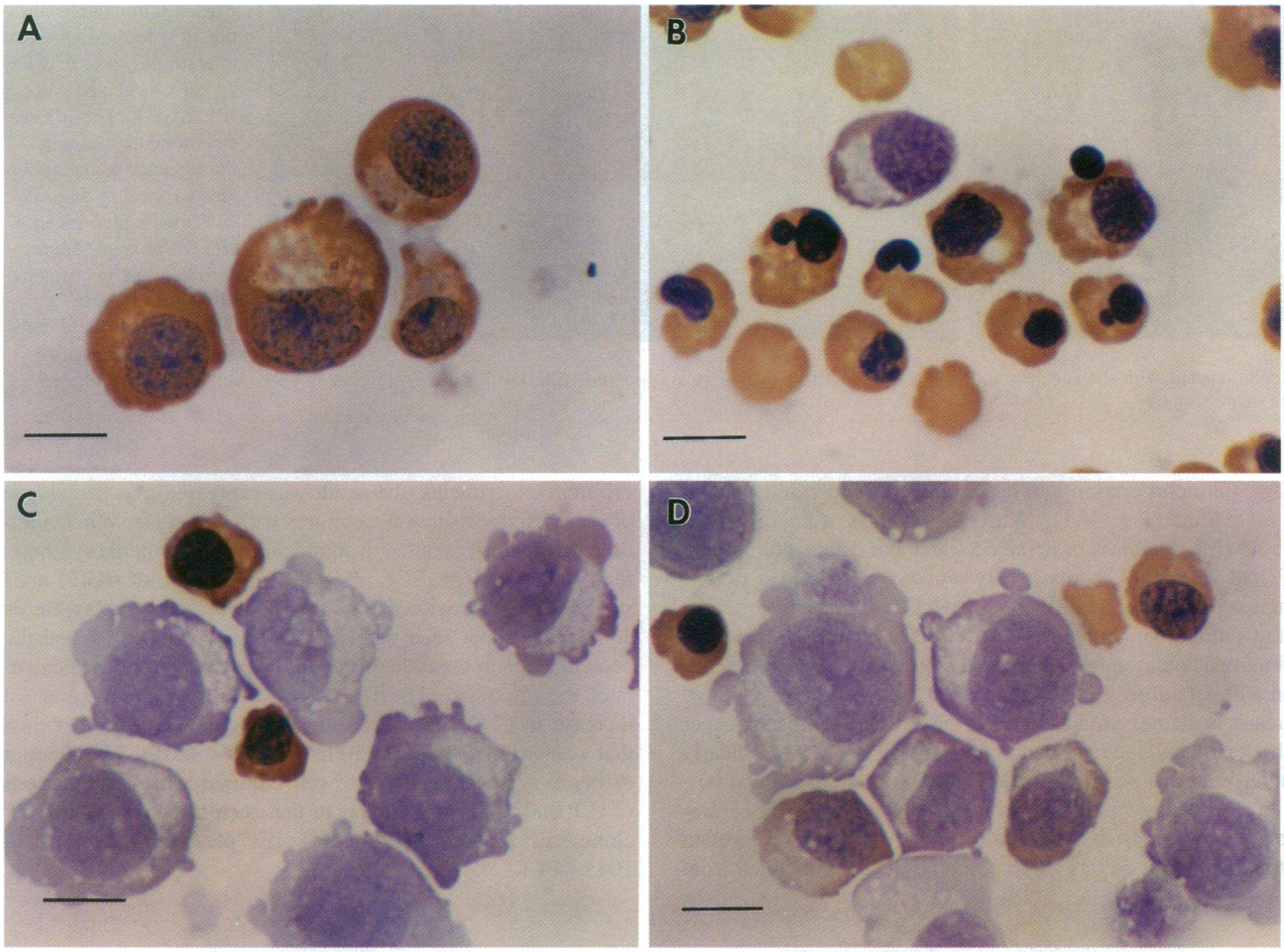

Figure 2. Morphology of ECFC on day 14 of serum-free liquid culture. ECFC (day 7 cells) were incubated with rEP (*A*), rEP plus rIGF-I (*B*), rEP plus rSCF (*C*), or rEP plus rIGF-I and rSCF (*D*) for 7 d, followed by staining with benzidine and hematoxylin. The purity of the ECFC was 56±5%. Bar, 10 μm.

cellular proliferation, the addition of rIGF-I ($P < 0.01$) or rSCF ($P < 0.01$) with rEP each resulted in a significantly greater number of viable cells compared to the cultures with rEP alone (Fig. 1 *C*). Analysis of the viable cell number in the cultures with rEP plus rIGF-I from six experiments revealed a 4.3-fold increase on day 11 ($P < 0.01$) and an 8.0-fold increase on day 14 ($P < 0.01$) compared to the viable cell number on day 7. The addition of rSCF plus rEP resulted in a much larger number of viable cells than that of rIGF-I plus rEP ($P < 0.05$), and the addition of both factors together with rEP produced an even greater number of viable cells than that seen when each factor alone was added to rEP ($P < 0.05$; days 9–11). The increase in the number of benzidine positive cells was parallel to the increase in viable cell number during the later days of culture (Fig. 1 *D*) and the addition of rIGF-I and/or rSCF plus rEP resulted in a greater number of benzidine positive cells than that with rEP alone ($P < 0.05$ on day 11).

