Effect of Human Recombinant Granulocyte Colony-Stimulating Factor on Induction of Myeloid Leukemias by X-Irradiation in Mice

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Hematopoietic suppression is one of the serious problems induced by whole body irradiation. Granulocyte colony-stimulating factor (G-CSF) stimulates the progenitors of granulocytes and accelerates their recovery from bone marrow suppression induced by cytotoxic chemotherapy or radiation. On the other hand, G-CSF stimulates proliferation of myeloid leukemia cells as well as normal granulocytes in vitro. We designed a method to determine if G-CSF affects the incidence of myeloid leukemias induced by irradiation and the types of leukemias induced according to the French-American-British (FAB) classification in RFM/MsNrs mice. Administration of G-CSF (2 μg/d for 7 days) after a single 3-Gy irradiation significantly increased the number of peripheral blood neutrophils as compared with those in control mice. Even after discontinuation of G-CSF, both the total leucocyte and neutrophil counts increased to day 10, and their levels remained elevated until day 14. The incidence of myeloid leukemia in mice exposed to a single 3-Gy irradiation was 18.6% (38 of 204), and treatment with G-CSF did not increase the incidence (15.7% [32 of 204]). In the mice with radiation-induced leukemia, those receiving G-CSF had a mean survival time of 367 days, whereas those not receiving the factor survived for 349 days. There was no significant difference of survivals between the two groups. Most of the radiation-induced leukemias in the two groups were M1 or M2, according to the FAB classification; no characteristic difference was observed among the types of leukemias. Although G-CSF stimulated the leukemia cells in vitro, G-CSF administration after irradiation did not increase the occurrence of radiation-induced myeloid leukemias. Our results show that administration of G-CSF effectively accelerates neutrophil recovery from irradiation-induced hematopoietic injury and does not enhance the induction of myeloid leukemia in RFM/MsNrs mice by irradiation.

G-GRANULOCYTE colony-stimulating factor (G-CSF) stimulates myeloid precursor cells and selectively expands the neutrophils.1-3 This cytokine also activates mature neutrophils and increases their functions.6,7 On the other hand, it induces terminal differentiation of myeloid leukemia cells.7,9 Recently, G-CSF has been purified,10 and its cDNA has been cloned.11 The availability of recombinant G-CSF has enabled it to be used clinically, where it has been found to enhance the recovery of granulocytes from bone marrow (BM) suppression after chemotherapy or radiation therapy in patients with cancer and to increase host resistance to infections, either locally or systemically.11-16

The mechanism(s) by which CSF treatment accelerates BM recovery remains elusive. Many studies suggest that G-CSF acts on surviving myeloid progenitor cells and enhances the recovery from decreased BM function.13 BM suppression is one of the serious problems induced by irradiation. Studies have shown that G-CSF can promote hematopoietic recovery and survivals in lethally irradiated mice.14-22 However, there are potential risks in using G-CSF for BM suppression induced by irradiation. Irradiation induces a variety of malignancies including myeloid leukemias.13 Hematopoietic growth factors, including G-CSF, decrease the cell-cycle time and increase the proliferation of progenitor cells.17 Therefore, it is possible that G-CSF increases irradiation-induced BM toxicity by shortening the interval from irradiation to cell division. Moreover, G-CSF has been reported to stimulate the proliferation of leukemia cells and self-renewal in vitro.23,24 Recent studies have shown that macrophage-CSF and granulocyte/macrophage-CSF accelerated the incidence of irradiation-induced myeloid leukemias and reduced their latency in SJL/J mice.25,26 In contrast, interleukin-6 inhibited the development of transplanted myeloid leukemia cells and reduced the incidence of irradiation-induced myeloid leukemias in these mice.27 These studies suggest that G-CSF may potentially have an adverse effect on the induction of myeloid leukemias by irradiation. For the clinical use of G-CSF as a myelorestor-
mA, 0.5-mm Al 0.5-mm Cu filters, and focus sample distance (FSD) 56 cm). Unirradiated mice served as the controls.

Reagents.
Human recombinant G-CSF (hr-G-CSF) expressed in Escherichia coli was kindly provided by Kirin Brewery Co, Ltd (Tokyo, Japan). The specific activity of the hr-G-CSF was $1 \times 10^8$ U/mg protein. This G-CSF contained less than 0.5 ng/mg of endotoxin by the Limulus amoebocyte lysate assay.

Administration of G-CSF.
Mice were intraperitoneally injected with 2 µg of G-CSF in 0.2 mL saline immediately after irradiation and then received an additional 6 shots, 1 per day (2 µg/shot/d) for the following 6 consecutive days. Control mice were injected with saline (0.2 mL) instead of G-CSF. The leukocyte counts were determined by a microcell counter (Sysmex F-800; TOA Medical Electronics Co, Ltd, Tokyo, Japan), and the absolute neutrophil count (ANC) was calculated from the Wright-Giemsa-stained blood smears.

