Radiosensitivity of Mouse Myeloid Stem Cells in Culture after Stimulation by Macrophage Colony-Stimulating Factor

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Mouse bone marrow cells were plated in a semi-solid medium containing an excess amount of macrophage colony-stimulating factor (M-CSF). The stimulated macrophage colony-forming cells (CFU-M) took about 40 hours before dividing into two cells. X-Ray irradiation of the cells shortly after plating decreased the survival of CFU-M, while the quasithreshold dose (Dq) and the 37% dose slope (Do) of the dose-response curve were 0.54 Gy and 1.27 Gy. Irradiation 5-50 hours after plating decreased CFU-M survival to the same extent as irradiation shortly after plating. Furthermore, the survival rate of CFU-M was similar to the above when the cells were irradiated in a liquid medium containing no M-CSF. These results suggest that the radiosensitivity of CFU-M does not notably change after their stimulation by M-CSF.

INTRODUCTION

The present study examines the radiosensitivity of monocyte/macrophage progenitor cells (CFU-M) in vitro by using monocyte/macrophage colony-stimulating factor (M-CSF, or CSF-1). Previous workers who studied the radiosensitivity of colony-forming cells in culture (CFU-C: myeloid stem cells) used feeder cell layers1-4) or crude cell-conditioned media5,6) as the source of the hematopoietic factors and did not characterize the cells that were produced in the culture. In the present study, we used a purified M-CSF preparation which stimulated proliferation only of CFU-M and thus were able to determine the radiosensitivity specifically of CFU-M.

We also examined time-dependent change of radiosensitivity of CFU-M after their stimulation by M-CSF. It is known that CFU-M are absolutely dependent on M-CSF for their prolifera-

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Abbreviations: CSF, colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; CFU-C, colony forming units in culture (myeloid stem cells); CFU-M, macrophage CFU (progenitor cells of macrophages); Do, 37% dose slope of the straight portion of the cell survival curve; Dq, quasithreshold dose of the cell survival curve.
eration. In the absence of M-CSF, unstimulated CFU-M spontaneously lose their proliferative capability, while a delay in supplying M-CSF by several hours results in a marked retardation of subsequent formation of colonies of the progeny cells of CFU-M. It is also known that M-CSF binds rapidly to CFU-M\(^{7}\), whereas the stimulated CFU-M enter into S phase after a lag period of 12 hours\(^{8}\). The supposition that radiosensitivity of CFU-M may change after stimulation by M-CSF suggested us to carry out the present study, which would provide us basal conditions for further detailed radiobiological studies on the myeloid stem cells in culture.

**MATERIALS AND METHODS**

**Culture of mouse bone marrow cells:** Femoral marrow cells from 6–8 month old male mice of the C3H/He strain were suspended at a density of 5–20 \(\times 10^4\)/ml in McCoy's 5A medium containing 0.3% agar and 20% horse serum. The CSF solution was added in the culture medium in various concentrations before the agar was solidified at room temperature. The cell suspension was then plated in 1.0-ml aliquots in 35-mm plastic petri dishes and incubated at 37°C for 7 days under air containing 5% CO\(_2\). The number of the developed cell colonies consisting of 50 cells or more was scored under a microscope after staining of the cells in situ with hematoxylin.

**Human urine M-CSF:** An M-CSF was purified from normal human urine to near homogeneity by high-performance liquid chromatography similar to that described by Hatake et al.\(^{9}\) One unit of M-CSF was defined as the amount required to produce one colony per 1 \(\times 10^5\) bone marrow cells. Morphological (hematoxylin staining) and histochemical (esterase staining by 1-naphthyl butyrate) examination showed that all the colonies developed both in the culture of normal and irradiated cells were composed solely of monocyte/macrophages.

**Irradiation of the bone marrow cells in culture:** At various time intervals, the bone marrow cells in the semi-solid agar medium were irradiated with X-rays in air at room temperature as previously described\(^{10}\). Briefly, the X-ray generator (Shimazu Shin-ai 250-II) was operated at 200 kVp and 20 mA with 0.5-mm Cu plus 0.5-mm Al filters (HVL: 1.16 mm Cu). The dose rate was 1.0 Gy/min (FDS; 50 cm) as monitored by a Radocon Model 575A ionization gauge. The dishes were returned to the CO\(_2\) incubator immediately after irradiation. In one experiment, the bone marrow cells were suspended at a density of 3 \(\times 10^6\) cells/ml in McCoy’s 5A medium containing no M-CSF, placed in 1.5-ml portions in 5-ml Nunclon N-1409 tubes and irradiated with X-rays. The cells were then brought to the semi-solid agar culture as described above.

