In Vitro Repopulation of Haemopoietic Stem Cells after Irradiation

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A culture system was designed in which proliferation of the haemopoietic stem cells was supported by adherent 'stromal' cell colonies. Application of the culture system to studies on kinetic behaviour of the haemopoietic stem cells after irradiation revealed: i) bone marrow stromal cells were radiosensitive with $D_0=95\,\text{R}$, when measured as the capability to proliferate and form adherent cell colonies in vitro, ii) radiosensitivity of the pluripotent stem cells (CFUs) in vitro was within the range of the in vivo sensitivity, iii) irradiated bone marrow cells under in vitro condition could repopulate at the same rate as those under in vivo condition, thereby suggesting that the function related to the support of haemopoiesis was radioresistant, iv) concentrations of both CFUs and granulocyte-macrophage precursor cells (CFUc) were higher in the irradiated cultures than those in unirradiated control culture at 3 weeks after irradiation.

Survival of sublethally irradiated animals depends mainly on the recovery of haemopoietic function, particularly the haemopoietic stem cells. Regeneration of the haemopoietic stem cells appears to be controlled by direct cell-cell interaction or by humoral factors, presumably released from the stromal elements in the haemopoietic system.

Recent development of an in vitro system for the longterm maintenance of the haemopoietic stem cells has provided a promising model for further studies on the control mechanisms of stem cell proliferation. The culture system consists of adherent 'stromal' cell colonies and haemopoietic cells proliferating in close contact to the stromal cell colonies in the culture. We have also established a quantitative culture system for the maintenance of haemopoietic stem cells and succeeded in inducing and maintaining granulocytic and erythrocytic cell production. Application of such a culture system to studies on stem cell kinetics after irradiation would promote a better understanding of the control mechanisms of the stem cell proliferation related to the stromal and haemopoietic cell interactions, either through direct cell-cell interaction or through humoral factors.
In the present experiments, we reproduced an in vivo situation in our bone marrow cell culture, and studied the effect of X-irradiation on the stem cell kinetics in vitro. We found that the stromal elements in our culture system could support an enhanced recovery proliferation of the haemopoietic stem cells after irradiation.

MATERIALS AND METHODS

**Mice.** Eight week-old male DDY strain mice were used for bone marrow cell culture, and 10 to 12 week-old male mice of the same strain were used as recipients for the assay of the pluripotent stem cells (CFUs). All mice were kept under conventional conditions with free access to drinking water.

**Culture of Bone Marrow Cells.** Femoral marrow cells were flushed directly into a screw-capped culture flask (C 25100, Corning, U. S. A.) in 10 ml Fischer's medium supplemented with 20% horse serum (lot No. 29211037, Flow Laboratories, U. S. A.) and antibiotic penicillin-streptomycin-neomycin (PSN, Gibcobio cult., Scotland), and the culture was carried out at 37°C in 5% CO₂ in humidified air. One half of the growth medium containing the cells in suspension was replaced with the same volume of fresh growth medium, at weekly intervals. Three weeks later, when adherent cell colonies had developed, cells in suspension were washed out and freshly harvested bone marrow cells (10⁶ cells/flask) were re-inoculated on the adherent cell colonies. Twenty-four hours later, various doses of X-rays were applied and the culture was continued as before. The cells in the altered medium were counted and used for the assay of CFUs and granulocyte-macrophage precursor cells (CFUc). In one experiment, bone marrow cells were irradiated with various doses of X-rays and then put into culture in order to study the effect of irradiation on the formation of the adherent cell colonies. The number of adherent cell colonies were determined at 3 weeks after irradiation (i.e., 3 weeks in culture).

**Assay for CFUs and CFUc.** The suspension cells removed during changes of the medium were assayed for CFUs and CFUc, according to the methods described by Till and McCulloch and by Bradley and Metcalf, respectively. Briefly, 5-30 × 10⁴ cells were injected into 800 R irradiated recipient mice and the colonies on the spleen were counted 8 days later (CFUs). One hundred thousand cells were incubated in the semi-solid culture medium, containing 15% mouse heart- and lung-conditioned medium as colony-stimulating activity, at 37°C for 7 days in 5% CO₂ in air, and the number of the colonies consisting of more than 50 cells was counted as CFUc. All results were expressed as average of 2-3 replicate experiments.

