Genistein Stimulates Hematopoiesis and Increases Survival in Irradiated Mice

Yong ZHOU and Man-Tian MI *

Genistein/Radioprotection/Hematopoiesis/Bone marrow.

Radiation protection from death and stimulating hematopoietic recovery by oral administrations of genistein, 160 mg/kg b.w., once daily for seven consecutive days before whole-body γ-rays irradiation, were confirmed by tests with adult male BALB/c mice. Moreover, the protective action of genistein was compared to that of diethylstilbestrol (DES). Based on the studies of survival, behavior of hematograms, endogenous hematopoietic spleen colony formation (endoCFUs), and numbers of nucleated cell, granulocyte-macrophage colony forming units (CFU-GM) in bone marrow following irradiation, it was demonstrated that genistein was an effective radioprotector. The survival of irradiated mice protected by genistein was significantly increased and statistically higher than that of mice pre-treated with DES. Stimulated recovery of leukocytes, erythrocytes, lymphocytes and thrombocytes were observed in mice pre-treated with genistein or DES, however, the effects of genistein on promoting recovery of bone marrow nucleated cells, leukocytes and lymphocytes were significantly higher than those of DES. Enhanced endoCFUs, numbers of bone marrow nucleated cells and CFU-GM were also found in mice pre-treated with genistein as well as DES. Meanwhile, endoCFU numbers in mice pre-treated with genistein was 3.47-fold higher than that in the irradiated control group, although no significant difference was found between genistein administration and DES administration. It could be deduced that the radioprotective action against death is induced by a possible process of enhanced regeneration of the hematopoietic stem cells due to not only strengthened radioresistance and increased numbers of remained hematopoietic cells, but also enhanced post-irradiation repair or promoted proliferation of the hematopoietic stem cells. These effects of genistein may have some therapeutic implications for radiation-induced injuries.

INTRODUCTION

The hematopoietic system as well as the hematocytes is known to be sensitive to radiation, and low doses of radiation can induce damage. Radioprotective agents are those that are administered before exposure to ionizing radiation to reduce the damaging effects, including radiation induced lethality. Many synthetic as well as natural agents have been investigated on whether they have the efficacy as a protector against radiation injuries in the recent past years. Among the radioprotective compounds, estrogens have been extensively studied. Both the natural estrogens like estradiol and the synthetic estrogens like diethylstilbestrol (DES) exerted radioprotective actions on radiation sickness of experimental animals including improving the survival and accelerating the recovery of hematopoiesis. Moreover, estrogens also ameliorated hematopoietic suppression induced by cancer radiotherapy or chemotherapy in the clinic. However, the inherent toxicities of these agents at the radioprotective concentration warranted further search of safer and more effective radioprotectors.

Genistein (4', 5', 7'-trihydroxy-isoflavone), a naturally occurring isoflavone found in soybeans, has structural similarity to 17β-estradiol but rather weaker estrogenic activities (10⁻² to 10⁻³-fold). Many studies have demonstrated that genistein, as one of the most important phytoestrogens, had no toxicity on human health at the pharmacological concentration and possessed potential properties to act as both an estrogen and anti-estrogen, inhibit the activities of tyrosine kinase and DNA topoisomerase II, and improve immune system. Consequently, it has gained increasing attentions because of its association with beneficial effects on persons with breast cancer, prostate cancer, cardiovascular disease, high cholesterol levels and osteoporosis. Moreover, the isoflavone was an effective antioxidant, which could eliminate the free radicals and boost the antioxidant enzymes activities, so it may provide protection against...
ultraviolet-B radiation when applied to the skin of hairless mice 1h before exposure. Genistein also reduced the frequency of micronucleated reticulocytes and increased survival of sublethally irradiated mice without exhibiting estrogenic actions on reproductive systems. The purpose of this paper was to study in vivo radioprotection of genistein on hematopoietic recovery contributing to improve survival of sublethally irradiated mice.

METHODS AND MATERIALS

Materials
Male BALB/c mice (10–12 weeks old, weighing 25 ± 2g) were purchased from the Center of Laboratory Animal of the Third Military Medical University. All materials were purchased from Sigma Aldrich (St.Louis, Missouri, USA), except for the materials stated here. Genistein was purchased from Baoshai Biotechnology Co. of Xi’an Jiaotong University (Shanxi, China). Iscoves Modified Dulbecco’s Medium (IMDM) and fetal bovine serum were purchased from Hyclone (Logan, UT, USA). Culture plastic flasks and dishes were purchased from Coring Incorporated Life Sciences (Acton, MA, USA).

