Further Evidence of the In Vivo Role of Erythropoietin or Companion Molecules Induced by Hypoxia on Proliferation and Continuing Differentiation of BFU-e in PCDC

By Kenichi Harigaya, Eugene P. Cronkite, Marilyn E. Miller, and Giuseppe Moccia

Normal and plethoric bone marrow cells were grown in plasma clot diffusion chambers (PCDC) implanted into the peritoneum of normal mice or mice submitted to 7 hr of hypoxia (23,000 ft) daily, on a single day or on 2 consecutive days at different times after implantation of the PCDC’s. Daily discontinuous hypoxia (DDH) produced more 6-day bursts than other treatments. Hypoxia on days 1 and 2 after implantation was nearly as effective as DDH on day-6 bursts. Later bouts of hypoxia or a single hypoxic exposure on day 1 or 2 was less effective. Erythropoietin (Ep) levels were measured by bioassay on both diffusion chamber (DC) contents and serum. Serum Ep levels peaked at 160 mU/ml after a 7-hr hypoxic exposure while the DC content Ep levels were in the nondetectable range (less than 50 mU/ml). The data implies that either higher than normal Ep levels or a companion molecule(s) produced by hypoxia are required for 1-2 days early in the culture period to force an increasing number of BFU-d-e down the erythrocytic pathway and thus increase red cell production at times of need in vivo.

ERYTHROPOIESIS in liquid diffusion chambers was first described by Boyum et al. but erythrocytic growth in that system is minuscule. Steinberg et al. demonstrated the growth of erythrocytic and granulocytic colonies in plasma clot diffusion chambers (PCDC). The PCDC provides a more physiologic environment than do in vitro systems and offer the possibility of manipulating the host animals by various treatments. Investigators using the PCDC system have used a large cell inoculum and obtained fewer erythrocytic colonies and bursts than are found in in vitro culture systems. It is not known whether the colonies and bursts seen in PCDC originate from the same progenitors, i.e., CFU-c, CFU-e, BFU-e, or CFU-m, that produce colonies and bursts in vitro in the agar, methylcellulose, or plasma clot systems. Accordingly, we call the progenitor cells of colonies and bursts in the PCDC; CFU-d-e, BFU-d-e, CFU-d-c, and CFU-d-m until it is determined if they are the same cells.

Our PCDC system stimulated by daily discontinuous hypoxia (DDH) produces larger numbers of bursts despite a smaller cell inoculum than that used in other PCDC studies. Discontinuous hypoxia produces a high plasma Ep level that declines to nondetectable levels a few hours after mice are returned to sea level. An earlier study showed that plethoric rat bone marrow BFU-d-e expressed larger bursts when hosts were stimulated by DDH. This study was undertaken in mice to see if BFU-d-e required daily stimulation by hypoxia or whether hypoxia and its concomitant pulse of erythropoietin was required only at specific times after implantation of PCDC to induce bursts at later intervals. The results clearly indicate that daily hypoxia is not required. In fact, the results show that hypoxia is required only on days 1 and 2 and that the ambient Ep levels are then sufficient to continue stimulation of most of the BFU-d-e to form bursts, although a fraction appear to need daily hypoxia.

MATERIALS AND METHODS

Diffusion Chamber Study

Male mice of the Hale-Stoner-Brookhaven (Swiss-Webster) strain (BNL mice) aged 8-11 wk were used as donors for bone marrow cells and as recipients in all experiments. The donor cells were prepared from hypertransfused plethoric mice in most experiments. Plethora was induced by the intraperitoneal injection of 1 ml of packed homologous red cells on two consecutive days, 5 days before the preparation of donor marrow for inoculation of the PCDC. The blood was collected aseptically by cardiac puncture with heparin as the anticoagulant. The red cells were washed in saline three times, and the hematocrit of the packed red cells was adjusted to 70%. Bone marrow cells were prepared by grinding the femoral bones with mortar and pestle. Cells were freed by gentle washing in ice-cold McCoy’s 5A medium (Microbiological Associates, Bethesda, Md.) with 20% fetal calf serum (Grand Island Biological Co., Long Island, N.Y.). The cell suspension was transferred into a sterile tube. After 20 sec agitation on a Vortex vibrator and 5 min settling, the upper suspension was passed through 27-gauge needles a few times and the cell concentration determined by a Coulter electronic particle counter. The working suspension was McCoy’s 5A medium supplemented with 20% fetal calf serum, 17% citrated bovine plasma (GIBCO) and antibiotics (50 U/ml of...
Fig. 1. (A) Erythrocytic burst that is composed of relatively discrete clusters. Most of erythrocytic cells are heavily stained with benzidine. Granulocytic-macrophage aggregates are seen in right upper portion. There is a slight to moderate growth of fibrocytic cells and macrophages in the background. Day 6. (x 120). (B) Erythrocytic burst that contains uncountable numbers of clusters, but the border of burst is well-defined. Most of erythrocytic cells are heavily stained with benzidine. Granulocytic-macrophage aggregates are seen in left upper portion. Day 10. (x 120). (C) Huge erythrocytic burst that is composed mainly of large clusters in the upper middle of the picture and many small erythrocytic clusters are scattered around them. The border is ill-defined and overlapped granulocytic-macrophage aggregates in right lower portion. There is a moderate growth of fibrocytic cells and macrophages in the background. Day 10. (x 120).
penicillin + 50 mcg/ml of streptomycin). Dilutions were subsequently made so that 120 μl of the working suspension would contain 20,000 cells for inoculation into PCDC.

