Human Colony-forming Units-Erythroid Do Not Require Accessory Cells, but Do Require Direct Interaction with Insulin-like Growth Factor I and/or Insulin for Erythroid Development

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Abstract

The presence of heterogeneous erythroid progenitor cells, contaminant cells, or serum may alter erythroid colony development in vitro. To obtain highly purified colony-forming units-erythroid (CFU-E), we cultured partially purified human blood burst-forming units-erythroid (BFU-E) in methylcellulose with recombinant human erythropoietin (rHuEPO) for 7 d and generated cells that consisted of 30–60% CFU-E, but no BFU-E. A serum-free medium was used that allowed development of the same number of erythroid colonies as serum containing medium, but with a greater percentage of larger colonies. This medium consisted of delipidated crystalline bovine serum albumin, iron saturated transferrin, lipid suspension, fibrinogen, thrombin, Iscove's modified Dulbecco's medium/F-12[HAM], and insulin plus rHuEPO. When CFU-E were cultured in a limiting dilution assay and the percentage of nonresponder wells was plotted against cell concentration, both serum-free cultures and serum-containing cultures yielded overlapping straight lines through the origin indicating that CFU-E development did not depend on accessory cells and that insulin acted directly on the CFU-E. Human recombinant interleukin 3 (IL-3) and/or granulocyte-macrophage colony-stimulating factor had no effect on CFU-E growth, while they markedly enhanced BFU-E growth. Physiological concentrations of recombinant human insulin-like growth factor I (IGF-I) enhanced CFU-E growth in the absence of insulin and, together with rHuEPO in serum-free medium, provided a plating efficiency equal to that of serum-containing medium. Limiting dilution analysis in serum-free medium with IGF-I showed a straight line through the origin indicating that IGF-I also acted directly on the CFU-E and not through an effect on accessory cells. These data demonstrate that CFU-E do not require accessory cells, but do require IGF-I and/or insulin which act directly on the CFU-E.

Introduction

Knowledge of the specific requirements for the growth and development of colony-forming units-erythroid (CFU-E)[1] has

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1. *Abbreviations used in this paper:* BFU-E, burst forming units-erythroid; BM, bone marrow; CFU-E, colony-forming units-erythroid;

been very limited up to the present time. Iscove et al. (1) described a serum-free medium containing delipidated albumin, iron-saturated transferrin, and a mixture of an unsaturated fatty acid, lecithin and cholesterol, that supports murine CFU-E development, but not burst-forming units-erythroid (BFU-E). Other investigators demonstrated that a similar medium supports human CFU-E as well (2, 3). However, another unresolved question which was posed by Iscove et al. (1) concerns the effect of numerous contaminant accessory cells and their products. The presence of BFU-E of diverse maturity might also influence the CFU-E count through the production of metabolic products (4), and a variable interpretation of the classification and count of the erythroid colonies.

It is now possible to obtain highly purified human CFU-E which provide a well-defined experimental system that avoids the effects of additional uncontrolled cells on CFU-E growth and interference with the interpretation of that growth (5). This makes it possible to study the direct effect of hormones and growth factors on CFU-E development and to define the necessary requirements for the growth of these cells. In this paper, these requirements have been studied using the combination of a serum-free medium, highly purified human CFU-E, and the technique of limiting dilution analysis.

Methods

Blood and marrow

Peripheral blood and marrow were obtained from normal adult volunteers who had previously signed consent forms approved by the Vanderbilt Committee for the Protection of Human Subjects and the Veterans Administration Research and Development Committee. Approximately 400 ml of peripheral blood or 10 ml of marrow was collected in sodium heparin (Upjohn Co., Kalamazoo, MI) at a final concentration of 20 U/ml.

BFU-E purification

This method has been previously described (5). In brief, the peripheral blood cells were separated over Ficoll-Hypaque (FH; 1.077 g/cm³; Pharmacia Fine Chemicals, Piscataway, NJ; Winthrop-Breton Laboratories, NY) at 400 g for 25 min at 24°C. The interface mononuclear FH cells were collected, washed twice with α-minimum essential medium (α-MEM) and were resuspended in 50% Iscove's modified Dulbecco's medium (IMDM)/50% α-MEM (Sigma Chemical Co., St. Louis, MO). The FH cells were incubated with sheep erythrocytes as described previously (5), and nonrosetted cells were separated over FH at 400 g for 15 min at 24°C. These cells were washed twice and surface immunoglobulin-positive cells were depleted by panning with dishes that had been previously coated with affinity-purified sheep anti–

EACA, epsilon aminocaproic acid; EPO, erythropoietin; FH, Ficoll-Hypaque; GH, recombinant human growth hormone; GM-CSF, recombinant human granulocyte-macrophage colony-stimulating factor; IGF, insulin-like growth factor; IMDM, Iscove's modified Dulbecco's medium; MC, methylcellulose; rHuEPO, recombinant human erythropoietin; PDGF, platelet-derived growth factor.

human IgG specific for the F(ab)₂ fragment (Cappel Laboratories, Inc., Malvern, PA). The nonadherent cells were collected and incubated overnight in polystyrene flasks, at 37°C in a 5% CO_2 atmosphere with 20% FCS and 10% giant cell tumor–conditioned medium (Gibco, Grand Island, NY). Nonadherent day 1 cells were suspended at 70 × 10⁶ cells/ml, 3°C, in a mixture of four monoclonal antibodies: 25 μl of CD11b/OKM*1 (20 μg/ml), 25 μl of CD2/OKT*11 (10 μg/ml) (Ortho Diagnostic Systems, Inc., Raritan, NJ), 50 μl of CD45R/MY 11, and 50 μl of MY 23 to coat granulocytes, monocytes, CFU-granulocyte-macrophage (CFU-GM), T and B lymphocytes and natural killer cells (5). After 60 min the cells were washed three times and were incubated at 4°C for 90 min on plastic tissue culture dishes that had been coated with affinity-purified goat anti–murine IgG (Boerhringer Mannheim Biochemicals, Indianapolis, IN). The antibody-negative, nonadherent cells were then collected for tissue culture.

