Evaluation of Erythropoiesis in Long-Term Hamster Bone Marrow Suspension Cultures: Absence of a Requirement for Adherent Monolayer Cells

By Christine E. Eastment and Francis W. Ruscetti

In long-term hamster bone marrow cultures, proliferation and differentiation of hematopoietic stem cells occurs for several months without need for hydrocortisone or adherent stromal elements, which are requirements for bone marrow growth in all other species studied. Only the most primitive erythroid progenitors (BFU-E) are produced in the cultures. Following treatment of the cells with erythropoietin, these progenitor cells undergo differentiation into mature hemoglobinized red blood cells. Concomitant addition of erythropoietin (Epo) and prostaglandin-E (PGE), results in the production of large numbers of maturing red blood cells. In cultures stimulated with Epo and PGE, as many as 70% of the cells are benzidine-positive, while Epo alone stimulated as many as 45% of the cells to become erythroid. Epo and PGE, do not have any apparent deleterious effect on the continuous hemopoiesis occurring in these cultures. Under identical conditions, syngeneic adherent cell cultures do not produce any erythroid elements. The development of mature red blood cells from primitive erythroid precursors occurs in the presence of Epo alone and without any apparent need for adherent stromal elements. These cultures provide a useful in vitro model for dissecting the positive and negative signals that regulate erythropoiesis.

Adult bone marrow is a cell renewal system in which all the mature functional hematopoietic cells are derived from multipotent hematopoietic stem cells. These stem cells have the distinguishing characteristic of extensive self-renewal and production of multiple lines of differentiation. An understanding of the events involved in this proliferation and differentiation will require experiments on isolated cell populations under well defined conditions.

Such conditions can be most easily achieved in vitro. Recent developments have allowed the maintenance of hematopoietic stem cell proliferation and differentiation in vitro for several months in the presence of an appropriate adherent stromal environment. The addition of hydrocortisone increases the efficiency of developing these cultures in murine and human systems. In these long-term murine cultures, production of an adherent layer of cells consisting of endothelial cells, fibroblasts, fat cells, and macrophages is an absolute requirement for hemopoiesis. While continuous production of mature granulocytes and macrophages occurs in these cultures, erythroid development is blocked at a primitive stage. More recent work using murine long-term cultures demonstrated that mature erythroid development can be achieved in the presence of erythropoietin (Epo) and a second factor present in mouse serum and adherent stromal cells.

Recently, one of us (C.E.) has developed a system using hamster bone marrow in which extensive proliferation and differentiation of hematopoietic stem cells occurs for several months in the absence of identifiable stromal elements and hydrocortisone. The development of an adherent stromal layer appears essential for the initiation of stem cell replication in vitro but not the long-term maintenance. As in the murine system, there is continuous production of macrophages and granulocytic cells as well as megakaryocytes, but no erythroid development is seen after the initial 3 wk of culture. The ability to grow hematopoietic stem cells in suspension culture provides an excellent model for analysis of the role of various accessory cells and humoral factors in the generation and maturation of primitive erythroid progenitors.

In this article, it is shown that erythropoiesis can be initiated and maintained for several months in long-term suspension cultures of hamster bone marrow cells devoid of any recognizable stromal elements. The addition of Epo is the only requirement for the initiation of erythropoiesis. Erythropoiesis is enhanced in this system by the addition of prostaglandin-E. Interestingly, the addition of Epo to hamster bone marrow stem cells growing in the presence of a marrow adherent layer does not produce erythroid differentiation. This system can be useful for evaluation of extrinsic humoral factors that regulate the ability of stem cells to differentiate along different cellular pathways.