PCDC were constructed by heat-sealing a 0.22 μm millipore membrane (Millipore Corp., Bedford, Mass.) on one side of a lucite ring and a 0.2 μm nucleopore membrane (Nucleopore Corp., Pleasanton, Calif.) on the other side. They were sterilized overnight in a 70°C oven. PCDC were loaded with 120 μl of the suspension. A nylon plug was dipped in sterile high vacuum silicone grease (Dow Corning Corp., Midland, Mich.) and was inserted firmly into the hole of the PCDC ring. The PCDC's were then placed in medium at 37°C to clot. Clot formation occurred in less than 15 min and was tested by rotating the PCDC. The PCDC's were placed in medium on ice until implanted into host animals. Four PCDC's were processed to check for uniformity of cell distribution in the clot and absence of cell clumps. After incubation, the plasma clots were removed, fixed in 10% buffered-formalin and stained with benzidine and hematoxylin.

Each mouse was implanted with 2 PCDC’s in the peritoneal cavity. Eight PCDC’s were harvested for each point in all experiments except experiment 2.

Intermittent hypoxia was induced by a simulated altitude of 23,000 ft in a low pressure chamber for 7 hr/day, after which the hosts were kept at ambient atmospheric pressure (sea level).

**Colony Counts**

The CFU-d-e is defined as the cell that produces a single colony of eight or more benzidine-positive cells and fewer than 65 erythrocytic cells. The BFU-d-e is defined as the benzidine-positive cell that produces a single colony of greater than 65 cells or consists of 2 or more clusters of erythrocytic cells (Fig. 1). Granulocytic and/or macrophage cell aggregates containing 4 to 19 cells were scored as clusters and those larger than 19 cells as colonies.

**Assays of Erythropoietin**

The sequential changes in erythropoietin concentration in the serum of BNL mice and diffusion chamber (DC) contents after a single exposure to 7 hr hypoxia were measured by the bioassay method. DC contents were aseptically harvested with Pasteur pipette and then pooled for each time point. Transfused CF1 virgin female mice, 12–16 wk old and weighing 25–30 g (Charles River Breeding Laboratories, Wilmington, Mass.) were used for the assay. The animals were killed 17 hr after termination of hypoxia and the plasma erythropoietin level of each mouse receiving daily discontinuous hypoxia was assayed by the Ep radioimmunoassay technique. Details of both Ep assays have been described previously.9

**RESULTS**

The first experiment was designed to determine the formation of erythrocytic colonies and bursts in PCDC inoculated with normal bone marrow cells or hypertransfused plethoric bone marrow (HPBM) cells for 11 days (Fig. 2). The hosts received daily discontinuous hypoxia. Normal bone marrow CFU-d-e produced 10 colonies on day 3 or one CFU-d-e derived colony per 2000 marrow cells inoculated. Thereafter the colonies declined in number to near zero following the 6th day. The HPBM cells produced very few colonies showing that CFU-d-e were strikingly decreased by plethora. The colonies consisted primarily of late poly-and orthochromatophilic normoblasts. Bursts commenced appearance on the 3rd day from BFU-d-e of normal and HPBM cells. The plateau in burst formation appeared from day 6 to day 9 for the BFU-d-e of normal bone marrow or HPBM. The burst number decreased after the 9th day but the

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**Table 1. Erythrocytic Burst Formation in PCDC with Hypoxic Exposure on a Single Day Compared to Daily and Days 1 Plus 2**

