Stem Cell Factor Amplifies Newborn and Sickle Erythropoiesis in Liquid Cultures

By Rona S. Weinberg, JoAnn C. Thomson, Roger Lao, Guogang Chen, and Blanche P. Alter

A two-phase liquid-culture system was used to substantially amplify and differentiate erythroblasts, starting with mononuclear cells from the blood of normal adults, newborn infants, and patients with sickle cell anemia. After the first 7 days (phase 1), in medium plus fetal bovine serum (FBS) alone, or in combination with stem cell factor (SCF) or conditioned medium (CM), the cell number was unchanged, and the cells all looked like lymphocytes. These cells were then diluted into medium with erythropoietin (Ep) alone, with Ep and either SCF or CM, or in methylcellulose with the same factors (phase 2). After 14 days in liquid phase 2 with SCF and Ep, the cell numbers increased an average of 30-fold in the sickle, 24-fold in the newborn, and 4-fold in the normal adult cultures; almost all the cells were erythroblasts and erythrocytes. SCF in phase 1 increased the number of late progenitors (CFU-E) assayed in methylcellulose, with the largest number in sickle, followed by newborn cultures and then adult cultures. We conclude that erythroid progenitor cells survive for at least 7 days without Ep (but with FBS). Progenitor cells are amplified, particularly with SCF. Later in culture, SCF with Ep increases the final number of differentiated erythroid cells. Both the early and the late effects of SCF are most effective in sickle, followed by newborn cultures and then adult cultures.

MATERIALS AND METHODS

Peripheral blood (PB) was obtained from three normal adults (all males) and three patients (2 males and 1 female) with sickle cell anemia (Hb SS); all were between 23 and 34 years of age. Umbilical cord blood was obtained after delivery from three normal newborn infants. All procedures were approved by the Institutional Review Board at Mount Sinai Medical Center. Blood samples were collected into syringes containing 30 U heparin (Liquaemin, Organon Inc., West Orange, NJ)/mL blood or into heparinized Vacutainer blood-collection tubes (Becton Dickinson, Rutherford, NJ). To prepare mononuclear cells (MNC), blood was diluted 1:1 with α medium (GIBCO Laboratories, Grand Island, NY), layered onto an equal volume of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), and centrifuged at 450g for 30 minutes at 18°C.

Phase 1 liquid cultures (phase 1L). The culture methods used were a modification of those previously reported. Cells per milliliter, 1 × 10^6, were cultured in a medium containing either 2% fetal bovine serum (FBS; Armour Pharmaceutical Intergen Co., Purchase, NY) (sickle cultures only, too few cells were obtained from normal adult and newborn samples for this variable to be included), or 10% FBS, and one of the following: 5% phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM; Terry Fox Laboratories, Vancouver, Canada), 10% U5637-conditioned medium (CM; preparation described below), or stem cell factor (100 ng/mL; courtesy of Dr. K. Zsebo, Amgen Biologics, Thousand Oaks, CA). All cultures contained 0.1 U penicillin and 0.1 μg streptomycin/mL (GIBCO Laboratories). Tissue culture flasks (Falcon number 3013; Becton Dickinson, Lincoln Park, NJ) were inoculated with 10 mL of the mixture of marrow and blood.

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of cell suspension in medium. Phase 1L cultures were incubated for 7 days at 37°C, 4% CO₂, high humidity, and either room air (Napco Incubator; Heinecke Co, Portland, OR) or 5% oxygen (here ability to gas balanced by nitrogen; Hereaus Inc, South Plainfield, NJ). On day 7, cells were recovered by centrifugation at 450g for 10 minutes at 18°C, and replaced in fresh medium in either liquid or methycellulose phase 2L (2L or 2L) cultures.

