Hematopoietic Precursors Resistant to Treatment With 4-Hydroperoxycyclophosphamide: Requirement for an Interaction With Marrow Stromal in Addition to Hematopoietic Growth Factors for Maximal Generation of Colony-Forming Activity

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We tested the ability of CD34+lin− precursor cells isolated from marrow after treatment with 4-hydroperoxycyclophosphamide (4HC) to generate colony-forming cells (CFC). In liquid cultures, recombinant human stem cell factor (SCF), in combination with interleukin-1 (IL-1), IL-3, IL-6, granulocyte-macrophage colony-stimulating factor, or granulocyte colony-stimulating factor caused untreated, but not 4HC-treated, CD34+lin− cells to form CFC. However, generation of CFC from CD34+lin− cells treated with 60 μg/mL of 4HC was possible in the presence of an irradiated allogeneic stromal cell layer. This generation was increased when combinations of hematopoietic growth factors including SCF and IL-3 were added. Maximal generation of CFC was seen after 11 to 21 days of culture. At that time, generation of CFC from CD34+lin− 4HC-treated cells equalled that from untreated cells. The phenotype of these 4HC-resistant CD34+lin− precursors was also further defined as CD38−. These studies show that the generation of CFC from the 4HC-resistant, highly immature population of CD34+lin− cells requires an as yet undefined interaction with marrow stroma in addition to known hematopoietic growth factors.

The nature of the stem cells responsible for hematopoietic engraftment, and the factors that influence their proliferation and differentiation, are not fully understood. Studies in mice have shown that the cell responsible for long-term marrow repopulation can be identified based on differences in physical properties (density and size), expression of cell surface antigens, and cell cycle status.1,4 These stem cells are thought to be mainly quiescent, relatively dense cells with a phenotype of Thy-1+ SCA-1+ and to lack expression of lineage-associated antigens (lin−). In humans, these precursors are thought to be CD34+ and CD33− and are presumed to lack other antigens associated with committed lymphoid or myeloid cells (lin−).5,12 This is based on the observations that isolated CD34+ cells are capable of reconstituting the marrow after marrow ablative therapy in human and nonhuman primates,5,6 and that hematopoiesis can be reconstituted with marrow depleted of CD33+ colony-forming cells (CFC).7 (unpublished observations) or depleted of cells expressing T- or B-cell-associated antigens.8,9

In vitro studies of normal marrow, the CD34+lin− population has been shown to contain precursors of CFC, and the more mature CD34+lin− progenitor population to contain CFC, but not their precursors.10,11 These CD34+lin− precursors have been shown to generate colony-forming units-granulocyte-macrophage (CFU-GM) and/or burst-forming units-erythroid (BFU-E) when cultured in the presence of an allogeneic irradiated marrow stromal cell layer or the ligand for the c-kit tyrosine kinase receptor (stem cell factor [SCF]) in combination with other growth factors such as interleukin-3 (IL-3).10,12

In other in vivo studies in humans, treatment of marrow with 4-hydroperoxycyclophosphamide (4HC), a derivative of cyclophosphamide, has been used to purge myeloid leukemia cells from marrow as part of an approach for autologous marrow transplantation.13,14 This treatment has been shown to spare, in vitro, the more primitive cells required for in vivo hematopoietic reconstitution. This treatment has also been shown to eliminate CFC, including CFU-GM, BFU-E, and CFU-mix, in a dose-dependent fashion while sparing relatively more primitive hematopoietic precursors.15-20 These latter cells have been detected by their ability to form blast cell colonies or to give rise to CFU-GM in long-term marrow culture.20,21

In the present study we determined the requirement for marrow stroma and/or other growth factors to induce the 4HC-resistant subpopulations of CD34+lin− precursors to give rise to CFC progeny. We show that the 4HC-resistant precursors, unlike CD34+lin− cells from untreated marrow, require an interaction with stroma to permit a maximal response to hematopoietic growth factors, including IL-3 and SCF.

