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The expansion of murine bone marrow cells preincubated in hypoxia as an *in vitro* indicator of their marrow-repopulating ability

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In liquid cultures of murine bone marrow cells stimulated with interleukin-3 and granulocyte/macrophage colony-stimulating factor, hypoxia (1% oxygen) induced a reversible block of hematopoiesis, maintaining the progenitors' expansion potential unreduced. Progenitors repopulating day-14 hypoxic cultures with cells or granulocyte/macrophage colony-forming units (CFU-GM) were found, on the basis of their maintenance in hypoxia (12% and 76%, respectively), to belong to different subsets, the latter being much more efficiently maintained. The maintenance in hypoxic cultures of progenitors detectable by marrow-repopulating ability (MRA) assay was 18% for MRA_{cell} progenitors and 69% for MRA_{CFU} progenitors. Thus, the repopulation of hypoxic cultures with cells or CFU-GM closely reflected the presence of progenitors capable of repopulating, with cells or CFU-GM, the bone marrow of lethally irradiated syngeneic animals. Progenitors repopulating hypoxic cultures were, like MRA progenitors, significantly resistant to 5-fluorouracil, progenitors repopulating cultures with CFU-GM being two-fold more resistant than those repopulating cultures with cells. We concluded that the repopulation of day-14 hypoxic cultures occurring after their transfer to air is to be considered an indicator of the maintenance of MRA progenitors in hypoxia. The relevance of these results to stem cell biology and their potential practical applications are discussed. *Leukemia* (2000) 14, 735–739.

Keywords: hypoxia; culture-repopulating cells; marrow-repopulating ability; 5-fluorouracil; hypoxic culture repopulating ability (HCRA)

Introduction

We previously reported that hematopoietic progenitors from murine bone marrow (BM) or human cord blood are variously sensitive to the incubation at low oxygen (1%) tension,^{1,2} in a liquid culture system initially designed by Sumner *et al*³ and later developed in our laboratory and by others.^{4,5} We found that the more primitive the progenitors, the lower their sensitivity to low oxygen, so that incubation in hypoxia selectively concentrates more immature progenitors. In particular, progenitors detected by a marrow repopulating ability (MRA) assay based on the count of granulocyte–macrophage colony-forming units (CFU-GM) in the recipients' BM (MRA_{CFU} progenitors) were better maintained at low than at normal oxygen (18–20%). A normal oxygen tension, on the contrary, was necessary for the generation of CFU-GM *in vitro* and the overall numerical expansion of cultures, whereas progenitors more primitive than CFU-GM and less primitive than MRA_{CFU} progenitors exhibited intermediate degrees of sensitivity to hypoxia. These results well supported the findings previously obtained *in vivo* that: (1) in murine BM, hematopoietic progenitors are distributed along the oxygen gradient, MRA progenitors being more concentrated where oxygen tension is

lower while CFU-GM reside preferentially in the best-oxygenated subendosteal areas, and (2) the probability of progenitors to be recruited into the mitotic cycle is inversely related to their distance from those areas.^{6–8}

In the experiments reported in this paper, liquid cultures of murine BM cells (BMC) supplemented with granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL3) were used to: (1) determine whether the inhibition of hematopoiesis in hypoxia is reversible; (2) characterize the hypoxia-maintained progenitors on the basis of their *in vitro* expansion potential, as measured by transferring hypoxic cultures to air; (3) compare these progenitors with progenitors detectable by the MRA assays. The results indicated that the hypoxia-dependent block of the expansion of hematopoietic clones is reversible. Taking advantage of this reversibility, two types of culture-repopulating progenitors were identified measuring the generation of CFU-GM *in vitro* and the overall numerical expansion of cultures. The maintenance of these progenitors in hypoxia closely paralleled that of two subsets of MRA progenitors. The progenitors of CFU-GM, like MRA_{CFU} progenitors, exhibited a significantly higher maintenance in hypoxia and resistance to 5-fluorouracil (5FU). These results point to the repopulation of hypoxic cultures after their transfer to air as an *in vitro* indicator of MRA. The potential practical applications of these findings in studies of normal and neoplastic hematopoiesis are discussed.

Materials and methods

Cell recovery

Femoral BMC from 8- to 12-week-old CBA mice were flushed and pooled in RPMI-1640 medium (HyClone Europe, Cramlington, UK). Cells were then centrifuged (250 g for 10 min), 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; HyClone). After a 3 h incubation, nonadherent cells were recovered, counted and plated. Cell counts were carried out in a hemocytometer and cell viability estimated by the trypan blue exclusion test, diluting cell suspensions 1:1 with a 0.4% wt/v trypan solution in 0.87% saline.