**Phase 2 liquid cultures (phase 2L).** Each milliliter of culture contained: 1 X 10⁵ adult or 0.5 X 10⁵ newborn cells recovered from phase 1L cultures, 30% FBS, 1% bovine serum albumin (BSA) (Cohn fraction IV; Sigma Chemical Co, St Louis, MO), 10⁻⁴ mol/L 2-mercaptoethanol (Sigma), 0.1 U penicillin and 0.1 µg streptomycin/mL (GIBCO Laboratories), and 2 U Ep/mL (kindly provided by Amgen Biologics). Some phase 2L cultures also contained either 10% PHA-LCM, 10% CM, or 100 ng SCF/mL. Cultures (1.2 mL) were incubated in Falcon-6-well culture dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at 37°C, 4% CO₂, high humidity, and either 20% or 5% oxygen. On day 14 and 21 (from the first day of phase 1L cultures) cells were counted using a Coulter ZBI counter (Hialeah, FL). Cytocentrifuge slides were prepared from each culture. Slides were stained with benzidine–Wright-Giemsa and differential counts were determined. Percent erythroblasts (number of erythroblasts/total number of nucleated cells) X 100. Percent red blood cells (RBCs) = (number of RBCs/number of nucleated cells) X 100.

**Phase 2 (phase 2M) and day 0 methycellulose cultures.** Methycellulose cultures were performed as described previously. Each milliliter of culture contained 1 X 10⁵ adult or 0.5 X 10⁵ newborn cells. In phase 2M cultures the cells were those recovered from phase 1L cultures. In day 0 cultures, the cells were mononuclear cells. The constituents of the media were the same as liquid cultures except for the addition of 0.8% methycellulose (Fisher Scientific Co, Pittsburgh, PA). Some Phase 2M cultures also contained either 10% PHA-LCM, 10% CM, or 100 ng SCF/mL. Cultures (0.3 mL) were incubated in Nunc 4-well culture dishes (Intermed, Roskilde, Denmark) at 37°C, 4% CO₂, high humidity, and either 20% (room air) or 5% oxygen. On days 14 and 21 (from the first day of phase 1L cultures), colonies were counted using a Bausch and Lomb Stereozoom dissecting microscope (Bausch & Lomb, Rochester, NY). Colonies that resembled CFU-E–derived colonies are referred to as CFU-E and contained 8 to 64 cells; similarly, those resembling BFU-E–derived colonies (BFU-E) contained as many as 1,000 cells and had at least three subcolonies. We also counted colonies that were larger than BFU-E, but lacked subcolonies, which we called “large colonies.”

**U5637 CM.** A modification of previously reported methods was used to prepare CM. Bladder-cell carcinoma cells from cell line U5637 (American Type Culture Collection, Rockville, MD), 5 X 10⁶, were cultured in 15 mL of α medium with 10% FBS, in 75-cm² culture flasks (Falcon). Cultures were maintained at 37°C, 4% CO₂, high humidity, and either room air or 5% oxygen, for approximately 14 days until cells became confluent. Supernatant medium was removed, centrifuged at 450g for 10 minutes, and filtered through a 0.2 µm Millipore filter (Millipore, Bedford, MA). The CM were stored at 4°C until used in cultures. CM prepared in room air and 5% oxygen had similar growth-promoting activities, and data presented here were obtained using CM prepared in 5% oxygen.

**Statistical analysis.** Data from replicate experiments were compared by one-tailed Student’s paired t-tests using log-transformed data.

**RESULTS**

**Phase 1 liquid cultures (phase 1L).** In cultures of normal adult, newborn, and sickle mononuclear cells, the number of cells in each culture on day 7 (the day cells were transferred to phase 2 cultures) was similar to that on day 0. The cells looked like lymphocytes, and there were no erythroblasts or mature RBCs in any cultures (Fig 1, A through C). There were no apparent differences between culture conditions or between cultures from normal adults, newborn infants, and sickle adults.