MATERIALS AND METHODS

Separation of marrow cells. Marrow samples were obtained with informed consent from healthy bone marrow donors undergoing marrow harvesting, and cells were separated by density-gradient centrifugation.10 A portion of the cells was treated with 4HC as described below. Initial enrichment of 4HC-treated or untreated lin− cells was achieved by immunomagnetic separation using beads bound to goat antimouse IgG (Dynal A.S., Oslo, Norway) after labeling the cells with IgG antibodies 35.1 (CD2), 24.1 (CD10), H37 (CD19), I5 (CD20), p67 (CD33), 60.1 (CD11b), R10 (anti-tiglycoporphin A), and 7B9. The specificity of each of these antibodies has been described, except for 7B9, which reacts with more than 70% of CFC, including virtually all BFU-E and CFU-GM, but not with precursors of CFC detectable in long-term marrow culture.11

The enriched lin− cells were then stained simultaneously with the IgG antibodies listed above followed by an anti-y chain-specific

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goat antiserum labeled with fluorescein isothiocyanate (FITC; Kirkegaard & Perry, Gaithersburg, MD), and with the IgM antibody 12-8 (CD34) followed by an anti-μ chain-specific goat antiserum labeled with phycoerythrin (PE; Biomeda, Foster City, CA). As controls, cells were labeled with isotype-matched antibodies of irrelevant specificities.22 Cells were analyzed and sorted using a modified FACS II equipped with a modified single cell deposition unit (Becton Dickinson, Mountain View, CA).11 To verify the sorting of single events in cloning experiments, single fluorescent beads were sorted into microtiter wells and directly visualized in each well.11 CD34+ cells with low or negative staining with IgG antibodies (1%) were separated from the negative population by 5 channels.12 Isolation of the CD34+ subset of CD34+lin- cells was accomplished by restaining these cells with CD34 (Becton Dickinson, San Jose, CA), followed by an FITC-labeled second step, and then resorting the cells into CD34+ and CD34- populations.

Treatement of marrow cells with 4HC. Light-density cells were incubated with 60 μg/mL freshly dissolved 4HC (gift of a Pharmaceutic Corporation, Baltimore, MD) for 30 minutes at 37°C at a cell concentration of 2 × 10^6 cells/mL in RPMI-1640 (GIBCO Laboratories, Grand Island, NY) containing 20% fetal bovine serum (FBS; Hyclone, Logan, UT). At the end of this incubation, the cells were diluted with cold RPMI-1640 and washed twice before staining and cell separation.

CFC assays. Isolated cells from liquid cultures of single CD34+lin- cells (see below) were cultured in 1 mL of Iscove’s modified Dulbecco’s medium (IMDM) and 0.9% methylcellulose (Terry Fox Cancer Center, Vancouver, BC, Canada) supplemented with 20% FBS (Intergen Co, Purchase, NY), 1% bovine serum albumin (BSA), 10^-4 M 2-mercaptoethanol (BioRad Laboratories, Richmond, CA), 20% human placental conditioned medium (HPCM), IL-3 (100 ng/mL), and erythropoietin (Epo; 2 U/mL) as previously described, unless otherwise specified.22 Cultures were incubated at 37°C in 5% CO2 in air for 14 days.

Liquid cultures. Single CD34+lin- cells were cultured in microtiter wells either in IMDM supplemented with 20% FBS and recombinant human growth factors (Tables 1 and 2) or in modified α-minimal essential medium (α-MEM) supplemented with 5 × 10^-5 M 2-mercaptoethanol, 10^-4 M hydrocortisone, 12.5% FBS, and 12.5% horse serum ( Irvine Scientific, Santa Ana, CA). Cultures were fed once weekly with appropriate media. At designated times the number of cells in each well was enumerated, and the cells (from wells with proliferation) were then harvested and plated in one dish for colony formation as described above.

