Effects of Cell Concentrations on the Survival and Repopulation of Haemopoietic Stem Cells in Irradiated Bone Marrow Cell Culture in Vitro

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Effects of cell concentrations on the survival and repopulation of haemopoietic stem cells after irradiation were studied in the long-term culture of mouse bone marrow cells in vitro.

No difference was observed in the survival of the stem cells among cultures in which 0-10^7 cells were re-inoculated on the adherent cell colonies in the culture flask. Stem cells showed a significant proliferation within 1 week and the number of the stem cells exceeded the control in 3 weeks after irradiation in the cultures with less than 10^6 re-inoculated cells per flask. In contrast, there was a considerable delay in the onset of stem cell proliferation after irradiation in the culture with 10^7 cells per flask.

Based on these results, a possibility that a stimulator of stem cell proliferation, released from irradiated stromal cells, is cancelled by an inhibitory factor produced by irradiated or unirradiated haemopoietic cells is postulated.

The clonal assay technique detecting a cell with extensive self-renewal capacity described as in vivo spleen colony assay by Till and McCulloch has promoted the studies on the behaviour of haemopoietic stem cells in response to various stimuli. Dexter's culture system in which an in vitro environment supports long-term proliferation and differentiation of haemopoietic stem cells offers a useful model for the study of the interactions between the microenvironment and the stem cells in vitro. The ability to sample the same culture repeatedly will also allow detailed kinetic studies on cells in vitro.

Substantial recovery of haemopoietic system in vitro was observed after irradiation in the long-term culture of the murine bone marrow cells. Irradiation of the stromal cell colonies (haemopoietic environment in vitro) resulted in the stimulation of the self-renewal of the stem cells in the re-inoculated normal bone marrow cells on the stromal.

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cell colonies until the stem cell concentration exceeded the control level\(^9\), while normal stromal cell colonies did not affect the balance between self-renewal and commitment of the stem cells to differentiation pathway\(^9\). However, a difference in the survival or repopulation of the stem cells in the culture after irradiation was noted depending on the concentration of the bone marrow cells re-inoculated\(^9\).

The purpose of the present experiments is to study in detail the effect of cell concentrations on the survival and recovery proliferation of the stem cells after irradiation and to obtain a system which mimicks best the in vivo situation. This would also help the understanding of the control mechanisms involved in the stem cell proliferation.

MATERIALS AND METHODS

Mice. Eight to twelve week-old male mice of DDY-strain were used under specific pathogen free condition.

Culture of the Bone Marrow Cells. Culture technique was described in detail in the previous reports\(^3\)-\(^8\). Bone marrow cells were cultured in 10 ml Fischer's medium supplemented with 20\% horse serum and antibiotics at 37°C in 5\% CO\(_2\) in air with weekly changes of a half (5 ml) of the growth medium. Three weeks later, when adherent stromal cell colonies had developed well, cultures were washed and freshly harvested bone marrow cells were re-inoculated onto the adherent colonies at various cellular concentrations. Cultures were continued as before, and the cells in the altered medium were counted and assayed for CFUs and CFUc. In one group of cultures, the culture medium was gently decanted at 3 weeks, and fresh growth medium was immediately introduced. This allowed a repopulation of the haemopoietic cells from the stem cells trapped among the adherent cell colonies.

Assay for CFUs and CFUc. Appropriate numbers of the cells in the altered culture medium were injected i. v. into lethally irradiated mice, and the colonies on the spleen were counted as CFUs 8 days later\(^9\). Cells were also inoculated in the semi-solid culture medium, containing 20\% mouse abdominal wall-conditioned medium as colony-stimulating activity, cultured at 37°C for 7 days in 5\% CO\(_2\) in air, and the colonies consisting of more than 50 cells were counted as CFUc\(^10\). Proportion of granulocytic, macrophage and mixed colonies did not vary significantly and is therefore not presented in the results.

