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Long-lasting Radioprotective Effects of a Combination of Pharmaceutical Drugs on the Survival of Mice Exposed to Lethal Ionizing Radiation

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To reduce radiation-related casualties, it is important to establish an easy-to-use therapeutic protocol for the emergency medical care of patients involved in radiation accidents. The present study aimed to establish an optimum therapeutic protocol using currently approved pharmaceutical drugs to increase the survival of victims exposed to lethal radiation and aimed to estimate the long-lasting radioprotective activities of the present therapeutic protocol. The present combination of four drugs—recombinant human erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF), c-mpl receptor agonist romiplostim (RP) and nandrolone decanoate (ND)—was administered to mice within 2 h after exposure to a lethal 7 Gy dose of γ-irradiation. On day 100 after irradiation, the health of the mice was analyzed using various hematological parameters, such as the number of peripheral blood cells, bone marrow cells and hematopoietic progenitor cells. Approximately 12.5% of the untreated irradiated control mice survived for 100 days. The combined administration of G-CSF, EPO and RP for 3 consecutive days and ND for 1 day immediately after irradiation led to an 87.5% survival of the irradiated mice until day 100. Hematological analyses showed that the number of most of the hematopoietic cells in the treated surviving mice had recovered to the levels of the non-irradiated mice on day 100. The present findings show that the combination of G-CSF, EPO, RP and ND may be a useful countermeasure for victims exposed to accidental lethal irradiation.

Key words: combination of approved pharmaceutical drugs, lethal dose of γ-irradiation, C57BL/6JJcl mice, long-lasting high radioprotective effects

1. Introduction

Radiation-related casualties following exposure to a lethal dose of ionizing radiation show severe acute radiation syndromes (ARS) involving bone marrow death and gastrointestinal death. ARS cause decreases in red blood

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cell count, white blood cell count, platelet count and gastrointestinal dysfunction, finally leading to death caused by systemic bleeding[1]. Half of individuals exposed to 3 Gy of total body irradiation die from bone marrow death within 60 days. Therefore, reconstitution and restoration of hematopoiesis is a top priority.

The Safety Reports Series 2 "Diagnosis and Treatment of Radiation Injuries" published by the International Atomic Energy Agency (IAEA) describes the potential effectiveness of granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) for accelerating the bone marrow recovery of victims exposed to lethal doses of radiation[2]. Although bone marrow transplantation (BMT) is also available for recovery from radiation-induced bone marrow damage[3–5], BMT for victims in radiation accidents has many limitations, including histocompatibility, age constraints, HLA type and the fact that immunosuppression would be required to reduce the risk of graft versus host rejection. Therefore, there are problems associated with current readiness protocols. In contrast, pharmacological approaches can accommodate a large number of victims with few limitations. It is important to develop an effective medication or combination of medications to protect victims of radiation emergencies and accidents. It is especially important to use commercially available pharmaceutical drugs, because they are both widely available and approved for use in humans.

Human recombinant hematopoietic cytokines, G-CSF and erythropoietin (EPO), are used clinically worldwide[6, 7], and human recombinant GM-CSF and IL-3 are used in a few countries. In addition, a few previous reports have shown that the myeloproliferative leukemia virus proto-oncogene (c-mpl) ligand thrombopoietin and c-mpl receptor agonist act as radioprotective agents[8–10], One human c-mpl receptor agonist, romiplostim (RP), was recently approved for idiopathic thrombocytopenic purpura in several countries, including Japan. In addition to hematopoietic cytokines, Ishihara *et al*. reported that nandrolone decanoate (ND; 19-nortestosterone), a pharmaceutical drug used for surgery and thermal injuries, accelerates the regeneration of the mucosa of irradiated small intestines[11]. These pharmaceutical drugs may be potential candidates for the treatment of victims of radiological accidents. However, the effectiveness of the combination of pharmaceutical drugs for long-term survival is unknown.

In the present study, to estimate the activity of a combination of four commercially available drugs (G-CSF, EPO, RP for 3 consecutive days and ND for 1 day) in mice exposed to a lethal dose of γ-irradiation, we assessed the long-lasting radioprotective activities of this combination with respect to the survival rates for 100 days after γ-irradiation and hematological parameters, including the number of mature or immature cells detected in peripheral blood and bone marrow.