Culture of BFU-E

Purified BFU-E were cultured at 3 × 10⁵ cells/ml in 0.9% methylcellulose (MC) with 1% deionized (6) human serum albumin (American Red Cross Blood Service), 10⁻⁴ M 2-mercaptoethanol (Eastman Kodak Co., Rochester, NH), 30% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT), penicillin at 500 U/ml, streptomycin at 40 μg/ml, 10% giant cell tumor–conditioned medium, and 2 U/ml of recombinant human erythropoietin (rHuEPO, 10,000 U/mg protein; AMGen Biologicals, Thousand Oaks, CA) by the method of Iscove et al. (7). The cells were plated in flat-bottomed, 12-well, tissue culture plates (Linbro, Flow Laboratories Inc., McLean, VA) at 1.0 ml per well and were incubated at 37°C in a high-humidity 5% CO_2, 95% air incubator.

CFU-E purification

After 7 d of BFU-E culture, the cells that proliferated in MC were collected and washed as previously described (5). Adherent cells were removed as described above. The nonadherent cells were collected and a 2-ml cell suspension was overlaid on 2 ml of FH. After centrifugation at 450 g for 15 min at 24°C, the interface cells (MC FH) were collected and washed three times with α-MEM, and resuspended in 50% IMDM/50% F-12[HAM] (Sigma Chemical Co.) containing 0.1% crystalline BSA (nuclease- and protease-free; Calbiochem-Behring Diagnostics, La Jolla, CA) that had been delipidated, deionized, and dialyzed (C-BSA-3D) as described below.

Preparation of bone marrow (BM) cells

BM cells were diluted with an equal amount of α-MEM and separated over FH at 400 g for 25 min at 24°C (8). The interface mononuclear cells (BM FH) were collected, washed three times with α-MEM, and were then resuspended in 50% IMDM/50% F-12[HAM] containing 0.1% C-BSA-3D.

Preparation of media

BSA, transferrin, and lipid suspension were prepared using minor modifications of the methods of Iscove et al. (1). Dextran T40 (Pharmacia, Uppsala, Sweden), 200 mg, was dissolved in 200 ml of endotoxin-free, deionized, and distilled water, which was used for all preparations. Activated charcoal (Sigma Chemical Co.), 2 g, was suspended in this solution and kept for 30 min at room temperature with occasional agitation. Crystalline BSA, 10 g, was added to the suspension and allowed to dissolve without stirring. The pH was then adjusted to 3.0 by slow dropwise addition of 5 M HCl with rapid stirring. The mixture was heated to 56°C for 30 min with frequent agitation. Charcoal was then partially removed by centrifugation at 2,700 g for 30 min. The pH was adjusted to 5.5 with 1 M NaOH, and the remaining charcoal was removed using 0.8 and 0.45-μm Millipore filters (Millipore Corp., Bedford, MA). The solution was deionized by resin AG 501-X8(D) (Bio-Rad Laboratories, Richmond, CA) (6) and concentrated to 50 ml over a Diaflo UM-10 membrane (Amicon Div., W. R. Grace & Co., Danvers, MA). After dissolving 1.8 g of IMDM and 0.3 g of $NaHCO_3$ into the albumin solution, the pH was adjusted to 7.4 and

it was diluted to 100 ml with water (10% wt/vol). The BSA solution was filter-sterilized and dialyzed in 2 liters of IMDM overnight at 3°C. The C-BSA-3D was filter-sterilized again, stored at −20°C, and routinely used for serum-free media. Cohn Fraction V BSA (Armour Pharmaceutical Co., Kankakee, IL; purity 95%) was treated by the same procedures as those of C-BSA-3D and used for serum-containing media. In experiments with L-triiodothyronine (T₃) 1% C-BSA-3D, containing 5.7 × 10⁻¹¹ M T₃, was replaced by crystalline BSA (Sigma Chemical Co., essentially fatty acid-free, globulin-free) in which T₃ was not measurable.

Human transferrin (iron-saturated, purity 98%, Sigma Chemical Co.), 360 mg, was dissolved in 4 ml of IMDM, pH 7.4, and then was totally saturated with $FeCl_3$. A 1.5-ml solution of 7.9 mM $FeCl_3$ in 1 mM HCl, was added to the transferrin solution and the mixture was dialyzed in 1 liter of IMDM to remove free iron and preservatives. The solution was then filter-sterilized and the concentration was adjusted to 30 mg/ml with IMDM before storage at −70°C.

Oleic acid (Sigma Chemical Co.), 28 mg, L-α-phosphatidylcholine (Sigma Chemical Co.), 40 mg, and cholesterol (Sigma Chemical Co.), 39 mg, were dissolved in 0.5 ml of chloroform at room temperature. The solvent was then completely evaporated until no chloroform odor remained, leaving a lipid film on the bottom of the beaker. 50 ml of phosphate-buffered saline (PBS), pH 7.4, containing 1% C-BSA-3D, was added and the beaker was placed in ice water for sonication at 0.45 W/in², 60 kHz, for 30 min (Ultrasonic Cleaner, Heat Systems-Ultrasonics, Inc., Plainview, NY). The contents were filtered through Millipore filters of pore-size 0.8 and 0.45 μm and stored at 4°C.

Porcine insulin (26.3 USP U/mg; Calbiochem-Behring Corp.), 20 mg, was completely dissolved in 1 ml 10 mM HCl and was centrifuged at 15,300 g for 4 min at 4°C. The supernatant was diluted to 40 ml with IMDM containing 0.3% C-BSA-3D and was then stored at −70°C. 5 ml of recombinant human insulin (100 U/ml, 26 U/mg, Eli Lilly & Co., Indianapolis, IN) with 0.6% C-BSA was dialyzed twice with 500 ml of IMDM at 3°C for 12 h each time and was then centrifuged and stored like the porcine insulin.

rHuEPO (AMGen Biologicals, 10,000 U/mg protein) was dissolved in IMDM containing 0.1% C-BSA-3D at a concentration of 50 U/ml, and stored at −20°C.

Human fibrinogen (grade L, coagulability 90% of total protein content, KabiVitrum, Stockholm, Sweden), 100 mg protein, was dissolved in 5 ml of water and dialyzed with 1 liter of PBS, pH 7.4, for 1 h and with 1 liter of α-MEM for another 2 h. This solution was filter-sterilized and was kept at 24°C for use within 24 h.

