Effects of 5-Fluorouracil on Hematopoietic Stem Cells in Normal and Irradiated Mice

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(Received January 9, 1992)
(Revision Received June 15, 1992, Accepted June 19, 1992)

Hematopoietic stem cells (CFU-S)/5-Fluorouracil/X-irradiation/Mice

The effects of 5-fluorouracil (5-FU) on hematopoietic stem cells (CFU-S) and nucleated cells in mouse femur and spleen were studied in normal and X-irradiated mice (ddY-SLC male, 8–10 weeks old). Changes in the number of circulating blood cells also were investigated in mice treated with 5-FU. A single dose of 5-FU (150 mg/kg) was injected i.p. The femoral CFU-S decreased after 5-FU treatment up to day 3 when it reached 2% of the control value. The cells then increased, reaching a maximum about twice that of control by day 12. A very similar profile was found for splenic CFU-S. Post-irradiation recovery for femoral and splenic CFU-S in mice treated with 5-FU 3 days before X-irradiation (1.9 Gy) was faster than in mice given irradiation alone. Regrowth rates of CFU-S were almost the same as in mice treated with 5-FU alone. The radiosensitivity of the CFU-S population in mice treated first with 5-FU at different times before X-irradiation (1.9 Gy) changed day-by-day after treatment. The maximal survival for femoral CFU-S was found in mice treated with 5-FU 5 days before irradiation, and for splenic CFU-S in mice treated 12 days before irradiation.

INTRODUCTION

The hematopoietic stem cell population is heterogeneous in terms of its ability to produce progenitors of different lineages. A fraction of undifferentiated cells from stem cell populations of hematopoietic systems can be obtained by the elimination of proliferating cells during cell cycling which are killed by such inhibitors of DNA synthesis as hydroxyurea and 5-fluorouracil (5-FU)1–3. Stem cells that survive treatment with hydroxyurea or 5-FU are more primitive than cells in the untreated stem cell population and have a marked capability for generating precursors of myeloid, erythroid and megakaryocyte lineages1,2,4–6. No information is available, however, on the radiation response of undifferentiated subpopulations of hematopoietic stem cells. Evaluation of the radiosensitivity of primitive stem cells is important to our understanding of the development of bone marrow death in radiation-exposed animals.

The effects of 5-FU on spleen colony-forming units (CFU-S), nucleated cells in bone
marrow and spleen, and circulating blood cells were examined in mice treated with 5-FU. The post-irradiation recovery of CFU-S populations in mice that had been treated with 5-FU and variation in the radiosensitivities of CFU-S populations at different times after 5-FU treatment also were investigated.

MATERIALS AND METHODS

Animals

The animals used were male ddY-SLC mice of closed colony stock, 8–10 weeks old and weighing 30–36 g. They were housed individually in small compartments separated from each other and were fed laboratory chow and tap water acidified with 0.1 N HCl *ad libitum*. The cages were kept in an air-conditioned room at a temperature of 24±1°C throughout the experimental period.

5-FU treatment

A stock solution of 5-FU (Wako Pure Chemical Industries) was prepared in physiological saline at 5 mg/ml immediately before use. Mice were treated with a single i.p. injection of 5-FU at a dose of 150 mg/kg body weight, except when differently described.

Irradiation

The mice were irradiated with X-rays (200 kVp and 20 mA) through 0.5 mm copper and 0.5 mm aluminum filters. The dose rate was 0.67–0.69 Gy/min. The X-ray dose delivered was measured with an AE-1320 exposure ratemeter. Irradiation was done in an irradiation chamber placed under an X-ray machine at a focus surface distance of 58-cm. Several mice in individual lucite chambers were given a single whole-body exposure simultaneously.

Assay of exogenous CFU-S

The exogenous CFU-S technique of Till and McCulloch was modified and used to estimate the number of hematopoietic pluripotential and transplantable stem cells. Femurs and spleens from 3–8 donor mice were used to make the cell suspension preparation. Femoral bone marrow and spleen cell suspensions were prepared separately in ice-cold TC-199 medium. These cells were injected intravenously through the lateral tail veins of the recipients (10 mice per experimental point), who had been irradiated with 7.6 Gy of X-rays 3 to 4 h before the injection. Nine days after transplantation of the donor cells, the recipients were killed and their spleens removed and fixed with Bouin’s solution. A few days later, the number of colonies that had formed on the surface of the spleen was counted under an illuminating magnifier at 6x magnification. The total numbers of CFU-S in the femur and spleen were calculated from the frequency of CFU-S per number of transplanted cells and from the total of nucleated cells in the same tissues.
Nucleated cell counting

To count the numbers of nucleated cells in the femur and spleen, we stained cell suspensions prepared in TC-199 medium with Türk solution and counted the cell numbers with a hemocytometer.