2. Materials and Methods

2.1. Exposure of mice to a lethal dose of γ-irradiation

Female C57BL/6JJcl mice were delivered at 6 weeks of age from the breeding facilities of Clea Japan (Tokyo, Japan). At 8 weeks of age, the mice were exposed to 7 Gy of ^{137}Cs γ-rays at a dose rate of 0.9 Gy/min and subjected to the previously specified drug regimen. All of the mice were housed in a conventional animal room with 12-hour light/dark cycles. All experiments were conducted according to the legal regulations in Japan and followed the Guidelines for Animal Experiments of the Institute for Environmental Sciences.

2.2. Treatment regimen using a combination of four pharmaceutical drugs

The irradiated mice were divided into two groups. One group was untreated after irradiation ($n = 8$), and the other group was treated with a combination of four pharmaceutical drugs ($n = 8$). All four drugs were administrated within 2 hours after γ-irradiation. Similarly, non-irradiated mice were administrated four drugs and were estimated as control. The present regimen was composed of the following combination of four commercially available drugs: recombinant human G-CSF, a Neutrogin® (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), recombinant human EPO, Espo® (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan), human c-mpl receptor agonist, Romiplate® (Kyowa Hakko Kirin, Co., Ltd., Tokyo, Japan), and ND Deca-Dumamin® (Fuji Chemicals Industrial Co., Ltd., Tokyo, Japan). EPO, G-CSF and RP were administered intraperitoneally for 3 days after irradiation, and ND was injected subcutaneously for 1 day immediately after irradiation. This suitable regimen was obtained by various preliminary experiments based on the 30-day survival rate of mice exposed to lethal γ-irradiation. Doses of EPO, G-CSF and ND were 100 U/kg of body weight/day, 100 μg/kg of body weight/day, 50 μmol/kg of body weight/day, respectively. The G-CSF and RP doses were determined by considering previous reports[8, 10, 12, 13]. Regarding RP, the applied doses in the present combination were 50 μg/kg of body weight/day. High doses of G-CSF (100 μg/kg body weight/day) and RP (50 μg/kg of body weight/day) were administered in the present study. These doses were 10- and 50-fold greater than those administered in a clinical setting. The treated mice were kept until day 30 and day 100, and weighed every 5 days. On day 30 and day 100, the numbers of white blood cells, red blood cells and

platelets in peripheral blood of the surviving mice were counted using Celltac-α (Nihon Kohden, Tokyo, Japan). The surviving mice were anesthetized with diethyl ether. Total nucleated cells in the femur were counted using Burker-Turk solution (Nacalai Tesque, Kyoto, Japan). The harvested bone marrow cells were analyzed by cell surface markers using a colony-forming assay.

2.3. Methylcellulose culture

Colony-forming cells (CFCs), including colony-forming unit-granulocyte/macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte (CFU-Mix) cells, were assayed by the methylcellulose method using MethoCult (StemCell Technologies Inc). One hundred or 450 cells from the bone marrow were poured into each well of a 24-well plate with 300 μl of culture medium containing 100 ng/ml of human recombinant IL-3 (Biosource, Tokyo, Japan), 100 ng/ml of human recombinant stem cell factor (Biosource), 10 ng/ml of GM-CSF (PeproTech, NJ, USA), G-CSF (10 ng/ml), EPO (4 U/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Each plate was incubated at 37℃ in a humidified atmosphere containing 5% CO_2 for 7 days. Colonies containing more than 50 cells were counted under 4 × magnification using an inverted microscope (Olympus, Tokyo, Japan). After benzidine staining, blue and colorless colonies were scored as BFU-E and CFU-GM, respectively.

2.4. Statistical analysis

Statistical analysis was performed using the Origin software package (OriginLab® Pro v8.1; Northampton, MA, USA) for the Windows operating system. Data were compared between the control and experimental groups by the paired *t*-test. The data were analyzed by two-sided Student's *t*-tests and the Mann-Whitney U-test. P values of less than 0.05 were defined as significance thresholds.