**Table 1. Formation of CFC by Single CD34+ lin- Cells in Liquid Culture (7-day culture without stroma)**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of Wells With CFC/Total (% wells)</th>
<th>4HC-Treated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8/72 (11.1)</td>
<td>0/72 (0)</td>
<td>&lt;.004</td>
</tr>
<tr>
<td>2</td>
<td>6/72 (8.3)</td>
<td>2/144 (1.4)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>3</td>
<td>6/72 (8.3)</td>
<td>3/360 (0.8)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Single CD34+lin- cells were cultured in the presence of 100 ng/mL of IL-3, G-CSF, SCF, and, for experiment no. 3, 4 U/mL Epo. After 7 days of incubation, the contents of wells containing proliferating cells were tested for CFC activity in semisolid medium. The number of proliferative wells containing CFC compared with the total number of wells seeded is shown. Experiments no. 2 and 3 are the same marrow samples shown in Table 2.

**Stromal cells.** Adherent stromal cell layers were established in 75 cm² flasks from allogeneic marrow using techniques previously described.23 The stromal cells were harvested by trypsinization of confluent 3- to 4-week-old adherent layers and added to microtiter wells. After 2 to 3 days to allow regrowth to confluence, the stromal cells were irradiated (1,500 cGy). Cells and growth factors were added as for the liquid cultures above. The presence of stroma precluded accurate determination of wells with proliferation. Therefore, all wells with stroma were subcultured for colony formation after harvesting of the cells by mechanical means.

**Growth factors.** Recombinant human c-kit ligand (SCF), IL-3, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), IL-1, IL-6, and Epo were used as purified material at 100 ng/mL, shown previously to represent optimal concentrations, unless otherwise specified. (All growth factors were provided by Amgen, Thousand Oaks, CA.)

**Statistical analysis.** Results of liquid culture are expressed as the number of wells (single cells) giving rise to CFC in secondary cultures, and the number of CFC generated per 100 CD34+lin- cells initially separated for single-cell culture. The numbers of CFC generated are expressed as the mean ± SEM. Comparisons between groups for the number of cells generating CFC were made using χ² statistics.

**RESULTS**

**Generation of CFC from 4HC-treated CD34+ precursors in liquid culture.** In previous studies we showed the ability of single CD34+lin- precursors to give rise to multiple CFC when cultured in the presence of hematopoietic growth factors that included SCF.22 We therefore first tested the ability of isolated CD34+lin- precursors from marrow that had been treated with 4HC at 60 μg/mL, a dose equivalent to that used to treat marrow from patients with acute myeloid leukemia (AML) before autologous transplantation.25 Single CD34+lin- cells were isolated from 4HC-treated and untreated marrow and deposited in individual wells of microtiter plates using fluorescence-activated cell sorting. The cells were then cultured for 7 days with a combination of hematopoietic growth factors including SCF, IL-3, G-CSF, and, in one of three experiments, Epo (Table 1).

In each of three experiments, microscopic examination of the wells showed that cellular proliferation had occurred in a significant proportion of the wells established with a single CD34+lin- cell from untreated marrow (13.9% to 38.9% of wells [data not shown]). In contrast, proliferating cells were infrequently noted in wells established with precursor cells from marrow that had been treated with 4HC (1.4% to 2.8%). In the absence of proliferation, cells were not detected in the culture wells, indicating cell death.

The contents of each of the wells in which proliferation had occurred were plated in semisolid medium and tested for their colony-forming ability. As shown in Table 1, CFC were generated in 8.3% to 11.1% of the cultures initially established with a single precursor from untreated marrow. The vast majority of these CFC were CFU-GM and less than 10% were BFU-E. These numbers are consistent with our previous experience, but lower than those previously reported in studies in which only the blast-sized cells and not small lymphocyte-sized cells were collected.10 In contrast to the results obtained with the control cells, few of the
wells established with 4HC-treated precursors gave rise to CFC (0% to 1.4% of wells).

In these experiments, untreated precursor cells gave rise to 3.4 ± 2.6, 4.7 ± 1.0, and 4.3 ± 2.0 CFC (mean ± SEM), respectively, in the three experiments. However, the few 4HC-treated precursors that gave rise to CFC formed a significantly greater number of CFC than did the precursors detected in untreated marrow (11.5 ± 0.5 and 10.0 ± 6.1 CFC in experiments no. 2 and 3, respectively). Thus, the ability of CD34\(^+\)lin\(^-\) precursors from 4HC-treated marrow to proliferate and form CFC in liquid culture in the presence of hematopoietic growth factors is diminished compared with that of precursors from untreated marrow. However, the few precursors detected in the 4HC-treated marrow did display a higher proliferative potential than those detected in untreated marrow as they gave rise to a greater average number of CFC.

