The Role of Hypoxia in the Maintenance of Hematopoietic Stem Cells

By Maria Grazia Cipolleschi, Persio Dello Sbarba, and Massimo Olivotto

Bone marrow cell liquid cultures were incubated at various oxygen concentrations ranging from 0% to 18% (air). The total number of cells in culture (CT) at the end of a 6-day incubation was found to be directly proportional to the oxygen concentration. As compared with air-incubated controls, cells recovered from severely hypoxic (1% oxygen) day-5 liquid cultures showed (1) the same day-7 colony-formation efficiency in semisolid culture (neutrophilic/mixed colonies) or in spleen; (2) a higher day-14 spleen colony-formation efficiency; (3) an enhanced radio-protection ability; and (4) an increased marrow repopulation ability, as measured by determining either total cell number in recipient marrow MRA

ALTHOUGH developmental and cytokinetic properties of hematopoietic stem cells have been intensively studied, the in vivo organization of the hematopoietic tissue remains largely unknown. In particular, the mechanisms regulating the life-long maintenance of stem cells are still unexplained. According to a widely accepted view, the conditions for this maintenance are realized in physiologically segregated areas of bone marrow (BM) ("niches"), wherein stem cells are restrained from commitment to extensive proliferation and differentiation.

In a previous report, we proposed that these niches are hypoxic areas, in which only oxygen-independent cells are able to survive. This proposal was based on the following rationale. Arterial blood enters the highly branched network of medullary sinuses only after circulating in the cortical canalicular system, so that a relatively desaturated blood reaches the marrow. Indeed, average oxygen tension in BM blood is significantly lower than in other organs and tissues, being similar to that in the jugular vein. Moreover, it is likely that cells crowding around sinuses strongly compete for the already scarce oxygen supplied, resulting in an even lower oxygen tension within the core of cell mass.

It is highly plausible that in such areas the proliferation of stem cells is blocked without threatening their survival. In fact, while mitochondrial respiration seems a prerequisite for cells to enter the mitotic cycle, growth factors have been shown to sustain cell survival through the stimulation of glycolysis. Therefore, the deeply hypoxic areas of BM appear to be particularly suitable for the long-term maintenance of stem cells, whereas the better oxygenated areas would allow proliferation of more differentiated progenitors.

In keeping with our hypothesis, hematopoietic progenitors actually appear to be aligned along the oxygen gradient of the BM. Progenitors responsible for BM repopulation in fact have been shown to be more concentrated in the low oxygen areas of BM, whereas fast cycling neutrophilic/mixed colony-forming units (CFU-NM) and day-7 colony-forming units in spleen (CFU-S) reside preferentially in subendosteal areas, where the blood enters the BM circulation. Furthermore, the probability of progenitors to be recruited into the mitotic cycle seems inversely related to the distance from those areas.

MATERIALS AND METHODS

Cell recovery and preparation. BMC were obtained from pooled 8- to 12-week-old CBA T6T6 mice by forced injection of RPMI-1640 medium (GIBCO Ltd, Paisley, UK) into femoral bone...
shafts. Cells were then centrifuged (250g for 10 minutes), resuspended in 0.87% NH₄Cl in H₂O to lyse erythrocytes, washed, and plated in cell culture dishes in RPMI-1640 supplemented with 10% heat-inactivated horse serum (HS; Flow Laboratories, Irvine, UK). After 3 hours of incubation, nonadherent cells were recovered, counted, and transferred into liquid or semisolid cultures (see below). Cell counts were performed in a hemocytometer and viability estimated by the trypan blue exclusion test, diluting cell suspensions 1:1 with a 0.4% wt/vol trypan solution in 0.85% saline (Flow Laboratories).

Spleen conditioned medium (SCM). Mouse SCM was prepared and tested for BMC growth-stimulatory activity as described elsewhere.² In brief, 10⁶ mouse splenocytes were cultured in 50 mL of RPMI-1640 supplemented with 5% pooled, heat-inactivated human serum and 0.4% pokeweed mitogen solution (GIBCO); after 7 days of incubation, culture medium was recovered, filtered, and frozen in small aliquots to be used without further manipulation.