Thrombin (Parke-Davis, Morris Plains, NJ) was dissolved in α-MEM at a concentration of 10 U/ml, filter-sterilized, and stored at −20°C. The solution was thawed and diluted to a concentration of 2 U/ml with 50% IMDM/50% F-12[HAM] just before its use.

Aminocaproic acid (EACA) (Elkins-Sinn, Inc., Cherry Hill, NJ) was diluted to a concentration of 100 mM with α-MEM, filter-sterilized, and stored at 4°C.

Culture of CFU-E

Serum-free medium. MC FH cells were plated into 0.5 ml mixture of C-BSA-3D (10 mg/ml), containing iron-saturated transferrin (150 μg/ml), lipid suspension (oleic acid, 2.8 μg/ml; L-α-phosphatidylcholine, 4.0 μg/ml, cholesterol, 3.9 μg/ml), insulin (10 μg/ml), rHuEPO (1 U/ml), fibrinogen (2 mg/ml), thrombin (0.2 U/ml), EACA (1.5 mM), and 50% IMDM/50% F-12[HAM]. In some experiments the above concentration of transferrin and lipid suspension was doubled. The cells were then cultured for 7 d at a concentration of 500 cells/ml in 24-well flat-bottomed tissue culture plates (Linbro) at 37°C in a high-humidity 5% CO_2-95% air incubator. The clots were fixed and stained with benzidine, and erythroid colony-forming cells which gave rise to colonies of 2 or more hemoglobinized cells were scored as erythroid colony-forming cells, while colonies of 8–49 cells were scored as CFU-E according to previous criteria (9, 10). BM FH cells were cultured in the same serum-free mixture at a concentration of 5 × 10⁴ cells/ml.

Serum-containing medium. C-BSA-3D was replaced by Cohn fraction V BSA and 30% (vol/vol) FCS was added to a culture medium like that above, but without transferrin, lipid suspension, and insulin. In some experiments FCS was added directly to the mixture prepared for serum-free culture.

Culture of BFU-E

Serum-free medium. The serum-free medium prepared for CFU-E was supplemented by the addition of 2-mercaptoethanol (5×10^{-5} M) and various concentrations of recombinant human interleukin 3 (IL-3) (Genzyme, Boston, MA; 10^8 CFU/mg) and/or recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Genetics Institute, Cambridge, MA; 50% desialated, 2.1×10^7 U/mg). The cells were cultured with 2 U/ml of rHuEPO for 14 d and then the clots were fixed and stained with benzidine. Enumeration of BFU-E was performed in accordance with the criteria of Clarke and Housman (8).

Serum-containing medium. The cells were plated into a 0.5-ml mixture of Cohn fraction V BSA (10 mg/ml), that had been delipidated, deionized, and dialyzed as described above, 20% FCS (vol/vol), 10% pooled human AB serum (vol/vol), rHuEPO (2 U/ml), 2-mercapthoethanol (5×10^{-5} M), fibrinogen (2 mg/ml), thrombin (0.2 U/ml), IL-3 (25 U/ml), and IMDM, and were then cultured for 14 d.

Specific binding of radioiodinated insulin and insulin-like growth factor I (IGF-I) to CFU-E

Human insulin, ^{125}I-labeled at tyrosine-A14 (2,013 Ci/mmol), and human recombinant (3-[^{125}I]iodotyrosyl)IGF-I, [Thr^{59}] (2,013 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL.

Before binding, the cells were incubated with 1 ml of 50% IMDM/50% F-12 [HAM] containing 0.1% C-BSA-3D, 75 μg/ml iron-saturated transferrin, 10 μl/ml lipid suspension, and 1 U/ml rHuEPO for 60 min at 37°C in a high-humidity 5% CO_2-95% air incubator. The cell suspension was then overlaid on 3 ml of FH and was centrifuged at 600 g for 15 min at 24°C. The interface cells were collected, washed three times with α-MEM, and then resuspended in 50% IMDM/50% F-12[HAM] containing 0.1% C-BSA-3D. 100 μl containing 4×10^5 cells was incubated with 0.66 nM radioiodinated insulin or IGF-I in 1-ml conical tubers (SARSTEDT, Numbrecht, Federal Republic of Germany) for 5 h at 37°C in the above incubator. Replicate tubes had a 5,000-fold concentration of insulin or IGF-I, respectively, to measure nonspecific binding. After this time, ice-cold IMDM containing 0.25% BSA was added to the tube and 1 ml of the cell suspension was overlaid on 3.5 ml of 10% BSA/PBS in a 15-ml tube. The tube was centrifuged at 600 g for 5 min at 3°C and the supernatant was removed at the level of the BSA. The tube wall was washed twice with 4 ml of IMDM and the BSA was removed. The cells were then washed again with 10 ml of IMDM containing 0.25% BSA. The cells were resuspended in IMDM with 1.0% BSA at a concentration of 10^6/ml and cytocentrifuge (Shandon, Southern Products Ltd., Astmoor, Runcorn, Cheshire, England) preparations were made from 100-μl aliquots.

Duplicate cytospin slides were fixed in 2.0% glutaraldehyde in PBS. Slides were washed three times with PBS, dehydrated through a series of ethanol washes, and allowed to air dry. The slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), diluted 1:1 with distilled water, dried, and exposed for 6 wk at 3°C. Autoradiograms were developed in Kodak D-19 developer diluted 1:1 with distilled water, rinsed briefly in distilled water, and fixed using Kodak fixer. Autoradiograms were washed with running water for 20 min before staining with hematoxylin and eosin. Photomicrographs were taken using Plus-X film and developed in Dialine.

To quantitate the number of cells positive for ^{125}I-insulin or ^{125}I-IGF-I binding, the following procedure was used. On each slide, the number of grains per cell were counted for 100 cells. More than 95% of the cells with nonspecifically bound ligand possessed five or fewer grains per cell. Therefore, on the slides containing cells that had total binding of the ligand any cell having six or more grains was considered

a positive cell for specific binding. The percent of positive and negative cells was calculated for each of the duplicate slides.