Materials and Methods

Long-Term Bone Marrow Cultures

Long-term bone marrow cultures were established according to the method of Dexter et al. with modifications. Bone marrow was obtained from the femur and humerus of LVG hamsters (Charles

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River) by flushing and scraping the marrow cavity with cold unsupplemented RPMI. No attempt was made to achieve a homogeneous cell suspension. Bone marrow cells (10^7) were placed in 10 ml of RPMI 1640 + 20% horse serum (HS) (Flow, Detroit, Mich.) with antibiotics. Cultures were placed at 37°C and 5% CO₂ in a humidified atmosphere. Cultures were fed every 3-4 days by removal of half the growth medium and addition of an equal volume of fresh medium.

Establishment of Suspension Cultures

Hamster bone marrow suspension cultures were established as previously described. Suspension cells were removed from continuous bone marrow adherent cultures at 1.5-3 wk, at which time the adherent layer was fully developed and clumps of “blast” cells were present. The cells were innoculated into fresh 25 or 75 cm² flasks at 10^6 cells/ml by complete removal of conditioned medium and addition of fresh RPMI + 20% HS. Thereafter, cells were subcultured in fresh flasks once or twice weekly in the same manner as the parent adherent culture. Once established, suspension cultures could be subcultured weekly. Some cultures have been maintained for more than 6 mo in this manner.

Chemicals

The stock solution of prostaglandins (Sigma, St. Louis, Mo.) used were prepared by dissolving them directly in RPMI-1640 at 10⁻³M. The solution was sterilized by Millipore filtration (0.45 μ) and aliquots were stored at -80°C until use. Epo (Step III) was purchased from Connaught Labs. The hormone was prepared by dissolving the lyophilized material in RPMI-1640 at a concentration of 25 U/ml. The solution was sterilized by Millipore filtration and stored in 1-ml aliquots at -80°C until use.

Cytochemistry

Slides were prepared by cytocentrifuge (Shandon Southern). Differential morphology was analyzed on Wright stained preparations. Benzidine-positive cells were determined as described by Meytes et al. with methyl green as a counterstain. Nonspecific esterase, acetylcholinesterase, and oil red-O were performed as previously described. Immunofluorescent staining for spectrin (kindly performed by Dr. Sandra Ruscetti) was done as previously described. 

BFU-E Assay

Erythroid progenitors were assayed in agar as follows. Suspension cells were placed in 2x RPMI plus 40% horse serum with antibiotics. A quantity of 10⁴-2x 10⁶ cells were added to each 35-mm Petri dish containing various concentrations of Epo and, in some cases, PGE₂. Melted agar was added to a final concentration of 0.3%.

Colonies were evaluated on day 7 by fixation of the entire agar disk according to the method of Salmon and Buick. Benzidine-positive colonies were determined as previously described.

CFU-C Assay

Myeloid progenitors were assayed in agar as follows. Suspension cells at 10⁶ cells/ml were placed in 2x RPMI medium containing 40% horse serum, 40% spleen conditioned media, and antibodies. This mixture is added to a 35-mm Petri dish with an equal volume of melted agar (0.6%). Colonies were counted on day 7. Spleen conditioned medium was prepared by incubating 2 x 10⁶ fresh hamster spleen cells/ml in RPMI-1640 media containing 5% human

### Table 1. Comparison of Erythrocytes in Adherent and Suspension Cultures of Hamster BM

<table>
<thead>
<tr>
<th>Weeks in Culture</th>
<th>Cells/ml* x 10⁶</th>
<th>Differential (%)*</th>
<th>BFU-E*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blast</td>
<td>Myeloid</td>
</tr>
<tr>
<td>Adherent cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.5 ± 0.3</td>
<td>10 ± 3</td>
<td>60 ± 8</td>
</tr>
<tr>
<td>4</td>
<td>2.4 ± 0.3</td>
<td>2 ± 1</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>Suspension cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2.3 ± 0.3</td>
<td>12 ± 4</td>
<td>28 ± 10</td>
</tr>
<tr>
<td>12</td>
<td>7.2 ± 1.7</td>
<td>16 ± 6</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>16</td>
<td>3.8 ± 1.0</td>
<td>24 ± 3</td>
<td>75 ± 6</td>
</tr>
</tbody>
</table>

*Means ± SE of 3 experiments. Cell counts represent total present 7 days after seeding at 2 x 10⁶ cells/mL. Differentials were performed on 200 cells stained with Wright-Giemsa. BFU-E were determined using 5 x 10⁶ cells/plate as described in Materials and Methods.
ERYTHROPOIESIS MINUS ADHERENT CELLS

plasmas and 0.05 ml of pokeweed mitogen/ml of medium for 7 days.

