Evaluation of Short-term Myelotoxicity Study in Dietary Reduced Rats

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Abstract: This study attempted to prove our hypothesis that a short-term toxicity study, using a 4-day dosing regimen as an example, is suitable for evaluating myelotoxicity in rats. We compared the hematological, bone marrow cytological and histopathological results of 5-fluorouracil (5-FU) treated and pair-feeding groups after a 4-day administration period. Several experimental groups were defined for this 4-day study as well as for our previously reported 14-day study (Miyata et al., 2009); these included 5-FU treated groups receiving 12, 15 and 18 mg/kg/day (FU12, FU15 and FU18), pair-feeding groups (R12, R15 and R18 receiving the same amount of food as the FU12, FU15 and FU18 groups, respectively) and a nontreated control group. Although severe reductions in body weight gain and food consumption were reported in the 14-day study, only slight reductions were observed in the 4-day study. In the 4-day study, a decrease in blood reticulocytes and a decreasing trend of marrow erythroid cells were only observed in the FU18 group, and no effects were observed in the pair-feeding groups. The erythroblastic changes observed in this 4-day study were thought to reflect the direct influence of 5-FU administration. Since concerns regarding the influence of secondary changes related to undernutrition were minimized in the 4-day study, it was thought to clarify the direct influence of 5-FU administration on erythroblastic cells. Thus, a 4-day study protocol might be helpful for distinguishing secondary changes related to undernutrition. (J Toxicol Pathol 2010; 23: 31–37)

Key words: myelotoxicity, short-term toxicity study, dietary restriction, 5-fluorouracil, pair-feeding, rat

Introduction

The rat is a widely used animal model for safety assessments of such things as pharmaceuticals. Dietary restriction is currently one of the most effective methods for extending the lifespan of rodents and delaying the onset of age-related diseases¹–³. Meanwhile, many reports concerning hematological changes in dietary restricted rats have been published⁴–⁹. Young rats exhibiting remarkable growth are generally used in short-term repeated-dose toxicity studies, and suppression of body weight gain and decreased food consumption are often recognized in the drug administration groups in such toxicity studies. Consequently, whether hematological, bone marrow cytological or histopathological changes are caused directly by the drug being tested or indirectly by suppression of body weight gain and decreased food consumption can be difficult to evaluate. We previously reported that many of the influences of dietary restriction on hematological examination values were comparable to those caused by 5-fluorouracil (5-FU) administration for 14 days in young rats (6 weeks old at the start of experimentation)¹⁰. Furthermore, we also reported that adult rats (12 weeks old at the start of experimentation) with a minimal body weight gain were more suitable than young rats for hematological evaluations in a 14-day study period¹¹. However, the age-related differences between the young and adult studies were not very large, possibly because these age groups were rather close. Thus, we suspected that further rat studies were needed to evaluate myelotoxicity. Though a 14-day period was selected in our previous studies, we hypothesized that a shorter period, such as 4 days, might be useful for evaluating myelotoxicity, since the influence of undernutrition would be reduced.

The anti-cancer drug 5-FU belongs to a category of chemotherapy agents called antimetabolites and functions as a pyrimidine analog¹²,¹³; the myelotoxic effect of 5-FU on the bone marrow is a serious adverse effect associated with
its clinical use\textsuperscript{14}. DNA and RNA synthesis is inhibited by 5-FU not only after repeated dosing, but also after single dosing\textsuperscript{15}. In this study, we selected 5-FU as a positive control for myelotoxicity.

In the present study, 5-FU treated and pair-feeding groups (animals that were given the same amount of food as the average amount of food consumed by the 5-FU treated animals) were used. We compared the hematological, bone marrow cytological and histopathological results of the 5-FU treated and pair-feeding groups after a 4-day administration period. Here, we discuss the differences in the hematological and/or myelotoxic effects under decreased food consumption based on the common general toxic parameters. Our hypothesis that a 4-day period may be suitable for evaluating myelotoxicity was verified by referring to our previous 14-day study results.