**Irradiation.** Recipient mice for CFUs assay were irradiated with 800 R of X-rays operating at 180 kVp-20 mA with a filter of 1.0 mm Al+0.5 mm Cu at a dose rate of 50 R/min, and the bone marrow cells at a rate of 250 R/min through 1.0 mm Al+0.3 mm Cu filter. One Roentogen is approximately equivalent to 0.97 rad under the
RESULTS

Effect of X-Irradiation on the Formation of Adherent Cell Colonies.

Culture of the bone marrow cells resulted in formation of adherent cell colonies within 3 weeks of the culture. Irradiation of the bone marrow cell suspension immediately before the start of the culture caused a dose-dependent reduction in the number of adherent cell colonies formed per culture flask (Fig. 1). The sensitivity of the bone marrow cells measured by the capability to form adherent cell colonies was within the range of the sensitivity of the normal marrow cells irradiated in vivo, $D_0$ being $95 \pm 6$ R$^{10,12}$. 

Radiosensitivity of Haemopoietic Stem Cells under Culture Condition.

Survival of the bone marrow cells in culture was assayed immediately after irradiation. Survival of CFUs showed a linear curve reduction, according to the dose of
irradiation, with $D_0$ of $88 \pm 11$ R, which is in the range of the in vivo sensitivity of normal bone marrow cells (Fig. 2). The radiosensitivity of CFUc under culture conditions differed from that under in vivo conditions or from that in bone marrow cells irradiated as single cell suspension (Fig. 3). Irradiation with 85 R of X-rays resulted in a 50% reduction of the survival but irradiation with more than 85 R did not alter the survival any further up to 255 R. Ten percent of CFUc survived the dose of 510 R.

**Fig. 2.** Effect of X-irradiation on the survival of CFUs under culture condition. Bone marrow cells were irradiated 24 hrs after secondary inoculation and immediately thereafter injected into lethally irradiated mouse to assay the survival of CFUs.
Bone marrow cells were irradiated 24 hrs after secondary inoculation and immediately thereafter seeded in the semi-solid medium to assay the survival of CFUc.

Recovery of Haemopoietic Stem Cells in Vitro after Irradiation.

To elucidate whether or not the irradiated bone marrow cells can undergo a recovery proliferation in culture, the bone marrow cell cultures were irradiated and further incubated in vitro for 3 weeks, and the numbers of CFUs and CFUc per flask were assayed weekly.

Survival of CFUs after 1 week in culture was about the same as that assessed immediately after irradiation (Fig. 4). Recovery thereafter was maximum in the range of 100 to 200 R-irradiation (Fig. 4). CFUc also showed a significant recovery proliferation as in the case of CFUs. However, the recovery of CFUc was rather poor in the culture of 50 R-irradiated cells (Fig. 5).

Effect of the irradiation was also profound when it was expressed as the decrease in the concentration of CFUs at 1 week after irradiation, suggesting that CFUs are more sensitive to the radiation or are slow in recovery for the first 1 week after irradiation than the total differentiated cells in suspension (Fig. 4, 1 Week). Increase in the concentration of CFUs, however, was more rapid than increase in the total cell number, i.e., the concentration of CFUs exceeded 100% of the control at 3 weeks after irradiation at any doses (Fig. 4, 3 Weeks). Such was also the case with CFUc (Fig. 5).

To illustrate the recovery pattern more clearly, the number of CFUs per flask and that of CFUc per flask were plotted as % of control against the time after irradiation. Irradiation of the bone marrow cells in culture did not significantly alter the number of suspension cells per flask, the number being about $1.5 \times 10^6$ cells per flask from 1.
Fig. 4. The dose effect on the number of CFUs at various times after irradiation in culture. Bone marrow cells in culture were irradiated and then kept in culture. Number of CFUs was assayed weekly after irradiation, and the results were expressed as % of the number of CFUs in control culture. The number of CFUs per 10^6 cells in control culture was 388±46, 400±72 and 385±51 at 1, 2 and 3 weeks, respectively (8 flasks per point). Open circles represent the number of CFUs per flask, and the solid ones that per 10^6 cells.
Fig. 5. The dose effect on the number of CFUc at various times after irradiation in culture. Experiments were similar to those in Fig. 4, and the number of CFUc was expressed as % of the control value, which was 620±40, 608±62 and 642±38 per 10^5 cells at 1, 2 and 3 weeks, respectively (8 flasks per point). Open circles represent the number of CFUc per flask, and the solid ones that per 10^6 cells.
week after irradiation until the end of the experiments. Irradiation of the cells in culture induced a significant enhancement of the proliferation of CFUs. *in vitro*. The recovery was evident at 1 week after irradiation with more than 200 R, whereas the recovery proliferation was delayed by 1 week after 50 or 100 R (Fig. 6). Similar recovery was observed with CFUc after irradiation (Fig. 7). Irradiation with 50 R of X-rays slowed the initiation of the recovery in this case as well. Thus, both the numbers of CFUs and CFUc *in vitro* reached near-normal levels 3 weeks after irradiation.