Radiation and Administration
According to our preliminary studies, genistein was dissolved in sesame oil and administered orally of 160 mg /kg b.w., once daily for seven consecutive days before irradiation. DES was subcutaneously injected 24 h before irradiation, a single dose of 5 mg/kg b.w. Control animals received sesame oil orally or saline for injection, respectively, in the same volume and at the same time as the treated group. Therefore, mice used for this study were divided in five groups: normal nonirradiation control (Group N), sesame oil plus irradiation control (Group O), genistein plus irradiation group (Group G), saline plus irradiation control (Group S), and DES plus irradiation group (Group D). Mice were quarantined for a period of 2 weeks and were housed in rodent cages with five to seven animals per cage at about 23°C with a relative humidity of 50%, and they were maintained under controlled condition and standard mouse food and water ad libitum. After treatments, Mice were placed in Plexiglass containers and the whole body exposed to 6.0 Gy of gamma rays (98.01–98.68 cGy/min) from a Co source.

Hematologic examinations
Whole blood was collected from the tail ends of mice on different days following irradiation and the fluctuation of hematograms, including leukocytes, erythrocytes, lymphocytes and thrombocytes, were automatically counted by a hematocyte counter. The blood count response was expressed as a percentage of the normal count determined 1 day before irradiation. Average values for each group were obtained from eight mice per group and the same eight mice were not sampled until 10 days later to avoid the influence of infection. If the hemogram changes of expired mice could not be counted, the treatment was repeated to increase the number of mice for experimental statistical analysis. Each treatment group consisted of 250–300 mice and surviving mice were euthanized by cervical dislocation on day 31.

Haemopoietic stem cell assays
Endogenous hematopoietic spleen colony formation (endoCFUs) was done according to method of Till and McCulloch. Briefly, endoCFUs were determined on day 10 after radiation. Mice were sacrificed by cervical dislocation; their spleens were removed and fixed in Bouins fixative for 24 h. The number of macroscopic spleen colonies was then scored. Bone marrow cells were obtained from anesthetized mice

Fig. 1. (A) Thirty-day survival of mice in various treatment groups. The results were a compilation of three separate experiments. Differences in the 30-day survival rates were calculated by the chi-square test. * p < 0.05, compared with group O; † p < 0.05, compared with group S; ‡ p < 0.05, compared with group G. (B) Survival curves of mice in various treatment groups (n = 30 per group)
by aseptic isolation of the femurs followed by a flushing of the marrow with IMDM medium, using a 25-gauge needle. The cells were suspended in the medium, and single cell suspensions were made.

To determine the CFU-GM (colony forming unit of granulocyte/macrophage progenitors) derived colonies, $2 \times 10^5$ bone marrow nucleated cells were cultured in triplicate in 24-well dishes in the presence of methylcellulose (3.0%), IMDM medium, L-glutamine (2.0mM), fetal bovine serum (15%), 2-mercaptoethanol (0.1 mM), penicillin (100 U/ml), streptomycin (100 ug/ml), and GM-CSF (10 ug/ml). The cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO$_2$ in air for 7 days. Colonies of at least 50 cells were scored at $30 \times$ magnifications.

**Survival assays**

Survival was monitored daily and reported as the percentage of animals surviving 30 days after irradiation. Each treatment group consisted of 30 mice. The dying animals in this experiment were killed when moribund. On day 31, surviving mice were euthanized by cervical dislocation. Data were expressed as % survival.

**Statistical analysis**

All experiment data were expressed as mean ± standard deviation and statistically analyzed with ANOVA test fol-
Results

Survival rate of mice after irradiation

It followed from results that mortality increased markedly in all irradiated groups and most mice were dead within the 7–14 days following irradiation. As illustrated in Fig. 1A, the percentages of mice surviving after 30 days, by group, were group S, 15.56%; group O, 16.67%; group G, 53.33%; group D, 45.56%. It showed that the 30-day survival of group G was significantly higher than other groups. The survival curve illustrated that, compared with the control group data, the time to death was significantly shifted to the right for mice pre-treated with genistein and DES, respectively (Fig. 1B). Those results demonstrated that genistein possessed highly radioprotective efficacy on prevention of mortality in sublethally irradiated mice and its protective action was superior to that of DES.

Fig. 4. Thrombocyte counts on different days after irradiation in various groups (%). Percentages leukocyte were calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation. a $p < 0.05$, compared with group N; b $p < 0.05$, compared with group O; c $p < 0.05$, compared with group S; * $p < 0.05$, compared with group D

Fig. 5. Lymphocyte counts on different days after irradiation in various groups (%). Percentages lymphocyte were calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation. a $p < 0.05$, compared with group N; b $p < 0.05$, compared with group O; c $p < 0.05$, compared with group S; * $p < 0.05$, compared with group D
Hematologic Examinations

Leukocyte, erythrocyte, lymphocyte and thrombocyte counts are shown in Fig. 2–4, respectively. As indicated by the percentages of the normal count (determined 1 day before treatment), hemograms of peripheral blood changed markedly because of the hematopoietic damage caused by irradiation in all irradiated groups.