Cell culture system. BMC culture in liquid medium was performed in gas leak-proof culture vessels that allowed us to establish, and maintain throughout the incubation, atmospheres composed of 5% CO₂ and different proportions of oxygen (ranging from 0% to 10%) and nitrogen (95% to 85%).²⁸²⁹ BMC were plated in 250-mL glass flasks, in RPMI-1640 supplemented with 20% HS and 10% SCM (5 × 10⁶ cells in 50 mL per flask). After flushing the sterile-filtered gas mixture, the air-tight inlet and outlet were closed and the flask transferred into an incubator at 37°C. Control cultures were established in the same type of flask, but kept in direct communication with the atmosphere of the incubator: 95% air, 5% CO₂, water-saturated (incubation "in air"). The pH of the hypoxic cultures remained within physiologic limits (7.2 to 7.4) throughout the incubation.

After 5 to 6 days of incubation in hypoxia or in air, cells were washed, counted, and diluted appropriately for the in vitro or in vivo assays, as indicated below. BMC recovered directly from animals ("time-zero" of the cultures) were assayed under the same conditions in a larger set of separate experiments.

In vitro clonal assay. To estimate the number of CFU-NM in BMC populations, semisolid cultures were established by plating 10⁶ viable cells per 3-cm petri dish in 1 mL of RPMI-1640 supplemented with 20% HS, 10% SCM, and 0.3% (wt/vol) agar (Bacto Agar; DIFCO, Detroit, MI). Assays were performed in triplicate and always incubated in air. After 7 days of incubation, the number of colonies (aggregates of 50 or more cells) per dish was scored at 25 × magnification. Colony-formation efficiency (CFE) was expressed as the ratio of the number of colonies developed to the number of cells plated per dish.

In Vivo Assays

Cells were transplanted into pooled 8- to 12-week-old, syngeneic mice that had been lethally irradiated with a total dose of 10 Gy at a rate of 1 Gy/min from a 60Co, 1.2 MeV source (Theratron 780; Atomic Energy of Canada Ltd, Ottawa, Canada). Cell suspensions in serum-free culture medium were injected into the lateral tail vein within 3 hours after irradiation. Irradiated mice injected with culture medium only were used to determine the background level for each parameter studied.

Spleen colony-formation assays. Cells were transplanted into 3 to 5 mice per group and spleens recovered from recipient mice 7 (CFU-S₇) or 14 days (CFU-S₁₄) after transplantation. Spleens were then fixed in Bouin's solution, and macroscopic surface colonies counted at 2 × magnification. To determine the number of CFU-S₇, chosen as an assay for the "delayed" spleen colony-forming activity,²²²³ various cell suspensions were transplanted (2.5 × 10⁴ to 5 × 10⁵ cells/0.2 mL). Counts of large day-14 spleen colonies, a possible source of inaccuracy,²³ were confirmed by determining spleen weight (not shown); CFE was expressed as the ratio of the colony number to the number of cells transplanted.

Radioprotection and marrow repopulation assays. The radioprotective ability (RPA) of BMC populations, ie, the capacity to confer long-term survival to lethally irradiated mice, was measured by transplanting various cell suspensions (2 × 10⁶ to 2 × 10⁶ cells/0.2 mL) into 10 mice per suspension. The fraction of recipient mice surviving 30 days after transplantation was determined for each suspension. RPA was expressed as the ratio of the percentage of surviving mice to the number of transplanted cells.²⁴²⁵

BM repopulating ability (MRA) of BMC populations was measured basically as described by Hodgson et al.,²⁷²⁸ by transplanting various cell suspensions (5 × 10⁴ to 2 × 10⁶ cells/0.2 mL) into 3 to 5 mice per suspension. Femurs were recovered from recipient mice 14 days after transplantation and individually processed. The total number of nucleated cells per femur was determined and aliquots of cells were assayed for the presence of CFU-NM, as described above. MRA was expressed as the ratio of the total number of cells (MRA₈₇₉, or the number of CFU-NM (MRACFU-NM) per recipient femur, to the number of transplanted cells. MRACFU-NM was chosen as an assay for MRAPE.²⁷²⁸²⁹

Data obtained with increasing dose of transplanted cells were plotted in log/log scale and best fitted by linear regression, with the exclusion of plateau values, according to Hodgson et al.²³

Cells responsible for RPA or MRA₈₇₉CFU-NM were referred to as RPA cells or MRA₈₇₉CFU-NM cells.²³²⁸²⁹

RESULTS

Dependence of BMC Growth on Oxygen Tension

Total number of cells (CT) was determined in BMC liquid cultures incubated for 6 days at different oxygen tensions. CT resulted directly proportional to oxygen concentration of the incubation atmosphere, increasing, as compared with the time-0 value, within an oxygen concentration range of 3% to 18% (Fig 1). There was no increase in cell number at 3% oxygen, whereas cell loss occurred at lower concentrations.