Diagnosis.
All mice were monitored throughout their life time. Mice that showed evidence of advanced leukemia, such as anemia or palpable splenomegaly, were killed by cervical dislocation. These mice were examined hematologically and pathologically, and aliquots of their spleen cells were transplanted into syngeneic female mice to confirm the diagnosis.

3H-thymidine incorporation. Cell proliferation was measured by 3H-thymidine incorporation. Briefly, $1 \times 10^6$ cells/100 µL were cultured in 96-well flat-bottomed microtiter trays in α minimum essential medium (MEM) supplemented with 10% fetal calf serum, $5 \times 10^{-3}$ mol/L 2-mercaptoethanol, 15 mmol/L HEPEs, and antibiotics for 30 hours with or without the addition of G-CSF. Eight hours before harvesting, 0.1 µCi (37 kBq) of tritiated thymidine (3H-Tdr; 6.7 Ci/mmol [248 GBq/mmol]; New England Nuclear, Boston, MA) was added. The cells were harvested onto a glass filter, and radioactivity was determined with a scintillation counter (LS6000SC; Beckman, Palo Alto, CA). All experiments were performed in triplicate, and data are expressed as the mean cpm ± SE. Data obtained from the cultures with G-CSF were compared with those from the cultures without G-CSF.

RESULTS

Effect of G-CSF on the recovery of white blood cell count in irradiated mice. We studied the effects of G-CSF on the recovery of leukocytes in irradiated mice (Fig 1). The counts of leukocytes and ANC in untreated male mice were 3,600 ± 320 and 1,400 ± 140 cells/µL (n = 4; mean ± SE), respectively. After a single 3-Gy irradiation, the leukocyte count transiently increased (n = 5, 4,370 ± 190 cells/µL, P < .05), whereas the ANC did not change (1,420 ± 60 cells/µL, P > .05). After irradiation, both the leukocyte count and ANC drastically decreased in both groups; on day 3, the leukocyte count was less than 800 cells/µL, and the ANC was less than 400 cells/µL in mice injected with saline. Both the counts remained at the low value during the observation period. In contrast, the leukocyte count and ANC of mice injected with G-CSF increased remarkably on day 5 (n = 5, 1,000 ± 160 cells/µL and 530 ± 160 cells/µL, respectively), continued to increase, and reached levels similar to the preirradiation levels on day 7 (3,400 ± 120 cells/µL and 1,150 ± 60 cells/µL, respectively). Statistical analysis by Student’s t-test showed that there were significant differences in the counts of leukocytes and ANC between mice treated with G-CSF and mice receiving saline alone on days 7 (P < .005 and P < .005), 10 (P < .005 and P < .005), and 14 (P < .05 and P < .025, respectively).

Effect of G-CSF on the incidence of radiation-induced myeloid leukemias and survival. To study the effects of G-CSF on the early phase of the myeloid leukemogenesis by radiation, mice received a single exposure to 3-Gy irradiation. No occurrence of myeloid leukemias was observed in unirradiated mice (n = 98). The hematopoietic malignancies other than myeloid leukemia were thymic lymphoma (2 mice, 1.9%) and nonthymic lymphoma (74 mice, 75.5%). On irradiation, 38 myeloid leukemias (18.6% [38 of 204]) were observed in mice receiving saline (Table 1), and they were found in 32 of the mice (15.7% [32 of 204]) injected with G-CSF. There was no statistical difference in the incidence of myeloid leukemia between the two groups ($\chi^2$ test).

Mean survivals after irradiation in mice with saline and G-CSF were 412 ± 143 days (range, 120 to 710 days) and 398

Table 1. Frequency of Myeloid Leukemia and Mean Survivals of Male RFM/MsNrs Mice After a Single Irradiation With 3 Gy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frequency (%)</th>
<th>All Mice Mean Survivals (days, mean ± SD)</th>
<th>Leukemia Mice Mean Survivals (days, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Gy + saline</td>
<td>18.6*</td>
<td>411.5 ± 142.9 (N = 204)</td>
<td>356.9 ± 115.1 (N = 38)</td>
</tr>
<tr>
<td>3 Gy + G-CSF</td>
<td>15.7†</td>
<td>397.5 ± 161.9 (N = 204)</td>
<td>348.7 ± 118.0 (N = 32)</td>
</tr>
</tbody>
</table>

