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Radioprotection of mice by recombinant rat stem cell factor

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ABSTRACT **Treatment with recombinant rat stem cell factor (rSCF) protects mice from the lethal effects of irradiation. Mice treated with a single dose of rSCF prior to irradiation of up to 1150 rads [given as a split dose (1 rad = 0.01 Gy)] resulted in >80% long-term survival, whereas a single injection given after the last dose of irradiation was not radioprotective. The combination of pre- and posttreatment (−20 h, −2 h, and +4 h) with rSCF resulted in 100% survival of otherwise lethally irradiated mice. Using this optimum schedule of rSCF administration, a radioprotective factor of 1.3–1.35 was achieved. The major cause of death in the control animals was massive bacteremia consisting of enteric organisms. The rSCF-treated animals had a much lower frequency of septicemia, due primarily to a rapid hematopoietic recovery of bone marrow function not evident in control animals.**

Radiation-induced septicemia and death are primarily due to damage of the intestine and hematopoietic system. Enhanced susceptibility to infections due to hematopoietic damage occurs in parallel with progressive radiation-induced damage of the epithelium lining of the intestines allowing for an influx of pathogens into the blood stream (1). A number of substances have been shown to protect animals from irradiation and include thiol compounds (2), interleukin 1 (IL-1) (3), tumor necrosis factor α (4), granulocyte colony-stimulating factor (G-CSF) (5), and granulocyte/macrophage colony-stimulating factor (GM-CSF) (5). DNA repair mechanisms, scavengers of free radicals, and the induction of resting bone marrow cells into cell cycle have been proposed as possible mechanisms for reduced damage by radiation (6–8). Transplantation of normal bone marrow cells into lethally irradiated animals and humans results in long-term survival and provides proof that the hematopoietic system is crucial for defense against the lethal complications induced by radiation.

Stem cell factor (SCF) is the product of the steel (*Sl*) locus of the mouse and is the ligand for the c-*kit* tyrosine kinase receptor encoded by the white spotting (*W*) locus of the mouse (9–13). The biological properties of SCF demonstrate that the action of SCF is on very primitive cell populations that have been described as stem cell populations (14–16). The action of SCF on stem cells suggested that it may act as a radioprotective agent similar to IL-1 (8). In this report we examined the effect of treating lethally irradiated mice with recombinant rat SCF (rSCF) and demonstrate marked stimulation of hematopoietic recovery leading to survival of the mice.

MATERIALS AND METHODS

Mice. Ten- to 12-week-old female (C57BL/6J × DBA2)F_1 mice were obtained from Charles River Breeding Laboratories and climatized in our institute for a minimum of 5 days prior to use. Mice were irradiated at various doses using a cesium source, given as a split dose of equal intensity, 4 h apart. Mice were injected i.v. in the tail vein or i.p. with rSCF at a dose of 100 μg/kg or saline containing 0.1% fetal bovine serum (excipient for rSCF dilutions) at various time intervals.

rSCF. rSCF was prepared as described by expression in *Escherichia coli* containing the appropriate plasmid (10). The rSCF was purified to homogeneity (10) and coupled with polyethylene glycol (PEG) as described (17). As a control, bovine serum albumin was also modified by PEG and tested for radioprotective properties. A solution of 0.92 mg of purified rSCF per ml contained <0.5 ng of endotoxin per ml as determined by the *Limulus* amoebocyte assay (18).

Bone Marrow Analysis. Bone marrow cells were collected by flushing femoral shafts with Hepes-buffered solution (BSS) containing 2% fetal calf serum. Bone marrow cellularity was determined by scoring nucleated cells on a hemocytometer after staining with crystal violet. High proliferative potential colony-forming cell (HPP-CFC) and granulocyte/macrophage colony-forming cell (GM-CFC) assays were performed as described (19, 20). Cultures were incubated for 14 days, after which time colony formation was determined using a dissecting microscope. HPP-CFCs were scored as colonies >0.5 mm in diameter containing at least 50,000 cells. EMT-6 conditioned medium (21) was used as a source of factors stimulating HPP-CFCs (22, 23); however, similar results were obtained using a combination of recombinant factors [namely, rSCF, rmIL-3, rhIL-6, and rmGM-CSF (m- and h- indicate mouse and human, respectively)]. GM-CFCs were scored as colonies containing >50 cells in response to macrophage colony-stimulating factor (rhCSF-1, 1600 units per dish; kindly supplied by Cetus).