Irradiation. Recipient mice for CFUs assay were irradiated with 800 R of X-rays operating at 180 kVp-20 mA at a rate of 50 R/min through a 1.0 mm Al+0.5 mm Cu filter. The cells in the culture were irradiated with 200 R 24 hr after the re-inoculation of the bone marrow cells at a rate of 250 R/min through a 1.0 mm Al+0.3 mm Cu filter. One roentgen is roughly equivalent to 0.97 rad under the present conditions.

RESULTS

In the first experiments, bone marrow cells were irradiated with 200 R of X-rays in culture, and the survival of the stem cells were determined for each cellular con-
CFUs PROLIFERATION AND CELL DENSITY IN IRRADIATED CULTURE

concentration immediately after exposure. Results are shown in Table 1.

There was no difference in the survival of the stem cells, either CFUs or CFUc, among the cultures at the concentrations of 10⁴-10⁶ cells/ml. The survival was about 5% for CFUs and about 10% for CFUc in any cultures tested. These results are in the range of the previous observations⁹.

As the cultures without the secondary inoculation of the bone marrow cells had few cells in suspension at the time of the irradiation, survival of the stem cells in such cultures was not determined.

To see if there is any differences in the patterns of repopulation of the stem cells in cultures in relation to cell density, the irradiated cultures were kept for 3 weeks as before.

Data at 1 week after irradiation are presented in Table 2. All the control, unirradiated cultures had similar numbers of CFUs, CFUc and total cells in suspension regardless of the concentration of the bone marrow cells re-inoculated. This was in good agreement with the previous observation⁹. There was, however, a considerable difference in the number and concentration of the stem cells in irradiated cultures between the cultures at 10⁶ cells/ml (high density culture) and those at less than 10⁶ cells/ml (low density cultures). The concentration of CFUs was around 50% of the control in low density cultures indicating a significant increase had occurred within 1 week after irradiation. In high density cultures, the concentration of CFUs as well as that of CFUc still remained very low. Thus, the recovery proliferation or repopulation of the stem cells from surviving CFUs was considerably slow in high density cultures.

Such was also found at 3 weeks after irradiation (Table 3). There was a significant increase in the concentration as well as in the number of total CFUs per flask in low density cultures. Although substantial increase in the concentration of CFUs was observed in high density cultures as well, the degree of the increase was less than that in low density cultures. In contrast, the number of CFUc per 10⁶ cells was less in low density cultures than that in high density cultures at this time.

Table 1.

<table>
<thead>
<tr>
<th>No. of cells re-inoculated</th>
<th>Dose of X-rays (R)</th>
<th>No. of CFUs/10⁶ cells (% control)</th>
<th>No. of CFUc/10⁶ cells (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁵ cells/ml</td>
<td>0</td>
<td>577±103</td>
<td>165±32</td>
</tr>
<tr>
<td>(10⁶ cells/flask)</td>
<td>200</td>
<td>31±4.6</td>
<td>19±4.2</td>
</tr>
<tr>
<td>10⁶ cells/ml</td>
<td>0</td>
<td>572±92</td>
<td>143±18</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>28±5.4</td>
<td>14±2.3</td>
</tr>
</tbody>
</table>

Bone marrow cells were overlayed on the adherent cell colonies in culture, irradiated and assayed for CFUs and CFUc immediately.