RESULTS

In both adherent and suspension cultures of hamster bone marrow, mature erythroid elements are absent after 3–4 wk of culture (Table 1). This occurs in spite of the observation that stem cell replication as well as mature myeloid-macrophage and megakaryocyte production continue for several months8 (Table 1). The erythroid progenitors in these cultures appear to be blocked at a primitive stage of erythropoiesis, since BFU-E can be generated in agar from the suspension cells after several months in culture (Table 1, Fig. 1). The number of BFU-E (6–12/5 x 10⁴ cells) produced did not vary greatly when the suspension cultures were established from 2–3-wk-old adherent cultures, but no BFU-E could be generated from the adherent cultures after the disappearance of “blast” cells. Similar numbers of BFU-E were produced from 2–4-mo-old suspension cultures (Table 1). However, considerable variation in the number of erythroid progenitors was observed in cultures derived from different hamsters. As many as 35 BFU-E per 5 x 10⁴ cells were observed. Untreated suspension culture cells were negative for both benzidine and spectrin from week 4 to week 24.

The number of erythroid bursts generated in agar was proportional to the concentration of Epo added, reaching a peak at 2.0 U Epo/plate (Fig. 2). Burst formation was also directly dependent on cell concentration over the range of 2 x 10⁴ to 2 x 10⁵ cells/plate. However, at concentrations greater than 10⁵ cells/plate, the increase in BFU-E number was not linear. The bursts produced from cultured marrow cells (Fig. 1) were very similar to those generated from fresh marrow, in that they were composed of grape-like clusters of hemoglobinized cells. When these agar plates were fixed and stained using the modified Papanicolaou stain as described in Materials and Methods, the majority of the cells in these bursts resembled late stage normoblasts (Fig. 3). An occasional burst was also found to contain morphologically identifiable megakaryocytes.

Erythropoietin-Dependent Erythropoiesis in Long-Term Suspension Cultures

Addition of Epo to hamster bone marrow suspension cultures that have been proliferating in the absence of an adherent layer for up to 5 mo results in the generation of benzidine-positive cells within 4–5 days and the production of burst-like clumps of cells possessing amounts of hemoglobin visible using phase

Fig. 2. Burst formation in agar is proportional to the concentration of erythropoietin added. Optimal numbers of erythroid bursts were obtained at 1.5–2.0 U of erythropoietin per 35-mm Petri dish. Cells used at a concentration of 5 x 10⁴ cells/plate were from 8-wk-old suspension cultures. Colonies were scored on day 7.
Within 7 days after the addition of Epo alone, cell aggregates containing from 8–10 up to several hundred nucleated erythrocytes are found floating in the culture medium (Fig. 4). Examination of these clumps on Wright stained cytospin preparations reveals both early and late stage nucleated erythroblasts as well as occasional nonnucleated red cells. In general, the size of these aggregates reflects their degree of erythroid differentiation, with the larger clumps containing the more mature erythroid elements. Examination of these huge erythropoietic “bursts” generated in the suspension cultures for other cell types was negative. In most pure erythroid bursts, 100% of the cells were benzidine-positive and spectrin-positive.