Peripheral Blood Analysis. Peripheral blood samples were obtained using cardiac puncture and collected into EDTA-containing tubes. Analyses of erythrocyte (RBC) number, leukocyte (WBC) number, hematocrit, and platelet numbers were performed on a Sysmex Microcell counter (TOA Medical Electronics, Kobe, Japan).

Blood Cultures. Blood was collected at days 8, 10, and 11 postirradiation. The mice were sacrificed by CO_2 asphyxiation, and cardiac punctures were performed in a sterile hood. The mice were washed with 70% ethanol and incised laterally across the abdomen to expose the chest cavity. Blood was collected in sterile 1-ml insulin syringes (Becton Dickinson) coated with 70 μl of a 3.8% trisodium citrate solution (used as an anticoagulant that does not inhibit aerobic growth). An aliquot (0.1 ml) of blood was inoculated into tubes containing (*i*) trypticase soy broth or (*ii*) fluid thioglycolate medium and incubated at 35°C for 7 days, after which time the presence

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Abbreviations: SCF, stem cell factor; rSCF, recombinant rat SCF; HPP-CFC, high proliferative potential colony-forming cell; GM-CFC, granulocyte/macrophage colony-forming cell; IL, interleukin; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; WBC, leukocyte; RBC, erythrocyte.

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of bacteria was determined using standard techniques (24, 25).

Serum Chemistry. Whole serum was collected without the addition of anticoagulants, and serum chemistry values were calculated using an automated serum chemistry analyzer (Astra 8; Beckman). Values for blood urea nitrogen, potassium, glucose, albumin, total protein, and creatinine were analyzed for 10 animals per group per day.

Histopathology. Mice were sacrificed at various time points postirradiation and the heart, lungs, liver, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach, and lymph nodes were studied by gross and microscopic examination. In a few mice, the gallbladder, peripheral nerves and autonomic ganglia, and skin were examined. In addition, multiple sections of the small and large intestine and cecum were made at various levels and cut in cross section and longitudinal section. Tissues were preserved in 10% buffered formalin, sectioned at 3 μm thickness, stained with hematoxylin/eosin, trichome, and Gram stains, and examined microscopically.

RESULTS

Effect of Treatment Schedules on Radioprotective Role of rSCF. Mice were irradiated at various doses ranging from 900 to 1200 rads (1 rad = 0.01 Gy) to determine a lethal dose of irradiation for the BDF_1 mice used in these studies. Fifty percent of mice survived 30 days given an irradiation dose of 1000 rads ($LD_{50/30}$). Similarly, the $LD_{90/30}$ was obtained at 1100 rads, and the $LD_{100/30}$ was obtained at 1150 rads. Subsequent studies were therefore performed at 1150 rads to obtain a lethal dose of irradiation. Since IL-1 radioprotection is only manifest after i.p. injection, we compared the i.p. versus i.v. routes of injection for rSCF. Both routes of administration resulted in comparable radioprotective effects (data not shown) and the i.v. route was used for subsequent experiments.

Treatment of irradiated mice with rSCF resulted in increased survival compared to irradiated controls and was dependent upon the treatment schedule administered as shown in Fig. 1. A single injection of rSCF given 4 h postirradiation did not protect against lethal irradiation; however, 50% of mice were alive at 12 days postirradiation, whereas 100% of control animals were dead by day 12 (Fig. 1). Treatment with rSCF at 20 h and 2 h prior to irradiation resulted in >80% long-term survival of mice (Fig. 1). The combination of pre- and posttreatment with rSCF (−20, −2, and +4 h) resulted in 100% survival of mice irradiated at 1150 rads (Fig. 1). In subsequent experiments, it was determined