Mean±S.E. (8 mice per point for CFUs, 4 cultures per point for CFUc)
Table 2.
Number of Haemopoietic Cells in 1-Week Culture of Irradiated Bone Marrow Cells

<table>
<thead>
<tr>
<th>Dose of irradiation</th>
<th>No. of cells overlaid (×10⁶)</th>
<th>Cells per flask (×10⁶)</th>
<th>CFUs per 10⁶ cells</th>
<th>CFUs per flask</th>
<th>CFUc per 10⁶ cells</th>
<th>CFUc per flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td>1.3</td>
<td>402 ± 56</td>
<td>553 ± 73</td>
<td>331 ± 21</td>
<td>4303 ± 273</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>1.8</td>
<td>388 ± 46</td>
<td>698 ± 83</td>
<td>365 ± 20</td>
<td>6570 ± 360</td>
<td></td>
</tr>
<tr>
<td>200 R</td>
<td>1.3</td>
<td>227 ± 31</td>
<td>250 ± 34</td>
<td>143 ± 10</td>
<td>1573 ± 110</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(56.5%)</td>
<td></td>
<td>(43.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>204 ± 13</td>
<td>306 ± 20</td>
<td>126 ± 21</td>
<td>1890 ± 315</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(53.4%)</td>
<td></td>
<td>(40.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>18 ± 12</td>
<td>22 ± 14</td>
<td>35 ± 11</td>
<td>424 ± 130</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.5%)</td>
<td></td>
<td>(9.5%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bone marrow cells were irradiated 24 hours after the re-inoculation at various concentrations in culture. Percentage of the corresponding control value is shown in the parenthesis. Gross mean ± S.E. of 2 separate experiments. *; No cells were overlaid.

Table 3.
Number of Haemopoietic Cells in 3-Week Culture of Irradiated Bone Marrow Cells.

<table>
<thead>
<tr>
<th>Dose of irradiation</th>
<th>No. of cells overlaid (×10⁶)</th>
<th>Cells per flask (×10⁶)</th>
<th>CFUs per 10⁶ cells</th>
<th>CFUs per flask</th>
<th>CFUc per 10⁶ cells</th>
<th>CFUc per flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.4</td>
<td>359 ± 62</td>
<td>503 ± 87</td>
<td>381 ± 58</td>
<td>5334 ± 812</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>1.1</td>
<td>380 ± 97</td>
<td>418 ± 107</td>
<td>342 ± 27</td>
<td>3773 ± 309</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>1.4</td>
<td>385 ± 51</td>
<td>539 ± 71</td>
<td>328 ± 28</td>
<td>4592 ± 392</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.2</td>
<td>840 ± 87</td>
<td>1008 ± 104</td>
<td>335 ± 50</td>
<td>3996 ± 600</td>
<td></td>
</tr>
<tr>
<td>200 R</td>
<td>0.9</td>
<td>828 ± 115</td>
<td>745 ± 104</td>
<td>247 ± 20</td>
<td>2223 ± 180</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(218%)</td>
<td></td>
<td>(72.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>537 ± 64</td>
<td>383 ± 57</td>
<td>476 ± 39</td>
<td>4184 ± 351</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(139%)</td>
<td></td>
<td>(145%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See Table 2. for explanation.

DISCUSSION

Studies on the effects of cell concentration before, during or after irradiation on the radiosensitivity have revealed that the survival of the cells depends on the degree of intercellular contact, and that the response of the cells to radiation in the stationary growth phase is different from that in logarithmic growth phase. Toxic substances produced by dead cells and/or nutrient depletion was proposed to be partly responsible for the lower survival in the culture at high cell density.

Most of the in vitro experiments, however, have so far been performed with established cell lines in which no intercellular interactions between stromal and parenchymal cells were reconstituted. These systems therefore may not validly represent in vivo situation.
In the long-term culture of the murine bone marrow cells, haemopoietic cells grow under a control of the stromal cell colonies. This culture system may exclude the possibility of contact inhibition of the cells playing an essential role in haemopoiesis. In fact, there was little difference in the survival of haemopoietic stem cells among cultures at different cell densities. In contrast, the pattern of the recovery following irradiation was quite different between cultures at high density and those at low densities. The difference appears essentially to be manifested as a delay of the onset of CFUs proliferation in high density cultures.