In Fig. 2, leukocyte counts declined rapidly and elevated gradually from day 9 following irradiation. Within the whole post-irradiation period, the recovery of leukocytes in mice of group G was significantly raper than those of group O and group S. On day 30 after irradiation, leukocyte counts of group G returned to normal level, as compared with group O values of 78.5% and group S values of 75.4%. Stimulating leukocyte recovery was also found in mice protected by DES, but its stimulating actions seemed to be lower than that of genistein at days 21 and 30 following irradiation, namely 90.5% and 102.8% with group G compared with group D values of 77.2% and 93.8%.

Figure 3 showed that the sublethal dose of acute irradiation caused erythrocyte numbers decreased slowly and reached a minimum value at day 14 after irradiation. The prescriptions genistein as well as DES before irradiation exerted some active effects on recovery of radiation damage by increasing the number of erythrocyte. Significant differences from the irradiated controls were seen at days 14 and 21 following irradiation, namely 90.5% and 102.8% with group G compared with group D values of 77.2% and 93.8%.

Figure 4 illustrated that thrombocyte counts decreased in a time-dependent manner and reached a minimum value at day 14 after irradiation. Reduction of the decrease and stimulated recovery of thrombocytes were found in mice pre-treated with genistein and DES, respectively. From day 6 after irradiation, the number of thrombocytes in group G was statistically higher than that in group O and it rendered approximately 96.24% of normal range compared with 80.45% of group O on day 30 after irradiation. Enhanced thrombocyte numbers was also found in mice of group D. Actually, there were significant differences between group G and group D at days 14 and 21 after irradiation. It showed that protective effect of genistein on thrombocytes was lower than that of DES.

Figure 5 showed that lymphocyte numbers decreased rapidly and reached a minimum value at day 6 after irradiation. Reduction of the decrease and stimulated recovery of lymphocytes were found in mice pre-treated with genistein and
DES, respectively. Interestingly, protective effect of genistein on stimulating recovery of lymphocytes was stronger than that of DES. At days 21 and 30 following irradiation, the number of lymphocytes in group G was much more than that in group D, namely 75.4% and 94.5% with group G compared with group D values of 65.5% and 83.7%.

**Numbers of bone marrow nucleated cells, endoCFUs and CFU-GM**

Radiation decreased numbers of bone marrow nucleated cells and induced hematopoiesis suppression obviously in all irradiated control groups, and recovery of nucleated cells started at day 6 after irradiation. Compared to irradiated groups, the consecutive administrations of genistein clearly accelerated hematopoietic recovery by the increase of bone marrow nucleated cell numbers. At day 21 after irradiation, number of nucleated cells in group G rendered 89.96% of normal range comparison with 56.02% of group S and 56.7% of group O. Furthermore, numbers of bone marrow nucleated cells in group G were significantly higher than those in group D at day 914 and 21 (Fig. 6). The numbers of endoCFUs, by group, were group S, 4.21; group O, 3.78; group G, 16.91; group D, 13.56. Number of endoCFUs in mice of group G was approximately 3.47-fold higher than that in group O and no further significant differences between group G and group D were found (Fig. 7). In addition, enhanced numbers of CFU-GM were also observed in mice pre-treated with genistein as well as DES, and there seemed to be no significant difference between group G and group D (Fig. 8).

**DISCUSSION**

One of the major syndromes of hematopoietic system damaged by high dose total-body exposure to ionizing radiation is bone marrow aplasia. It is generally agreed that radiation death in the sublethal dose range is due to impairment of bone marrow hematopoietic function and that the leucopenia, erythropenia and thrombocytopenia which ultimately develop predispose to infection, hemorrhage and death. Survival brought about by radioprotectors after potentially lethal irradiation is thought to be due primarily to their effect on hematopoietic cells. Accordingly, we used peripheral blood cell count as indicators of bone marrow function in order to assess the effect of radioprotection on normal tissue which is critical for survival. The present study revealed that pre-irradiation administrations of genistein or DES could increase the survival and stimulate recovery of peripheral hematocytes, falls of bone marrow nucleated cells induced by radiation. Interesting, the efficacy of genistein on enhancement of the survival and promoting recovery of leukocytes, lymphocytes and bone marrow nucleated cells were stronger than those of DES in our experiments, although its protection against the decrease of thrombocyte counts was weaker than that of DES. According to the conclusions of Floersheim et al., it would appear that genistein affords hematological protection by both preventing the destruction blood cells and enhancing hematopoietic recovery. That...
means not only that the circulating blood cells but also the progenitor cells may be protected under irradiation by prior genistein administration.