Liquid cultures

BMC were cultured in RPMI-1640 supplemented with 20% HS, 5 ng/ml recombinant murine GM-CSF (PeproTech EC, London, UK) and 20 ng/ml recombinant murine IL3 (PeproTech). Cells were plated at 5 × 10⁶ cells in 5 ml per flask (see below) and incubated at 37°C in water-saturated atmosphere, as described.¹ Incubation at low oxygen tension was carried out in gas-tight 250 ml glass flasks containing an

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atmosphere composed of 1% O₂, 5% CO₂, 94% nitrogen ('hypoxic cultures'). Control cultures at normal oxygen tension ('cultures in air') were established in similar flasks incubated in a 95% air, 5% CO₂ atmosphere. The pH of the hypoxic cultures remained within physiological limits (7.2–7.4) throughout the incubation.

In other experiments, cells directly recovered from animals, or pre-incubated for 14 days in hypoxia, were treated for 2 days in air or in hypoxia, respectively, with 100 µg/ml 5FU (Roche, Neuilly-sur-Seine, France), washed twice to remove 5FU, and finally incubated in air for a further 7 days. In some cases, α-tocopherol (Sigma-Aldrich, Milan, Italy) was added to cultures at a 10 µM final concentration, to prevent cellular reoxygenation damage.

Semisolid cultures

To determine the number of granulocyte–macrophage colony-forming units (CFU-GM) in BMC liquid cultures, *in vitro* clonal assays were established by transferring cells from these cultures into a semisolid medium composed of RPMI-1640, 20% HS, 0.3% (wt/v) agar (Bacto Agar; Difco, Detroit, MI, USA), 5 ng/ml GM-CSF and 20 ng/ml IL3. Cells were plated at 10⁴ viable cells/1 ml/dish in quadruplicate 3 cm petri dishes, always incubated in air. After 7 days, the number of colonies (aggregates of 50 or more cells) per dish was scored at a 25× magnification.

MRA assay

The MRA of liquid cultures was measured as described.¹ In brief, CBA mice (8- to 12-week-old) were lethally irradiated with one dose of 10 Gy, at a rate of 1 Gy/min, from a ⁶⁰Co, 1.2 MeV source (Theratron 780; Atomic Energy of Canada, Ottawa, Canada). Cells recovered directly from donor animals or from day-14 cultures incubated in hypoxia were suspended in 0.2 ml of serum-free medium and transplanted (1 × 10⁵ cells) into six irradiated recipients per experimental condition. At day 14 after transplantation, femoral nucleated cells were individually counted, pooled and processed for CFU-GM assays as described above. MRA of cultures was calculated by the product of (1) the ratio of total cell number in culture to the number of transplanted cells with (2) the number of cells (MRA_{cell}) or of CFU-GM (MRA_{CFU}) recovered per recipient's femur.

Results

In order to determine whether the inhibition in hypoxia (1% O₂) of the expansion of hematopoietic clones is reversible, murine BMC were incubated in hypoxia or in air from time-zero, or in hypoxia and then in air (Figure 1). In control cultures (in air from time-zero), total cell number underwent a six-fold increase over the first 6 days of incubation, and then decreased progressively to approach zero in about 4 weeks. By contrast, in hypoxic cultures, total cell number decreased from the beginning of incubation to approach zero in about 1 week. When these cultures were transferred to air (at day 7 or day 14), total cell number increased by six or 7.5 times, respectively, with respect to the values at the time of transfer. However, the peak of total cell number reached by cultures reoxygenated at day 14 was significantly lower than that of