After reculture in fresh medium on day 11, the cells were further incubated through day 14 with rEP alone or with rEP plus rIGF-I and/or rSCF for examination of morphology (Fig. 2, *A–D*). At the beginning of the cultures on day 7, ECFC showed the characteristic morphology of immature erythroid cells as previously described (1). While the majority of the cells cultured with rEP plus rIGF-I were hemoglobinized (86%; Fig. 2 *B*) and disclosed the features of terminal erythroid maturation such as reduction in cell size, nuclear condensation and enucleation, the majority of cells cultured with rEP alone (Fig. 2 *A*) were larger in size, and the nuclear condensation and enucleation in those cells were far less prominent (26% mature erythroblasts plus reticulocytes). Addition of rSCF to the cultures in the presence of rEP or rEP plus rIGF-I (Fig. 2, *C* and *D*) resulted in a large proportion of immature erythroid cells that did not have benzidine staining positivity (42% benzidine negative with rEP plus rSCF and 49% with rEP plus rIGF-I and rSCF). To exclude a possible expansion of non-erythroid contaminant cells under stimulation by rSCF, expression of membrane erythroid spectrin was determined by immunofluorescence using anti-erythroid spectrin antibody to examine day 14 cells incubated with rEP plus rIGF-I and rSCF (Fig. 3, *A–C*). In contrast to the negative control evident with day 3 BFU-E (Fig. 3 *C*), most of the immature day 14 cells (Fig. 3 *B*) strongly expressed erythroid spectrin (84%; Fig. 3 *A*), indicating that the immature cells that had proliferated in the presence of rSCF were erythroid. Additional strong evidence that these immature cells are erythroid is that their appearance in culture is absolutely dependent upon rEP plus rSCF.

Effect of rEP, rIGF-I, and rSCF on heme synthesis. Heme synthesis during ECFC culture was measured on days 6–9,

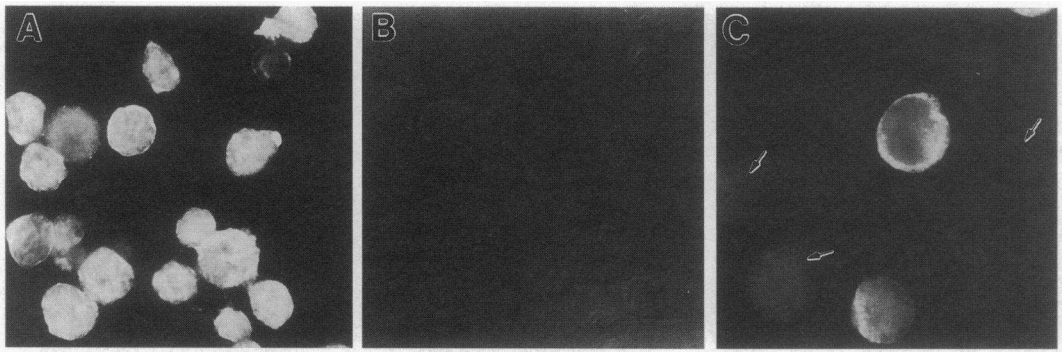

Figure 3. Immunolocalization of spectrin. Expression of erythroid spectrin in day 14 cells incubated with rEP, rIGF-I, and rSCF was determined by immunofluorescence using anti-erythroid spectrin antibody. (*A*) Immunofluorescence. (*B*) Differential interference contrast microscopy of the same immunofluorescence field demonstrating that most of the cells reacted with the spectrin antibody. (*C*) Spectrin negative cells observed with day 3 BFU-E preparation (*arrows*). The purity of the ECFC was 56±5%.

using ^{59}Fe incorporation into heme, and was expressed as cpm/ml of culture (Fig. 4 *A*). When the ECFCs were incubated without rEP, synthesis of heme declined, but cells cultured with rEP had a gradual increase between day 6–7, and a drastic increase beginning on days 8 through 9, without any increase in the number of viable cells. Addition of rIGF-I plus rSCF maintained heme synthesis between days 6 and 9 above the level seen without added growth factors ($P < 0.01$). The remarkable increase in heme synthesis beginning on day 8 did not occur without rEP, and the rate seen with rIGF-I plus rSCF was clearly smaller than that seen with rEP ($P < 0.01$ on days 8 and 9).