Materials and Methods

Chemicals and dose selection rationale

The 5-FU was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and was dissolved in water for injection for dosing. Since this 4-day study was a comparative experiment, the same dosages as used in our previous 14-day study\textsuperscript{10} were chosen; therefore, dosages of 12, 15 and 18 mg/kg/day were selected.

Animals and housing conditions

A total of 42 male CrI:CD(SD) rats were purchased from Charles River Japan, Inc. (Tsukuba, Ibaraki, Japan). The animals were housed individually in stainless steel cages (W:225 mm $\times$ D:350 mm $\times$ H:200 mm) with an artificial lighting cycle of 12 hours (7:15 to 19:15), a temperature of 23 $\pm$ 3°C, a relative humidity of 50 $\pm$ 20% and a ventilation cycle of 10 to 20 times/hour. Before group assignment, all the animals were allowed free access to a standard laboratory animal chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water. After group assignment, the R12, R15 and R18 groups described below were given restricted diets. At the start of dosing and pair-feeding, the animals were 6 weeks of age.

All the animals were treated in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Taisho Pharmaceutical Co., Ltd.

Study groups

The animals were divided into the following 7 groups: NT, FU12, FU15, FU18, R12, R15 and R18 (6 rats/group). The animals in the NT group were nontreated and were given restricted diets. The animals were allowed free access to a standard laboratory animal chow; therefore, dosages of 12, 15 and 18 mg/kg/day were selected. If all the food was not consumed in any of the R12, R15 or R18 groups, the next allotment of chow was supplied without removing the food that had not been consumed. The next day of initial administration of 5-FU was set as the start date of pair-feeding.

Examinations and methods

The starting day of 5-FU administration or pair-feeding was designated as Day 0 in this study. Body weight and food consumption were measured every day in each animal. The ratio of the amount of limited food was calculated using the following formula: $fu / nt - 100$, where $fu$ represents the total amount of food consumption in each 5-FU group $\times$ 100 and $nt$ represents the total amount of food consumption in the NT group.

Significant differences between the NT and 5-FU treated groups or between the NT and pair-feeding groups were analyzed using the following procedure. The homogeneity of the variance among the groups was first tested using a Bartlett’s test. When a homogenous variance was noted, all the groups were compared using a one-way analysis of variance. When a heterogeneous variance was noted, the Kruskal-Wallis test was subsequently performed. Finally, the Dunnett’s test (if homogeneous) or Dunnett-type multiple comparison test (if heterogeneous) was used if
a significant difference was noted between the groups.

Significant differences between the 5-FU treated and pair-feeding groups (i.e., FU12 vs. R12, FU15 vs. R15 or FU18 vs. R18) were analyzed using the following procedure. The homogeneity of the variance among the groups was first tested using the F-test, and then the Student’s t-test (if homogeneous) or Aspin-Welch’s t-test (if heterogeneous) was performed.

The Bartlett’s test, one-way analysis of variance, Kruskal-Wallis test and F-test were conducted using a significance level of 5% (two-tailed), while the other tests were conducted using significance levels of 1% and 5% (two-tailed). Statistical analyses of the clinical observations and necropsy and histopathology results were not performed.

Results

Mortality and clinical signs

No deaths and no abnormalities were seen in the present 4-day study.

Food consumption and body weight

In the 5-FU treated groups, a decrease in food consumption was seen at the end of the administration period. The ratios of the total amount of food consumption in each 5-FU treated group (FU12, FU15 and FU18), compared with the NT group, were –8%, –7% and –11%, respectively (Fig. 1A). In the pair-feeding groups, one rat left some food on Day 2 but subsequently consumed all the available food thereafter.

Body weight in the FU18 group was lowest at the end of the administration period, but it was not a marked change since it was only less than 10% compared with the corresponding NT group. No statistically significant difference was observed between the 5-FU treated and pair-feeding groups (Fig. 1B).