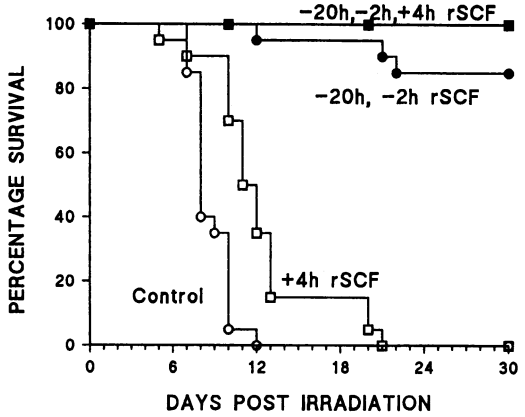

FIG. 1. Radioprotective effect of rSCF on mice irradiated at 1150 rads with different schedules of administration. The rSCF was injected i.v. at 20 h and 2 h prior to irradiation and 4 h after the last dose of irradiation. The results presented are from 20 mice in each group.

that −2-h administration of rSCF was not radioprotective, but −20 h alone resulted in 50% survival of irradiated mice (data not shown).

To determine the extent to which SCF could protect against otherwise lethal irradiation, mice administered rSCF at the optimum schedule (−20, −2, and +4 h) were subjected to escalating doses of irradiation from 1150 to 1550 rads as a split dose 4 h apart. The $LD_{50/30}$ of these mice given rSCF was 1300 rads and the $LD_{100/30}$ was 1550 rads (data not shown). Therefore, using the optimal schedule of administration of rSCF, a radioprotective factor of 1.3–1.35 could be achieved.

To determine if hematopoietic recovery was crucial in the recovery from these doses of irradiation, mice irradiated at 1150 and 1350 rads were transplanted with bone marrow cells from syngeneic normal donors, 4 h postirradiation. i.v. injection of 0.1 of a femur resulted in 100% survival of mice at both doses of irradiation compared to 0% survival of control animals that did not receive a bone marrow transplant.

To control for the possible radioprotective effects of PEG, which is covalently attached to the rSCF used in these studies, PEG-modified albumin and free PEG were tested for their radioprotective properties using the same optimal dosing schedule. Neither PEG-albumin nor free PEG was able to radioprotect (0% survival after 30 days) mice given 1150 rads (data not shown).

Role of rSCF on Hematopoietic Recovery in Lethally Irradiated Mice. To study the effect of rSCF on hematopoietic recovery, mice were irradiated at 1150 rads and injected with rSCF or carrier. At days 0, 1, 3, 6, 8, 10, 14, and 21, mice from either the rSCF-treated or the control group were sacrificed and peripheral blood and bone marrow were harvested. By day 11, 0% of control animals survived. In comparison, none of the rSCF-treated animals died postirradiation, and the animals continued to survive past the 30-day study period. In excess of 300 mice have been treated with rSCF using this regime, and 100% of mice receiving rSCF survive 1150 rads given as a split dose. Some animals have been followed in excess of 6 months, and the only effect observed has been early greying of the hair, which is common for mice given high doses of irradiation.

Peripheral blood hematology showed no significant difference between rSCF-treated and control mice over the first 8 days postirradiation (Fig. 2). At day 10, in the rSCF-treated mice, there was a small but variable increase in WBCs, followed by a rapid rise in WBCs between days 14 and 21 (Fig. 2). No significant difference in RBC levels was seen until day 10, at which point a sudden drop in RBC levels in control animals was observed (Fig. 2). In some control animals internal bleeding was observed, which may account for this sudden drop in RBC levels. The RBC levels in the rSCF mice had returned to preirradiation values by day 21 (Fig. 2). Hematocrit levels followed the pattern seen for RBCs in the control and rSCF-treated mice (Fig. 2). Platelet values dropped rapidly 3 days postirradiation (Fig. 2). In control mice the levels continued to drop; at day 10, four of five mice had platelet levels <20,000 per μl, with the fifth mouse having only 29,000 platelets per μl. In contrast, the platelet counts in rSCF-treated mice never dropped below 50,000 per μl. The nadir occurred at day 8 postirradiation, followed by a subsequent increase back to an average of 600,000 platelets per μl by day 21 (Fig. 2*C*). At day 10, rSCF-treated mice had 6-fold higher platelet levels than control mice.