Male RFM/MsNrs mice were exposed to a single 3-Gy x-irradiation and then treated with 2 µg of G-CSF intraperitoneally for 7 days.
* Not significant, $\chi^2$ test.
† Not significant, generalized Wilcoxon test.
Effect of G-CSF treatment on the myeloid leukemia type induced by radiation. To further characterize the myeloid leukemias induced by irradiation, myeloid leukemias in the two groups were classified according to the French-American-British classification for humans. Most of the leukemias were M1 and M2 in both groups. The occurrence of M1 + M2 was 61% (23 cases; 11 cases of M1 and 12 cases of M2) of all myeloid leukemias in mice receiving saline (38 cases). In addition to M1 and M2, other types were M3 (1 case), M4 (1 case), M5 (2 cases), and others (11 cases) in the

± 162 days (range, 56 to 752 days), respectively. On the other hand, mean survivals in mice with leukemia administered saline and G-CSF were 357 ± 115 days (range, 145 to 615 days) and 349 ± 118 days (range, 181 to 673 days), respectively (Table 1 and Fig 2). There were no significant differences in mean survivals after irradiation between the two groups of irradiated mice and also between mice with leukemia receiving or not receiving G-CSF (generalized Wilcoxon test).

Distribution and cumulative incidence of myeloid leukemias in male RFM/MsNrs mice after irradiation. All mice were monitored throughout their life time. The pattern of incidence of myeloid leukemia at various times after irradiation in both groups, with and without treatment of G-CSF, were very similar (Fig 3A). The earliest onset of myeloid leukemia was observed at 201 to 300 days after irradiation; thereafter, the number of mice with myeloid leukemias increased and plateaued at 401 to 500 days.

We also compared the cumulative incidence of radiation-induced myeloid leukemia between both groups (Fig 3B). In the control mice, the incidence of myeloid leukemia at 1 year was 12.3%, whereas that in the G-CSF-treated mice was 10.8%; treatment with G-CSF did not increase the incidence.
irradiated control mice. On the other hand, in the irradiated mice treated with G-CSF, the percentage of M1 + M2 was 72% among all the mice with leukemia. Of the 32 cases in total, 8 were M1, 15 were M2, 1 was M4, 1 was M5, and 7 were other types. No significant difference between the two groups was observed in the incidence of either M1, M2, or M1 + M2 ($\chi^2$ test). Myeloid leukemia classified into "others" included myelodysplastic syndrome, leukemias similar to chronic myeloid leukemia (CML), and unclassified cases that were diagnosed by the histopathologic examinations because fresh materials such as peripheral blood or BM cells were not available. Four mice with "CML" in the two groups had prominent leukocytosis in their peripheral blood, and their leukemia cells were at varying stages of differentiation, from immature cells to mature ones (leukocyte count, 12.8 to 38.4 $\times$ 10$^9$ cells/$\mu$L; hematocrit [Ht], 19.9% to 33.7%; and hemoglobin, 5.8 to 10.0 g/dL).

Proliferative response to G-CSF in irradiation-induced myeloid leukemia cells in vitro. Myeloid leukemia cells derived from the spleen were cultured with 100 U/mL of G-CSF for 30 hours and assayed for $^3$H-thymidine incorporation. In most cases, the leukemia cells significantly proliferated in response to G-CSF, except for those in 2 M1 cases (Table 2).

**DISCUSSION**

Radiation induces the transformation of a variety of cells as well as cell death. Thymic lymphomas, myeloid leukemias, or osteosarcomas are well known as radiation-induced malignancies. Myeloid leukemias are induced with relatively high incidence by irradiation in certain mouse strains such as RFM/Un, CBA/H, and SJL/J.29-31 and studies showed that the incidences were significantly higher in male mice.29,30 Previously, we and others reported that whole-body irradiation induced myeloid leukemias in RFM/MsNrs male mice with relatively high incidence.27,32 Using these mice, we studied the effect of G-CSF on the induction of myeloid leukemias by irradiation.

Many studies have indicated that myeloid leukemias can best be induced with 2- to 3-Gy irradiation in mice. Higher doses did not increase the myeloid leukemia frequency because of the cell-killing effect of the irradiation.29,33 We irradiated RFM/MsNrs mice with a single dose of 3 Gy. This dose of irradiation induced severe BM suppression and induced myeloid leukemias with relatively high incidence in these mice. On the administration of G-CSF, the neutrophil count recovered to the preirradiation level on day 7 or later, and it later overshot this level, increasing up to a threefold excess level. These data suggest that G-CSF acts on BM myeloid progenitor cells and effectively promotes hematopoietic recovery.