Furthermore, the date from our experiments showed that mice protected with genistein demonstrated much more powerful recovery of endo CFUs and CFU-GM numbers after irradiation, but no significant differences were found compared to those of DES pre-treated mice. The enhancement of endoCFUs counts in genistein pre-treated irradiated mice in comparison to irradiated control indicates the role of genistein in protecting the stem cells and/or stimulating the proliferation of survival cells. Measures of CFU-GM are good indications of myeloid haemopoietic activity in animals recovering from exposure to radiation. We accordingly infer that the mechanism of stimulating hematopoiesis recovery by genistein may involve the enhancement of bone marrow stem cell radiotolerance to inhibit of the decrease of bone marrow stem cell number and the promotion of proliferation of survival cells.

Various mechanism such as prevention of damage through inhibition of free radical generation or their intensified scavenging, enhancement of DNA and membrane repair, replenishment of dead hematopoietic and other cells and stimulation of immune cell activity are considered important for radioprotection. Genistein has several of the above-mentioned properties under different experimental conditions, which might attribute to its stronger radioprotective efficacy than that of DES.

Most radiation damages arise from and interaction of the radiation-induced free radicals with the biomolecules. Free radical interactions may relate to DNA and other cellular macromolecules damage. Molecules with the ability to scavenge free radicals, therefore, can prevent radiation damage. Evidences demonstrated that genistein has stronger antioxidant actions combining with its capacity to activate the antioxidant systems that results in reduction of the level of free radical lipid peroxidation products and stabilization of cellular membrane structure.\(^\text{27,28}\) Wei et al. reported that genistein provided protection against non-ionizing ultraviolet-B radiation through either direct quenching of reactive oxygen species or indirect anti-inflammatory effects when it was applied to the skin of hairless mice 1h before exposure.\(^\text{16}\) Arora et al. found that soy isoflavonoids could hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions, which might help to stabilization of cellular membrane structure.\(^\text{29}\) Genistein also reduced the frequency of micronucleated reticulocytes in the peripheral blood of mice receiving a sublethal dose of ionizing radiation.\(^\text{17}\) Thus, the antioxidant activity of genistein, its ability to protect against radiation-induced cytogenetic damage could contribute to its radioprotective action.

Genistein pre-irradiation administration rendered a significant increase in the number of leucocytes and lymphocytes that was reduced by irradiation. The increase in CFU counts in spleen associated with the increase in leucocyte and lymphocyte counts in genistein pre-treated mice in comparison to untreated control mice indicated that the immunostimulatory role of genistein. Some previously reports also demonstrated that genistein has immunomodulatory activity in different experiments.\(^\text{30,31}\) Since immunosuppression following radiation exposure and subsequent opportunistic infections are the major drawbacks of radiation damage, the immunomodulatory roles of genistein maybe another important mechanism of its radioprotective efficacy. In addition, genistein possesses some other biological properties that may relate to its radioprotective efficacy. These include its estrogenic activity and its role in signal transduction pathways where it is an inhibitor of topoisomerase, protein kinase and caspases involved in apoptotic pathways.\(^\text{21,22}\) These properties have been associated precisely with radioprotection.\(^\text{23-26}\)

On the other hand, genistein has gained increasing attentions because of its association with beneficial effects on persons with cancer, cardiovascular disease, high cholesterol levels and osteoporosis, specially its benefits in tumor prevention and therapy.\(^\text{13-15}\) Some reports showed that genistein using alone in vivo as well as in vitro could delay the growth of tumors and induce apoptosis of cancer cells.\(^\text{32,33}\) Now, radiotherapy and chemotherapy are two important methods in cancer therapy. Recently, many studies demonstrated that genistein showed additive benefits in tumor radiotherapy or chemotherapy, resulting in greater therapy efficacy. Some authors indicated that genistein in combination with other agents could delay tumor growth by its antiangiogenic activity.\(^\text{34,35}\) Yan et al. reported that genistein could enhance the radiosensitivity of DU145 prostate cancer cells.\(^\text{36}\) Hillman et al. also showed that genistein combined with prostate tumor irradiation led to a greater control of the growth of the primary tumor and metastasis to lymph nodes than genistein or radiation alone.\(^\text{37}\) Therefore, the use of genistein in radiotherapeutic or chemotherapeutic applications can also be exploited.

In summary, the results of the present study demonstrated that genistein administration before irradiation has effects of increasing the survival and providing the intensification of post-irradiation hematopoiesis recovery of irradiated mice. Although our investigations might provide some information basis for the possible use of genistein as a radioprotector of hematopoietic system, further studies are necessary to determine the mechanism of its radioprotective action.

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