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>23,000 ft: 7 hr/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td>1–8</td>
<td>1 + 2</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>1–8</td>
<td>4.0 ± 1.8*</td>
<td>4.0 ± 1.6</td>
<td>40.3 ± 10.3</td>
<td>37.0 ± 9.4</td>
<td>31.5 ± 10.9</td>
<td></td>
</tr>
<tr>
<td>1 + 2</td>
<td>0.3 ± 0.3</td>
<td>7.0 ± 5.7</td>
<td>25.8 ± 14.4</td>
<td>8.8 ± 5.6</td>
<td>8.5 ± 6.9</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>2.3 ± 1.9</td>
<td>2.8 ± 1.1</td>
<td>5.3 ± 3.2</td>
<td>20.0 ± 9.7</td>
<td>6.3 ± 5.8</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>0.3 ± 0.3</td>
<td>2.0 ± 2.0</td>
<td>4.0 ± 3.0</td>
<td>0.8 ± 0.5</td>
<td>3.3 ± 2.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>3</td>
<td>1.8 ± 0.8</td>
<td>1.0 ± 0.6</td>
<td>3.5 ± 2.8</td>
<td>2.3 ± 1.4</td>
<td>6.8 ± 4.5</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>0.5 ± 0.5</td>
<td>9.8 ± 5.6</td>
<td>0.3 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>0.8 ± 0.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>2.5 ± 1.7</td>
<td>5.5 ± 3.7</td>
<td>15.3 ± 11.6</td>
<td>2.3 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.0 ± 0.7</td>
<td>0.3 ± 0.3</td>
<td>0.0 ± 0.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>None</td>
<td>0.0 ± 0.0</td>
<td>2.3 ± 1.3</td>
<td>0.7 ± 0.7</td>
<td>1.8 ± 1.8</td>
<td>1.3 ± 0.9</td>
<td>0.0 ± 0.0</td>
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</table>

*Mean ± SEM, n = 4.
size of bursts increased, some becoming enormous by 9 days of culture.

Subsequent experiments were designed to determine if DDH was required for the maximal expression of BFU-d-e or whether hypoxia and its concomitant pulse of Ep and/or other factors produced by hypoxia were required only at specific times during the culture period. The control groups received either DDH or were maintained in room air throughout the culture period. DDH produced peak burst formation on day 6 (Tables 1 and 2). The number of bursts on day 6 (Table 1) was greater when the mice were subjected to DDH than when hypoxia was administered only on days 1 and 2. This difference is not highly significant (p > 0.10). There was no difference in burst formation on day 6 whether there was DDH or hypoxia only on days 1 + 2 (Table 2). Hypoxic exposure on a single day or two successive days other than days 1 + 2 produced fewer bursts. However, earlier hypoxic exposure (on day 1 and days 2 + 3) induced a little more burst formation than the other groups. In the control group of mice maintained at sea level, burst formation was minimal and the bursts were smaller in size. The combined data of the groups subjected to DDH, hypoxia on days 1 + 2, and those maintained at sea level is presented in Fig. 3. The numbers of bursts on day 6 in the groups treated with DDH or hypoxia on days 1 + 2 is not statistically different (p > 0.10) and thereafter show a dissociation on day 7 and 8. The numbers of BFU-d-e that formed bursts on day 6 in DDH are 25.8 ± 5.4/2 × 10^4 HPBM cells. The combined data on the proliferation of granulocytic and/or macrophage clusters and colonies in the same experimental groups are shown in Table 3. The numbers of clusters and colonies were not different in the 3 treatment groups but granulocytic and/or macrophage colonies in the groups subjected to hypoxia were generally smaller than the ones in the group maintained at sea level. The number of granulocytic and/or macrophage colonies could not be enumerated after day 6 of culture because they became larger and fused to one another.

In order to determine if delaying the time of onset of DDH would lead to maximal burst formation 6 groups of mice with PCDC were exposed to DDH starting on days 1, 2, 3, 4, or 5 of the culture period and compared to mice maintained at sea level (Table 4). DDH from day 1 of culture produced a larger number of erythrocytic bursts and maintained the growth of BFU-d-e for a longer period than DDH beginning on days 2, 3, 4, or 5 of culture. However, these groups produced more BFU-d-e derived bursts than those maintained at sea level.

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**Table 2. Erythrocytic Burst Formation in PCDC with Hypoxic Exposure on Two Consecutive Days**

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>23,000 ft: 7 hr/day</th>
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<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>1-8</td>
<td>2.9 ± 1.3*</td>
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<td>1 + 2</td>
<td>4.4 ± 1.4</td>
</tr>
<tr>
<td>2 + 3</td>
<td>3.8 ± 1.9</td>
</tr>
<tr>
<td>3 + 4</td>
<td>6.0 ± 1.8</td>
</tr>
<tr>
<td>4 + 5</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td>5 + 6</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2.3 ± 1.0</td>
</tr>
</tbody>
</table>

*Mean ± SEM, n = 8.