**Microscopic examination of the growing colonies:** The cultures were photographed at a magnification of 40 under an inverted tissue culture microscope (Olympus Model CK). One field covered about 0.05 cm\(^2\) or about 1/150 of the total area of the 35 mm dish. Each field was identified by a mark on the bottom of the dish and photographed once a day for several consecutive days. The number of cells in individual growing colonies was scored on the photograms.
RESULTS

Specified locations of seven different dishes of normal bone marrow cell culture were photographed daily to follow the time course of the development of the macrophage colonies, and it was found that the number of cells in each colony increased exponentially after the CFU-M began to proliferate (Fig. 1). Extrapolation of the growth curves to the axis of abscissa indicated that there was a lag period of about 40 hours before individual CFU-M divided into two cells. In addition to these colonies, clusters of 2–8 cells were formed during the first 2 or 3 days, but they did not grow to the size of a colony.

In the subsequent study, bone marrow cells were irradiated with X-rays 30 minutes after plating in the semi-solid agar medium containing various concentrations of M-CSF. Figure 2 shows the increase in the number of developed macrophage colonies as a function of initial M-CSF concentration. The dose-response curves coincidentally leveled off at the M-CSF con-
concentration of about 200 units/ml for both non-irradiated and X-irradiated groups.

Table 1 shows that the radiosensitivity of stimulated and unstimulated CFU-M were similar. No significant difference was observed when the cells were irradiated in a liquid medium containing no M-CSF or irradiated after suspended in a semi-solid medium containing an excess amount of M-CSF. This result shows that M-CSF has no apparent effect on the radiosensitivity of CFU-M with respect to either sensitization or protection.

The effects of X-ray doses on CFU-M were studied in cultures containing an excess amount of M-CSF. The data shown in Fig. 2 and those similarly obtained by our other experiments were combined to give the cell survival curve shown in Fig. 3. The experimental data were fitted to a multi-target model to obtain $D_0$ (1.27 Gy), $D_q$ (0.54 Gy) and $n$ (1.54) with a correlation coefficient of 98.8%. The cell survival values observed are those expected using the multi-target model. Fitting of the same data to the linear-quadratic model gave $-4.82 \times 10^3$/rad and
The effect of X-rays on the colony number was slightly enhanced when radiation was applied 20-30 hours after plating of the cells, whereas it was reduced when the time interval between cell plating and irradiation was 50 hours or longer (Fig. 4). This pattern was most clearly observed with 1 Gy of X-rays; immediate irradiation decreased the colony number by 39% whereas irradiation 20 or 95 hours after cell plating resulted in 50% or 20% decrease of the colony number, respectively.

- 4.42 x 10^6/rad for the constants alpha and beta, respectively.

Table 1. Radiosensitivity of CFU-M in the absence and in the presence of M-CSF.

<table>
<thead>
<tr>
<th>Condition of irradiation</th>
<th>CFU-M survival (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Gy</td>
</tr>
<tr>
<td>in a liquid medium containing no M-CSF</td>
<td>62.5 ± 2.8</td>
</tr>
<tr>
<td>in a semi-solid medium containing 200 units/ml M-CSF</td>
<td>61.6 ± 3.5</td>
</tr>
</tbody>
</table>

Each figure in the table represents the average ± S.E. of 12 dishes from 3 experiments. Controls were not irradiated and produced 107.3 ± 3.6 colonies per dish. Separately, it was shown that the rate of survival of the non-irradiated CFU-M was not changed significantly when the bone marrow cell suspension was left without M-CSF for the period of time (about 15 min) required for the irradiation procedure.

Table 2. Summary of the data on the radiosensitivity of CFU-C irradiated in vivo or in vitro.