Fig. 4 *B* shows the changes in heme synthesis after serum-free culture for 40 h with multiple combinations of growth factors beginning on day 6. To combine the data obtained from different experiments, the result was expressed as a percentage of radioactivity seen in the cultures with rEP alone. While addition of rEP alone resulted in significantly greater heme synthesis than rIGF-I and/or rSCF ($P < 0.01$), addition of rIGF-I and/or rSCF together with rEP markedly increased heme synthesis compared to that seen with rEP alone ($P < 0.01$). When the percentage increase of heme synthesis per viable cell was calculated the combination of rEP plus rIGF-I resulted in the greatest increase in (207±92%), which was significantly greater than that seen with rEP alone (100±17%; $P < 0.01$). The amount of heme synthesis per viable cell cultured with combinations of rEP and rSCF was similar to that seen in cultures with rEP alone (rEP plus rSCF; 122±46%; rEP plus rIGF-I plus rSCF: 104±44%).

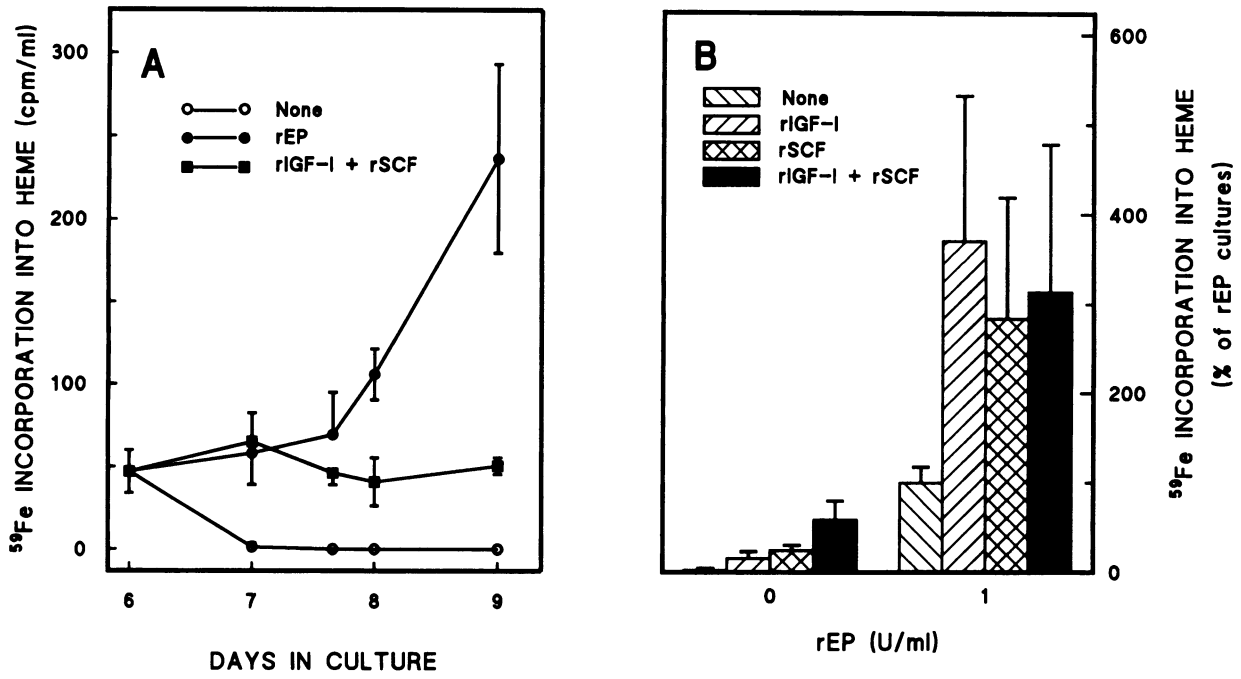

Figure 4. Effect of rEP, rIGF-I, and rSCF on heme synthesis. (*A*) ECFC (day 6 cells) incubated with indicated growth factors were pulsed with ^{59}Fe-transferrin for 4 h at indicated times, followed by determination of ^{59}Fe incorporation into heme. Heme synthesis is expressed as cpm/ml of culture and data are the mean±SD of triplicate determinations. The purity of the ECFC was 54±5%. (*B*) ECFC (day 6 cells) were incubated with indicated growth factors for 40 h, followed by similar measurement of heme synthesis. The mean value obtained from cultures with rEP was designated as 100% so that results of different experiments could be compared. Each bar is the mean±SD of eight replicates from four experiments. The purity of the ECFCs was 58±2%, 41±5%, and 42±6%.

