Effects of Irradiation on Marrow Stromal Cells with Respect to Committed Granulocyte-Macrophage Progenitor Cells

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The effects of irradiation on the growth regulatory function of marrow stromal cells (MSC), and on the committed granulocyte-macrophage progenitor cells (GM-CFC), were investigated using a liquid culture system. CSF activity in the supernatant of irradiated MSC (0–900 rads) increased markedly with the increase of MSC irradiation dose. CSF-inhibitory activity in the supernatant of irradiated MSC (0–900 rads) also increased with the increase of MSC irradiation dose. Furthermore, the activity of exogenous CSF added to the supernatant of 900 rad-irradiated MSC was lost more slowly than that of non-irradiated MSC. These data suggest that irradiation affected CSF production, inhibitor production and consumption of CSF by MSC.

INTRODUCTION

Previous studies have shown that bone marrow stromal cells play an important role in the proliferation and differentiation of hemopoietic stem cells in vitro. Adherent marrow stromal cells provide an environment for sustaining pluripotent stem cells and progenitor cells, by producing regulatory factors and by cell/cell interaction. Lord et al. and Toksoz et al. reported that stromal cell conditioned medium contained stimulatory and inhibitory factors for pluripotent stem cells. Shadduck et al. reported the production of CSF, the stimulatory factor of GM-CFC, in a long-term bone marrow culture system. Oblon et al. showed the existence of isofferin, the inhibitor of GM-CFC, in the same culture system. These data indicate that marrow stromal cells produce both stimulatory factors and inhibitory factors in hemopoiesis. Marrow stromal cells have a function other than the production of regulatory factors. Gualteri et al. proposed that inhibitory cells in marrow stromal cells inactivate CSF. Heard et al. also showed that CSF was consumed by marrow stromal cells in a bi-layer agar culture system. Previously, we reported that marrow stromal cells have three different functions with respect to the effects on the GM-CFC in vitro culture system. Thus, it is apparent that marrow stromal cells

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have both a positive function, such as CSF production, and negative functions, such as inhibitor production and CSF consumption (as their own growth factors). In the present study, we investigated the effects of irradiation on these functions of marrow stromal cells.

**MATERIALS AND METHODS**

**Mice**

Eight-to-12 week old male DDY strain mice were purchased from Japan Laboratory Animals Co., Ltd. and were maintained in cages containing 8 to 10 mice each; the mice were provided with standard mouse food and acidified water.

**Bone marrow cells**

Bone marrow cells were drawn through a #23 gauge needle from the femora and tibiae into alpha medium (Flow Labs.). Single cell suspensions were then prepared in alpha medium by repeated pipetting, and the suspensions were adjusted to the desired concentration using alpha medium.

**Culture of marrow stromal cells (MSC)**

Two-ml samples of pooled bone marrow cell suspension in alpha medium, supplemented with 20% fetal calf serum (FCS), containing $2 \times 10^6$ nucleated cells per ml, were placed onto 35-mm plastic dishes (Falcon #3001). The dishes were incubated at 37°C in fully humidified air containing 5% CO$_2$. On day 10, the medium and the non adherent cells were removed, and MSC adhering to the dishes were washed three times with alpha medium. These dishes were employed for preparing supernatant.

**In vitro irradiation of MSC.**

The MSC layers were exposed to 0, 150, 500 or 900 rads of X-rays (MBR-1505R, Hitachi Co. Ltd. Japan) operated at 140 KVP 40 mA, filtered by 0.5mm Cu plus 1.0 mm of Al, at a target distance of 40 cm.