The number of benzidine-positive cells (BPC) produced in these cultures in the presence of Epo varied but never exceeded 45% of the suspension cell population (Table 2). The dose–response of formation of BPC in response to Epo was similar to that of BFU-E in response to Epo (Fig. 2), with the optimal concentration of Epo being 1.5 U/ml and a plateau observed at higher concentrations (data not shown.) Erythropoiesis was maximal on day 7 or 8 after addition of Epo. Refeeding the culture every 3–4 days with Epo did not increase the total number of maturing red cells.

**Fig. 3.** Appearance of an erythroid burst that has been fixed and stained using the modified Papanicolau stain as described in text. The majority of the cells are normoblasts (x220).

**Fig. 4.** Erythropoiesis in long-term suspension cultures. These aggregates are found floating in situ 7 days after the addition of erythropoietin. Note their similarity to burst formation in agar as shown in Fig. 1 (x380).

**Effect of Prostaglandins on Epo-Dependent Erythropoiesis in Suspension**

Several investigators have reported that prostaglandins modulate the effects of erythropoietin on erythroid differentiation both in vivo15–16 and in vitro16–17.

Prostaglandins were added to hamster bone marrow contrast microscopy. Within 7 days after the addition of Epo alone, cell aggregates containing from 8–10 up to several hundred nucleated erythrocytes are found floating in the culture medium (Fig. 4). Examination of these clumps on Wright stained cytospin preparations reveals both early and late stage nucleated erythroblasts as well as occasional nonnucleated red cells. In general, the size of these aggregates reflects their degree of erythroid differentiation, with the larger clumps containing the more mature erythroid elements. Examination of these huge erythropoietic “bursts” generated in the suspension cultures for other cell types was negative. In most pure erythroid bursts, 100% of the cells were benzidine-positive and spectrin-positive.

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ERYTHROPOIESIS MINUS ADHERENT CELLS

Table 2. Effect of Erythropoietin on Hemopoiesis in Hamster Bone Marrow Suspension Cultures

<table>
<thead>
<tr>
<th>Culture* Additions</th>
<th>Cells/ml†</th>
<th>Blasts</th>
<th>Immature Myeloid</th>
<th>Mature Myeloid</th>
<th>Erythroid</th>
<th>Macs</th>
<th>BFU-E‡</th>
<th>CFU-C‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.8 ± 1.2</td>
<td>24</td>
<td>34</td>
<td>40</td>
<td>0</td>
<td>2</td>
<td>16 ± 3</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>Epo</td>
<td>6.2 ± 1.0</td>
<td>4</td>
<td>14</td>
<td>35</td>
<td>45</td>
<td>1</td>
<td>47 ± 4.5</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Epo (7 days) + none (7 days)</td>
<td>10.8 ± 1.8</td>
<td>23</td>
<td>18</td>
<td>50</td>
<td>6</td>
<td>3</td>
<td>18 ± 2.7</td>
<td>41 ± 8</td>
</tr>
</tbody>
</table>

*Epo at 1.5 U/ml as added to the cultures for 7 days. After 7 days, Epo was removed and cells were incubated in regular medium for 7 days.
†Cell counts represent total present 7 days after seeding at 2 x 10⁵/ml. Differentials were performed on 200 cells stained with Wright-Giemsa.
‡BFU-E and CFU-C were determined using 5 x 10⁵ cells/plate as described in Materials and Methods.

Table 3. Effect of Various Prostaglandins on In Vitro Erythropoiesis in the Presence of Erythropoietin

<table>
<thead>
<tr>
<th>Prostaglandin*</th>
<th>Percent of Benzidine Positive Cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo alone</td>
<td>21.5 ± 3.5</td>
</tr>
<tr>
<td>E₁</td>
<td>43.5 ± 5.0</td>
</tr>
<tr>
<td>E₂</td>
<td>30.0 ± 0.1</td>
</tr>
<tr>
<td>F₁α</td>
<td>18.0 ± 1.4</td>
</tr>
<tr>
<td>F₂α</td>
<td>19.0 ± 2.8</td>
</tr>
<tr>
<td>B₁</td>
<td>14.0 ± 1.4</td>
</tr>
<tr>
<td>B₂</td>
<td>16.0 ± 2.8</td>
</tr>
<tr>
<td>A₁</td>
<td>27.0 ± 1.4</td>
</tr>
<tr>
<td>A₂</td>
<td>26.5 ± 2.1</td>
</tr>
</tbody>
</table>