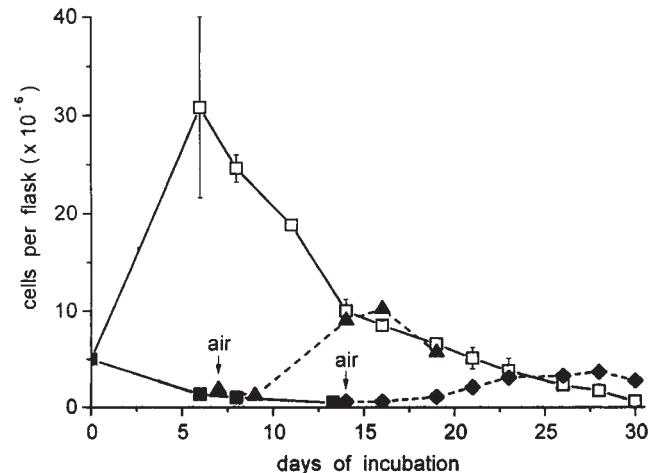


Figure 1 Effects of hypoxia on total cell number in liquid cultures of murine BMC. Cells were incubated in hypoxia (1% O₂) or in air (20% O₂) directly after recovery from animals (solid lines), or in hypoxia for 7 or 14 days and then in air (dashed lines). Points are means ± s.e.m. of five independent experiments (some error bars are concealed by the symbols). (□) Air from time-zero; (■) hypoxia from time-zero; (▲) hypoxia from time-zero, transfer to air at day 7; (◆) hypoxia from time-zero, transfer to air at day 14.

cultures reoxygenated at day 7, this being lower than that of control cultures incubated in air from time-zero.

The time-course of CFU-GM in culture is shown in Figure 2. In control cultures incubated in air from time-zero, the number of CFU-GM increased by 2.5 times over the first 6 days, and then decreased progressively to approach zero in about 2 weeks. In hypoxic cultures, the number of CFU-GM started to decrease immediately after the beginning of incubation, to approach zero in about 1 week. The transfer at day 14 of hypoxic cultures to air and their further incubation for 9 days resulted in a six-fold increase of the number of CFU-

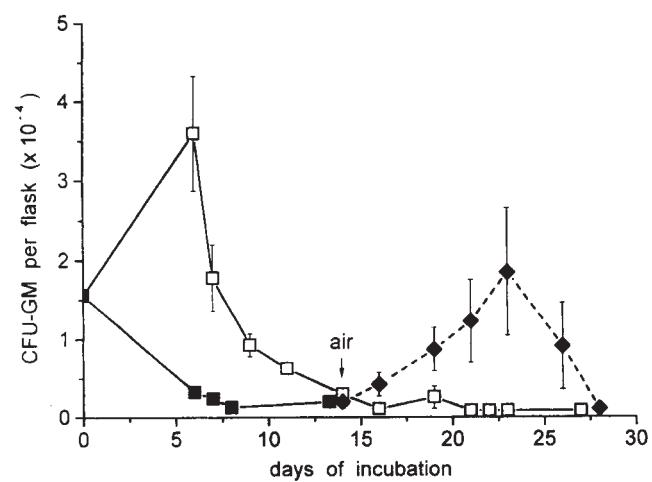


Figure 2 Effects of hypoxia on the time-course of CFU-GM in liquid cultures of murine BMC. Cells were incubated in hypoxia or in air directly after recovery from animals (solid lines), or in hypoxia for 14 days and then in air (dashed line). At selected incubation times cells were replated in semisolid medium and CFU-GM-derived colonies counted 7 days later. Points are means ± s.e.m. of five independent experiments (some error bars are concealed by the symbols). (□) Air from time-zero; (■) hypoxia from time-zero; (◆) hypoxia from time-zero, transfer to air at day 14.

GM over the value at the time of transfer. This reversibility of the inhibition of the hematopoietic expansion occurring in hypoxia was not enhanced by the complete renewal of culture medium or the addition of antioxidants, such as α -tocopherol, to prevent cellular reoxygenation damage (not shown).

The data shown in Figures 1 and 2 were computed in Table 1 to quantify, and compare with each other, the potentials of hypoxia-maintained cells for culture repopulation with cells or CFU-GM. Lines A and B indicate that after 14 days of incubation in hypoxia the maintenance of time-zero ability of repopulating cultures with cells was 12% and that with CFU-GM 76%. Table 1 also shows (lines C and D) that in cultures incubated in hypoxia for 14 days MRA_{cell} was 18% of the time-zero value, and MRA_{CFU} 69%. These values were very similar to those of lines A and B, ie the maintenance in hypoxia of progenitors capable of repopulating, with cells or CFU-GM, the aplastic BM of lethally irradiated animals closely matched the maintenance of progenitors capable of repopulating, with cells or CFU-GM, hypoxic cultures after their transfer to air.