3. Results

3.1. Alteration of survival rates and body weight

To evaluate the radioprotective effects of pharmaceutical drugs on the survival of mice exposed to lethal 7 Gy γ-irradiation, a combination of four pharmaceutical drugs (EPO, G-CSF, and RP for 3 consecutive days and ND for 1 day) was administered to the mice within 2 hours after irradiation. The survival of each mouse was measured until day 100 (Fig. 1). The survival rate of the irradiated control mice was 12.5% on day 100. In contrast, the administered combination improved the survival rate up to 87.5%. Next, the body weight of each mouse was measured after irradiation every 5 days until day 100 (Fig. 2). The body weight of the irradiated mice with medication gradually increased from 18.51 ± 0.67 to 21.73 ± 1.40 g in the experimental periods as well as that of non-irradiated treated mice, whose increase was from 18.13 ± 0.80 to 22.38 ± 1.06 g. Although the body weights of the irradiated mice was gradually reduced to 15.48 g by day 30, the weight of one surviving mouse showed a similar increase compared with the treated mice.

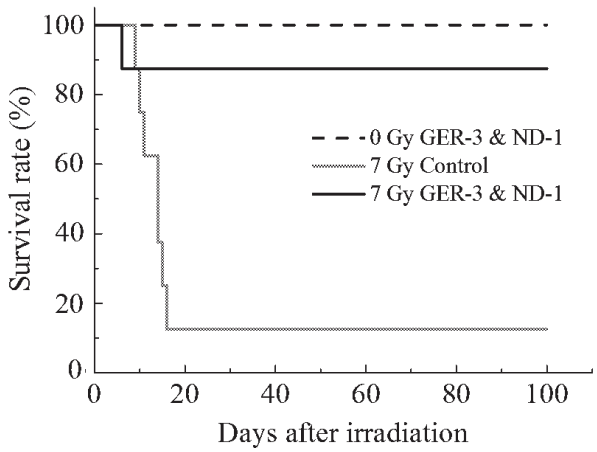

Fig. 1. Kaplan-Meier plots for the survival of female C57BL6/JJcl mice treated with combinations of four drugs compared with placebo controls. Mice were treated for 3 consecutive days, starting within 2 hours after 7 Gy total body irradiation. The medications were administered in the following combination: G-CSF, EPO, and RP were administered for 3 consecutive days, and ND was administered for 1 day after γ-irradiation (n = 8). Untreated mice exposed to 0 Gy or 7 Gy of radiation served as controls (n = 8).

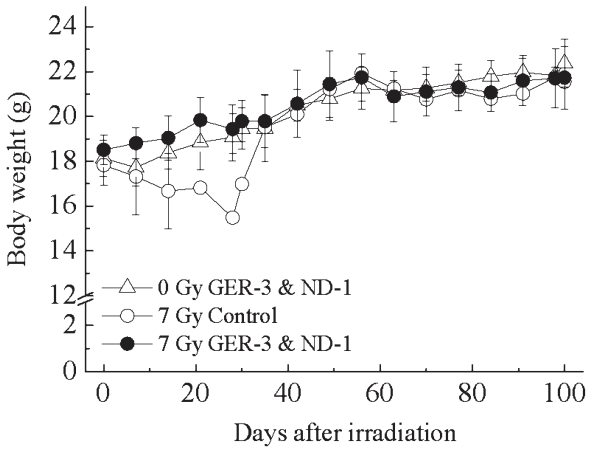

Fig. 2. Body weight changes of irradiated groups every 5 days until day 100. This shows the treatment results of the present combined regimen (n = 8). Untreated mice exposed to 7 Gy of radiations served as controls (n = 8).

3.2. Hematopoietic parameters in surviving mice compared with non-irradiated control mice on day 30 and day 100

The numbers of white blood cells, red blood cells and platelets detected in the peripheral blood of the surviving mice were analyzed. As shown in Figure 3, the number of white blood cells in the irradiated group insufficiently recovered on day 30 and day 100 (Fig. 3A). At this time, treated non-irradiated control mice showed a decrease in cell counts compared with the untreated non-irradiated control mice. On day 30 and day 100, red blood cell counts of the irradiated group were also less than that of the non-irradiated control group (Fig. 3B). Similarly, a significant suppression was also found in the platelet counts of treated irradiated mice (Fig. 3C), showing an insufficient recovery of blood cell counts on day 100.