Production of a stimulator of CFUs proliferation by the bone marrow cells was reported previously\textsuperscript{15,16}. The cells responsible for the production of such stimulator have been shown to be those other than haemopoietic cells\textsuperscript{17}. We have also shown that irradiation of the stromal cell colonies alone resulted in an accelerated proliferation of the stem cells, and have suggested that the stromal cells are reponsible for the production and release of the stimulator\textsuperscript{9}.

If the haemopoietic cells in suspension killed by the irradiation released a stimulator of CFUs proliferation, there should be a higher concentration of the stimulator in the culture at high cell density. This does not seem to be the case, since the degree of the recovery was quite the same between the culture with $10^6$ cells/ml and that without re-inoculation of the bone marrow cells. Thus, our present observations also support the concept that the CFUs stimulator is produced by the stromal cells\textsuperscript{9}.

Endproduct inhibition plays a key role in the regulation of the proliferation of single cell organisms\textsuperscript{18}. Haemopoietic system could be taken as an assembly of single cells in suspension in vivo migrating freely in the blood stream or trapped by the stromal cells in haemopoietic tissues. In fact, polycythemia, induced by injections into mice of large numbers of erythrocytes, is known to result in the arrest of the proliferation and differentiation of their precursor cells\textsuperscript{19,20}. An inhibitor of CFUs proliferation was also detected in the normal bone marrow in which CFUs is dormant\textsuperscript{21,22}. Wright and Lord fractionated the mouse bone marrow cells and found the inhibitory activity in a fraction other than CFUs\textsuperscript{17}. Schofield found that the number of CFUs remained subnormal when total differentiated cells in haemopoietic tissues as well as in the peripheral blood recovered to normal level in mice following exposure to 50 rads of X-rays (personal communication). Hirashima also found a similar phenomenon (Publication in Japanese, 1979).

These findings indicate that CFUs proliferation is at least partly controlled by endproduct inhibition and that mature cells in the haemopoietic tissues are responsible for the production of the inhibitory factor. Suppression of the CFUs proliferation in high density cultures may well be explained by this concept. Since all the stromal cell colonies were irradiated under a similar condition, the same amount of the stimulator should have been released from these cells in all the cultures. Yet, there was a considerable delay in the onset of the proliferation, or suppression of the stem cells after irradiation was observed in the cultures at high cell density. If a fixed amount of the inhibitor is released from a single cell, there will be more than 10 times as much
inhibitor in the culture with $10^6$ cells/ml as in the cultures with less than $10^4$ cells/ml. This increased amount of the inhibitor in the high density cultures might have cancelled the stimulatory activity produced by the irradiated stromal cells. Following changes of the growth medium, then, would reduce the inhibitory factor by diluting out at each medium change, while stimulator is produced continuously in the culture. This would certainly allow the CFUs to start proliferation at later times. Such may be mimetic with the effect of post-irradiation bleeding which is known to stimulate the recovery proliferation of CFUs after irradiation $^{21,23}$.

Accordingly, it may not be too unreasonable to conclude that the stromal cells release a stimulator of CFUs proliferation in response to irradiation whereas irradiated or unirradiated haemopoietic cells other than CFUs release an inhibitory factor. Even if such is the case, further studies are necessary to elucidate whether the inhibitor acts directly on CFUs in competitive manner with the stimulator as suggested in our previous report$^6$, or indirectly by suppressing the production or release of the stimulator by the stromal cells.

Other possibilities should not be neglected, however, that irradiated bone marrow cells simply release non-specific toxic substances hence resulting in the deterioration of the culture condition, or nutrient depletion in the cultures at high cell density$^{12,14}$. At least the latter seems to be negligible since there were no significant differences in the numbers of CFUs, CFUc and total cells in suspension in the control, unirradiated cultures at any cell density.

The long-term culture system of the mouse bone marrow cells has thus been shown to offer an in vitro model for the kinetic studies on the proliferation and differentiation of the stem cells and on the role of the stromal cells in haemopoiesis.

ACKNOWLEDGEMENT

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