*All prostaglandins were added on day 0 to a final concentration of 10⁻⁶ M in the presence of 1.5 U of erythropoietin. This concentration of prostaglandin produced the largest number of benzidine-positive cells over the dose range tested (10⁻⁸ - 10⁻⁶ M). No benzidine-positive cells were present in the absence of erythropoietin.
†Means ± SE of 2 experiments.

In hamster suspension cultures, the number of mature erythroid elements increases, the absolute number of immature myeloid cells decreases (Fig. 6: Table 2). Removal of the erythroid stimulus results in the return of the number of myeloid cells to their normal level by day 7 (Table 2). The number of BFU-E and CFU-C present in the cultures also returned to the same levels. Maximal stimulation of erythropoiesis does not have toxic effects on the hemopoiesis in the culture, since similar numbers of mature erythroid cells could be generated in the same culture over a period of several months with subsequent regeneration of granulopoiesis when the erythroid stimulus was removed.

Role of the Adherent Layer in Erythropoiesis in Hamster Marrow Cultures

The addition of Epo alone or in combination with PGE₁ to hamster adherent cultures capable of producing mature granulocytes and macrophages did not result in the production of benzidine-positive cells. The addition of syngeneic suspension cultures treated with Epo and PGE₁ to these adherent cultures produced only occasional benzidine-positive cells. When examined by electron microscopy, the adherent layer was found to be composed of macrophages and fibroblasts with no obvious endothelial or lipid-containing cells present (data not shown). Since it is conceivable that in the hamster, some marrow accessory cells are nonadherent, the developing erythroid "bursts" in suspension were examined for other cell types. Most bursts were 100% benzidine- and spectrin-positive. No macrophages or fat cells, as judged by nonspecific esterase and oil red-O cytochemistry, were observed.
Fig. 5. Effect of prostaglandin-E₁ on erythropoiesis stimulated by varying Epo concentrations. Suspension cells in culture for 8 wk were cultured at 2 × 10⁸ cells/ml. PGE₁ was added at 10⁻⁵M final concentration. After 7 days the number of benzidine-positive cells was determined. Data represent means ± SE of 3 experiments.

Fig. 6. Comparison of cell differentials in treated and untreated suspension cultures. Cells used at a concentration of 2 × 10⁸ cells/ml were from 8-wk-old suspension cultures. Five-hundred cells were counted on each Wright-Giemsa-stained cyto spin preparation.
Fig. 7. Effect of cell concentration on erythropoiesis. Varying numbers of suspension cells were cultured in the presence of 10^{-6} M PGE, and 1 U Epo/ml. Cells were harvested on day 7 and the percentage of benzidine-positive cells was determined. Cells in suspension culture for 8 wk were used. Data represent means ± SE for 3 experiments.

DISCUSSION

In recent years, development of long-term bone marrow cultures has allowed proliferation of stem cells and production of committed progenitors for many months. Although mature granulocyte and macrophage cells predominate in long-term marrow cultures of murine, primates, human, and hamster committed precursors of all hematopoietic lineages are present in the murine and hamster systems. Current ideas about early hemopoietic events are largely based on results obtained with colony-forming assays. The availability of long-term in vitro culture systems should facilitate biochemical studies of "normal" erythropoietic development as well as studies of cell-cell and hormonal interactions important in regulation of erythropoiesis. The systems have allowed the description of early progenitor cells like the "macroburst." The ability of hamster marrow to proliferate and differentiate in the absence of an adherent layer as required by the other systems makes the hamster culture system a useful one for investigation of humoral control of hemopoiesis.