Source and assays for multiple hormones and growth factors

Hydrocortisone was purchased from Calbiochem-Behring Corp. Testosterone and T_3 were purchased from Sigma Chemical Co. Recombinant human IGF-I was purchased from AMGen and recombinant human growth hormone (GH), 2 IU/mg, was purchased from Genetech, Inc., South San Francisco, CA. Human platelet-derived growth factor (PDGF), 312,500 half-maximal U/mg protein, and rat insulin-like growth factor II (IGF-II) were purchased from Collaborative Research Inc., Lexington, MA. Hydrocortisone and testosterone were dissolved in absolute ethanol at a concentration of 10 mM and stored at 3°C. T_3 was dissolved in 1 part HCl + 2 parts absolute ethanol at a concentration of 100 mM, and then diluted to 10 mM with absolute ethanol, and stored at 3°C. PDGF, IGF-I, and IGF-II were dissolved in 10 mM HCl at a concentration of 50 μg/ml and stored at -70°C. GH was dissolved in IMDM at a concentration of 10 mg/ml just before use. Filter-sterilization was avoided for the preparation of these reagents.

Radioimmune assays of diverse hormone concentrations in C-BSA-3D, C-BSA (Sigma Chemical Co.), fibrinogen, thrombin, and transferrin were made by the Vanderbilt Clinical Laboratory. Hydrocortisone was measured by the Coat-A-Count Cortisol procedure of Diagnostic Products Corp., Los Angeles, CA; T_3 by the ^{125}I-triiodothyronine procedure of Immuchem Corp., Carson, CA; testosterone by the method of Abraham et al. (11); GH by the method of Glick et al. (12); and insulin by the Pharmacia Insulin R1A 100 procedure of Pharmacia Diagnostics, Uppsala, Sweden.

Significance was calculated using the *t* test (13).

Results

Growth of CFU-E in serum-free medium. The appropriate concentration of each ingredient of the serum-free medium was determined with dose-response curves and comparison to growth in serum-containing medium (Fig. 1). C-BSA-3D, transferrin, lipid suspension, rHuEP, and insulin were essential for optimal CFU-E growth. Omission of insulin reduced the number of erythroid colonies by 60% whereas the combination of these ingredients allowed MC FH cells to express the same number of erythroid colonies as those developed in serum-containing cultures. Maximal stimulatory activity of each ingredient was seen at the following concentrations: rHuEPO, 1 U/ml; C-BSA-3D, 10 mg/ml; transferrin, 75 μg/ml; lipid suspension, 5 μl/ml (oleic acid, 2.8 μg/ml; L-α-phosphatidylcholine, 4.0 μg/ml; cholesterol, 3.9 μg/ml); and insulin, 10 μg/ml. Similar results were also observed when pure rHuEPO (AMGen; 160,000 U/mg protein) was used (data not shown).

Proliferative capacity of erythroid progenitors in serum-free medium. The size of the erythroid colonies that developed in serum-free and serum-containing media was compared as shown in Table I. When MC FH cells were plated in serum-free medium, they expressed the same number of erythroid colony-forming cells as those in serum-containing medium, but the number of CFU-E colonies, consisting of 8–49 cells, was 82% and greater than the number observed in serum-containing medium ($P < 0.001$). The serum-free medium supported increased proliferative capacity of the erythroid progenitor cells, either through a better nutrient mix or the removal of a serum inhibitor, and was better for CFU-E growth than the serum-containing medium. Almost no colonies of 50 or more cells were observed in either serum-free or serum-
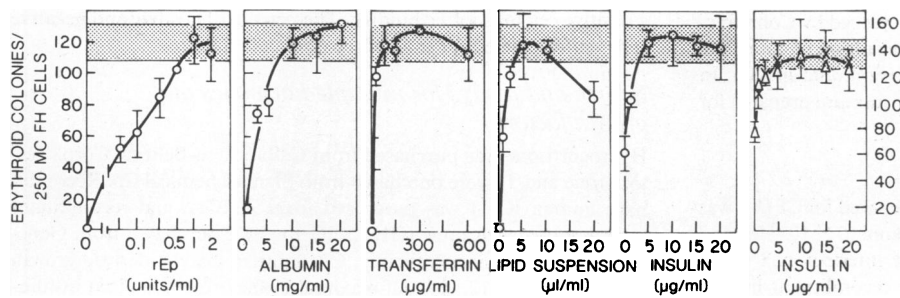
Figure 1. Effect of increasing concentrations of individual components of serum-free medium on CFU-E growth. The complete serum-free medium consisted of 50% IMDM/50% F-12[HAM] supplemented with delipidated crystalline BSA (10 mg/ml), iron-saturated transferrin (300 µg/ml), lipid suspension (oleic acid, 5.6 µg/ml; L-α-phosphatidyl choline, 8.0 µg/ml; cholesterol, 7.8 µg/ml), insulin (10 µg/ml), rHuEPO (1 U/ml), fibrinogen (2 mg/ml), and thrombin (0.2 U/ml). MC FH cells were plated in 0.5-ml fibrin clots at a concentration of 500 cells/ml. Each point (○) represents the mean±SD of triplicates. Shaded areas show the mean±SD of triplicates observed in serum-containing medium. In a separate experiment recombinant human insulin (×) was compared to porcine insulin (△) under the same conditions.

containing medium. When MC FH cells were incubated in serum-containing medium to assay for BFU-E, no BFU-E colonies were observed (data not shown) indicating that MC FH cells predominantly consisted of late stage EPO-dependent erythroid progenitor cells.

To examine whether the serum-free medium could support primary culture of bone marrow CFU-E, BM FH cells were plated in serum-free medium. These cells expressed only 61% of the number of erythroid colonies grown in serum-containing medium. At least half of the decrease in erythroid colony formation occurred with the disappearance of erythroid colonies of > 50 cells that were present in BM FH cells and not in MC FH cells. These larger colonies, with more proliferative capacity, arise from more immature erythroid progenitor cells such as late BFU-E. Their appearance demonstrates the heterogeneity of BM FH erythroid progenitors and their disappearance in serum-free medium indicates that it is not adequate for more immature erythroid progenitor cells.

Limiting dilution analysis of CFU-E growth. The clonal basis of CFU-E growth was studied using limiting dilution analysis (14). MC FH cells were plated in serum-free or serum-containing medium at a variety of concentrations as indicated in Fig. 2, and the percentage of non-responder wells was plotted against the number of cells per well. Both serum-free and serum-containing medium showed overlapping straight lines through the origin, indicating that both of the culture systems are completely adequate for growth of single

CFU-E and that CFU-E do not require accessory cells for their clonal development. This result also indicates that insulin acts directly on the CFU-E (14).