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**Table 3. Number of Granulocytic and/or Macrophage Clusters or Colonies in PCDC with Different Hypoxic Treatments**

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>23,000 ft: 7 hr/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>1-8</td>
<td>37.8 ± 6.5*</td>
</tr>
<tr>
<td>1 + 2</td>
<td>49.1 ± 3.9</td>
</tr>
<tr>
<td>None</td>
<td>31.0 ± 4.5</td>
</tr>
</tbody>
</table>

*Mean ± SEM, n = 12.
When the data from 4 PCDC experiments are combined, the number of BFU-d-e that formed bursts on day 6 in the group subjected to DDH from day 1 of culture is 21.3 ± 2.5 per 2 × 10^4 HPBM cells (mean ± SEM, n = 28) or 1 burst per 940 cells inoculated.

Ep levels of serum and DC contents were determined by bioassay in mice exposed to 23,000 feet immediately after 7 hr of exposure and at 2 hr-intervals thereafter for 17 hr (Table 5). Maximal serum Ep levels were observed immediately after the 7 hr exposure. The 24 hour % ^59Fe uptake of the serum was 3.4 ± 0.5%, which is equivalent to 160 mU/ml of Ep (international reference preparation of erythropoietin-standard B). There was no detectable Ep in the DC contents at any time point after the hypoxic exposure. Ep levels of serum or DC contents were not detectable by bioassay immediately prior to the hypoxic exposure. The limit of sensitivity of our Ep bioassay procedure is 50 mU/ml. There was a rapid decline in the serum Ep levels after cessation of the hypoxic exposure and levels were in the nondetectable range within 8 hr (Table 5).

Since serum Ep levels may be substantially elevated while still in the nondetectable range by bioassay, individual plasma samples were assayed by RIA immediately prior to hypoxic exposure (17 hr after the last hypoxic exposure). It may be seen in Fig. 4 that the Ep levels in the mice subjected to DDH as measured by RIA had returned to the levels that were similar to those maintained at sea level.

DISCUSSION

Axelrad et al.\textsuperscript{10} described erythrocytic burst formation in cultures repeatedly fed with high concentrations of erythropoietin. Burst formation did not occur in the absence of a high concentration of erythropoietin. Furthermore, the ability to form bursts was rapidly lost if erythropoietin was not present at the time of initiation of the cultures. Later, Iscove and Sieber\textsuperscript{11} showed that a single large dose of erythropoietin given at the initiation of the culture sufficed for the induction of erythrocytic bursts. In this case Ep is relatively constant throughout the culture. It has also been shown that in the whole mouse BFU-e numbers in the bone marrow are relatively unresponsive to sustained elevation or suppression of erythropoietin.\textsuperscript{10,12,13} From these data, the BFU-e are believed to be considerably less sensitive to erythropoietin than the CFU-e.

Udupa and Reissman\textsuperscript{14} have shown that daily injections of erythropoietin accelerates the regeneration of CFU-e and 4 day BFU-e after their depletion by an injection of 1,3-bis (2-chlorethyl)-1-nitosourea (BCNU). They concluded that regeneration of 8-day BFU-e depended upon inflow from the CFU-s, but regeneration of more mature erythroid progenitors required Ep.

Recent in vitro observations indicate that early BFU-e growth is modulated by a humoral factor, other than erythropoietin, which is produced in lectin stimulated spleen cell cultures.\textsuperscript{15,17} This substance has been...
Fig. 4. Plasma Ep levels of PCDC loaded mice by Ep radioimmunoassay. ---: plasma Ep levels of the mice with daily discontinuous hypoxia; - - - - : plasma Ep levels of the mice without hypoxic treatment. The sacrifice of mice was simultaneously performed at 17 hr after cessation of hypoxia in both groups. Mean ± SEM, n = 4.

termed burst enhancing factor (BEF). When murine bone marrow cells are cultured in the absence of added Ep for 4 days, there is a marked drop in the number of surviving BFU-e. However, when Ep is added after 4 days of culture, bursts develop during the next 5 days that are as large as bursts in the control cultures. If the culture had been initiated in the presence of lectin stimulated spleen cell conditioned medium, the administration of Ep could be delayed for as long as 3 days and burst formation would still ensue with undiminished efficiency. These data indicate that early BFU-e may not require Ep for survival or proliferation, but do require Ep for expression of hemoglobin synthesis and terminal differentiation. Our data shows a decline in BFU-d-e derived bursts after the 6th day in the PCDC that were stimulated by hypoxia on days 1 + 2 suggesting that some BFU-d-e derived bursts cease proliferation. This represents a failure of stimulation or the death of BFU-e destined to form colonies after day 6. Alternatively, it might represent a lack of preservation of differentiated red cell colonies once these had been formed.