Hematology and bone marrow cytology

The principal results are shown in Figs. 2 and 3. In the hematological analysis of the peripheral blood samples, a statistically significant decrease in the number of reticulocytes was observed in the FU18 group (Fig. 2A). In the bone marrow analysis, a decreasing trend of erythroid cells was observed in the FU18 group, although this change was not statistically significant (Fig. 3A). Meanwhile, no effects were observed in the pair-feeding groups in the 4-day study. Individual data regarding the numbers of blood reticulocytes and marrow erythroid cells in the FU18 group are shown in Fig. 4. Decreases in the numbers of these cells were particularly remarkable in 2 of the 6 rats. In addition, abnormal granulocytes with hypersegmented nuclei and/or polyploidy nuclei (with a frequency of 1% or less) were observed in one rat in the FU18 group. This rat was one of the two rats that exhibited remarkable decreases in the numbers of reticulocytes and erythroid cells, as mentioned above. A statistically significant difference in the number of marrow lymphoid cells was observed between the 5-FU treated and pair-feeding groups, but this change was not meaningful, as it was within the physiological range (Fig. 3C).

Organ weight

A decrease in the thymus weight was observed in the FU18 group (Fig. 5A). No change was observed in the spleen weight (Fig. 5B).

Necropsy and histopathology

No abnormalities were seen in either the macroscopical or microscopical examinations performed in all the groups. Representative tissue images of the bone marrow are shown in Fig. 6.

Discussion

We compared the hematological, bone marrow cytological and histopathological results of the 5-FU treated and pair-feeding groups after a 4-day administration period. Differences between the present study data (4-day period) and previously reported study data (14-day period) were then compared.

Although severe reductions in body weight and food consumption were reported in the 14-day study, only slight reductions were observed in the present 4-day study. A decrease in the numbers of blood reticulocytes and a decreasing trend of marrow erythroid cells were observed in the FU18 group in the 4-day study. Furthermore, the decreases in the numbers of these cells were particularly remarkable in 2 of the 6 rats. Meanwhile, no effects were observed in the pair-feeding groups in this 4-day study. On the other hand, no differences in these decreases of the erythroblastic cells were found in the 5-FU treated or pair-feeding groups in the 14-day study due to severe undernutrition. The erythroblastic changes observed in
this 4-day study were thought to reflect the direct influence of 5-FU administration. Ogawa et al. reported that dietary restriction strongly influences the reticulocytes in peripheral blood\(^5\). We previously reported that the influence of undernutrition on erythroblastic changes cannot be disregarded in 14-day toxicity studies\(^10, 11\). Moreover, the susceptibility of plasma erythropoietin levels to feeding conditions\(^16\) originates from the decrease in the dietary protein content\(^17–19\). We considered that undernutrition, especially a reduction in dietary protein, might also result in a decrease in blood reticulocytes and marrow erythroid cells. Since the influence of secondary changes related to undernutrition was reduced in the 4-day study, it was thought to clarify the direct influence of 5-FU administration on erythroblastic cells. Thus, evaluations performed at a time when the influence of undernutrition had not yet appeared were useful for evaluating the erythroblastic cells. In addition, the direct influence of 5-FU administration was also detected in the marrow cytological examination performed in the 4-day study. Abnormal granulocytes with hypersegmented nuclei and/or polyploid nuclei (with a frequency of 1% or less) were observed in one rat in the FU18 group; however, a reduction in myelo-lymphoid cells was not observed. Though those changes were diminutive, but they were considered the effects of 5-FU based on our previous 14-day study results. These cytological anomalies were not observed in any of the nontreated and pair-feeding regimens in our previous studies\(^10, 11\). The administration period might be short in dosage used in this 4-day study so that marrow cytological change may change clearly. The number of reticulocytes is larger in rats than in dogs or monkeys\(^20, 21\); thus, rats appear to exhibit erythrokinetic activity. This fact might explain the difference in cytopenia receptivity. In this 4-day study, no histopathological changes were detected, though telangiectasis and decreased hematopoesis in bone marrow were reported in the previous 14-day study\(^10\). The above pathological differences between the two studies were likely due to the short dosing period, low dosage of 5-FU and small influence of undernutrition in the 4-day study.