By day 3 postirradiation, the bone marrow cellularity dropped to ≈4% of preirradiation levels (Fig. 3). The bone marrow cellularity of control mice never recovered, whereas rSCF-treated mice had a dramatic increase in cellularity by day 6 and had almost returned to preirradiation levels by day 21 (Fig. 3). Decreases in GM-CFCs and HPP-CFCs were also

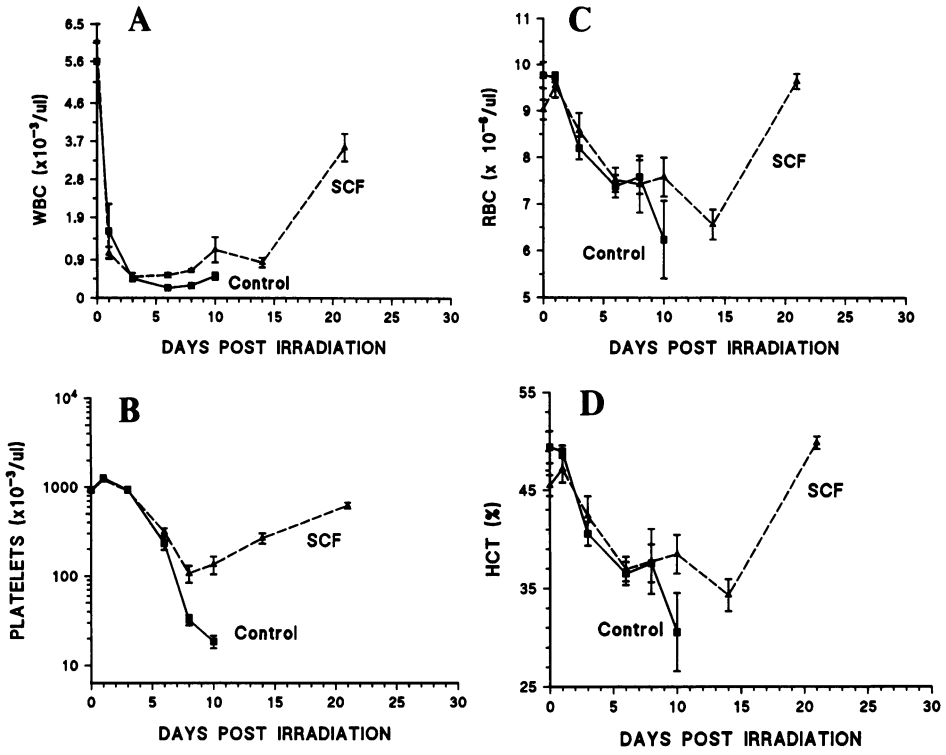

FIG. 2. Peripheral blood hematology of mice irradiated at 1150 rads and treated with excipient (——) or rSCF (– – –) at −20, −2, and +4 h. Data presented are the mean ± SEM for five mice at each time point. (*A*) WBC count. (*B*) Platelets. (*C*) RBC count. (*D*) Hematocrit (HCT).

observed following irradiation. HPP-CFCs and GM-CFCs dropped to <1% of pre-irradiation levels by day 1 postirradiation (Fig. 3). HPP-CFC and GM-CFC levels remained <1% of preirradiation values in control mice until their death. In contrast, in rSCF-treated mice, HPP-CFC and GM-CFC levels recovered postirradiation, and the levels at day 6 were 100-fold and 28-fold higher than in control mice, respectively. By day 21, GM-CFC levels had returned to normal, whereas the more primitive HPP-CFCs had only recovered to 50% of preirradiation levels (Fig. 4).

Cytospin preparations of bone marrow cells showed a marked decrease in neutrophil progenitors and mature neutrophils in control mice (0.5% of pretreatment levels at day 8), whereas rSCF-treated mice had an initial decrease in total numbers of neutrophils (6.8% pretreatment levels at day 8), followed by a return to preirradiation levels and an overshoot by day 21 (190% pretreatment levels). Of particular note is the fact that total neutrophil and neutrophil progenitor content of the bone marrow in rSCF-treated mice was 10-fold greater than control mice during the time period when control animals were dying (days 8–11).