**Phase 2 liquid cultures (phase 2L).** Figure 2A shows the growth of normal adult cells in a representative phase 2L culture on day 21 from initiation of cultures. The number of cells in phase 2L cultures increased approximately twofold by day 21 when phase 1L cultures contained either CM or SCF and phase 2L contained only Ep or Ep and CM. When SCF was in phase 2L (independent of the factor in phase 1L), the increases were up to eightfold. In this experiment the CM was from the bladder-cell carcinoma cell line U5637. Similar results were obtained in two other normal cultures, one with PHA-LCM and the other with CM. Data from the three experiments are summarized in Table 1. SCF in phase 2L significantly increased the number of cells on day 21 when compared with Ep alone or Ep and CM (Table 1). We found no reproducible differences between cultures incubated in room air or 5% oxygen for either phase. In the experiment shown in Fig 2 the number of cells recovered was highest when cells were cultured in 5% oxygen in both phases.

Erythroid differentiation of erythroid progenitor cells was assessed by determination of the percent erythroblasts and the percent mature (enucleated) RBCs. Examples of these erythroblasts are shown in Fig 1, D through F. Data from a representative normal culture are shown in Fig 2B. On day 21, erythroblasts ranged from 18% to 59%, and RBCs from 2% to 8% when phase 1L was with any growth factors and phase 2L was with any factors except SCF. When SCF was in phase 2L, erythroid differentiation was greater, with 58% to 86% erythroblasts and up to 13% RBCs.

Figures 3A and 4A show the growth of sickle erythroid progenitor cells in two different phase 2L cultures on day 21. Data from three sickle experiments are summarized in Table 1. The number of cells in sickle phase 2L cultures increased 2- to 15-fold by day 21 when phase 1L cultures contained either PHA-LCM or SCF and phase 2L contained either Ep alone or Ep and PHA-LCM. When SCF was in phase 2L (independent of the factor in phase 1L), the increases were 5- to 67-fold. SCF in phase 2L significantly increased the number of cells on day 21 when compared with Ep alone or Ep and CM. Thus, the amplification of erythroid cells was substantially greater in sickle than in normal phase 2L cultures. Results were similar when U5637 CM was used as a source of growth factors (data not shown). In the experiment in Fig 3 the best growth was with air in both phases, while in Fig 4 the best growth was with 5% oxygen in both phases.

Erythroid differentiation of sickle progenitor cells is shown in Figs 3B and 4B. On day 21, erythroblasts ranged from 63% to 94% and there were up to 15% RBCs, regardless of which growth factors were present in phases 1L or 2L. In contrast with normal cultures, erythroid differentiation in sickle phase 2L cultures was similar with and without SCF in phase 2L. It should be noted that in sickle phase 2L cultures, even cells maintained under suboptimal conditions of...
Fig 1. Morphology of erythroid differentiation in liquid cultures. All slides were stained with benzidine Wright-Giemsa, original magnification × 1,000. (A) Day 0 mononuclear cells before culture. (B) Cells recovered from phase 1L culture on day 7. (C) A dividing cell recovered from phase 1L culture on day 7. (D) Developing erythroblasts recovered from phase 2L culture on day 14. (E) Mature erythroblasts and RBCs recovered from phase 2L culture on day 21. (F) A “colony” of mature erythroblasts recovered from phase 2L culture on day 21.

2% FBS and no added growth factors in phase 1L were increased 3- to 36-fold (Table 1), and produced up to 93% erythroblasts and 8% RBCs in phase 2L cultures, (Fig 4, A and B).

Figures 5A and 6A show the growth of normal newborn erythroid progenitor cells in representative phase 2L cultures on day 21. Data from three newborn cultures are summarized in Table 1. The number of cells in phase 2L cultures increased 3- to 10-fold by day 21 when phase 1L cultures contained either CM or SCF and phase 2L contained either Ep alone or Ep and CM. When SCF was in phase 2L (independent of the factor in phase 1L), the increases were 7- to 48-fold. SCF
in phase 2L significantly increased the number of cells on day 21 compared with Ep alone or Ep and CM (Table 1). The amplification of erythroid cells in newborn cultures was intermediate between that in normal adult and sickle cultures. In these experiments, the best growth was with 5% oxygen in phase 1 and air in phase 2.