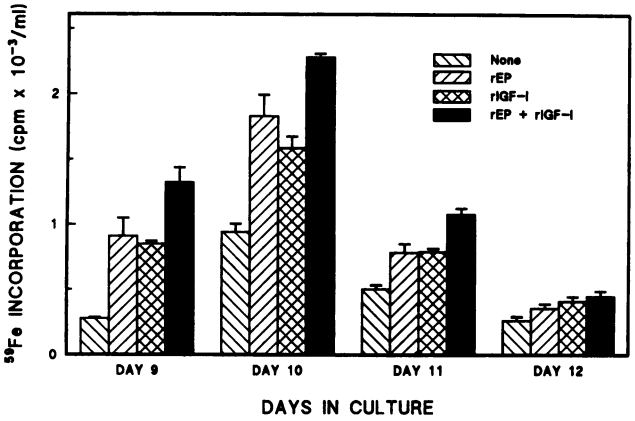

Figure 5. The requirement for IGF-I during late erythroid maturation. Day 7 ECFC were recultured in serum-supplemented liquid cultures with 2 U/ml rEP and were harvested on days 9–12 for 24 h incubations with indicated growth factors and ^{59}Fe-transferrin in serum-free medium. Heme synthesis was determined and each bar is the mean±SD of triplicate determinations. The purity of the ECFCs was 57±5%.

The requirement for IGF-I during the later stages of erythroid development was determined by measuring the accumulated amount of ^{59}Fe incorporation into heme over successive 24 h periods (Fig. 5). On days 9–11, the cells incubated with rIGF-I disclosed a significantly larger amount of heme synthesis than that seen in the cells cultured without the addition of growth factors ($P < 0.01$) and the effect of rIGF-I on accumulation of heme was equivalent to that of rEP throughout the culture period. The addition of rEP and rIGF-I together significantly increased the amount of heme synthesis beyond that seen with each factor alone ($P < 0.05$ on day 9; $P < 0.01$ on day 10 and day 11). The effect of rSCF was also examined in a similar experiment and showed little effect on days 9–11 (data not shown).

To determine the effect of rSCF on heme synthesis, liquid cultures in a serum-free medium were started with day 7 cells and incubated through day 16 in the presence or absence of rSCF (Table I). On days 10 and 14, the culture medium was replaced with fresh medium and the concentration of the cells was reduced to no more than 10^6/ml. Accordingly, the amount of heme synthesis was adjusted to reflect the dilution factor. The addition of rSCF to the cultures with rEP plus rIGF-I produced a remarkable increase in heme synthesis and resulted in a 21-fold greater total accumulation on day 16, compared with the peak value seen on day 12 with only rEP plus rIGF-I, reflecting a marked expansion of the erythroid mass as the cells stimulated by rSCF eventually matured.

Effect of rEP, IGF-I, and rSCF on DNA synthesis. The proliferative response of the ECFC to rEP, rIGF-I, and rSCF was determined by measuring DNA synthesis using [^{3}H]thymidine incorporation (Fig. 6 *A*). rEP alone maintained the [^{3}H]-thymidine uptake, and the amount was similar to the initial amount throughout most of the culture period. The addition of rIGF-I plus rSCF, in the absence of rEP, resulted in slightly greater DNA synthesis compared to the cultures with rEP alone ($P < 0.01$ on days 7 and 8). After rEP, rIGF-I and rSCF were added together, a very large increase in DNA synthesis occurred with a markedly enhanced proliferative response. When the amount of DNA synthesis per viable cell was calculated, addition of rIGF-I and rSCF to rEP resulted in a 3.6- (day 7), 3.8- (day 8), and 2.4- (day 9) fold increase, compared to the values seen with rEP alone.

Table I. Effect of rSCF on heme synthesis by ECFCs

Days of culture	Culture addition	
	rEP + rIGF-I	rEP + rIGF-I + rSCF
8	181±12*	125±27*
10	2155±57	1763±169
12	2322±69	5570±727
14	1644±39	32700±1699
16	318±25	49350±2229

ECFCs (day 7 cells) were incubated with indicated growth factors in liquid serum-free medium and pulsed with ^{59}Fe-transferrin over 4 h at indicated times. Heme ^{59}Fe was measured after extraction using cyclohexanone to determine the amount of heme synthesis. The data are the mean±SD of triplicate determinations. The purity of the ECFCs was 40±5%. * Heme ^{59}Fe (cpm/ml).