When Epo is added to long-term hamster liquid suspension cultures, benzidine-positive cells appear in 4–5 days and burst-like aggregates containing hundreds of hemoglobinized cells are visible in the suspension by day 7 (Fig. 5). Concomitant addition of Epo and PGE results in about a two-fold increase in the number of maturing erythroid cells. Spectrin, a specific protein marker for erythroid differentiation, is negative in untreated cultures and closely parallels the development of benzidine-positive cells. Multiple additions of PGE and Epo did not increase the amount of erythropoiesis observed.

The kinetics of erythrocyte induction were reproducible from week to week in these cultures. Morphologically recognizable benzidine-positive cells are first recognized by day 7, and peak erythropoiesis is complete by day 7 or 8. Interestingly, the kinetics of erythroid maturation in these cultures has the same time course in agar and in suspension. In addition, the development of BFU-E in this system through the various stages of erythroid differentiation and maturation requires only the addition of Epo. Whether the cells themselves in this culture system produce additional factors that are necessary for erythroid differentiation remains to be resolved. These data indicated that the addition of Epo and PGE to these cultures has no discernible deleterious effect on long-term hemopoiesis, and after removal of the erythroid differentiation stimulus, the numbers of BFU-E are maintained in the culture at the same levels observed before addition of Epo and PGE (Table 2).

Stimulation of erythropoiesis results in a consistent and reproducible depression in the number of immature myeloid cells produced at the time when the numbers of mature erythroid elements is increasing (Fig. 6). However, the addition of Epo to the marrow culture results in almost a doubling of the cell number, so that the total number of all myeloid cells combined remains unchanged. This suggests that myelopoiesis is only briefly affected by addition of Epo. When the erythroid stimulus is removed, the number of myeloid and other cell types returns to levels observed before
adding Epo and PGE. That the addition of Epo and PGE to agar did not inhibit growth of CFU-C suggested that these agents were not toxic for granulocyte progenitors. As in the case of erythroid progenitors, maximum stimulation of erythropoiesis does not affect regeneration of granulopoiesis in these cultures. Whether growth of these cultures in the presence of Epo and PGE, for long periods of time would alter hemopoiesis remains to be determined.

The ability of prostaglandins to significantly increase production of mature erythroid cells in vitro (Table 2) supports results that have been obtained in vivo. Previous results have indicated that PGE stimulates the stem cells to go into cell cycle, which could supply increased numbers of erythroid progenitors. However, prostaglandins have also been shown to stimulate production of burst-promoting activities necessary for differentiation of early erythroid progenitors. Finally, PGE, has been shown to inhibit myeloid differentiation. Obviously, much more work needs to be done to sort out which, if not all of these, are important in this system.

The most interesting and probably somewhat controversial finding is that the constant generation of erythroid progenitors and their differentiation to mature form can occur in the absence of a stromal adherent layer. This is clearly not the case in the mouse. Indeed, in the hamster system, adherent cultures developed from the same material inhibit the suspension cells from undergoing erythropoiesis. In the murine system, it is clear that monocytes and macrophages are found in close association with developing erythroid foci. In the hamster, neither macrophages, fat cells, nor fibroblasts, as judged by morphological and cytochemical criteria, are found in the developing erythroid bursts in suspension. These bursts are usually 100% benzidine- and spectrin-positive. However, it is interesting to note that even in suspension cultures, erythropoiesis occurs primarily in clumps. This is not true of granulopoiesis. Of course, it remains possible that a nonadherent stromal cell is necessary. Attempts to clone and identify such a cell are underway. These cultures provide a useful in vitro model for studying both the positive and negative regulation of erythropoiesis.

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Evaluation of erythropoiesis in long-term hamster bone marrow suspension cultures: absence of a requirement for adherent monolayer cells

CE Eastment and FW Ruscetti