Effect of IL-3 and/or GM-CSF on erythroid progenitor cell growth. MC FH cells and partially purified blood BFU-E were plated into serum-free medium with various concentrations of IL-3 and/or GM-CSF. As shown in Fig. 3, no effect of IL-3 or GM-CSF on CFU-E growth was observed, whereas IL-3 or GM-CSF markedly enhanced BFU-E growth, the former more than the latter. Addition of GM-CSF to cultures grown in the presence of an appropriate amount of IL-3 did not result in a significant enhancement of colony formation. These results indicate that CFU-E in contrast to BFU-E, do not require IL-3 and GM-CSF for colony development.

Effect of multiple hormones and growth factors on erythroid colony-forming cell development. Using MC FH cells, the effects of hydrocortisone, T_3, testosterone, PDGF, GH, IGF-I, and IGF-II, which have been reported to enhance or suppress growth of erythroid progenitor cells, were studied in serum-free medium. None of these reagents except IGF-I showed an effect on erythroid colony growth at physiological concentrations, and in the presence of insulin the effect of IGF-I was not apparent (Fig. 4). Because the marked stimulatory effect of IGF-I was only evident in the absence of insulin these experiments were repeated in serum-free medium without insulin. As shown in Fig. 5, none of these hormones, IL-3, or GM-CSF were able to replace insulin, except IGF-I, in providing a

Table I. Proliferative Capacity of Erythroid Colony-forming Cells in Serum-containing and -free Medium

Cells	Serum	Number of cells in erythroid colonies					Erythroid colonies
		2–4	5–7	8–16	17–49	>50	
		%	%	%	%	%	*Percentage of control*
MC FH	(+)	26±5	13±4	53±10	11±10	1±3	100
	(−)	9±2	8±1	49±5	33±3	1±1	103±5
BM FH	(+)	31±8	9±3	24±5	19±6	22±6	100
	(−)	23±4	7±4	53±6	16±5	0.4±0.5	61±12

MC FH cells and BM FH cells were cultured in serum-containing and -free 0.5-ml fibrin clots at a concentration of 500 cells/ml and 1×10^5 cells/ml, respectively. After 7 d of culture, clots were fixed and stained by benzidine, and the number of hemoglobin-containing cells per colony was counted. Mean values±SD for three experiments are shown. The number of erythroid colonies observed in serum-free medium was compared with the number in serum-containing medium, and the data expressed as % (±SD) of the latter. The absolute number of erythroid colony-forming cells observed in serum-containing medium in these three experiments was 137±35 per 250 MC FH cells and 522±212 per 5×10^4 BM FH cells.
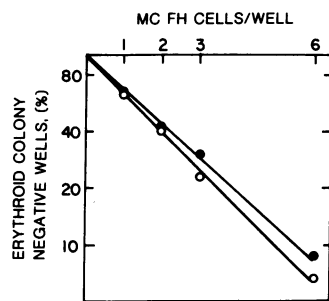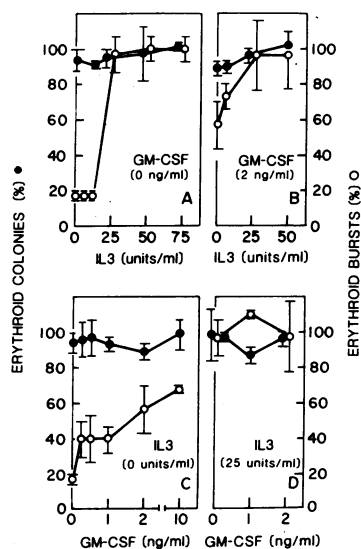
Figure 2. Limiting dilution analysis of CFU-E growth. MC FH cells were suspended in serum-containing (•) and serum-free (o) medium at concentrations of 20, 40, 60, and 120 cells/ml. Aliquots of 50 μl were plated into 96-well tissue culture flat bottom plates. After 7 d of incubation, the clots were fixed and stained by benzidine, and the number of clots in which erythroid colonies did not develop were counted as erythroid colony-negative wells. This was plotted against the number of MC FH cells plated into the wells. Each point represents the values obtained from 60 wells.

serum-free medium that was equivalent to serum-containing medium in supporting erythroid colony growth.
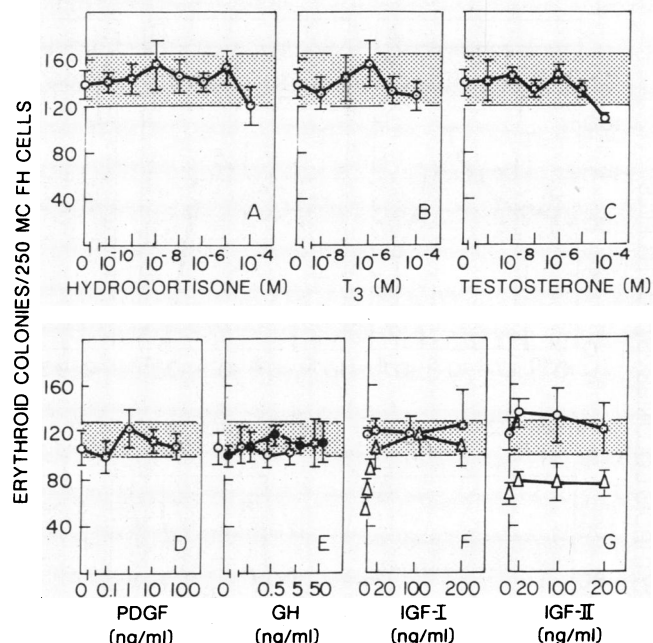
Since the extracted biological components of the serum-free medium might be contaminated with diverse hormones, the concentration of hydrocortisone, T_3, testosterone, GH, and insulin in these ingredients were measured by radioimmunoassay and in all cases the calculated maximum concentrations in the serum-free medium were < 1% of the concentrations in normal human serum.

Effect of insulin and IGF-I on CFU-E development. Using MC FH cells, the relationship of the concentration of insulin and IGF-I on erythroid colony growth was analyzed (Fig. 6). Only 46% of the maximum erythroid colony number was observed in the absence of insulin and IGF-I, but with increasing concentrations of insulin or IGF-I, the number of colonies

Figure 4. Effect of a variety of hormones and growth factors on erythroid colony-forming cell growth in standard serum-free medium which contained insulin (10 μg/ml). MC FH cells were plated in triplicate and each value represents the mean±SD (O). In some experiments, 10 ng/ml IGF-I was added (•) (E), whereas in others insulin was omitted from the culture medium (△) (F, G). Shaded areas show the mean±SD of triplicates observed in serum-containing cultures. This figure is a composite of two individual experiments (A–C and D–G).