**DISCUSSION**

Importance of the microenvironment in the recovery of haemopoietic stem cells after irradiation has been well documented. Trentin's group showed that the differentiation of haemopoietic stem cells was controlled by a haemopoietic inductive micro-

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**Fig. 6.** Repopulation of CFUs in culture after irradiation. Data in Fig. 4 (number of CFUs/flask) were plotted against time after irradiation. • : 50 R, □ : 100 R, ○ : 200 R and △ : 300 R—irradiated culture.
Mori et al. found that the recovery of haemopoietic stem cells could be enhanced by treating the reticuloendothelial system with particulate substances\(^{12}\).

For the study of cell-cell interactions between stromal and haemopoietic cells, an in vivo system may not be so appropriate since it is difficult to exclude undesirable effects other than haemopoiesis. It would, therefore, be rewarding if such a microenvironment could be established in the culture system. Friedenstein et al. developed a clonal culture of fibroblast-like cells from the bone marrow and spleen cells, and have shown that these cells can support haemopoietic cell replication\(^{10,10}\). More recent establishment of the in vitro culture system for the maintenance of haemopoietic stem cells will provide a good model for such study, especially because the culture system consists of the stromal cell colonies and haemopoietic cells, and the haemopoietic cell proliferation seems to be supported by the stromal cells\(^6,7\). We succeeded in a semi-quantitative culture of such a haemopoietic system\(^5\) and this led to the study of kinetic behaviour of haemopoietic cells after various treatments in vitro.

The present results demonstrate that this culture system actually represents the in vivo situation in that haemopoietic stem cells such as CFUs and CFUc did undergo an enhanced recovery proliferation following a decrease after irradiation. The recovery of CFUs in the mice irradiated with a low dose of X-rays is slow and incomplete, and this suppressed recovery is due to the lack of activation of the stromal elements which stimulate the proliferation of CFUs\(^{17}\). The delay in the initiation of the recovery

Fig. 7. Repopulation of CFUc in culture after irradiation. Data in Fig. 5 (number of CFUc/flask) were plotted against time after irradiation. Symbols as in Fig. 6.
proliferation in 50 R-irradiated cell cultures may be due to comparatively insensitive recognition mechanisms of the stromal elements. It is also possible that the stromal cell colonies have shown a particular response in vitro to irradiation with 50 R, since there was a dip at 50 R in the dose response curve of the formation of the stromal cell colonies (Fig. 1).

Dose response curve of CFUc in culture appears to indicate the presence of relatively radioresistant CFUc. This, however, requires further investigation.

Concentrations of both CFUs and CFUc were higher in irradiated cultures than in the unirradiated control culture at 3 weeks after irradiation, although the sensitivity of the stem cells was higher than that of total bone marrow cell population when determined immediately or 1 week after irradiation (Fig. 4, 5). This can possibly be due to an enhancement in the self-replication of the stem cells rather than differentiation as suggested by Chervenick and Boggs.16

It is tempting to postulate that haemopoietic stem cells can grow only under the direct control by adherent 'stromal' cells and that the irradiation of such stromal cells results in a physiological alteration of haemopoietic microenvironment which stimulates the proliferation of haemopoietic stem cells. We have already reported that a factor was released by irradiated mouse bone marrow cells which stimulates the proliferation of haemopoietic stem cells (CFUs).15 Chan and Metcalf also reported that radioresistant stromal cells in the bone or bone marrow released a colony-stimulating factor for the growth of CFUc in vitro30. Although marrow cells showed a radiosensitivity similar to that reported in previous studies19 when it was measured as the capability to form adherent cell colonies, the adherent cell colonies appear to support an enhanced proliferation of the haemopoietic stem cells in irradiated bone marrow cell culture in vitro. Whether the stimulation acts through direct cell-cell interaction or through short-range humoral factor(s) is now being investigated.

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