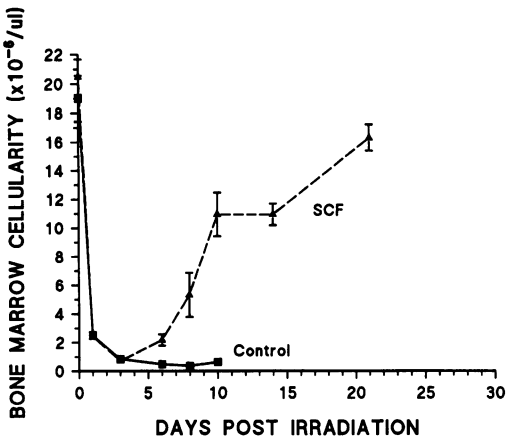

FIG. 3. Bone marrow cellularity of mice irradiated at 1150 rads and treated with excipient (——) or rSCF (– – –) at −20, −2, and +4 h. Data presented are the mean ± SEM for five mice at each time point.

Blood Cultures of Peripheral Blood to Test for the Presence of Bacteria. Blood from rSCF-treated and control mice was cultured under various conditions to test for disseminated bacteria. The organisms that grew were typed and grouped into either gut related or skin derived. Gut-related bacteria were detected in seven of seven control mice tested compared to only one of six rSCF-treated mice (representative of three separate experiments).

Autopsy Examination. Irradiated control mice died of bacterial infection. Multiple bacterial colonies were distributed within the liver parenchyma, spleen, oral mucosa, facial skin, and salivary glands. Bacterial emboli were sometimes noted in branches of the renal arteries. Although there were degenerative changes in the parenchymal cells surrounding the bacterial masses, cellular exocytic inflammatory changes were never present. In control and rSCF-treated mice, there was lymphocytic depletion in abdominal and thoracic lymph nodes, spleen, and submucosa of the small intestine.

Mucosal changes in the intestinal tract were carefully evaluated. There was blunting, shortening, and occasional superficial necrosis of the tips of the villi in the small intestine. Mitotic activity in the crypts was prominent. The flora and mucosal pattern of the cecum and large intestine were unremarkable. There was no evidence of perforation or peritonitis. No consistent differences were seen between the control or rSCF-treated mice in the small or large intestine using the qualitative methods employed in this study.

Serum Chemistry. Whole serum was analyzed for urea, nitrogen, potassium, glucose, albumin, total protein, and creatinine levels. Significant differences in albumin and glucose levels were observed between control and SCF-treated mice (data not shown). The other parameters measured showed no significant differences. The serum chemistry data