Figure 3. Effect of IL-3 and/or GM-CSF on erythroid progenitor cells. MC FH cells were cultured in serum-free medium at a concentration of 500 cells/ml in the presence of various concentrations of IL-3 and/or GM-CSF. After 7 d of culture, clots were fixed and stained with benzidine, and the number of erythroid colonies was counted. Cell aliquots were also plated into serum-containing medium without IL-3 and GM-CSF. Each value is the mean±SD of triplicates and is expressed as a percentage of the value obtained in serum-containing cultures which gave rise to 151±12 erythroid colonies/250 MC FH cells in each clot. For BFU-E growth the serum-free medium was supplemented with 5 × 10⁻⁵ M 2-mercaptoethanol, and BFU-E, partially purified to 0.4% (5), were cultured at a concentration of 1.5 × 10⁴ cells/ml in the presence of various concentrations of IL-3 and/or GM-CSF. After 14 d of culture, clots were fixed and stained with benzidine, and the number of erythroid bursts was counted. Cell aliquots were also plated into serum-containing medium with 25 U/ml of IL-3. Each value is the mean±SD of triplicates and is expressed as a percentage of the value obtained by serum-containing cultures which gave rise to 30±3 BFU-E/7,500 cells in each clot.

with eight or more cells increased and full erythroid colony development was observed at a concentration of 10 μg/ml of insulin or 100 ng/ml of IGF-I. Significant increases in the number of erythroid colonies were seen at 1 ng/ml of insulin or at 1.56 ng/ml of IGF-I, which are within the physiological range, and small concentrations of insulin significantly enhanced the effect of small concentrations of IGF-I. Although CFU-E respond to both insulin and IGF-I when small physiological concentrations are present, and reach normal growth potential within a range of normal serum concentrations of IGF-I, 100-fold greater concentrations of insulin, in the pharmacologic range, are necessary to equal this effect. No colonies formed in the absence of added rHuEPO.

We also performed limiting dilution analysis of MC FH cells in serum-free medium without insulin, but with 100 ng/ml of IGF-I, to determine if the IGF-I is acting directly on the CFU-E. This experiment demonstrated a single straight line through the origin, indicating that the effect of IGF-I on CFU-E growth is a direct effect on these cells and does not occur through an effect on accessory cells (Fig. 7). Although the number of CFU-E was slightly less than with serum-containing medium a wide variety of subsequent experiments have repeatedly confirmed that the effect with 100 ng/ml of IGF-I is not significantly different than the effect with serum-containing medium. Autoradiography confirmed direct specific binding of ¹²⁵I-insulin and ¹²⁵I-IGF-I to the CFU-E (Fig. 8).

Discussion

We improved the purity of three specific factors that relate to CFU-E growth and development. First, we developed a
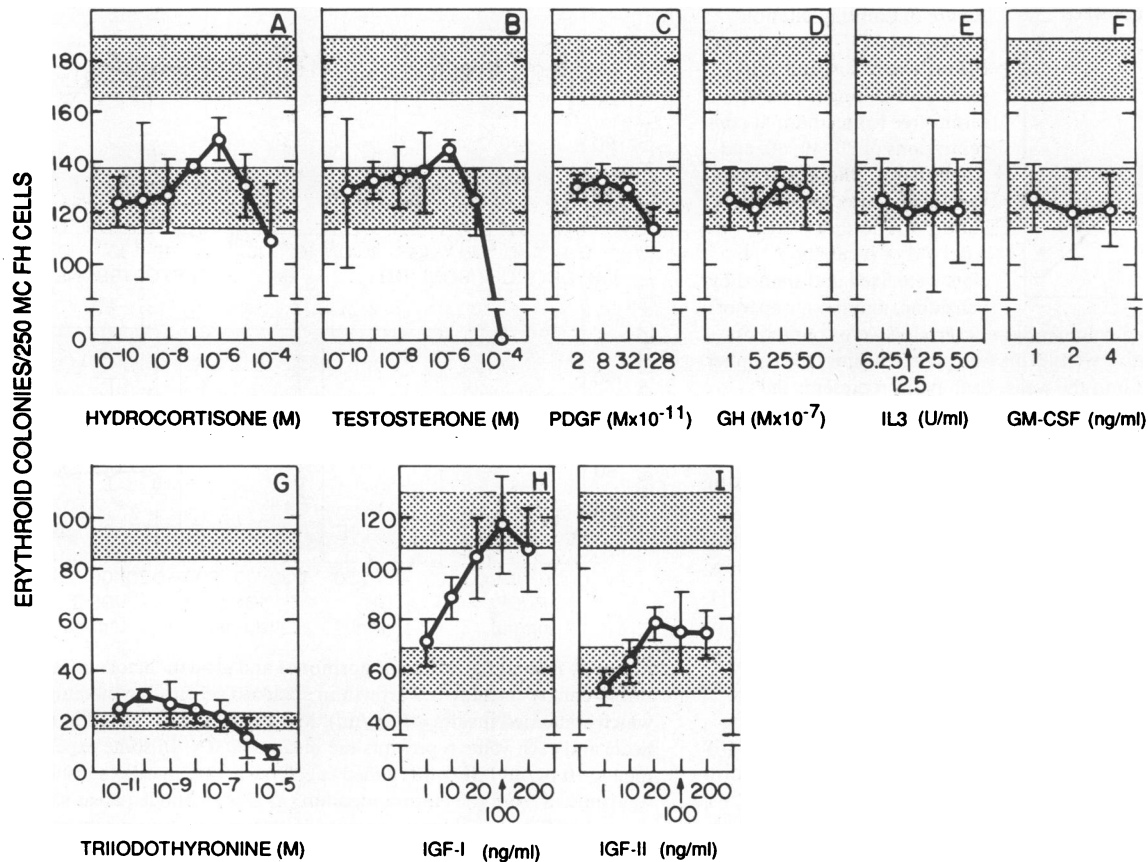
Figure 5. Effect of a variety of hormones and growth factors on erythroid colony-forming cell growth in serum-free medium without insulin. MC FH cells were plated in triplicate and each value represents the mean±SD of triplicates. Upper shaded area shows the mean±SD of erythroid colonies in serum-containing cultures, while lower shaded area shows the mean±SD of erythroid colonies in serum-free cultures without insulin. The figure is composed of three individual experiments (A–F, G, and H and I).