The mechanism(s) of myeloid leukemogenesis by irradiation remains uncertain. Deletion/rearrangement of chromosome 2 has been found in radiation-induced myeloid leukemias as a consistent cytogenetic feature in mice.34-36 However, Silver et al.37 reported that chromosome 2 rearrangement was induced in multipotential hematopoietic cells by irradiation. These studies indicate that deletion/rearrangement of chromosome 2 may be an initiating event in radiation-induced myeloid leukemias, but not sufficient for the development of overt leukemia.23 Furthermore, an in vitro study has shown that hematopoietic stem cells underwent malignant transformation on cocultivation with irradiated BM stromal cells.38 These studies indicate that induction of myeloid leukemias by irradiation involves a multiphase process.39 A model of irradiation-induced my-

<table>
<thead>
<tr>
<th>Type of Leukemia</th>
<th>Case No.</th>
<th>$^3$H-TdR Uptake (mean + SE cpm)</th>
<th>Medium</th>
<th>G-CSF</th>
<th>SI</th>
<th>$P$ Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>1</td>
<td>16,070 ± 330</td>
<td>25,180 ± 340</td>
<td>1.6</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>39,290 ± 840</td>
<td>30,700 ± 1,200</td>
<td>3.4</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26,200 ± 2,200</td>
<td>17,100 ± 210</td>
<td>2.0</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28,430 ± 850</td>
<td>12,660 ± 640</td>
<td>2.1</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>85,340 ± 3,400</td>
<td>100,680 ± 1,390</td>
<td>3.7</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>19,430 ± 630</td>
<td>72,670 ± 1,850</td>
<td>12.9</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>1</td>
<td>16,070 ± 330</td>
<td>25,180 ± 340</td>
<td>1.6</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>39,290 ± 840</td>
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<td>19,430 ± 630</td>
<td>72,670 ± 1,850</td>
<td>12.9</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>1</td>
<td>22,580 ± 600</td>
<td>69,040 ± 1,410</td>
<td>3.1</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td>CML</td>
<td>1</td>
<td>2,370 ± 30</td>
<td>3,550 ± 50</td>
<td>1.5</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13,050 ± 240</td>
<td>203,880 ± 2,780</td>
<td>15.6</td>
<td>&lt; .005</td>
<td></td>
</tr>
<tr>
<td>Normal Control</td>
<td>(n = 5)</td>
<td>1,630 ± 90</td>
<td>2,290 ± 110</td>
<td>1.4 ± 0.05</td>
<td>&lt; .005</td>
<td></td>
</tr>
</tbody>
</table>

A total of 10$^6$ cells derived from the spleen were cultured with or without 2 $\mu$g of G-CSF for 30 hours. Cells were pulsed with $^3$H-TdR for the last 8 hours. Data reported as the mean ± standard error (SE) of triplicate determinations.

$SI = \frac{3H-TdR \text{ Uptake With G-CSF}}{3H-TdR \text{ Uptake Without G-CSF}}$
eloid leukemias has been proposed involving two effects of irradiation, acute effects that result in stem cells carrying potentially leukemic lesions such as a deletion/rearrangement of chromosome 2 and late effects that result in changes in the organization of the stem-cell compartment such as abnormal stromal-cell function. Recent studies have shown that additional treatment with corticosteroids shortly after irradiation markedly increased the incidence of radiation-induced myeloid leukemias and reduced their latency. These indicate that radiation-induced initiated cells require additional influence to promote their neoplastic transformation, ultimately yielding a high myeloid leukemia incidence. In this study, we have examined the effects of changes in the organization of the stem-cell compartment of myeloid leukemias has been proposed involving two effects of CSF stimulated proliferation of leukemic cells in vitro. G-CSF enhances the survival of lethally irradiated mice. No difference was observed in survival between leukemic mice with and without G-CSF. Our results indicate that G-CSF, at least, does not enhance proliferation of radiation-induced malignant transformation or malignant clones in vivo that may be committed to leukemic processes.

Susceptibility to myeloid leukemia development after irradiation has been found in several strains of mice, such as SJL/J, CBA/H, and RFM. Studies have shown that M-CSF and GM-CSF accelerated the incidence of myeloid leukemias in a different model in which SJL/J mice were exposed to irradiation and corticosteroids. In the present study, G-CSF did not increase the rate of irradiation-induced myeloid leukemias in RFM/MsNrs mice. The mechanism(s) for these differences is not clear. However, other investigators have reported that cytokine responses can vary significantly in different strains of mice. These differences among strains and experimental systems may also lead to different results. Further studies are required.

In the irradiated mice, there was no difference in survival between the animals receiving G-CSF and those not receiving the factor. Irradiation of 3 Gy is optimal for the induction of myeloid leukemias but is not a lethal dose. Although 3-Gy irradiation induced BM suppression, this was transient and recovered without G-CSF treatment after 2 weeks. Death from infection-related neutropenia induced by 3-Gy irradiation may be relatively uncommon in the present study.

In summary, the present study provides a comparative analysis of the in vivo effect of G-CSF on the induction of myeloid leukemias in high-risk mice that were exposed to total body irradiation. There are accumulating reports that G-CSF enhances the survival of lethally irradiated mice. Our results show that G-CSF can be safely administered to individuals who have been in radiation accidents or to patients undergoing clinical radiation therapy without enhancing the incidence of myeloid leukemia.

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