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The data indicated that, whereas BMC expansion in vitro is oxygen-dependent, a sizeable number of hematopoietic cells is able to survive in severe hypoxia (1% to 3%). We decided to characterize the composition of this residual population with respect to several types of hematopoietic progenitors and stem cells. Incubation in 1% oxygen for 5 days, providing sufficient numbers of viable cells for secondary in vitro or in vivo assays, was chosen on the basis of preliminary tests (not shown) and used for all experiments reported below.

Effects of Hypoxia on BMC Cloning Efficiency

Table 1 shows the effect of a 5-day incubation of BMC in 1% oxygen (hypoxia) or in air (aerobiosis) on cloning efficiency, as detected 7 days after replating in vitro (CFU-NM) or transplantation in vivo (CFU-S). The in vitro clonal assays were performed in both cases in air, to avoid differences in yield caused by the reported influence of oxygen tension on colony formation in semisolid medium.30-34 Day-7 cloning efficiency, both in vitro and in spleen, was found to be significantly decreased, as compared with time-zero, independently of the incubation atmosphere. Furthermore, no significant effect on cloning efficiency was produced by incubation in 1% oxygen as compared with air. This indicated that hypoxia, although reducing CT (Fig 1), did not alter the concentrations of CFU-NM and CFU-S, and, therefore, that these progenitors are as sensitive to hypoxia as the overall BMC population.

On the contrary, BMC cloning efficiency in spleen, as detected 14 days after transplantation (CFU-S14), was significantly influenced by incubation in hypoxia. This is shown in Fig 2, in which the relationship between inoculum size and colony number is reported for all tested conditions. This relationship turned out to be linear in all cases, allowing for the calculation of the cloning efficiencies from the slopes of the lines. Cloning efficiency was found to be decreased in day-5 cultures, as compared with time 0, independently of the incubation atmosphere. However, this reduction was 5 times lower in hypoxia than in aerobiosis. Thus, CFU-S14 appeared concentrated in hypoxic as compared with aerobic day-5 cultures, showing a substantial difference between these progenitors and CFU-NM or CFU-S7.

Effects of Hypoxia on RPA

The percent survival of lethally irradiated mice is plotted in Fig 3 versus the inoculum size. To compare variously treated cell populations, we considered intercepts of the lines best fitting the data with the number of transplanted cells required to obtain 50% survival. This number was estimated to correspond approximately to 3.5 × 10⁴ cells at time zero, 12 × 10⁴ cells incubated in hypoxia, and 69 × 10⁴ cells incubated in air. Thus, RPA was found decreased in day-5 cultures, as compared with time 0, but this decrease was 5.7 times lower in hypoxia than in aerobiosis, paralleling the results obtained for CFU-S14.

Effects of Hypoxia on MRA

MRAcell was calculated by plotting the number of cells recovered from recipient femoral marrow versus the number of transplanted cells (Fig 4A). To compare variously treated cell populations, we considered intercepts of the lines fitting the data with a reference value of repopulation of recipient femoral marrow (2.5 × 10⁵ cells, corresponding to the dashed line in Fig 4A). It was estimated that, to obtain this value, one needs 8.7 × 10⁴ cells at time 0, 18.2 × 10⁴

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**Table 1. Effects of BMC Incubation in Air or Hypoxia on Day-7 Cloning Efficiency in Culture (CFU-NM) or in Spleen (CFU-S)**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Colonies Per Dish</th>
<th>Colonies Per Spleen</th>
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<tr>
<td>No (time 0)</td>
<td>34.9 ± 1.63 (39; 10)</td>
<td>15.2 ± 0.75 (56; 11)</td>
</tr>
<tr>
<td>In air</td>
<td>21.2 ± 5.39* (26; 7)</td>
<td>4.5 ± 0.80† (27; 6)</td>
</tr>
<tr>
<td>In 1% oxygen</td>
<td>22.3 ± 1.94* (19; 5)</td>
<td>6.8 ± 0.89† (17; 3)</td>
</tr>
</tbody>
</table>

BMC, recovered directly from donor mice (time 0) or from liquid cultures incubated for 5 days in either air (control) or hypoxia (1% oxygen), were replated in semisolid medium (10⁵ cells per dish) or transplanted into lethally irradiated syngeneic mice (10⁵ cells per mouse), and colonies counted 7 days later. Data are means ± intrasexperiment standard errors (total number of counts; number of separate experiments). Results were assessed by one-way analysis of variance. Differences between time 0 and day-5 cultures are defined by the symbols. Differences between hypoxia and control day-5 cultures were not significant.