MRA progenitors are relatively resistant to cell cycle-specific cytotoxic drugs such as 5FU.⁹ In order to assess whether culture-repopulating progenitors in our system are also 5FU-resistant, we determined the effects of 5FU *in vitro* on the increase of total cell number and CFU-GM number (Table 2). Cells directly recovered from animals, or preincubated for 14 days in hypoxia, were treated with 5FU for 2 days in air or hypoxia, respectively, and then incubated for 7 days after removal of 5FU, in both cases in air. In cultures treated with 5FU in hypoxia, substantial numbers of cells and CFU-GM were generated during the following incubation in air (column 2). On the contrary, few viable cells and no CFU-GM were found in cultures treated with 5FU in air (column 1). Thus, incubation in hypoxia during the treatment with 5FU was required for both total cell number and CFU-GM number increase in air after removal of the drug. This indicates that

hypoxia-incubated, but not air-incubated, cultures contained 5FU-resistant progenitors repopulating cultures with cells or CFU-GM. To estimate the percentage of 5FU-resistant progenitors within the hypoxia-maintained progenitors, the data of column 2 were compared with those obtained from hypoxic cultures incubated in the absence of 5FU (column 3). Column 4 shows that 16% of hypoxia-maintained progenitors sustaining the increase of total cell number and 32% of those responsible for the increase of CFU-GM number were 5FU-resistant. When the data of Tables 1 and 2 were combined to estimate the absolute percentage of 5FU-resistant hypoxia-maintained progenitors (Figure 3), it emerged that 24% of progenitors repopulating cultures with CFU-GM were 5FU-resistant, whereas a negligible percentage (<2%) of progenitors repopulating cultures with cells survived the treatment with 5FU.

Discussion

The first advancement we obtained from this work with respect to our previous study¹ was that the hypoxia-induced block of the expansion of hematopoietic clones is reversible (Figure 1). In hypoxic cultures, indeed, total cell number increase with respect to the time of transfer to air (at days 7 or 14; six- or 7.5-fold, respectively) was identical to that of controls with respect to time-zero (six-fold). However, as the longer the incubation in hypoxia, the lower the peak values of total cell number, we concluded that hypoxia determined a progressive loss of hematopoietic clones, yet combined with an unreduced expansion potential of hypoxia-maintained clones. This implies that the counts obtained from hypoxic cultures following their transfer to air reflect the number of progenitors whose expansion potential is preserved. When this conclusion was applied to the comparison of data of Figures 1 and 2, progenitors responsible for CFU-GM generation resulted more efficiently maintained in hypoxia than those sustaining total cell number increase. As the difference was marked (6.3 times, as calculated from column 3 of Table 1), the two types of progenitors clearly belong to different subsets, a conclusion in keeping with the previous finding that maintenance in hypoxia is a distinctive property of hematopoietic progenitors.¹

To characterize the two subsets of culture-repopulating progenitors identified via the reversibility of the effect of hypoxia, their maintenance in hypoxic cultures was compared with that of MRA progenitors, which are selectively enriched and well maintained in hypoxia.¹ In day-14 hypoxic cultures, MRA_{CFU} and MRA_{cell} resulted in 69% and 18% of the time-zero value, respectively (Table 1). These figures closely match those previously obtained¹ for day-5 hypoxic cultures (73% for MRA_{CFU} and 23% for MRA_{cell}) stimulated with a spleen conditioned medium providing conditions rather similar to the combination of IL3/GM-CSF used in this study. This indicates that the extension of the incubation in hypoxia from 5 to 14 days did not significantly reduce the percentage maintenance in hypoxia of MRA progenitors, confirming that this maintenance is a distinctive property of each progenitor subset, rather than being a function of incubation time. The most interesting result, however, emerging from the *in vivo* experiments was that the percentages of maintenance in hypoxia of MRA were similar to those of culture-repopulating progenitors (Table 1), the value obtained for MRA_{CFU} progenitors in particular being very close to that of cells generating CFU-GM *in vitro* (69% vs 76%). Thus, the maintenance in hypoxia of progenitors

Table 1 Maintenance in hypoxia of culture- and marrow-repopulating abilities

Culture content	1	2	3
	6 days in air	14 days in hypoxia +9 days in air	Maintenance in hypoxia
A cells	25.8 ± 11.3 × 10 ⁶	3.2 ± 0.2 × 10 ⁶	0.12
B CFU	20.4 ± 7.3 × 10 ³	15.4 ± 8.0 × 10 ³	0.76
	Time zero	14 days in hypoxia	Maintenance in hypoxia
C MRA _{cell}	12.7 ± 4.3 × 10 ⁶	2.3 ± 0.9 × 10 ⁶	0.18
D MRA _{CFU}	14.6 ± 3.8 × 10 ³	10.1 ± 6.1 × 10 ³	0.69

Lines A and B: column 1 reports the cell or CFU-GM number increase in cultures incubated in air for 6 days from time-zero (ie the differences between the peak and time-zero values of Figures 1 and 2); column 2 reports the cell or CFU-GM number increase in cultures incubated in hypoxia for 14 days from time-zero and then in air for 9 days (ie the differences of Figures 1 and 2 between the peak values reached by hypoxic cultures after their transfer to air and the values at the time of transfer). Lines C and D: the values are means ± s.e.m. of six independent experiments, each carried out using six mice per incubation time. Values represent the product between the number of cells or CFU-GM recovered per recipient's femur and the ratio of total cell number in culture at time-zero (column 1) or after 14 days in hypoxia (column 2) to the number of transplanted cells (1 × 10⁵ per recipient).