To further determine the interaction of rEP, rIGF-I, and rSCF in regulating the proliferation of ECFC, various combinations of these growth factors were added to the serum-free liquid cultures on day 6, followed by a 1-h assessment of [^{3}H]-thymidine incorporation after 40 h of incubation (Fig. 6 *B*). The addition of rEP, rIGF-I and/or rSCF significantly increased DNA synthesis compared to that seen without rEP ($P < 0.01$), but, as a single addition, rSCF had the greatest effect compared with rEP or rIGF-I ($P < 0.01$). The addition of rIGF-I and rSCF together resulted in a higher rate of DNA synthesis than each factor alone ($P < 0.01$). While rEP had little effect on DNA synthesis when added alone, the addition of rEP together with rIGF-I and/or rSCF resulted in a much larger increase compared to that seen with each factor alone ($P < 0.01$). Addi-

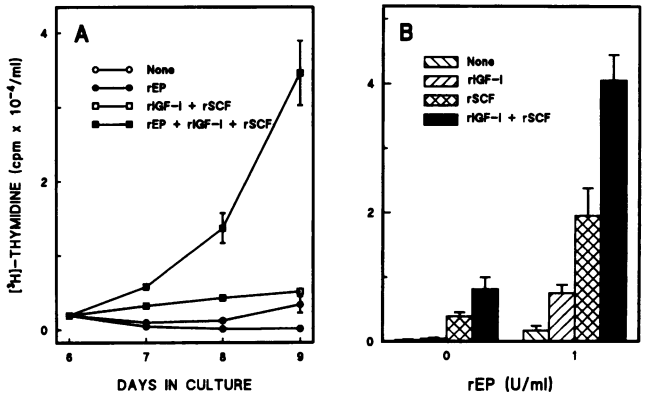

Figure 6. Effect of rEP, rIGF-I, and rSCF on DNA synthesis. (*A*) ECFC (day 6 cells) incubated with indicated growth factors in liquid serum-free cultures, were pulsed with [^{3}H]thymidine for 1 h at indicated times, followed by TCA precipitation to determine the amount of DNA synthesis, which is expressed as cpm per ml of culture. The data are the mean±SD. (*B*) ECFCs (day 6 cells) were incubated with indicated growth factors for 40 h, followed by measurement of DNA synthesis with [^{3}H]thymidine for 1 h. Each bar is the mean±SD of six replicates from two experiments. The purity of the ECFCs was 32±2%, and 41±2%.

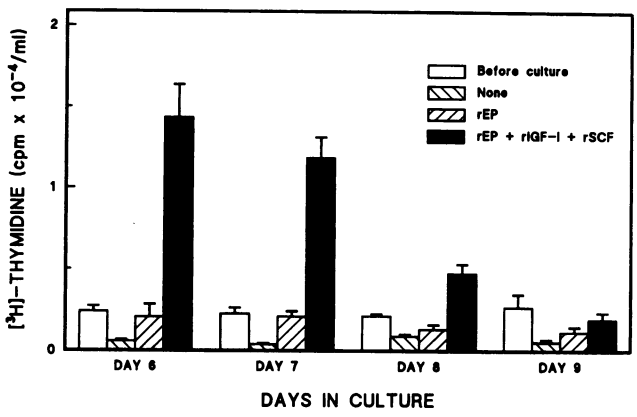

Figure 7. ECFCs (day 6 cells) were removed from methylcellulose and were recultured in liquid serum-supplemented cultures for harvest on succeeding days followed by incubation in serum-free medium with the indicated growth factors. The incubations were continued for 24 h, and DNA synthesis was determined before and after each 24 h of culture. The data are mean±SD of triplicate determinations. The purity of the ECFCs was 51±7%.

tion of rEP to the cultures with rIGF-I, rSCF, or rIGF and rSCF resulted in 3.5-, 3.5-, and 4.1-fold greater increases in DNA synthesis, respectively, compared to the sum of the increases observed with rIGF-I and/or rSCF in the absence of rEP plus the increase observed with rEP alone. Since these large increases depended on the presence of rEP, it is very likely that they represented the erythroid cells and not the contaminant cells. In the presence of rEP, addition of rSCF produced a greater increase than that of rIGF-I ($P < 0.01$), and the addition of rIGF-I and rSCF together to the cultures resulted in a greater increase than that of each factor alone ($P < 0.01$) or the sum of these factors. When DNA synthesis per viable cell was calculated in each incubation, addition of rEP plus rIGF-I and rSCF (3,578 cpm/10^5 viable cells) increased 2.2-fold compared with the sum of the cultures with rIGF-I and rSCF (1,176 cpm/10^5 viable cells) plus those with rEP alone (463 cpm/10^5 viable cells).

To understand the kinetics of growth factor action on DNA synthesis by the ECFC, day 6 cells were recultured in a serum-supplemented liquid medium, and were studied after serial harvests on succeeding days. DNA synthesis was measured before and after 24-h incubations with the indicated growth factors in serum-free liquid medium (Fig. 7). Throughout the culture period, DNA synthesis in the cultures with rEP alone was greater than in those without rEP, but similar or less than that observed before the 24 h of incubation. The marked effect of the addition of three factors together was noted in the earlier day 6 and day 7 cells, and declined by day 8.