serum-free culture system that supports human CFU-E growth with the same plating efficiency as serum-containing systems and even enhances erythroid progenitor cell proliferation. This

made it possible to reduce inhibitory and/or stimulatory effects of undefined factors present in the serum, and to see complete effects of added hormones, which might be obscured by high baseline serum concentrations. Secondly, erythroid progenitor cells were highly purified to avoid the effects of nonerythroid cells which produce various growth factors, and metabolites that might influence CFU-E growth (15–18). Thirdly, the presence of a single population of CFU-E, without
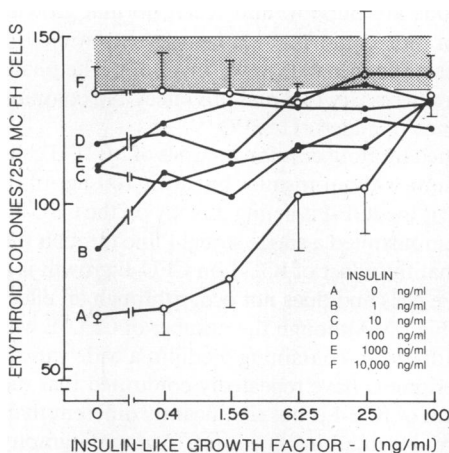

Figure 6. Effect of increasing concentrations of IGF-I and/or insulin on CFU-E growth. MC FH cells were cultured in serum-free medium with various concentrations of IGF-I and/or insulin for 7 d. Each value is the mean of triplicates (●) and for A (absence of insulin) and F (addition of 10 μg/ml insulin) ±SD (O). Shaded area shows the mean±SD of triplicates from serum-containing cultures.
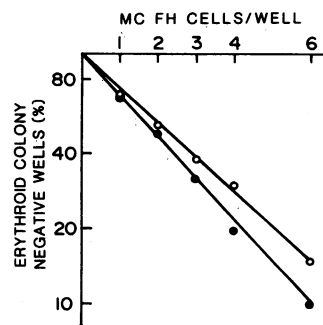

Figure 7. Limiting dilution analysis of CFU-E growth in serum-free medium with IGF-I. MC FH cells, at concentrations of 20, 40, 60, and 120 cells/ml, were suspended in serum-free medium without insulin, but with the addition of 100 ng/ml of IGF-1 (o), or in serum-containing medium (●). Aliquots of 50 μl were plated into 96-well tissue culture flat-bottomed plates. After 7 d of incubation, clots were fixed and stained by benzidine, and the number of clots in which erythroid colonies did not grow was counted as erythroid colony-negative wells. This was plotted against the number of MC FH cells plated into the wells. Each point represents the value obtained from 60 wells.
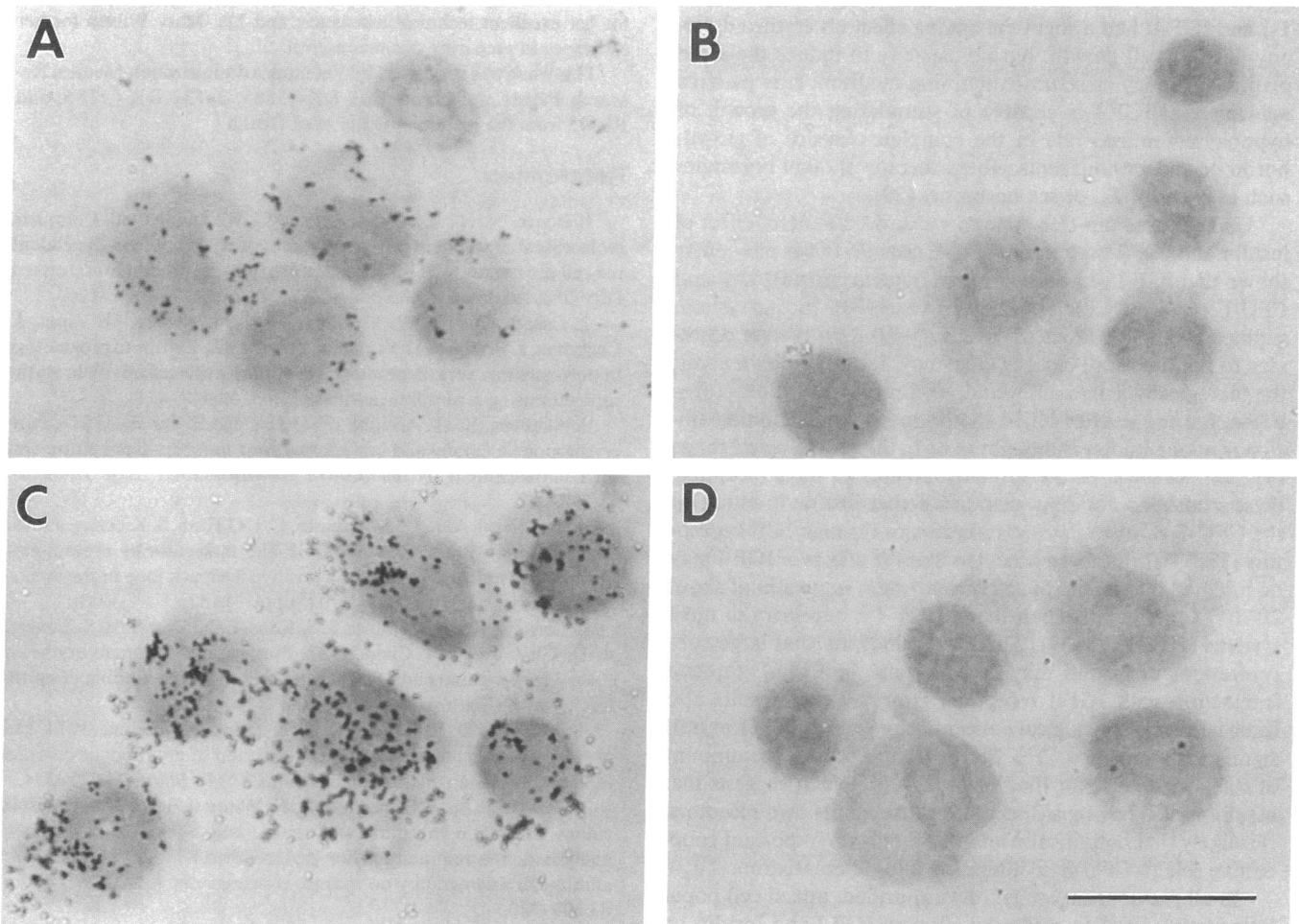
Figure 8. (*A*) Total and (*B*) nonspecific ^{125}I-insulin binding and (*C*) total and (*D*) nonspecific ^{125}I-IGF-I binding to human CFU-E. The percentage of cells positive for ^{125}I-insulin specific binding was 70–78% whereas 81–83% were positive for ^{125}I-IGF-I binding. CFU-E were 48±5% of the cells. Bar, 20 μm.