To estimate the potential of this treatment regimen on the long-term survival of each mouse, an analysis of the hematopoietic characteristics of bone marrow cells was performed (Fig. 4). Although the total number of bone marrow cells in the treated irradiated mice was less than that of the non-irradiated control mice on day 30, but there was no statistical significant difference. These numbers on day 100 had recovered to non-irradiated control levels. Similarly, the number of myeloid hematopoietic progenitor cells, CFCs, including CFU-GM, BFU-E and CFU-Mix, detected in the bone marrow cells were similar to the number of bone marrow cells. As shown in Figure 5, the number of CFCs was less than the non-irradiated control levels on day 30 (no statistical significant difference); however, it recovered to the control level on day 100. In these analyses, there were no significant differences regarding the number of CFU-GM, BFU-E and CFU-Mix in both days (data not shown).

4. Discussion

In the present study, the long-lasting radioprotective activities of the combination of four commercially available drugs, G-CSF, EPO, RP and ND, with respect to the survival rates at 100 days after γ-irradiation were assessed. Although untreated mice exposed to a lethal dose of radiation achieved a 12.5% 100-day survival rate, the survival rate improved up to 87.5% with the combination treatment (Fig. 1). However, insufficient recovery was found in the number of peripheral blood cells obtained from the treated mice on day 100 (Fig. 3), although the bone marrow and its hematopoiesis