**Influence of marrow stroma on the generation of CFC.** Because 4HC-treated marrow has been shown to generate CFC in the presence of marrow stroma in long-term cultures,\(^{15,20}\) we tested the ability of the isolated CD34\(^+\)lin\(^-\) precursors from 4HC-treated marrow to give rise to CFC when cultured over an irradiated allogeneic marrow stromal cell layer. As part of experiments no. 2 and 3 described in Table 1, single CD34\(^+\)lin\(^-\) cells from 4HC-treated and nontreated marrow were also deposited into individual microtiter wells containing a previously established, adherent, irradiated, allogeneic, feeder layer and cultured in the presence or absence of SCF. After a culture period of 11 days, the entire contents of each well were removed and tested for the presence of CFC by culturing in semisolid media.

A portion of wells containing stroma and a single 4HC-treated cell gave rise to CFC (2.8% and 4.2% of wells in the two experiments, respectively) that were virtually all (95% to 100%) granulocyte/monocyte precursors. Furthermore, as shown in Table 2, in the presence of SCF, there was an apparent increase in the proportion of wells giving rise to CFC (11.1% of wells in both experiments; experiment no. 2, \(P = .12\); experiment no. 3, \(P = .17\); combined results, \(P = .04\), as well as a 5- to 15-fold increase in the number of CFU-GM and BFU-E generated (predominantly myeloid colonies). In contrast to the result seen in the liquid cultures, the number of wells that generated CFC from the 4HC-treated CD34\(^+\)lin\(^-\) cells was not substantially different from the untreated CD34\(^+\)lin\(^-\) precursors (5.6% and 11.1% of wells without SCF and 0% and 19.4% for wells containing the growth factor).

**Influence of 4HC dose on CFC generation.** Cells were cultured with or without stroma in the presence of IL-3 and SCF. Cultures without stroma were harvested after 6 days when cell death was observed in wells in which proliferation had not occurred and after 13 days for cultures containing stroma. The contents of each well were subcultured for CFC. A dose relationship was observed in the absence of stroma (Fig 1). None of the cells treated with 120 \(\mu\)g/mL of 4HC produced CFC in the absence of stroma, and increased numbers of CFC were generated as the dose of 4HC was decreased. In the presence of stroma, no CFC were generated from cells treated with 120 \(\mu\)g/mL of 4HC. However, when stromal cells were present in the cultures with cells treated with 30 or 60 \(\mu\)g/mL of 4HC, the number that generated CFC significantly increased.

<table>
<thead>
<tr>
<th>Experiment No. 2</th>
<th>No. of Wells With CFC/Total Colonies/100 Cells</th>
<th>Colonies/100 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2/36 (5.6)</td>
<td>22.2 ± 19.6</td>
</tr>
<tr>
<td>Treated</td>
<td>3/72 (4.2)</td>
<td>5.6 ± 3.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment No. 3</th>
<th>No. of Wells With CFC/Total Colonies/100 Cells</th>
<th>Colonies/100 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4/36 (11.1)</td>
<td>38.9 ± 20.8</td>
</tr>
<tr>
<td>Treated</td>
<td>1/35 (2.8)</td>
<td>5.6 ± 5.6</td>
</tr>
</tbody>
</table>

* Shown are mean ± SEM number of CFC (CFU-GM + BFU-E) produced after 11 days of culture expressed as the number of CFC generated per 100 CD34\(^+\)lin\(^-\) cells seeded. Also shown is the total number of colonies formed per 100 cells seeded in initial culture. Experiments no. 2 and 3 are the same marrow samples shown in Table 1.

**Fig 1.** Effect of varying concentrations of 4HC on proportion of cells generating CFC (CFU-GM, BFU-E, and CFU-E) in liquid culture. Cells were cultured with or without a preformed adherent stromal layer. A total of 36 wells was seeded for each of the groups. One representative experiment is shown.
phosphatidylcholine was observed in two additional experiments (data not shown).