BFU-E, made the evaluation and interpretation of effects of erythropoietic factors more reliable and precise and removed the possible production of other growth factors by additional erythroid cells (4).

In our initial studies, we demonstrated the relative effect of a variety of nutrients on the growth of human CFU-E by their individual dose response curves in serum-free medium. The cloning efficiency is sharply affected by the removal of BSA, lipids, or transferrin. It has been reported that charcoal treatment of BSA reduces the capacity of BSA to sustain erythroid differentiation in vitro (19), and we encountered the same phenomenon. We do not know the precise reason for this, but the problem was completely resolved by changing the medium from IMDM alone to a mixture of IMDM and F-12[HAM]. Omission of insulin led to a 54% reduction of cloning efficiency and almost no erythroid colonies of eight or more cells. The dose-response curve of rHuEPO in the presence of insulin was like that of serum-containing cultures (5), and despite the addition of insulin and/or IGF-I, no erythroid colonies were seen in the absence of rHuEPO. Thus, our studies indicate that both insulin and IGF-I do not act like EPO since they do not stimulate CFU-E in vitro without the presence of the primary regulator of erythropoiesis. This is in contrast to the studies of Kurtz et al. (20, 21) who observed that murine bone marrow

and fetal liver CFU-E responded to IGF-I or insulin in a serum-free medium without the presence of EPO, but with a dose-response curve like that of EPO. It is possible that murine CFU-E have different requirements than human CFU-E, but additional possibilities for this difference include the stimulation of adjacent accessory cells to produce other factors that affect the proliferation and differentiation of these cells when they are in a mixed population.

An enhancing effect of hydrocortisone (22), T_3 (23), testosterone (24), PDGF (25), GH (26), and IGF-II (27) on CFU-E development has been reported. In this investigation, however, we could not detect a significant effect of these hormones or growth factors by repeated experiments. The difference in our results could be explained, in part, by the difference in the cell populations that were tested. In our system, the cells have been highly purified and maturity has been predominantly synchronized to the level of the CFU-E. Therefore, if the effect of a hormone was mediated through accessory cells it might be difficult to detect and if these factors acted mainly on immature erythroid progenitors, no effect would be seen in our system. The enhancing effect of T_3 on human BFU-E growth has indeed been shown to occur through the release of erythropoietic growth factors by bone marrow accessory cells (28). In the absence of insulin and IGF-I, hydrocortisone, testosterone,

T$_3$, and IGF-II had a slight enhancing effect on erythroid colony-forming cell growth, but no capacity to induce the same plating efficiency as serum-containing medium. It is perhaps relevant that IGF-I is capable of stimulating the growth of hypophysectomized rats in the complete absence of growth hormone and without replacement therapy of other hormones such as cortisol, T$_3$, or sex hormones (29).

Using this serum-free system, we showed a direct effect of insulin and IGF-I on human CFU-E growth. It was previously shown that IGF-I stimulates human bone marrow BFU-E and CFU-E erythroid differentiation in vitro (30). In our system, supraphysiological levels of insulin (5–10 μg/ml) were necessary to maintain full cloning efficiency. Little is known about the biologically active, unbound, serum concentration of free IGF-I, but the level of IGF-I that induced a high cloning efficiency in our studies is thought to be in the physiological range (31, 32). Because of the cross-reactivities to their receptors, these studies do not demonstrate whether insulin is acting on the CFU-E through its own receptors or through IGF-I receptors (33, 34). It has been accepted that the effects of IGF-I may be initiated by insulin, but a concentration of insulin of about 20–100 times greater than that of IGF-I is necessary in most systems (20, 21, 35–37). This would indicate that large concentrations of insulin may be acting on the CFU-E through interaction with IGF-I receptors. However, our results also indicate that physiological concentrations of insulin (1 ng/ml) significantly enhance CFU-E growth and reduce the amount of IGF-I necessary for that effect (Fig. 6), which suggests that insulin is also having a direct effect through its own receptors. It is likely that both insulin and IGF-I play an important cooperative role in CFU-E proliferation and differentiation.

In all previous investigations unpurified, mixed cell populations were used to study the effect of IGF-I and insulin on erythroid progenitor cells (3, 20, 21, 27, 30, 38, 39). Even though our CFU-E were purified to 30–60% of the cell population the effects of added hormones could still occur through a stimulation of the remaining contaminant cells. For this reason we applied the technique of limiting dilution analysis to identify the presence an accessory cell mechanism. When the number of MC FH cells in each serum-free well with IGF-I or insulin was plotted against the logarithm of the percentage of erythroid colony-negative wells, a straight line through the origin was apparent indicating that clonal growth was unaffected by the presence of additional cells and only dependent on the CFU-E itself. If the effect of the added hormone had occurred through an effect on accessory cells an increasing frequency of erythroid colonies would have been noted. The present limiting dilution studies, which show a single hit curve, indicate that a single cell population is involved and that IGF-I and insulin act directly on the CFU-E.

In conclusion, we have found that human CFU-E do not require accessory cells, but do require IGF-I and/or insulin, as well as EPO, for erythroid development. IGF-I and/or insulin are essential for full human CFU-E development and their effects are direct whereas IL-3 and GM-CSF had no effect on CFU-E growth and development.

Acknowledgments

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