Expansion of ECFC during liquid culture. To determine the effect of rEP, rIGF-I, and/or rSCF on the expansion of the ECFC, the number of erythroid colonies was assayed by the plasma clot method after incubating day 6 ECFC for 40 h in a serum-free liquid medium with rEP, rIGF-I and/or rSCF in various combinations (Fig. 8). When the cells were incubated with rEP alone, 36% of erythroid colony-forming capacity was lost over 40 h. Addition of rIGF-I and/or rSCF together with rEP significantly increased the number of erythroid colonies above that seen prior to the 40 h of culture (rEP plus rIGF-I: $P < 0.05$; rEP plus rSCF rEP plus rIGF-I and rSCF: $P < 0.01$). The number of colonies observed with rEP plus rSCF was greater than that seen with rEP plus rIGF-I ($P < 0.01$) and in the presence of both rEP and rSCF together, an additive effect of rIGF-I was not evident. The addition of rIGF-I and rSCF together resulted in a greater number of erythroid colonies compared to that seen in the cultures with each factor alone ($P < 0.01$).

The erythroid colonies were further differentiated into three groups according to the number of cells in each colony. While the addition of rIGF-I to rEP increased the number of colonies of 8–49 cells, addition of rSCF to rEP resulted in a remarkable increase in colonies of more than 50 cells, demonstrating a higher proliferative capacity for the ECFC. When rIGF-I and/or rSCF were added to the cultures in the absence of rEP, the number of erythroid colonies was greater than that seen in cultures without the addition of any growth factors, where no ECFC developed into erythroid colonies (data not shown). When the cells were incubated with rSCF alone, the erythroid colony-forming capacity was maintained at a similar capacity with regard to both colony number and colony size, like that seen before 40 h of culture, and maintenance of the capacity to form colonies of more than 50 cells was retained.

To characterize further the immature cells seen in the cultures with rSCF and rEP during the later days of incubation (day 14; Fig. 2, *C* and *D*), the cells, which had been incubated in liquid medium with various combinations of growth factors, were plated in plasma clots on day 14 and their erythroid colony-forming capacity was determined 7 d later. While the cells incubated with rEP alone, or rEP plus rIGF-I maintained < 2% of original colony-forming capacity on day 14, cells cultured with rEP plus rSCF, or rEP plus rIGF-I plus rSCF disclosed preservation of 14–20% of the original day 7 erythroid colony-forming capacity. 40–50% of these erythroid colonies contained > 8 cells (data not shown).

Combined measurement of apoptosis plus heme and DNA

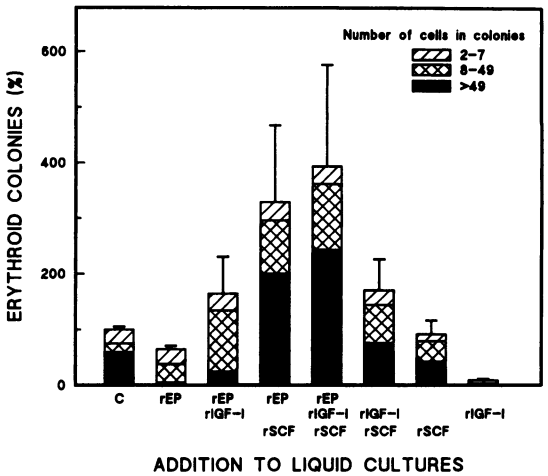

Figure 8. Effect of rEP, IGF-I, and rSCF on number of ECFCs in liquid cultures. After 40 h of incubation of day 6 cells with indicated growth factors, erythroid colony-forming capacity was determined by the plasma clot method. The control (*C*) number of erythroid colonies from cells plated before the incubation was designated as 100%. Erythroid colonies were further differentiated into three groups according to cell number, as indicated. Each bar is the mean±SD of eight replicates from two experiments. The purity of the ECFCs was 41±2%, and 30±2%.

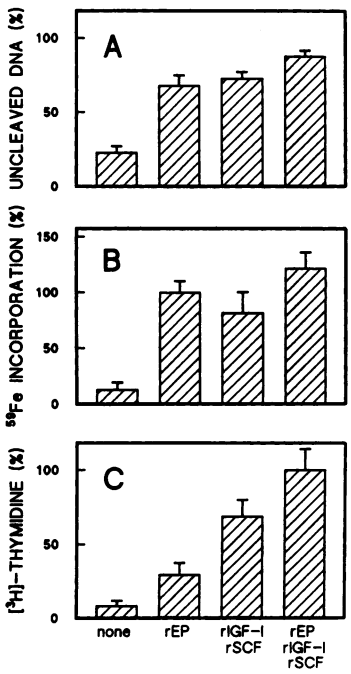

Figure 9. Combined measurements of apoptosis plus heme and DNA synthesis. ECFCs (day 7 cells) were incubated with indicated growth factors in serum-free medium for 16 h and the amount of uncleaved DNA (*A*), heme synthesis (*B*), and DNA synthesis was determined (*C*). The data are expressed as a percentage of total radioactivity (uncleaved DNA), ^{59}Fe incorporation into heme with rEP, or [^{3}H]thymidine cellular uptake with rEP, rIGF-I, and rSCF. Each bar is the mean±SD of four replicates from two experiments. The purity of the ECFCs was 53±4% and 39±2%.