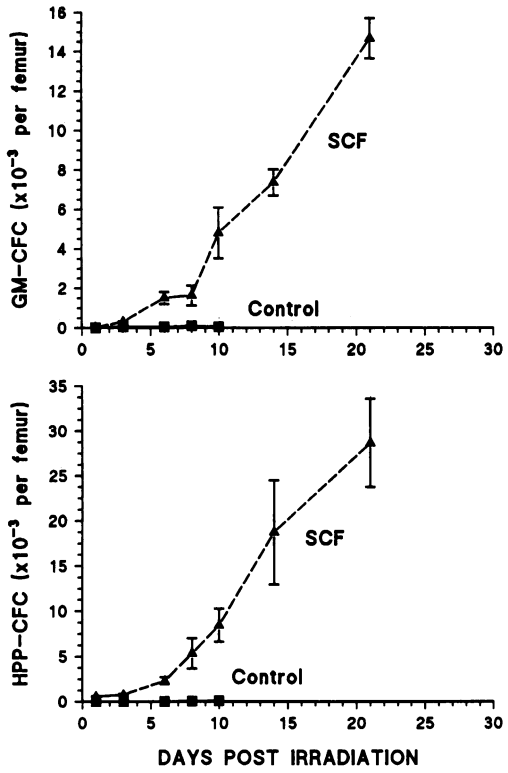

FIG. 4. Bone marrow progenitors of mice irradiated at 1150 rads and treated with excipient (——) or rSCF (– – –) at −20, −2, and +4 h. Data presented are the mean ± SEM for five mice at each time point. Mature progenitors (granulocyte-macrophage colony-forming cells; GM-CFC) and primitive progenitors (high proliferative potential colony-forming cells; HPP-CFC) were assayed as described in the text. At day 0 the levels of GM-CFC and HPP-CFC were 13,319 and 6981, respectively, for control mice and 13,541 and 6628, respectively, for rSCF-treated mice.

suggest that there were no major organ failures that could account for the differences in survival in the groups. The differences obtained are consistent with the control mice consuming less food and water than their much healthier rSCF-treated counterparts in the terminal phase of their life postirradiation.

DISCUSSION

The data presented in this study clearly demonstrate a significant radioprotective effect of rSCF in mice. All animals had radiation-induced damage in the bone marrow (Figs. 3 and 4). Previous studies have shown that damage to the lymphoid and hematopoietic systems is the primary cause of septicemia and death in irradiated animals (1). The most striking effects of rSCF are seen in the reduced frequency of infections as a result of rSCF administration. This reduction in infections appears to result from prompt hematopoietic recovery in the rSCF-treated animals. Primitive (HPP-CFC) and mature (GM-CFC) progenitor content as well as cellularity of the bone marrow increased markedly above the levels in control mice by day 6. Control mice died within 2 weeks of irradiation and blood cultures documented gut-derived septicemia.

The optimal radioprotective effect of rSCF requires administration pre- and postirradiation and suggests that SCF acts by sparing early hematopoietic cells from radiation-induced injury as well as stimulating hematopoietic recovery following irradiation. The role of SCF as a radioprotector is similar to IL-1 (3), which has been shown to enhance the cell cycling of bone marrow cells particularly into late S phase (8). The late S phase has been shown to be the most radioresistant phase of the cell cycle (26, 27). We propose that SCF acts in a similar fashion, synchronously stimulating the proliferation of radioprotective cells so that as a population they are more radioresistant. The fact that a −20-h administration, but not a −2-h administration, of rSCF results in radioprotection supports the notion that synchronizing cells into the S phase of the cell cycle is a probable mechanism for rSCF radioprotection. Following irradiation these cells repopulate the bone marrow and hematopoietic recovery follows. The magnitude of this phenomenon is striking, as control mice never recover hematopoiesis even up to 8–10 days postirradiation, whereas rSCF-treated mice have significant bone marrow regeneration detectable 5 days postirradiation. Although it is evident that the survival-enhancing effect of rSCF is in prevention of gut-derived septicemia, there were no significant differences in the peripheral blood absolute neutrophil counts (ANC) during the 8- to 10-day period postirradiation when most of the control animals were dying. Significant attempts have been made at determining a possible radioprotective effect of rSCF on the small intestine by histopathology; however, to date, no statistically significant differences have been observed. It is possible that at these doses of irradiation only small pockets of small intestine are damaged, making comparative analysis difficult. An additional factor in the protection against infection in the rSCF-treated mice may be radioprotection of tissue mast cells. rSCF is a potent proliferative signal for mast cells (28). Despite the lack of difference in peripheral blood ANC, there is a 10-fold higher level of neutrophils and neutrophil progenitors in the bone marrow of rSCF-treated mice. It is possible that the neutrophils are migrating to the tissues as rapidly as they produced in the bone marrow, to meet the severe bacterial challenge induced by the irradiation damage.

It is of interest to note that mice mutated at the *Sl* locus (SCF structural gene) are radiosensitive. *Sl* mutant animals have an LD_{100} of 200 rads, in contrast to an LD_{100} of their congenic counterparts being in the range of 700–900 rads (29, 30), suggesting that a lack of SCF may result in increased radiosensitivity.

The radioprotective effects of rSCF documented in this report suggest a potential therapeutic role in dose escalation against radiosensitive tumors such as lymphoma without bone marrow transplant support.

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