Erythroid differentiation of newborn cells is shown in Figs 5B and 6B. On day 21, erythroblasts ranged from 47% to 90% and there were up to 19% RBCs, regardless of which growth factors were present in phases 1L or 2L. In newborn, but not sickle or normal adult cultures, 10% to 30% of the erythroblasts were multinucleated. As in sickle cultures, erythroid differentiation in newborn cultures was similar with and without SCF in phase 2L.

Phase 2 methylcellulose cultures (phase 2M). Day 21 colonies in a phase 2M sickle culture are shown in Fig 7. CFU-E-like colonies were observed in phase 2M cultures by day 14 (ie, after 7 days in phase 2M with Ep) (Fig 7A). These colonies continued to increase in number until day 21. By day 21, Phase 2M cultures also contained BFU-E-like colonies (Fig 7B). In addition, some cultures contained large erythroid colonies that were always larger than CFU-E (limited to 64 cells) and were often larger than BFU-E (approximately 1,000 cells), but lacked the subcolony structure typical of BFU-E (Fig 7C). Colonies with similar morphology were also present in normal adult and newborn cultures.

Figure 2C shows the numbers of each type of colony in a representative normal phase 2M culture on day 21. Phase 2M cultures seeded with cells maintained in phase 1L with SCF developed up to twice as many colonies as those seeded with cells maintained in CM in phase 1L. U5637 CM was used in this experiment, but similar results were observed with PHA-LCM (data not shown). SCF in phase 2M increased the proportions of both large and BFU-E-derived colonies, regardless of the factor in phase 1L.

Table 1. Fold Increase in Cell Number on Day 21

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* Ep was in all phase 2L cultures, therefore in phase 2L CM = CM + Ep, SCF = SCF + Ep.
† P values compare phase 2L culture conditions.

Fig 2. A representative normal culture on day 21. (A) Cells recovered from phase 2L. (B) Percent erythroblasts and RBCs in phase 2L. (C) Colonies/10^6 MNC in phase 2L. (D) Colonies/10^6 cells in methylcellulose cultures seeded with mononuclear cells on day 0. All data shown are from cultures incubated in 5% oxygen in phases 1 and 2. The x axis shows additives in phase 2. Ep, Ep alone; CM, U5637 CM; cultures with CM and SCF also had Ep.
Fig 3. A representative sickle culture on day 21. (A) Cells recovered from phase 2L. (B) Percent erythroblasts and RBCs in phase 2L. (C) Colonies in phase 2M; no large colonies were observed in this experiment. (D) Colonies/10^6 cells in methylcellulose cultures seeded with mononuclear cells on day 0. All data shown are from cultures incubated in air in phases 1 and 2. The x axis shows additives in phase 2. *, Not determined; Ep, Ep alone; CM, PHA-LCM; cultures with CM, SCF, and FBS all had Ep.

Figures 3C and 4C show two sickle phase 2M cultures, one without large colonies (Fig 3C), and the other with large colonies (Fig 4C). Phase 2M sickle cultures had up to 30 times as many colonies as normal adult cultures. In phase 2M cultures of cells maintained in PHA-LCM in phase 1L, there were up to 273 CFU-E, 84 BFU-E, and 110 large colonies (per 10^6 cells) on day 21. Similar results were obtained with U5637 CM (data not shown). Incubation in SCF in...
Fig 5. A representative newborn culture on day 21. (A) Cells recovered from phase 2L. (B) Percent erythroblasts and RBCs in phase 2L. (C) Colonies in phase 2M; only a few large colonies were observed in this experiment. (D) Colonies/10⁶ cells in methylcellulose cultures seeded with mononuclear cells on day 0. All data shown are from cultures incubated in 5% oxygen in phase 1 and 20% oxygen in phase 2. The x axis shows additives in phase 2. Ep, Ep alone; CM, U5637 CM; cultures with CM and SCF also had Ep.