**Fig. 2.** Hematological changes in reticulocytes (A), red blood cells (B), hemoglobin (C), platelets (D) and white blood cells (E) at the end of the administration period. Data are expressed as means ± S.D. (n=6). Statistical significance was analyzed using the Dunnett’s test or Dunnett’s-type test (**\(p<0.01\)) compared with the NT group. Moreover, the differences in values between the 5-FU treated and pair-feeding groups were analyzed using a Student’s \(t\)-test or Aspin-Welch’s \(t\)-test (# \(p<0.05\)).
Matsumoto et al. reported that 4-day toxicity studies are effective based on the total generation time of marrow cells, and the occurrence of hemocytopenia is seen within about 4 days in tests for several myelotoxic chemicals. Since examinations performed within a 4-day period are not yet influenced by undernutrition, the influence of 5-FU on erythroblastic cells was successfully detected in our 4-day study. The pair-feeding examination performed in the present study also suggested that the 4-day toxicity study was useful for detecting 5-FU toxicity. Nonspecific stress responses commonly lead to erroneous identification of test compounds as immuno-myelotoxic. In conclusion, in repeated-dose toxicity studies of drugs in rats, weight loss and decreased food consumption are often recognized in drug administration groups. Consequently, 4-day toxicity studies for hematological evaluations might be helpful for distinguishing secondary changes arising from undernutrition.

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References

1. Pollard M and Luckert PH. Tumorigenic effects of direct- and indirect-acting chemical carcinogens in rats on a restricted diet. J Natl Cancer Inst. 74: 1347–1349. 1985.
2. Sheldon WG, Bucci TJ, Hart RW, and Turturro A. Age-related neoplasia in a lifetime study of ad libitum-fed and food-restricted B6C3F1 mice. Toxicol Pathol. 23: 458–476. 1995.
3. Jiang W, Zhu Z, and Thompson HJ. Dietary energy restriction modulates the activity of AMP-activated protein kinase, Akt, and mammalian target of rapamycin in mammary carcinomas, mammary gland, and liver. Cancer Res. 68: 5492–5499. 2008.
4. Oishi S, Oishi H, and Hiraga K. The effect of food restriction for 4 weeks on common toxicity parameters in male rats. Toxicol Appl Pharmacol. 47: 15–22. 1979.
5. Ogawa Y, Matsumoto K, Kamata E, Ikeda Y, and Kaneko T. Effect of feed restriction on peripheral blood and bone marrow cell counts of Wistar rats. Exp Anim. 34: 407–416. 1985.
6. Levin S, Semler D, and Ruben Z. Effects of two weeks of feed restriction on some common toxicologic parameters in Sprague-Dawley rats. Toxicol Pathol. 21: 1–14. 1993.
7. Hubert MF, Laroque P, Gillet JP, and Keenan KP. The effects of diet, ad Libitum feeding, and moderate and severe dietary restriction on body weight, survival, clinical pathology parameters, and cause of death in control Sprague-Dawley rats. Toxicol Sci. 58: 195–207. 2000.
8. Yoshii A, Shiraiishi Y, Ogawa S, Kinomoto T, Iino Y, Matsui A, Sawada M, Hamano H, Kuroda H, Hayashi Y, Nishi N, Mera Y, and Takei M. Effects of food restriction on result of hematology examination and urinalysis in CD(SD)IGS rats—a four-week restricted feeding examination with two-week recovery period in six-week-old rats— In: Biological Reference Data on CD(SD)IGS Rats - 2002/2003, Y Maeda and K Shibuya (eds). Best Printing, Tokyo. 73–84. 2003.
9. Seki M, Yamaguchi K, Marumo H, and Imai K. Effects of food restriction on reproductive and toxicological parameters in rats — in search of suitable feeding regimen in long-term tests. J Toxicol Sci. 22: 427–437. 1997.