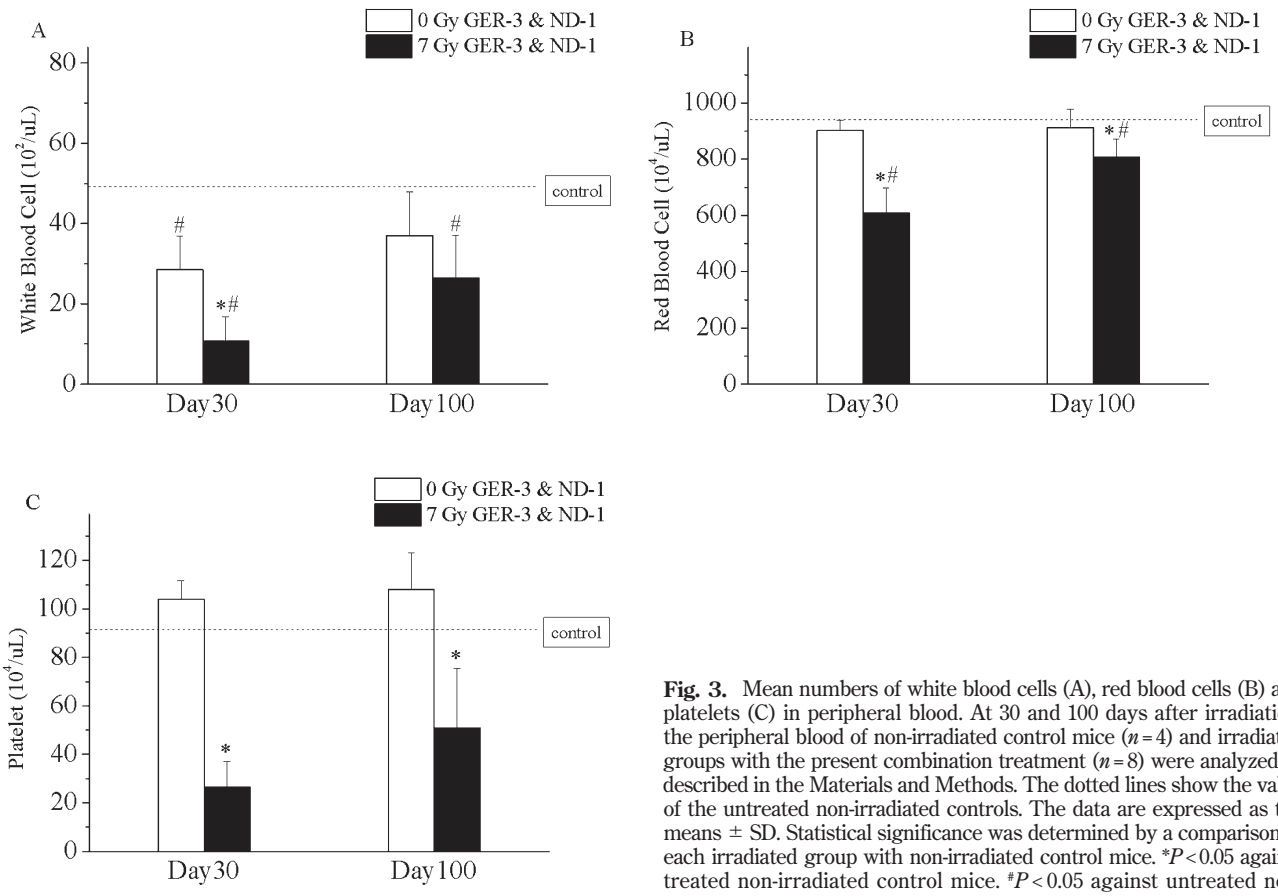

Fig. 3. Mean numbers of white blood cells (A), red blood cells (B) and platelets (C) in peripheral blood. At 30 and 100 days after irradiation, the peripheral blood of non-irradiated control mice ($n = 4$) and irradiated groups with the present combination treatment ($n = 8$) were analyzed as described in the Materials and Methods. The dotted lines show the value of the untreated non-irradiated controls. The data are expressed as the means ± SD. Statistical significance was determined by a comparison of each irradiated group with non-irradiated control mice. $^*P < 0.05$ against treated non-irradiated control mice. $^\#P < 0.05$ against untreated non-irradiated control mice.

appeared characteristically normal (Figs. 4 and 5). The treatment regimen suppressed the number of white blood cells in the non-irradiated mice (Fig. 3A). Although it is not possible to demonstrate precise mechanisms based only on the present data, the combination treatment may affect the hematopoiesis of treated mice because their administration doses were higher than that used in clinical settings and there was a possibility that pseudo bone marrow depletion occurred by more leukocytes producing. In addition, the number of platelets was especially lower at both 30 and 100 days after irradiation than those of controls (Fig. 3C). As one of a reason, CFU-Meg are more radiosensitive progenitor cells than other myeloid progenitors such as CFU-GM, BFU-E and CFU-Mix[14], it may be affected the state of these thrombocytopenia. Since a recent study reported that c-mpl receptor activation signaling by DNA damage activated the DNA repair function in hematopoietic stem and progenitor cells[15], RP may be activated the DNA repair function rather than platelet production. Additional analysis to determine the optimal dose of each drug as well as the long-lasting side effects and mechanism(s) involved in the increased survival is needed.

Because little information has been reported about whether a combination of currently-approved pharmaceutical drugs has the potential to induce radioprotective effects, the mechanism underlying the improvement of the survival rates of mice exposed to lethal γ-irradiation shown here is unknown. However, the present findings show that a combination of pharmaceutical drugs may promote hematopoietic recovery. The receptors for three effective drugs, G-CSF, EPO and RP, are classified into cytokine receptor class I (hemopoietin receptor), and they induce receptor dimerization and tyrosine phosphorylation, as well as a series of signaling events, including the activation of JAK/STAT, Shc/Ras/MAPK and PI3K/Akt[10, 16]. Cytokine receptors are constantly expressed on hematopoietic stem/progenitor cells and are relevant for red blood cell, megakaryocytic and granulocytic differentiation[17]. In addition, Ishihara *et al.* reported that ND (19-nortestosterone) used in the present study, a pharmaceutical drug used for surgery and thermal injuries, accelerates the regeneration of the mucosa of small intestines after irradiation[11].