Fig 6. A representative newborn culture on day 21. (A) Cells recovered from phase 2L. (B) Percent erythroblasts and RBCs in phase 2L. (C) Colonies in phase 2M; large colonies were observed in this experiment. (D) Colonies/10⁶ cells in methylcellulose cultures seeded with mononuclear cells on day 0. All data shown are from cultures incubated in 5% oxygen in phase 1 and 20% oxygen in phase 2. The x axis shows additives in phase 2. Ep, Ep alone; CM, U5637 CM; cultures with CM and SCF also had Ep.

Phase 1L resulted in as much as an eightfold increase in colonies with up to 1,020 CFU-E, 467 BFU-E, and 54 large colonies in phase 2M. Cells from sickle cell patients maintained in 2% FBS and no other growth factors in phase 1L also had significant numbers of colonies with up to 191 CFU-E, 81 BFU-E, and 110 large colonies. As in normal cultures, SCF in phase 2M increased the growth of large and BFU-E-like colonies.
Fig 7. Morphology of erythroid progenitor-cell–derived colonies from a representative sickle phase 2M culture. (A) CFU-E, original magnification × 80. (B) BFU-E, original magnification × 80. (C) Large colony, original magnification × 80.

Figures 5C and 6C show two newborn phase 2M cultures, one with very few large colonies (Fig 5C) and the other with many large colonies (Fig 6C). As in sickle, newborn phase 2M had up to 20 times as many colonies as normal adult cultures. In phase 2M cultures of cells maintained in CM in Phase 1L, there were up to 44 CFU-E, 49 BFU-E, and 15 large colonies on day 21. Incubation in SCF in phase 1L resulted in as much as a 14-fold increase with up to 429 CFU-E, 114 BFU-E, and 81 large colonies in Phase 2M. As in normal adult and sickle cultures, SCF in phase 2M increased the growth of large and BFU-E–like colonies.

Thus, SCF amplified the number of CFU-E when in phase 1L, and both the number and size of progenitor-cell–derived colonies in Phase 2M cultures; this amplification was more dramatic in sickle than normal cultures.

Day 0 methylcellulose cultures. Mononuclear cells were cultured on day 0 with either Ep alone or Ep and either PHA-LCM, CM, or SCF. Data from normal adult, sickle, and normal newborn cultures are shown in Figs 2D, 3, and 4D, and 5 and 6D, respectively. The numbers of colonies/10^5 cells in all cultures were similar to those previously reported by our laboratory. Sickle and newborn cultures were shown in Figs 2D, 3, and 4D, and 5 and 6D, respectively. The numbers of colonies/10^5 cells in all cultures were similar to those previously reported by our laboratory. Sickle and newborn colonies were more numerous than normal adult. SCF increased the number of colonies in 3 of 3 normal adult, 0 of 3 sickle, and 1 of 3 newborn experiments.

DISCUSSION

Normal adult, newborn, and sickle peripheral blood–derived erythroid progenitor cells can be maintained in liquid culture for at least 7 days without added Ep. Addition of growth factors such as SCF or those found in CM may not be necessary, because cells maintained in α medium with only 2% FBS (previously presumed to be suboptimal conditions) grew and differentiated in a manner similar to those incubated for the first week with growth factors. These results suggest that cells in the peripheral circulation with erythroid potential are very hardy; they either survive unchanged, or perhaps selectively multiply, compared with other cells derived from the blood that die in culture.

In methylcellulose cultures cells are fixed in space, dividing cells form colonies, and cellular interactions may be limited and localized. In contrast, in liquid cultures cells are free floating, dividing cells move apart, and cellular interactions are unencumbered. These differences enabled us to observe different phenomena in phase 2 methylcellulose and liquid cultures, with each providing unique information about the mode of action by which SCF amplifies erythropoiesis.