Blood cell counting

Whole blood drawn from mouse outer iliac arteries and veins was collected in tubes containing the anticoagulant EDTA-2K. Leukocytes, erythrocytes and thrombocytes in the circulating blood were counted with a Sysmex K-1000 30 min after collection.

RESULTS

Changes in circulating blood cells in mice treated with 5-FU

Leukocytes, erythrocytes and thrombocytes in the circulating blood were chased for 24 days after 5-FU treatment (Fig. 1). The number of thrombocytes were at a minimum (41% of the control value) on day 6, reaching a higher value than that for the untreated controls 9 to 15 days after 5-FU treatment (158% of the control value on day 10). A similar phenomenon was seen in the changes in the leukocyte counts, there being a lesser but more prolonged depression and a smaller overshooting than for the thrombocytes. The number of erythrocytes did not change significantly. It was lower than the control values for 5 to 13 days after 5-FU treatment, being about 80% of the value on day 10.

Fig. 1. Changes in the numbers of leukocytes (upper), erythrocytes (middle), and thrombocytes (bottom) in circulating mouse blood after 5-FU treatment. Day 0, untreated mice (n=32). Points on all the other days represent the means±SE for 5–15 mice treated with 5-FU. Standard errors of the mean (S.E.) are indicated by bars except when enclosed by the experimental points (symbols). Control values (mean±SE); leukocytes 70.3±3.7 (×10⁴), erythrocytes 977.3±6.1 (×10⁴), thrombocytes 143.0±3.3 (×10⁴).
Changes in nucleated cells in femurs and spleens of mice treated with 5-FU

Nucleated cells in the femur were at a minimum (17% of the value of the untreated controls) on day 4, but had recovered to nearly the control value on the 9th day after 5-FU treatment (Fig. 2). The nucleated cells in the spleen decreased, being 62–66% of the control values between days 2 and 4, but recovering to the control value by day 9 after treatment.

Fig. 2. Changes in the total numbers of nucleated cells in moust femur (○) and spleen (●) after 5-FU treatment. The areas enclosed by dots (Normal) represent the means ± SE for the untreated controls. Each point represents the mean ± SE for 2 to 4 experiments. In each experiment, 3–8 mice that had been treated with 5-FU were killed, then the average total nucleated cell numbers were determined for the femur and spleen. Control values (mean ± SE); femur 1.87±0.12 (×10⁷), spleen 1.72±0.06 (×10⁸).

Changes in the CFU-S population in mice treated with 5-FU

The patterns of change in the femoral and splenic CFU-S numbers in donor mice after 5-FU treatment are shown in Fig. 3. The number of femoral CFU-S decreased up to day 3 after treatment, to 2% of the untreated control value. Thereafter, the number increased to nearly the control value by day 9 and reaching the maximal value of 208% on day 12. A very similar profile was found for splenic CFU-S. The splenic CFU-S populations respectively were 0.3% and 230% of the control values on days 3 and 12. On day 3, the CFU-S response to 5-FU was dose dependent in the range of 19 to 150 mg/kg (Fig. 4). The dose survival curve for femoral CFU-S
after the single dose was exponential with a $D_{10}$ (the in vivo dose that produces 10% survival of CFU-S) of about 100 mg/kg after a shoulder at about 40 mg/kg. The effect on femoral CFU-S was less than on splenic CFU-S ($D_{10} \approx 60$ mg/kg).

**Effect of 5-FU on post-irradiation changes in nucleated cells**

Changes in nucleated cells in the femur and spleen after 1.9 Gy X-irradiation were examined in mice treated with 5-FU 3 days before irradiation (combined treatments) and in untreated, irradiated mice (irradiation alone) (Fig. 5). The nucleated cell recovery pattern in the spleens with combined treatments was similar to that for irradiation alone and was not affected by 5-FU alone. These results and those for the femur indicate that the combined effects of 5-FU
Effect of 5-FU on post-irradiation changes in CFU-S populations

Experiments similar to those for the nucleated cells were done on CFU-S populations in the femur and spleen. The number of femoral CFU-S in the combined treatments (the 5-FU prior treatment group; treatment with 5-FU 3 days before 1.9 Gy X-irradiation) had decreased to about 0.1% of the untreated control value 2 hours after irradiation (Fig. 6). The CFU-S number began to increase on day 1 after irradiation, earlier than for the irradiation alone cells which began to increase on day 2. The CFU-S numbers for the combined treatment group returned to the control value much earlier than in the irradiation alone group. Regrowth of femoral CFU-S...
during the recovery phase was more rapid in the combined treatment group than in the irradiation alone one. Moreover, the recovery rate was almost the same as for 5-FU treatment alone.