synthesis. To determine if suppression of apoptosis by rEP could solely account for the modulation of the amount of proliferation and maturation of ECFC, the capacity of the growth factors to protect day 7 cells from DNA fragmentation (Fig. 9 *A*), as well as to modulate heme synthesis (Fig. 9 *B*) and DNA synthesis (Fig. 9 *C*) was measured within the same experiment after incubation of the cells in serum-free cultures for 16 h. In these experiments, a relatively higher concentration of rEP (10 U/ml) was used to obtain the maximum effect on suppression of apoptosis (11). rEP significantly increased the amount of uncleaved DNA by suppressing apoptosis, compared to that seen without rEP ($P < 0.01$; Fig. 9 *A*). Addition of rIGF-I plus rSCF also increased the amount of uncleaved cellular DNA equivalent to the effect seen with rEP (Fig. 9 *A*). While the amount of heme synthesis was similar among the cultures with rEP and those with rIGF-I plus rSCF (Fig. 9 *B*), DNA synthesis seen with rEP was significantly less than that with rIGF-I plus rSCF ($P < 0.01$; Fig. 9 *C*). In addition the additive effect of rIGF-I plus rSCF to the effect of rEP on the amount of uncleaved DNA (20%), as well as the amount of heme synthesis (22%), was relatively small, but the effect observed on DNA synthesis with rEP plus rIGF-I and rSCF was 3.4-fold greater than that seen with rEP alone. Since the cell number only increased by 43%, this also represents a 2.4-fold increase per viable cell.

Discussion

Although it has been shown that EP protects cells from apoptosis (10, 11), whether EP acts solely as a survival factor by reducing apoptosis, or has additional functions as a mitogen and/or differentiation factor, has not yet been established. In early erythroid progenitor cells, it has been shown that EP increases DNA synthesis of bone marrow BFU-E in short term liquid culture (14), and the amplification of BFU-E in liquid culture in the presence of rEP (15) supports this observation. Using late erythroid progenitors, Koury and Bondurant (9, 10) demonstrated that the maintenance of DNA synthesis in FVA-infected erythroid progenitor cells could be explained entirely by the prevention of apoptosis and that it was not necessary to postulate a separate direct stimulation of DNA synthesis. However, studies have been reported with a murine erythroleukemia cell line which indicate that EP might act both as a mitogen and as a survival factor (16). All of these experiments were performed with a serum-containing medium and EP might be acting in a cooperative fashion with other serum-containing growth factors such as SCF.

Human ECFCs, generated from partially purified peripheral blood BFU-E and harvested on day 8, proliferate and mature from erythroid progenitor cells into late erythroblasts in serum-free cultures with EP and IGF-I, which act directly on the progenitor cells (2). No other growth factors appear to be necessary for this late process of proliferation and maturation. It is also known that SCF acts directly on BFU-E to promote their further development and that this necessary action extends from the earliest BFU-E to day 8 ECFCs (3). While these general effects have been observed, no attempt has been made to determine the relative role of each growth factor, at the same time, on the maintenance of viability, proliferation, and erythroid maturation by these cells. The capacity to provide highly purified progenitor cells and a serum-free medium has now allowed us to look at the relative effect of each of the growth factors on viability plus hemoglobin and DNA synthesis. When the ECFCs were cultured in serum-free media with EP alone the viability of the cells was maintained without enhanced proliferation, but the cells had increased heme synthesis indicative of enhanced erythroid maturation. Addition of IGF-I plus EP resulted in a significant increase in the viable cell number and DNA synthesis, associated with enhanced heme synthesis and complete erythroid maturation. However, viable cell number and DNA synthesis were markedly enhanced only in the presence of EP plus SCF, which promoted a marked increase in both. Assay for DNA fragmentation showed that EP protects ECFCs from apoptosis, without much additional effect by IGF-I and SCF (Fig. 9 *A*). Thus the large effect of SCF on cell proliferation and DNA synthesis cannot readily be explained by an anti-apoptosis effect, but rather appears to be due to stimulation of proliferation. Using murine erythroid cell lines, it has been shown that proliferation and maturation of erythroid cells could be uncoupled by phosphorylation inhibition (17), or by chemical induction of maturation instead of EP induction (18). Together with these reports, our observations indicate that EP produces a survival and maturation signal by a process independent of the stimulation of cellular proliferation, without a need for other growth factors, while the regulation of proliferation depends on additional intracellular signals initiated by SCF and/or IGF-I, and possibly other growth factors yet undiscovered. Since IGF-I and SCF are present in serum (19, 20, 21), the proliferative response of ECFCs promoted by EP that has been observed in vivo and in serum-supplemented cultures would depend on these hormones.