**Preparation of the supernatant of irradiated MSC**

After the establishment of MSC, the medium was replaced with fresh medium supplemented with 20% FCS, either with the addition of 15% abdominal wall conditioned medium (AWCM) which was used as a source of CSF to examine CSF consumption by MSC, or without the addition of AWCM to examine the production of CSF or inhibitor by MSC. The MSC were then exposed to 0, 150, 500 or 900 rads at room temperature. The irradiated MSC were cultured for another 7 days. The MSC supernatant was harvested every day for 7 days, passed through a 0.45-mm millipore filter, and then kept at -20°C until use.
Coculture of the supernatant of irradiated MSC and GM-CFC

GM-CFC was assayed by employing a modified double layer agar culture, based on the method of Pike and Robinson (1997). One and a half ml of alpha medium, containing 0.5% agar, 12.5% FCS, and 50% of the final concentration of MSC-conditioned medium as an underlayer, was set in 35-mm plastic dishes. Then 0.5 ml of 0.3% agar medium containing 12.5% FCS and $5 \times 10^4$ bone marrow cells, which were supplied with or without 7.5% (final concentration) of AWCM depending on whether the inhibitory activity of GM-CFC or CSF production was to be examined, was carefully overlaid on the agar base. In the study of inhibitor production and CSF consumption by MSC, the supernatants incubated without MSC were used as controls. The supernatants were maintained under the same condition as mentioned above for 7 days. Colonies consisting of 50 or more cells were counted using an inverted microscope.

Statistical analysis of the data

Student's t-test was used to determine the significance of the differences between values.

RESULTS

Colony stimulating activity in the supernatant of irradiated MSC

CSF from irradiated MSC were measured for 7 consecutive days beginning at the time of irradiation (Fig. 1). The time course of CSF production by non-irradiated MSC revealed maximum activity at day 2, which then decreased gradually. After exposure to 150 rad, CSF production by MSC was significantly enhanced with a peak at day 3 which was followed by gradual decline as in the case of non-irradiated MSC. However, the time course of CSF production by MSC exposed to 500 or 900 rads was quite different from that observed for 0- or 150-rad exposure groups, and the maximum activity was observed at day 7.

Inhibitory activity in the supernatant of irradiated MSC

Inhibitory activity was detected in the supernatant of 900-rad irradiated MSC at day 1 (Fig. 2). It was dose-dependent and significant, although the magnitude of the inhibition was at best 30%. The production of inhibitory factors in the supernatant of irradiated MSC was measured for 7 consecutive days, beginning the first day after irradiation (Fig. 3). The inhibitory activity of the supernatant of irradiated MSC at day 1 increased with the increase of MSC irradiation dose, compared with that of non-irradiated MSC. The time course of inhibitory activity in the supernatant of irradiated MSC revealed maximum activity at day 1, which then declined gradually.

The numbers of MSC at day 7 after irradiation, with 0, 150, 500 and 900 rads were $3.14 \times 10^5$, 2.63 $\times 10^5$, 1.53 $\times 10^5$, 1.33 $\times 10^5$ per dish, respectively.

Consumption of CSF by irradiated MSC

Consumption of CSF by irradiated MSC was investigated. Two ml of fresh medium, composed of alpha medium was supplemented with 20% FCS and 15% CSF with 0- or 900-rad-irradiated MSC for 3 days. The supernatant was collected every day and used as a source of
CSF for GM-CFC assay. CSF activity in the supernatant of non irradiated MSC declined linearly with the increase in incubating time (Fig. 4). The residual CSF activity in the supernatant of irradiated MSC was different from that in the supernatant of non-irradiated MSC. CSF levels in the supernatant of 900-rad-irradiated MSC at days 2 and 3 were significantly higher than that of non irradiated MSC.
Fig. 2. Inhibitor production activity by irradiated MSC. After establishing MSC, the medium was replaced with new medium. MSC were exposed to 900 rads and the supernatant was collected at day 1 after irradiation. To examine inhibitory activity in the supernatant, GM-CFC were assayed with various doses of the supernatant, in the presence of CSF. Data are values (± SEM) for 2 separate experiments.