* P < .01 (71 degrees of freedom).
† P < .01 (88 degrees of freedom).
cells incubated in hypoxia, and $138 \times 10^4$ cells incubated in air. Thus, again, MRA$_{cell}$ decreased in culture, but this decrease was 7.6 times lower in hypoxia than in aerobiosis, in keeping with the results obtained for CFU-S$_{14}$ and RPA.

The above procedure was applied to estimate MRA$_{CFU-NM}$ (Fig 4B), assuming $10^3$ CFU-NM as a reference value of repopulation of recipient femoral marrow (dashed line in Fig 4B). This value was obtained with $14.8 \times 10^4$ cells at time zero, $9.5 \times 10^4$ cells incubated in hypoxia, and $120 \times 10^4$ cells incubated in air. Strikingly, in hypoxic cultures, MRA$_{CFU-NM}$ not only was much higher than in aerobic cultures (12.6 times), but it also appeared unreduced, if not enhanced, in comparison to time 0.

**Effects of Hypoxia on the Absolute Number of Progenitors in Culture**

The effects of hypoxia on the absolute numbers of hematopoietic progenitors were tentatively quantified by taking into account the changes in CT under the various culture conditions (Table 2). It was shown that incubation in air for 5 days resulted in an increase in the number of CFU-NM and CFU-S$_7$, along with CT expansion. On the other hand, all the other progenitors decreased: CFU-S$_{14}$, RPA cells, and MRA$_{cell}$ cells (progenitors sustaining RPA and MRA$_{cell}$, respectively) to 26%, on the average, whereas MRA$_{CFU-NM}$ cells (progenitors responsible for MRA$_{CFU-NM}$) to about 50%. Incubation in hypoxia for 5 days reduced all types of progenitors to 21%, on the average, the only exception being MRA$_{CFU-NM}$ cells, that were about 75% conserved.

Comparison of columns 2 and 3 (or 4 and 5) of Table 2 led to the conclusion that the enrichment of hypoxic cultures with hematopoietic progenitors shown in Figs 2, 3,
and 4A was essentially accounted for by the suppression in these cultures of the CT increase that occurs in air. MRA_{CFU-NM} cells (Fig 4B) had the further advantage of being better preserved in hypoxic cultures, rather than in aerobic cultures.

**DISCUSSION**

The main information to emerge from this study was that hypoxic culture conditions clearly selected for certain types of hematopoietic progenitors in BMC cultures, as compared with aerobic cultures. This effect was maximal for MRA_{CFU-NM} cells (Fig 4B). A clearer outline of the fate of various progenitors in aerobiosis or in hypoxia was obtained from the computation of their total number in culture (Table 2), showing that (1) the concentration of most progenitors in hypoxic cultures was essentially accounted for by suppression of CT increase; and (2) MRA_{CFU-NM} cells were better preserved in hypoxia than in aerobicism.

When total numbers of progenitors in hypoxic cultures (Table 2) were reported as percentages of the corresponding number in aerobic cultures (Fig 5), it was possible to identify three main categories of progenitors: (1) oxygen-dependent progenitors, including CFU-NM and CFU-S; (2) oxygen-independent progenitors (CFU-S_{14}, RPA cells, and MRA_{cell} cells); and (3) hypoxia-preserved progenitors (MRA_{CFU-NM} cells).

The above categories fit very well with progenitor phenotypes identified on the basis of mitochondrial activity, as measured by rhodamine-123 (Rh) staining: (1) Rh-positive cells (CFU-S and persistently CFU-S_{12}); (2) Rh-weakly positive cells (delayed-CFU-S_{12}, RPA cells, and MRA_{cell} cells); and (3) Rh-dull cells (most MRA_{CFU-NM} cells).

The finding that CFU-NM and CFU-S depend on oxygen for survival and expansion in vitro is in keeping with the abundance of active mitochondria shown in group (1) cells by the Rh-positivity. This result is also consistent with the respiration-dependent step in the mitotic cycle shown for CFU-NM and suggests the existence of a similar step in CFU-S that, like CFU-NM, are actively cycling committed progenitors. On the other hand, the oxygen-dependence of CFU-NM contrasts with the reported enhancement of colony-formation efficiency in semisolid cultures incubated in hypoxia, an effect attributed to a reduced oxygen toxicity on clonogenic progenitors. However, sensitivity of these cells to oxygen toxicity is apparently lower in mass liquid cultures than in clonal assays in semisolid medium. Thus, it is conceivable that in our system the positive effects of oxygen on cell growth largely prevail over a low degree of oxygen toxicity.