<table>
<thead>
<tr>
<th>Marrow cells</th>
<th>Conditions of irradiation</th>
<th>CSF source</th>
<th>D_0 of CFU-C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>in vivo, 48 hrs before plating</td>
<td>kidney cell feeder layer</td>
<td>0.85 Gy</td>
<td>1)</td>
</tr>
<tr>
<td>Mouse</td>
<td>in vivo, 24 hrs before plating</td>
<td>kidney cell feeder layer</td>
<td>0.95 Gy</td>
<td>2)</td>
</tr>
<tr>
<td>Human</td>
<td>as suspended in solution</td>
<td>kidney cell feeder layer</td>
<td>1.37 Gy</td>
<td>3)</td>
</tr>
<tr>
<td>Mouse</td>
<td>as suspended in solution</td>
<td>kidney cell feeder layer</td>
<td>1.60 Gy</td>
<td>3)</td>
</tr>
<tr>
<td>Human</td>
<td>as suspended in solution</td>
<td>leukocyte feeder layer</td>
<td>0.45 to 1.65 Gy</td>
<td>4)</td>
</tr>
<tr>
<td>Mouse</td>
<td>as suspended in solution</td>
<td>mouse abdominal wall conditioned medium</td>
<td>1.55 Gy</td>
<td>5)</td>
</tr>
<tr>
<td>Human</td>
<td>as suspended in solution</td>
<td>PHA-stimulated leukocyte conditioned medium</td>
<td>1.14 Gy</td>
<td>6)</td>
</tr>
<tr>
<td>Mouse</td>
<td>as seeded in soft agar</td>
<td>purified M-CSF</td>
<td>1.26 Gy</td>
<td>Present study</td>
</tr>
</tbody>
</table>

- 4.42 x 10^6/rad for the constants alpha and beta, respectively.
DISCUSSION

We examined the effect of X-rays on the proliferative activity of CFU-M in the presence of an excess amount of purified M-CSF. The observed radiosensitivity of CFU-M, in terms of $D_0$, $D_q$, and $n$, was not much different from those reported by other workers who used unspecified stimulants for the colony-forming cells (Table 2). The reports by other workers also indicate that the colony forming cells are more radiosensitive when they are irradiated in vivo than when they are irradiated in vitro. The high radiosensitivity observed in vivo may be due to migration of CFU-M from the damaged bone marrow to spleen.

It has been reported that nearly all CFU-C in bone marrow are constantly regenerat-
Fig. 4. Effect of time interval between plating and irradiation on the number of colonies produced in the bone marrow cell cultures. A hundred thousand cells were plated in each dish, incubated at 37° in the presence of 200 units of M-CSF for the interval as indicated, and then irradiated with 1–3 Gy X-rays. Each point in the figure represents the average of 4–6 dishes from 2–3 experiments. Vertical bar on each point is the standard error.

ing\textsuperscript{11,12} in contrast to more primitive hematopoietic stem cells which are rather quiescent in proliferative activity. However, the CFU-M that were taken from the bone marrow and suspended in semi-solid agar obviously showed a considerable lag period before they started logarithmic growth (Fig. 1). The length of the lag period varied over a range of 36–44 hours, while doubling time of the cells belonging to different colonies also varied considerably (range: 8.9–13.3 hours) once they started to grow. We previously observed that \textsuperscript{3}H-thymidine uptake by the bone marrow cells in culture was negligible 24 hours after the stimulation by M-CSF, while it began to increase 2–3 days thereafter\textsuperscript{13}. This observation accords with the assumption that there is a lag period before the stimulated CFU-M enters the S phase. Nevertheless, we failed to observe time-dependent changes of radiosensitivity of the CFU-M during this lag
period (Fig. 4). It seems that M-CSF did not induce synchronous S entry of CFU-M in the culture. Alternatively, radio-sensitivity of CFU-M may not have cell-cycle dependency such as that observed in HeLa S3 cells\(^{14}\) or CHO cells\(^{15}\).

Moderation of the X-ray effect that was observed 50–95 hours after incubation (Fig. 4) may be ascribed to the fact that the seeded cells had multiplied by this time of incubation. If a few of the cells in a cluster were killed by X-rays, other cells that survived in the cluster were able to proliferate later to make a colony of normal size in 7 days. In this regard, the extent of moderation was much less pronounced than might be expected in view of the time-course of cell number increase. For example, the effect of radiation should be halved if the cells were irradiated 40 hrs after plating rather than shortly after plating, because individual CFU-M must have been divided into two cells by this time (Fig. 1) and both of the two must equally be capable of producing a colony in 7 days. However, no significant difference was observed whether the culture was irradiated shortly after plating or 40 hours after plating (Fig. 4). This suggests that the presence of the dead cell acted deleteriously on the proliferation of the surviving cell and that the cluster of two cells behaved like one single cell.

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REFERENCES