Iscove has also shown that early phases of BFU-e are dependent in culture on molecules that are distinct from erythropoietin and act not only on them, but also on pluripotent stem cells and early committed cells of each of the hemopoietic pathways. In contrast to the in vitro cultures of erythropoietic progenitors, the in vivo requirements for proliferation and continuing differentiation of the erythropoietic progenitors has not been established. However, the studies of Valentine et al. and Stohlman et al. clearly showed that sublethally irradiated animals regenerated their hemoglobin as rapidly as nonirradiated animals if they were bled. In addition, Udupa and Reissman have shown that injections of Ep accelerate the regeneration of 4-day BFU-e and CFU-e after depletion by injection of BCNU. These studies indicate that a burst of Ep through hemorrhage accelerates erythropoiesis even after irradiation and that Ep is required for mid- to late progenitors. Our studies suggest that increased concentration of Ep and/or companion substances produced by hypoxia may only be required early in the culture period in order to induce BFU-e proliferation. The later proliferation of the CFU-d-e and their hemoglobinization then take place at normal Ep levels.

Schooley et al. have shown that DDH is an effective stimulus for production of erythropoietin and that the animals eat normally and do not lose weight. When male BNL mice with PCDC are exposed to 7 hr of hypoxia, the serum Ep level is 160 mU/ml at end of the exposure. The serum Ep is cleared rapidly and is in the nondetectable range within 8 hr after return to sea level. Ep levels of DC contents by bioassay, however, are always in the nondetectable range (less than 50 mU/ml) during 17 hr after the cessation of hypoxia. These data do suggest that the in vivo Ep concentrations are much lower than that required for proliferation of BFU-e in vitro. Gregory et al., using a methyl cellulose culture system, found the optimum Ep concentration for CFU-e was 0.05 to 0.1 U/ml, for 3-day BFU-e, 2.5 U/ml, and for 8-day BFU-e, the number of bursts continued to increase when more than 10 U/ml of Ep was added. The frequency of CFU-e in vitro is 1/345 compared to 1/2000 in PCDC. This low frequency of CFU-e in PCDC was also found in previous papers. This might derive from the original nature of PCDC rather than our technical procedures, although CFU-e are fragile. The frequency of BFU-e in vitro on day 3 is 1/5400, on day 6, 1/5400, and day 8, 1/4000. In our study the frequency of day 6 BFU-d-e was 1/940. The frequency of burst forming cells on day 6 is greater than in other PCDC studies and in Gregory's study in vitro or Iscove et al. of 1 per 4000 bone marrow cells and is close to that of the CFU-e of 1/500 reported by Metcalf et al. Since Hara and Ogawa found an increase in bone marrow BFU-e (10 days) from 5000 per femur to 7000 per femur, it is reasonable to ascribe some of our greater frequency of...
BFU-e in PCDC to be due to plethora. Correcting for the increase in BFU-e in plethoric bone marrow there is still a substantially larger number of bursts grown in the PCDC when hosts are given DDH or hypoxic treatment on days 1 + 2 only. From these studies it can be concluded that the host submitted to hypoxia daily or on days 1 + 2 produces sufficient Ep and/or companion molecules that diffuse into the PCDC and provide a more effective stimulus of erythropoietic progenitors than can be obtained by the in vitro cultures reported to date by Gregory et al. Iscove et al., and others who use much higher concentrations of impure Ep that may also contain companion molecules influencing erythropoiesis. If results in the PCDC represent events taking place in vivo in bone marrow, it would appear that the hypoxic treatments recruit more BFU-e into proliferating and continuing differentiation down the erythrocytic pathway, thus increasing the production rate of red cells. These data suggest that, under normal steady state conditions, a large fraction of the BFU-e do not progress down the erythropoietic pathway. It is of interest that plethora suppresses the production of CFU-e and identifiable erythrocytic precursors, but BFU-e continue cycling as shown by tritiated thymidine cytocide. These observations strongly support our notion that under normal steady state conditions a fraction of BFU-e die in situ. Lastly, in this experimental model nature supplies all of the stimuli for proliferation and further differentiation.

ACKNOWLEDGMENT
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REFERENCES
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