Culturing day 7 cells in semisolid media with Ep provides an analysis of the numbers of progenitor cells that have developed. Several morphologically distinct erythroid colonies arise from these progenitor cells. Colonies having CFU-E and
BFU-E morphology were observed, as well as many large unilocular colonies. These large colonies (containing 1,000 to 5,000 cells) were far larger than CFU-E (limited to 64 cells), and were often larger than BFU-E (approximately 1,000 cells) in the same plates, but lacked the subcolony structure of typical BFU-E. The presence of several colony types suggests that PB contains progenitor cells with a range of erythroid maturity, each capable of a different degree of amplification.

The number of CFU-E in phase 2 methylcellulose cultures was increased when SCF was present in phase 1. The increase was small in normal cultures, larger in normal newborn, and largest in sickle. Thus, there was amplification of late erythroid progenitors by SCF. Amplification of erythroid colonies from newborn infants and patients with SCD was dramatic in phase 2 liquid cultures. When cultures contained Ep alone or with Ep and CM, the number of cells increased up to 10- and 15-fold, respectively. SCF combined with Ep substantially increased the number of cells up to 48- and 67-fold. Replication of normal cells in phase 2 liquid cultures was modest by comparison with cells from sickle cell patients or normal newborn infants. SCF in normal phase 2 liquid cultures resulted in an eightfold increase in the number of colonies compared with an approximately twofold increase with Ep or Ep and CM. With or without SCF in phase 2 the majority of cells became erythroblasts and mature RBCs.

The effects of SCF were different in each phase. SCF in phase 1 increased the number of colonies in phase 2M, implying that SCF early in culture increased the number of progenitor cells. SCF in phase 2M did not further increase the number of CFU-E-derived colonies but did increase the proportion of BFU-E and large colonies. This suggests that SCF can also act by shortening the time required for cells to divide, thereby increasing the number of divisions and the size of each colony.

Phase 2L cultures of cells maintained in SCF in phase 1 must also have had increased numbers of progenitor cells, but this difference was not apparent by the end of the phase 2L culture period. Perhaps in phase 2L other factors stimulated exuberant growth (such as increased cytokine production by accessory cells), and thus superseded differences resulting from SCF in phase 1. SCF in phase 2L dramatically increased the number of cells at the end of the culture period, clearly showing that SCF can act late in culture.

Even with increased colony size in phase 2M, amplification and differentiation was far greater in liquid than methylcellulose. Perhaps the time required for cells to divide is shorter in liquid than semisolid medium, resulting in more cells. In liquid cultures cells matured to RBCs while in methylcellulose cultures only erythroblasts were present.

The present studies support previous observations of increased numbers of circulating erythroid progenitor cells in cultures from newborn infants and sickle cell patients. In both phase 2 methylcellulose and liquid there was significantly more growth in sickle and newborn than normal adult cultures. SCF amplified the growth of sickle and newborn erythroid progenitor cells to a greater extent than normal. Whether these differences are caused by differences in progenitor or accessory cells or both remains to be determined.

Low oxygen has been shown to improve the growth of human erythroid progenitor cells in vitro. Other investigators incubated cultures at 5% to 6% oxygen in phase 2, but we found no consistent effect of oxygen concentration in either culture phase.

We conclude that sickle, normal adult, and newborn erythroid progenitor cells can be maintained in liquid cultures for at least 1 week without added Ep. Culture for an additional 2 weeks with Ep allows cells to multiply and differentiate to erythroblasts and RBCs. SCF increases the total number of mature erythroid cells in liquid cultures. It appears that SCF is able to influence erythroid growth and differentiation both early and late during the maturation process and to magnify differences between sickle and newborn compared with adult cultures.

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Stem cell factor amplifies newborn and sickle erythropoiesis in liquid cultures

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