DISCUSSION

There have been many previous reports of the effect of irradiation on the function of MSC in vitro\textsuperscript{19-20}. Tavassoli et al.\textsuperscript{21} and Dexter et al.\textsuperscript{22} have reported loss of the supportive function of hemopoietic stromal cells in vitro, after a radiation dose as low as 25–50 rads. However, Qusenberry et al.\textsuperscript{23} and Zuckerman et al.\textsuperscript{24} found that established stromal cells, irradiated in vitro with a higher dose of irradiation, supported hemopoiesis. The effects of irradiation on the
marrow microenvironment are controversial. In this study, we investigated the effect of irradiation on hemopoietic regulatory functions on GM-CFC. Firstly, the effect of irradiation on CSF production by MSC was investigated. The numbers of MSC decreased with the increase in irradiation dose. However, CSF in the supernatants of irradiated MSC increased with the increase in MSC irradiation dose. These data suggest that radio-resistant cells of MSC produce CSF, and that CSF production by MSC is enhanced by irradiation.

Gualteri et al.\textsuperscript{12} and Naparstek et al.\textsuperscript{25} also reported that CSF production by irradiated

\textbf{Fig. 3.} Time course of inhibitor production activity by irradiated MSC. After establishing MSC, the medium was replaced with new medium. The supernatant of MSC exposed to various doses was collected daily for 7 days. To examine the strength of inhibitory activity in the supernatant, GM-CFC were assayed with 50\% of the supernatant, in the presence of CSF. Data are mean values (± SEM) for 3 separate experiments.

( O 0 rad, Δ 150 rads, □ 500 rads, ◆ 900 rads)
MSC was dose-dependent. Our results reconfirmed those observations. Secondly, the effect of irradiation on inhibitor production by MSC was investigated. Inhibitor activity in the supernatants of 900-rad-irradiated MSC at day 1 was significant compared with that of non-irradiated MSC at day 1. Inhibitor activity in the supernatants of irradiated MSC at day 1 increased with the increase in the MSC irradiation dose. However, activity in the supernatants diminished with the increase in incubating time after irradiation. These data indicated that inhibitor production by irradiated MSC was dose-dependent and transient. Gualiteri et al.\textsuperscript{12} reported the result of an
experiment in which control and irradiated stromal cells were cocultured. This experiment revealed that hematopoietically active control stroma could block detection of irradiation-related CSF elevation. Heard et al.\textsuperscript{13}) reported that CSF was consumed by MSC in a bilayer agar culture system. Recently, we\textsuperscript{15}) also reported the same phenomenon in a liquid culture system. In that experiment, we could not detect the inhibitory activity produced by MSC, 2 to 7 days after the addition of exogenous CSF\textsuperscript{15}). Our preliminary study showed that the exogenous CSF, which was added to the cultured medium of MSC, stimulated the proliferation of MSC and increased the number of MSC one and half-fold in 7 days. Wang et al.\textsuperscript{20}) reported that GM-CSF stimulated proliferation of MSC, especially macrophages and endothelial cells. Yan et al.\textsuperscript{27}) reported that MSC-conditioned medium contained an autocrine factor, possibly IL-1, for bone marrow fibroblasts, and a paracrine factor (CSF-1) for macrophages and/or endothelial cells. These data suggested that CSF stimulated the proliferation of MSC and, in the process, consumed itself. Therefore, in our third experiment, the effect of irradiation on CSF consumption by irradiated MSC was investigated. When fresh medium containing exogenous CSF was cultured with 900-rad-irradiated MSC, the CSF in the supernatants at days 2 and 3 was higher than the CSF in the supernatants of non-irradiated MSC. These data suggest that irradiation disturbs CSF consumption by MSC. Zuckerman et al.\textsuperscript{28}) reported that total RNA, total protein and collagen synthesis in MSC were reduced by 35–60% within two days after 900-rad irradiation. In our preliminary study, the numbers of MSC cocultured with exogenous CSF for 7 days after exposure to 0- and 900-rad irradiation were $4.55 \times 10^5$ and $1.86 \times 10^5$ per dish, respectively. Our result might be due to the fact that irradiation suppressed the MSC proliferation stimulated by CSF. Further investigation is necessary to clarify the reason for this.

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