Kinetics of the generation of CFC in long-term culture. Individual CD34+lin- cells isolated from 4HC-treated and untreated marrow were cultured in the presence of a marrow stromal cell layer and hematopoietic growth factors including IL-3 and SCF. After a period of 1 to 4 weeks, the contents of each well were tested for CFC activity. The 4HC-treated cells generated significantly fewer CFC after 1 week of culture than did the untreated cells (Fig 2). However, by 3 weeks of culture, the 4HC-treated cells generated as many or greater numbers of CFC than did the untreated cells. (Similar results were observed with two other marrow samples; data not shown.) The CFC generated were virtually all of the CFU-GM type, except after only 1 week of culture, when 9% of CFC generated from untreated cells and 44% of those generated from treated cells were BFU-E. Overall, the data suggest that although treatment with 4HC eliminates cells responsible for the initial generation of CFC, it does not affect cells responsible for the longer term production of hematopoietic progenitors, suggesting their greater immaturity.

Influence of combinations of hematopoietic growth factors on generation of CFC in the presence of marrow stroma. The contribution of stromal cells to the generation of CFC from 4HC-treated cells may result from the production of various, known cytokines. To determine if the inclusion of cytokines in addition to SCF and IL-3 could replace or further amplify the effects of stroma, we tested CFC generation from single CD34+lin- cells treated with 60 μg/mL of 4HC. Cells were obtained from five donors (two representative experiments are shown). As shown in Fig 3, the combinations tested supported little or no generation of CFC in the absence of stroma. With all combinations, both the number of 4HC-treated cells generating CFC and the number of CFC generated were greater in the presence of stroma. We also did not observe a consistent effect of cytokine combination on the types of colonies produced, except that combinations, particularly those that also included GM-CSF or G-CSF, appeared more likely to induce erythroid expression.

Phenotype of 4HC-resistant precursors. We further determined the phenotype of the CD34+lin- precursors resistant to treatment with 60 μg/mL of 4HC. Because lack of CD38 expression has been associated with quiescent, immature precursors, we isolated CD34+lin- cells from 4HC-treated and control-treated marrows from two donors. These cells were then resorted into CD38- and CD38+ populations. The results in Table 3 show that most of the precursors that gave rise to CFC after incubation with IL-3, IL-1, and SCF in the presence of irradiated stroma were in the CD38- fraction. This finding was true for untreated as well as 4HC-treated marrow.

DISCUSSION

In the present study we examined the factors required to induce the formation of colony-forming progeny from the subset of marrow precursors that is resistant to treatment with 4HC at a dose known to spare stem cells capable of hematopoietic reconstitution. To examine these cells in purified form, we isolated CD34+ cells that are known to include hematopoietic precursors capable of in vivo engraftment. These cells were also depleted of those cells expressing surface antigens associated with cells committed to individual hematopoietic lineages, including those expressing antigens associated with developing lymphoid cells and myeloid cells. These highly purified CD34+lin- precursors, when obtained from normal marrow, have been shown to form few colonies except when cultured in the presence of combinations of growth factors including the e-kit ligand, or when cultured in the presence of marrow stroma in a long-term culture system.

The results of the current study show that 4HC-resistant precursors represent a distinct subset of the CD34+lin- population. This CD38- and presumably quiescent subset was found to require an as yet undefined interaction with stroma to enable their response to hematopoietic growth factors including IL-3 and SCF.
Fig 3. Effect of different cytokine combinations on the generation of (A) CFU-GM, (B) BFU-E, or (C) CFU-E by CD34⁺lin⁻ cells treated with 60 μg/mL of 4HC and cultured in the presence [a] or absence [b] of previously established stromal cell layers. A total of 36 wells was seeded for each group. Two representative experiments are shown.