One of the first priorities in managing exposure to lethal ionizing radiation is to reduce mortality and promptly recover damage to radiosensitive tissues, such as the hematopoietic system, intestinal mucosa and skin[1, 18]. Medications can be applied as primary care because hematopoietic stem cell transplantation is not appropriate for mass casualties who require prompt treatment. As recommended in the IAEA report, combinations of multiple cytokines have been suggested to have the potential to accelerate bone marrow recovery after radiation exposure in the lethal range[2]. However, human recombinant cytokines cannot be used globally owing to their unavailability or scarcity and high cost. Although various studies have been reported[19–21], the pharmaceutical drugs used for accidental irradiation must be approved for use in humans, which can take many years and will vary by country.

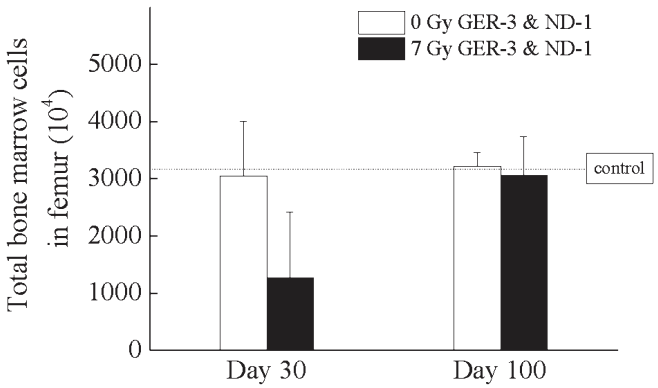

Fig. 4. The effects of the present medication on bone marrow of 7 Gy γ–irradiated mice were estimated by comparing the total number of nucleated bone marrow cells in the treated mice with the non-irradiated control mice at 30 and 100 days after irradiation. The total number of nucleated bone marrow cells in the femurs of non-irradiated control mice ($n = 4$) and irradiated groups with the present combined regimen ($n = 8$) were counted using Burker-Turk solution. The data are expressed as the means ± SD. Statistical significance was determined by the comparison of the irradiated group with the non-irradiated control mice.

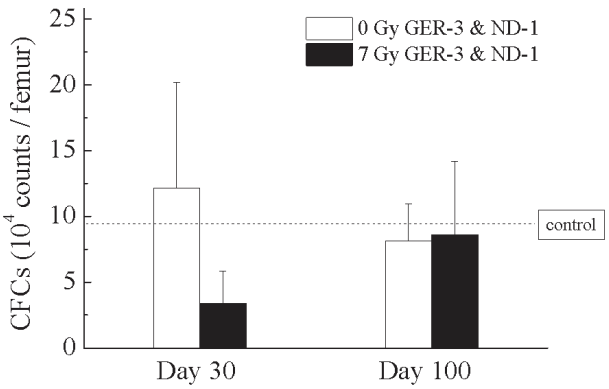

Fig. 5. Colony-forming assay of bone marrow cells collected on day 30 and day 100 after irradiation. As described in the Materials and Methods, we assayed the hematopoietic progenitor cells of non-irradiated control ($n = 4$) and irradiated groups treated with the present combined regimen ($n = 8$). This shows the results of the CFCs including CFU–GM, CFU–Mix and BFU–E. The data are expressed as the means ± SD.

Worldwide, the risk of radiation exposure due to radiological terrorism and radiological accidents caused by nuclear industry-related facilities that cause mass-casualty scenarios has been increasing. In March 2011, a serious radiological accident occurred at the Fukushima-Daiichi nuclear power plant in Japan following an earthquake and tsunami[22]. This accident shocked the world and is still affecting Japanese society. Highly effective and completely safe medical countermeasures are needed to provide better ways to prevent and control radiological accidents in the future. The present findings show that a combination of three pharmaceutical drugs, G-CSF, EPO, RP and ND, may be useful as a countermeasure for victims exposed to accidental lethal irradiation. However, a variety of issues should be addressed, such as the safety and efficacy of their application in humans, optimal doses of the drugs, the optimal duration and timing of administration and the applicable range of radiation doses that can be effectively countered. Studies to elucidate this information will be essential to establish a new medical countermeasure for accidental radiation exposure, and such studies will need to account for the medical circumstances of each country.

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