Fig. 5. Relative organ weight changes in the thymus (A) and spleen (B) at the end of the administration period. Data are expressed as means ± S.D. (n=6). Statistical significance was analyzed using the Dunnett’s test or Dunnett’s-type test (* p<0.05), compared with the NT group. Moreover, the differences in values between the 5-FU treated and pair-feeding groups were analyzed using a Student’s t-test or Aspin-Welch’s t-test (not significant: p>0.05).

Fig. 6. Histopathology of the bone marrow at the end of the administration period. Representative images of the NT group (A), FU18 group (B) and R18 group (C) are shown. No abnormalities were seen in the microscopical examinations performed in all the groups. Magnification: ×140. H&E stain.
10. Miyata H, Asanuma F, Iwaki Y, Kimura M, and Matsumoto K. Evaluation of myelotoxicity in dietary restricted rats. J Toxicol Pathol. 22: 53–63. 2009.
11. Asanuma F, Miyata H, Iwaki Y, Kimura M, and Matsumoto K. Importance of starting age for myelotoxicity study in dietary restricted rats. J Toxicol Pathol. 22: 153–166. 2009.
12. Hartmann KU and Heidelberg C. Studies on fluorinated pyrimidines. XIII. Inhibition of thymidylate synthetase. J Biol Chem. 236: 3006–3013. 1961.
13. Duschinsky R, Pleven E, and Heidelberger C. The synthesis of 5-fluoropyrimidines. J Am Chem Soc. 79: 4559–4560. 1957.
14. Macdonald JS. Toxicity of 5-fluorouracil. Oncology (Williston Park). 13: 33–34. 1999.
15. Tewari SP, Srivastava RK, Verma P, and Zaidi SH. Effect of 5-fluorouracil on rat bone marrow. Indian J Cancer. 21: 99–101. 1984.
16. Naeshiro I, Yoshioka M, Chatani F, and Sato S. Changes in the plasma erythropoietin level in rats following fasting, aging, and anaemia. Comp Haematol Int. 8: 87–93. 1998.
17. Fried W and Anagnostou A. The role of protein and other nutritional factors in the regulation of erythropoiesis. In: Current Concepts in Erythropoiesis, CDR Dunn (ed). John Wiley & Sons Ltd, New York. 233–244. 1983.
18. Borelli P, Blatt S, Pereira J, de Maurino BB, Tsujita M, de Souza AC, Xavier JG, and Fock RA. Reduction of erythroid progenitors in protein-energy malnutrition. Br J Nutr. 97: 307–314. 2007.
19. Barrio Rendo ME. Depressed response of the erythropoietin-responsive splenic cell population to erythropoietin in acutely protein restricted mice. In Vivo. 9: 71–73. 1995.
20. Matsuzawa T, Nomura M, and Unno T. Clinical pathology reference ranges of laboratory animals. Working Group II, Nonclinical Safety Evaluation Subcommittee of the Japan Pharmaceutical Manufacturers Association. J Vet Med Sci. 55: 351–362. 1993.
21. Hirashima K. Comparative hematology. In: Hematology of Experimental Animals. M Seki, K Hirashima, and K Kobayashi (eds). Soft Science, Tokyo. 192–204. 1981 (in Japanese).
22. Matsumoto K and Shirai T. Basic study on drug-induced myelotoxicity—An application of bone marrow testing to toxicity study—. Juntendo Med. J. 33: 74–86. 1987 (in Japanese).
23. Uehara T, Tsuchiya N, Masuda A, Torii M, Nakamura M, Yamate J, and Maruyama T. Time course of the change and amelioration of nedaplatin-induced nephrotoxicity in rats. J Appl Toxicol. 28: 388–398. 2008.
24. Torous DK, Hall NE, Murante FG, Gleason SE, Tometsko CR, and Dertinger SD. Comparative scoring of micronucleated reticulocytes in rat peripheral blood by flow cytometry and microscopy. Toxicol Sci. 74: 309–314. 2003.
25. Constable TB and Blackett NM. Comparison of effect of four cytotoxic agents on granulocytic and erythroid repopulating ability of rat bone marrow. J Natl Cancer Inst. 48: 941–948. 1972.