The low sensitivity to hypoxia of CFU-S_{14}, RPA cells, and MRA_{cell} cells (Fig 5) is in keeping with the weak Rh-positivity of group (2) cells, indicating a scarcity of active mitochondria. It is worthwhile to recall that these progenitors are mainly quiescent but readily recruitable and comittable by growth factors ("activated stem cells") to generate higher

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**Table 2. Computation of Total Number of Hematopoietic Progenitors in BMC Cultures**

<table>
<thead>
<tr>
<th>Type of Progenitor</th>
<th>Absolute Number of Cells in Culture</th>
<th>% of t = 0 Values</th>
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<tr>
<td></td>
<td>t = 0</td>
<td>Air</td>
</tr>
<tr>
<td>Culture no.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-NM*</td>
<td>17,450</td>
<td>44,944</td>
</tr>
<tr>
<td>MRA_{cell}</td>
<td>57</td>
<td>15</td>
</tr>
<tr>
<td>MRA_{CFU-NM} cells</td>
<td>34</td>
<td>18</td>
</tr>
</tbody>
</table>

Abbreviations: t = 0 (time 0), BMC recovered directly from donor mice; air, 5-day cultures incubated in air; hypoxia, 5-day cultures incubated in 1% oxygen.

* Values were obtained multiplying CT by the colony-formation efficiencies (see Table 1 and the legend to Fig 2).
† Values are expressed in arbitrary units, 1 U corresponding to the number of transplanted cells supporting survival of 50% recipient mice after 30 days.
§ Values were obtained dividing CT by the number of transplanted cells corresponding to 1 U, calculated from Fig 3 (RPA), Fig 4A (MRA_{cell}), or Fig 4B (MRA_{CFU-NM}).
numbers of less immature progenitors. Therefore, activated stem cells are likely to be depleted in our system as in any growth factor-stimulated culture. Indeed, in our experiments, these cells were found substantially decreased in both control and hypoxic cultures, pointing out the irrelevance of cell respiration for their survival.

Finally, within the limits that MRA<sub>CFU</sub>-LM cells (group 3) are actually Rb-dull, i.e., lacking of active mitochondria, these progenitors could be regarded as anaerobically adapted. In keeping with this view, MRA cells have been found to be more highly concentrated in the low oxygen areas of BM. Anaerobic adaptation of metabolism, usually accompanied by an enhanced cell sensitivity to oxygen toxicity, seems to be confirmed by our finding that MRA<sub>CFU</sub>-LM cells were better maintained in hypoxia than in air, a striking difference from all of the other types of progenitors. Interestingly, MRA<sub>CFU</sub>-LM cells are also mostly nongrowing, an essential attribute of the most immature hematopoietic progenitors, the quiescent stem cells. The latter are believed to be less easily depleted than activated stem cells under the action of growth factors, as confirmed by our data (see Table 2, column 4).

In this context, one can hypothesize that anaerobic adaptation of metabolism is a major feature distinguishing activated from quiescent stem cells. Assuming that the recruitment of stem cells to active hematopoiesis is respiratory-dependent, the lack of active mitochondria in some of these cells would prevent their recruitment and limit the action of growth factors to the support of survival. In other words, the anaerobic orientation of a subset of stem cells would reduce their sensitivity to proliferation/differentiation stimuli and play a crucial role in their maintenance in G<sub>0</sub> and long-term conservation.

On the whole, data presented seem to support the proposed hypothesis that quiescent stem cells survive in "hypoxic niches" of hematopoietic tissue, protected from commitment. An increase of number/activity of mitochondria may well be a prerequisite for the activation of stem cells. These activated stem cells could conceivably move to less hypoxic areas close to the niches, where the response to growth factors is no longer restrained and the generation of lineage-restricted progenitors is possible. The latter, in better oxygenated areas, would then undergo the extensive proliferation responsible for clonal expansion.

Whatever the merit of these considerations, it is a fact that various subsets of hematopoietic progenitors are differently affected by oxygen tension. While this effect needs to be further explored by BMC fractionation procedures, incubation in severe hypoxia or anoxia might prove to be a useful and "physiologic" tool to select quiescent stem cells from normal and leukemic BM.

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