Table 2 Effects of 5FU *in vitro* on progenitors repopulating cultures with cells or CFU-GM

Culture content	1	2	3	4
	Time zero +5FU		14 days in hypoxia	
		+5FU	-5FU	+5FU/-5FU
Cells	$4.0 \pm 3.0 \times 10^3$	$180 \pm 50 \times 10^3$	$1150 \pm 260 \times 10^3$	0.16
CFU-GM	0	$2.6 \pm 0.8 \times 10^3$	$8.3 \pm 2.2 \times 10^3$	0.32

Cells directly recovered from animals (time-zero) or incubated for 14 days in hypoxia were treated with 5FU for 2 days in air or hypoxia, respectively, and then incubated for 7 days in air after removal of 5FU (columns 1 and 2). Cells were incubated in the absence of 5FU for 16 days in hypoxia and then for 7 days in air (column 3). The number of CFU-GM was determined in secondary clonal assays. Values are means \pm s.e.m. of three (columns 1 and 2) or five (column 3) separate experiments. Differences between values of columns 2 and 3 were found statistically significant according to the Student's *t*-test (cells: $P < 0.02$; CFU-GM: $P < 0.05$).

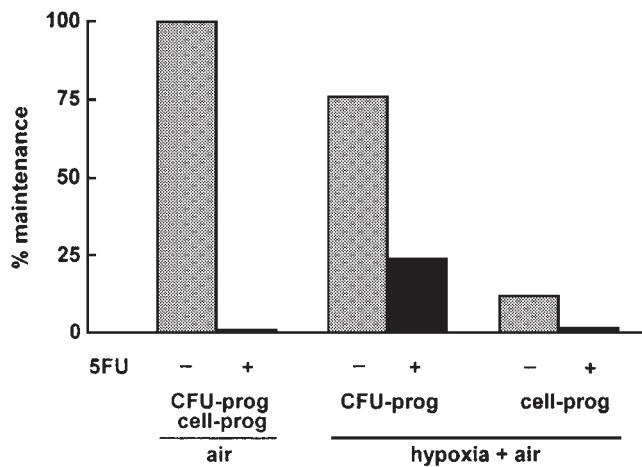


Figure 3 Maintenance in hypoxia and 5FU-resistance of culture-repopulating progenitors. The Figure summarizes the results reported in Tables 1 and 2. The data are expressed as percentages of those obtained for 5FU-untreated cultures incubated in air for 6 days from time-zero (as in Table 1, column 1, lines A or B). As for cultures incubated in hypoxia, grey histograms (without 5FU) correspond to the values of Table 1, column 3, lines A or B, while black histograms (with 5FU) represent the product of these values by the corresponding values of Table 2, column 4. CFU-prog, progenitors repopulating cultures with CFU-GM; cell-prog, progenitors repopulating cultures with cells.

capable of repopulating cultures, with cells or CFU-GM, closely reflected that of progenitors capable of repopulating, with cells or CFU-GM, the bone marrow of lethally irradiated animals. In other words, the two parameters we measured in day-14 hypoxic cultures after their repopulation in air appeared suitable as indicators of the maintenance in hypoxia of two subsets of MRA progenitors.

Strong support of the above conclusion, as well as of the identification of two different subsets of hypoxia-maintained culture-repopulating progenitors, came from the experiments based on 5FU (Table 2). The rationale of these experiments was that, as MRA progenitors are relatively resistant to cell cycle-specific cytotoxic drugs such as 5FU,⁹ to determine the 5FU resistance of culture-repopulating progenitors would help in pursuing their characterization. In 5FU-treated hypoxic cultures, substantial numbers of cells and CFU-GM were generated during the following incubation in air, indicating that sizeable percentages of culture-repopulating progenitors were 5FU-resistant. However, progenitors responsible for CFU-GM generation were two times more resistant than progenitors sus-