IGF-I clearly supports the development of erythroid progenitor cells in semi-solid cultures (2, 22). We previously demonstrated that IGF-I acts on the ECFCs without requirement for accessory cells (2), and can maintain ECFC viability and colony-forming capacity by decreasing apoptosis (11). Furthermore, we have presented here evidence that IGF-I plays an essential role in cellular proliferation and maturation at a late

stage of erythroid development. In the presence of EP, IGF-I markedly enhanced heme synthesis (Fig. 4 *B*). In addition, a markedly defective morphology of the cells cultured with EP alone (Fig. 2 *A*), compared with that of normal erythroblasts seen in the cultures with EP plus IGF-I (Fig. 2 *B*), supports this observation. Thus a reduction of apoptosis by EP is not adequate for complete erythroid maturation which requires direct stimulation by IGF-I. Using murine purified CFU-E, it was reported that IGF-I enhances erythroid maturation only when EP levels are low (10 mU/ml) (23). In our experimental system, even in the presence of high concentrations of EP (1 U/ml), the stimulating effect of IGF-I on heme synthesis was prominent. Difference in the cell system, cell maturity, and/or culture conditions, such as BSA preparation, might account for this discrepancy.

The proto-oncogene c-kit encodes a transmembrane tyrosine kinase receptor, and its ligand, SCF (24), stimulates colony formation by hematopoietic progenitor cells of diverse lineages (25, 26). We previously demonstrated that SCF, as well as EP and IGF-I, reduces apoptosis of ECFC between days 7–8 of culture (11). While the effect of SCF on suppression of apoptosis was less prominent than EP (11), the evidence presented here indicates that SCF stimulates proliferation of ECFCs substantially. In contrast to the cellular proliferation associated with erythroid maturation seen in the cultures with EP plus IGF-I, addition of SCF together with EP resulted in an expansion of colony-forming cells with high proliferative capacity (Fig. 8) and an increase in immature erythroid cells that were evident during later days of culture (Fig. 2, *C* and *D*). While it has been reported that SCF stimulates maturation of mast cells (27), this work was performed in a mixed cellular system, with the presence of serum, and several reports have indicated that SCF preferentially enhances proliferation, but not differentiation of a variety of precursor cells (28, 29, 30). In our experiments SCF enhanced ECFC proliferation by itself, although the effect was substantially greater in the presence of EP. SCF also enhanced maturation itself during days 8–9 of culture, but the effect was limited to the very early phase of heme synthesis, after which EP was required as the principal maturation factor. SCF and EP, with, or without, IGF-I produced a marked proliferation of erythroid cells through day 14, which were benzidine negative and this was coupled with a maintenance of colony-forming capacity. This shows a difference in the maturation stage of the cells generated by SCF from those generated without SCF. There remain the following two possible mechanisms for this: (*a*) SCF greatly enhanced the proliferation of a relatively immature subpopulation of the erythroid progenitor cells within the ECFCs; or (*b*) the maturation process of many of the ECFCs was delayed during the marked cellular proliferation stimulated by SCF. Experiments to clarify this issue are now underway in our laboratory.

It could be maintained that SCF might allow enhanced proliferation by preventing apoptosis of progenitor cells that are in an earlier stage of development and were more prone to cell proliferation rather than hemoglobin synthesis. However, day 7 cells, which are responsive to both EP and SCF, had a marked suppression of apoptosis by EP with a stimulation of DNA synthesis that was only ⅓ of the maximum response (Fig. 9). While the suppression of apoptosis by EP was only minimally affected by the addition of SCF, DNA synthesis was markedly enhanced. This discrepancy between the large increase in DNA synthesis and minimal effect on the amount of uncleaved (i.e., protected) DNA, suggests that the proliferation of ECFC is not only "maintained" by suppressing apoptosis, but also stimulated by SCF in the presence of EP.

In conclusion, it appears that EP prevents apoptosis and supports erythroid viability and maturation. IGF-I enhances erythroid maturation and supports limited proliferation, while SCF stimulates extensive cellular proliferation to expand the number of erythroid progenitor cells. These findings indicate that proliferation and maturation of erythroid cells are regulated in a separate manner by these hormones. While the intracellular molecular mechanisms which mediate these actions are still unknown and need to be clarified, further molecular work will have to be performed under conditions that segregate the effects of these growth factors.

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