Table 3. Formation of CFC by Single CD34⁺lin⁻ Cells Classified by CD38 Expression

<table>
<thead>
<tr>
<th></th>
<th>With stroma</th>
<th>Without stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>4HC-Treated</td>
</tr>
<tr>
<td>CD38⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp 1</td>
<td>0/32 (0%)</td>
<td>3/32 (9.4%)</td>
</tr>
<tr>
<td>Exp 2</td>
<td>3/32 (9.7%)</td>
<td>3/32 (9.4%)</td>
</tr>
<tr>
<td>CD38⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp 1</td>
<td>16/32 (50%)</td>
<td>12/32 (37.5%)</td>
</tr>
<tr>
<td>Exp 2</td>
<td>9/32 (28.1%)</td>
<td>12/32 (37.5%)</td>
</tr>
<tr>
<td>CD38⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp 1</td>
<td>2/32 (6.3%)</td>
<td>4/32 (12.5%)</td>
</tr>
<tr>
<td>Exp 2</td>
<td>0/32 (0%)</td>
<td>2/32 (6.3%)</td>
</tr>
<tr>
<td>CD38⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp 1</td>
<td>5/32 (15.6%)</td>
<td>4/32 (12.5%)</td>
</tr>
<tr>
<td>Exp 2</td>
<td>2/32 (6.3%)</td>
<td>0/32 (0%)</td>
</tr>
</tbody>
</table>

* Single CD34⁺lin⁻ cells were cultured in the presence of 10 ng/mL of IL-1 and 100 ng/mL of IL-3 and SCF. After 13 days of incubation, the contents of wells containing proliferating cells were tested for CFC activity in semisolid medium. The number of proliferative wells containing CFC compared with the total number of wells seeded is shown.† The difference between the proportion of CD38⁺ and CD38⁻ cells proliferating in stroma-containing wells in experiments no. 1 and 2 was significant for untreated (P < .001 and P < .055, respectively) and 4HC-treated (P < .008 for both experiments) cells. Differences between CD38⁺ and CD38⁻ cells, 4HC-treated or untreated, cultured without stroma were not significant.

The contribution of the stroma required to induce the 4HC-resistant precursors to grow was not determined in these studies. Potential signals provided by the stroma include ones delivered by known or novel growth factors presented on their surface or elaborated into the medium, adhesive interactions, or other as yet undefined cellular interactions. It is also possible that the initial events induced by the stroma can occur spontaneously under the appropriate culture conditions in the absence of stroma, as suggested by studies in which it was possible to form blast cell colonies from 4HC-resistant cells. The precise sequence of events leading to the formation of CFC is also not known. The results of the present study suggest that the 4HC-resistant subset of CD34⁺lin⁻ cells require an initial interaction with marrow stromal cells to attain the capacity to respond to the combinations of soluble growth factors known to elicit CFC formation from non-4HC-treated CD34⁺lin⁻ precursors. Consistent with this notion is the observed increased generation of CFC from 4HC-treated precursors when these hematopoietic growth factor combinations were added to the stroma-containing cultures. On the presumption that the 4HC-resistant cells are quiescent ones, these results suggest a model in which the hematopoietic stem cell is a 4HC-resistant CD34⁺lin⁻CD38⁺ precursor that, under the influence of marrow stromal cells, enters the cell cycle and, under the influence of combinations of growth factors such as IL-3 and c-kit ligand, proliferates and differentiates into multiple CFC that are CD34⁺lin⁻. These latter cells are distinguishable from their lin⁻ precursors as they do not require the addition of c-kit ligand to other individual factors to induce CFC formation.

There are a number of practical implications of the pres-
ent study. It is known that treatment of marrow with 4HC to eliminate AML cells also eliminates CFC and leads to a significant delay in engraftment for patients with AML in particular. Because it is thought that the CFC are responsible for the initial recovery of blood cells, it may be possible to generate in vitro CFC from an aliquot of a 4HC-treated marrow and infuse these cells along with the remaining treated marrow cells. However, further improvement in culture conditions will be required to allow the generation of adequate numbers of CFC for use in clinical trials.

REFERENCES


Hematopoietic precursors resistant to treatment with 4-hydroperoxycyclophosphamide: requirement for an interaction with marrow stroma in addition to hematopoietic growth factors for maximal generation of colony-forming activity

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