The same trend was found for changes in the number of splenic CFU-S (Fig. 7). Recovery of the number of splenic CFU-S in the combined treatment group (mice treated with 5-FU 3 days before 1.9 Gy X-irradiation) was faster than that for the irradiation alone group. Overshooting for the survival of CFU-S was seen for the combined treatment group on day 9 after irradiation, but was not seen in the irradiation alone group throughout the observation period.

![Fig. 8. Radiosensitivity of CFU-S in mouse femur and spleen at different times after 5-FU treatment. Open symbols: femoral CFU-S; solid symbols: splenic CFU-S. Day 0, untreated mice (Control) only irradiated with 1.9 Gy. All other donor mice were irradiated with 1.9 Gy of X-rays at different times after 5-FU treatment. Radiosensitivities were calculated from the equation: Survival (%) = \( \frac{\text{CFU-S for irradiated mice with 5-FU}}{\text{CFU-S for mice with 5-FU alone}} \times 100 \). The bars were calculated from the means ± SE of both factors: \( \Delta N = N \cdot \sqrt{\left( \frac{\Delta A}{A} \right)^2 + \left( \frac{\Delta B}{B} \right)^2} \).](image)

**Effect of 5-FU priortreatment on the radiosensitivity of the CFU-S populations**

The radiosensitivities of the CFU-S populations in the femur and spleen were tested for 15 days after 5-FU treatment. Donor mice were irradiated with 1.9 Gy of X-rays at specified times after 5-FU treatment, and their CFU-Ss assayed 2 hours later. Maximal survival for femoral CFU-S occurred in the mice treated with 5-FU 5 days before irradiation (Fig. 8), being 21.6% as compared to the 13.3% for the untreated controls (given 1.9 Gy). By contrast, an increase in the survival of splenic CFU-S was found in mice treated with 5-FU 12 days before irradiation; 16.4% survival as compared to the 4.9% for the untreated controls (irradiation alone). These results demonstrate that the radiosensitivity per se of the CFU-S populations that survived 5-FU treatment varied daily after treatment.
DISCUSSION

The kinetics of the leukocytes and thrombocytes in the circulating blood gave minimum values on about day 6 after 5-FU treatment then increased, reaching even higher values than those for the controls by day 12. The increase in thrombocyte numbers was much more marked than that for leukocytes. The number of erythrocytes, however, remained slightly less than that for the controls for about 2 weeks after treatment. The results reported here are in general agreement with previous reports\(^{8-11}\). The minimum number of nucleated cells in the femur was about 20\% of the control value on day 4 after 5-FU treatment (Fig. 2), whereas the minimum value for femoral CFU-S in the treated mice was 2\% of the control value on day 3 (Fig. 3). The difference seen in the values for the nucleated cells and CFU-S populations may depend on the proliferation of these cells in cell fractions. That is, depletion of the CFU-S population by 5-FU accounts for more than 90\% of the cell killing in the entire hematopoietic stem cell population. By contrast, the surviving CFU-S fraction after 5-FU treatment reached the control value by day 9 then overshot on day 12. This complete recovery of the CFU-S population is evidence that the CFU-S fraction that survived 5-FU treatment did not retain the damage induced by 5-FU and had a marked ability for repopulation.

The effects of 5-FU treatment and X-irradiation on the killing of nucleated cells and of CFU-S were additive (Figs. 5, 6 and 7). Recovery from the radiation-induced injury to femoral and splenic CFU-S in mice previously treated with 5-FU was faster than in mice given X-irradiation only. Moreover, the recovery rates for CFU-S in mice given the combined treatment were almost the same as, or somewhat faster than, those for mice treated with 5-FU alone. The enhanced post-irradiation recovery of CFU-S population in irradiated mice first treated with 5-FU can not be considered to be the result of the direct action of 5-FU because 5-FU inhibits the thymidine synthesis that leads to the death of proliferating cell fractions. Conceivably, 5-FU acts indirectly on the regrowth of CFU-S. One possibility is that changes in the CFU-S microenvironment caused by 5-FU's elimination of proliferating cell fractions lead to the secretion of unknown factors efficacious for the promotion of the proliferation or regrowth of CFU-S. Cytotoxic agents such as cytosine arabinoside\(^{12}\), vincristine\(^{13,14}\) and cyclophosphamide\(^{15,16}\) have been shown to cause marrow depletion, the result being enhanced recovery from radiation-induced damage to hematopoietic stem cell populations. Our results also indicated that the radiosensitivities of CFU-S populations in the femur and spleen changed daily for 15 days after 5-FU administration to mice (Fig. 8). This suggests that CFU-S populations that survived 5-FU treatment are heterogeneous for radiosensitivity as a function of time after treatment and that a small number of radioresistant cells exist in any given CFU-S population.

ACKNOWLEDGEMENTS

We thank Mrs. T. Uekusa and Mrs. N. Suzuki for their skilful technical help and their care
of the animals used in the experiments.

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