taining total cell number increase, the percent 5FU-resistance values (32% and 16%, respectively) being comparable to those obtained by Hodgson and Bradley⁹ (42% and 23%) for the survival of MRA_{CFU} and MRA_{cell} progenitors after a 24 h treatment with 5FU *in vivo*. On the whole, the 5FU experiments indicated that the two subsets of culture-repopulating progenitors we identified react differently to 5FU and are most probably related to the two main subsets of MRA progenitors. The effects of 5FU *in vitro* on the maintenance of MRA progenitors could not be directly determined in our system, as even large hypoxic cultures were unable to support survival of lethally irradiated mice. The early death of transplanted mice suggested that this was most probably due to the insufficient content of late hematopoietic progenitors and maturing precursors, highly sensitive to 5FU, rather than to an insufficient MRA, which, inferring from Table 2 by comparison with culture-repopulating progenitors, was reduced to 16–32%. The demonstration of a significant 5FU resistance of hypoxia-maintained progenitors represents another advancement over our previous study.¹

When the data relative to the maintenance of progenitors in hypoxia and to their 5FU-resistance were taken together (Figure 3), the subset of progenitors repopulating cultures with CFU-GM appeared functionally heterogeneous. Out of four of these progenitors present in culture at time-zero, indeed, one was hypoxia-maintained and 5FU-resistant, two were hypoxia-maintained and 5FU-sensitive, and one was hypoxia- and 5FU-sensitive. As for the comparison, among hypoxia-maintained cells, of 5FU-resistant with 5FU-sensitive progenitors, the hypothesis that the former are quiescent, while the latter are in cycle, is a very straightforward one. This hypothesis is also in keeping with the fact that in air, where all cells are presumably cycling, no progenitor survived the treatment with 5FU. Thus, hypoxia appears as a necessary, although not sufficient, condition for quiescence of short-term repopulating (STR) cells, such as MRA_{CFU} progenitors or progenitors repopulating cultures with CFU-GM. In hypoxia, in other words, STR progenitors would be able to remain in a quiescent state, yet being recruitable to commitment and clonal expansion.^{10,11} On this basis, the 5FU experiments reported here provide experimental support to our hypothesis that, in relatively hypoxic areas of BM, the progenitors' response to recruitment/proliferation/differentiation stimuli is modulated in favor of their maintenance and long-term conservation.¹ To generate lineage-restricted progeny and then undergo extensive clonal expansion, STR progenitors would be required to move to areas of BM at higher oxygen tensions, a process mimicked in our experimental system by culture transfer to air.

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The above scenario is certainly influenced by the available growth factors. The combination of IL3 and GM-CSF used in our study supports proliferation of multipotent progenitors¹² and survival in culture, as well as 'proliferation competence',¹³ but not cell cycling^{14–16} of quiescent stem cells. The latter is, indeed, triggered by 'proliferation progression' factors, which include stem cell factor, interleukin-6, interleukin-11, granulocyte colony-stimulating factor, leukemia inhibitory factor and FLT3-ligand. Therefore, under the conditions described here, only STR progenitors activated *in vivo* would be recruitable, but not long-term repopulating (LTR) quiescent stem cells imported as such to cultures. We are currently studying, by means of the experimental system presented here, the effects of 'proliferation progression' factors on LTR progenitors in hypoxia, examining in particular the possibility that hypoxia favors the generation of STR progenitors in culture.

In conclusion, we showed that incubation at a rather low oxygen tension (1%) reversibly blocks the expansion of hematopoietic clones. The expansion potential of these clones is unreduced, so that the counts obtained from hypoxic cultures following their transfer to air reflect the number of hypoxia-maintained progenitors. A sizeable percentage of these progenitors is 5FU-resistant. The complex of these data represents a significant advancement over the results of our previous study.¹ Furthermore, two subsets of culture-repopulating progenitors were identified, whose percentages of maintenance in hypoxia and resistance to 5FU were similar to those of the two main subsets of MRA progenitors. Thus, the repopulation of hypoxic cultures after their transfer to air appeared suitable as an indicator of MRA maintenance in hypoxia. The experimental system described here can be useful, in studies of murine hematopoiesis, to overcome problems due to the low probability of survival of animals transplanted with *in vitro* manipulated cell populations; in human studies, it is significantly easier and more rapid to perform than the long-term culture-initiating cell assay. We plan to use this system for the characterization of maintenance in hypoxia, response to growth factors and sensitivity to cell cycle-specific cytotoxic drugs, of progenitors from acute or chronic myelogenous leukemia explants.

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