Effect of 5-Fluorouracil on "Primitive" Hematopoietic Stem Cells That Reconstitute Whole Erythropoiesis of Genetically Anemic W/W' Mice

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The potential to reconstitute the whole erythropoiesis of a genetically anemic (WB × C57BL/6)F₁-W/W' (WBB6F₁-W/W') mouse for at least 8 weeks was compared between 5-fluorouracil (5FU)-treated and nontreated bone marrow cells. C57BL/6-Pgk₁/Pgk₁ female mice, in which each stem cell had either A-type or B-type phosphoglycerate kinase (PGK) owing to the random inactivation of one of two X chromosomes, were used as donors. As a marker of the reconstitution, electrophoretic pattern of hemoglobin was used. The concentration of the stem cells that reconstitute the whole erythropoiesis of WBB6F₁-W/W' mouse was higher in the marrow of donors that had received an injection of 5FU two days previously (two-day 5FU-treated) than in the marrow of nontreated donors. In the marrow of four-day 5FU-treated mice, however, the concentration was comparable to that of nontreated mice.

The PGK electrophoretic pattern of WBB6F₁-W/W' mice reconstituted by nontreated marrow cells was comparable to the PGK pattern of WBB6F₁-W/W' mice reconstituted by four-day 5FU-treated marrow cells. Thus, a single stem cell with extensive proliferative potential rather than multiple spleen colony-forming units appeared to be responsible for the erythropoietic reconstitution in the transplantation of nontreated healthy marrow cells as well as 5FU-treated marrow cells.

MATERIALS AND METHODS

Mice. C57BL/6-Pgk₁/Pgk₁ and WBB6F₁-W/W' mice were raised in our laboratory. The original stock of mice with Pgk₁ gene was introduced from Dr Vern M. Chapman of Roswell Park Memorial Institute to the National Cancer Center Research Institute in Tokyo by Dr Hiroshi Tanooka. We obtained C3H mice with the Pgk₁ gene from Dr Tanooka and introduced the Pgk₁ gene to the C57BL/6 strain of our inbred colony by repeated backcrosses (11 backcrosses at the time of the present study). Because the original C57BL/6 strain has the usual type of PGK gene (Pgk₁), C57BL/6-Pgk₁/Pgk₁ mice were produced by mating C57BL/6-Pgk₁/Pgk₁ female mice with C57BL/6-Pgk₁/Y male mice. Resulting C57BL/6-Pgk₁/Pgk₁ mice were used as donors of bone marrow cells at 2 to 3 months of age. The ratio of A-type to B-type PGK in erythrocytes of C57BL/6-Pgk₁/Pgk₁ mice is 50/50.

Although the W' mutant gene was derived from the Jackson Laboratory, Bar Harbor, ME, it is now maintained in our inbred C57BL/6 mouse colony. WB-W'/+ strain, which was also obtained from the Jackson Laboratory, has been maintained by brother-sister mating. WBB6F₁-W/W' mice which had been produced by a cross between WB-W'/+ and C57BL/6-W'/+ mice were used as recipients at 4 months of age.

Hemoglobin of WBB6F₁-W/W' mice (Hbb'/Hbb') can be distinguished from that of C57BL/6-Pgk₁/Pgk₁ mice (Hbb'/Hbb') by electrophoresis.

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5FU treatment and bone marrow transplantation. On various days after intravenous (IV) injection of 5FU (150 μg/μg weight), C57BL/6-Pgk-1P/Pgk-1P mice were anesthetized with ether and killed by cervical dislocation. Bone marrow cells were collected from at least five mice and suspended in Eagle’s medium according to the method described previously.15 Cells were counted with a standard hemocytometer.

Recipient WBB6F1-W/W+ mice were irradiated (200 rad) with a Shimadzu X-ray machine (180 kV and 20 mA, with 2-mm Al filter, 50 rad/min) to facilitate bone marrow reconstitution. Various numbers of bone marrow cells from 5FU-treated or nontreated mice were injected through the lateral tail vein.

Assay of CFU-S. The method of Till and McCulloch14 was used; the recipient WBB6F1-W/W+ mice were killed eight or 14 days after the transplantation of bone marrow cells; spleens were removed and fixed in Bouin’s solution, and colonies were counted with a dissection microscope (×7).

Blood sampling. Mice were anesthetized with ether, and blood samples were obtained from the retroorbital sinus with heparinized microhematocrit tubes. Care was taken to remove small volumes (≤50 μL) of blood to minimize possible hematologic stress. The microhematocrit tube was centrifuged, and the tube was cut beneath the level of the buffy coat; packed erythrocytes were divided into two parts; one part was used for examination of hemoglobin and another part was used for examination of PGK.

Examination of hemoglobin. Electrophoresis of hemoglobin was performed after modification with cysteine according to the method described by Whitney15; the hemoglobin pattern was determined visually using known mixtures as standards. By this method, hemoglobin of WBB6F1-W/W+ mice (HbbS/HbbS) mixed with hemoglobin of C57BL/6-Pgk-1P/Pgk-1P mice (HbbP/HbbP) is detectable if the former contributes ≥5% of the total. We assumed that WBB6F1-W/W+ recipient mice were reconstituted by bone marrow cells of C57BL/6-Pgk-1P/Pgk-1P mice only when the hemoglobin pattern of the former could not be distinguished from that of the latter; i.e., >95% of erythrocytes in the reconstituted WBB6F1-W/W+ mice were derived from the bone marrow of C57BL/6-Pgk-1P/Pgk-1P mice.

Limiting dilution analysis. The principle of the method has been described by Porter and Berry,17 Breivik,18 and Boggs et al19 and was performed as follows. Random samples from a homogeneous cell population were assayed for the presence or absence of stem cells. Such cells should be distributed among samples in a Poisson fashion. The finite probability that a sample will not contain a stem cell is defined by $P_n = e^{-n}$, where $n$ is the fraction of stem cells in the sample of $x$ cells. $P$ is the proportion of samples devoid of stem cells and estimates $P_0$. The concentration of stem cells in the sample can then be expressed at $\phi = -\ln P/x$.

Electrophoresis of PGK. Small pieces of filter paper were soaked with blood samples and placed in small plastic tubes; the tubes were kept at –80°C. PGK pattern of erythrocytes was examined only when WBB6F1-W/W+ recipient mice were assumed to be reconstituted by bone marrow cells of C57BL/6-Pgk-1P/Pgk-1P mice. After thawing, pieces of filter papers with blood samples were inserted into the gel plate. The electrophoresis was performed on a gel plate (10 × 20 cm) of starch (Wako Pure Chemical, Tokyo, Japan) for 17 hours at 5 V/cm and 4°C according to the method described by Tanooka and Tanaka.11 The gel plates were then cut into two slices, and PGK was detected as nonfluorescent spots owing to conversion of NADH to NAD in the PGK assay system. The spot of A-type PGK was usually 6 cm from the origin, and that of B-type was 4 cm from the origin. According to the report of Reddy and Fialkow,19 the relative activity of PGK alloenzymes was estimated visually using known mixtures as standards. With this technique, a minor population in a mixture of blood is detectable if it contributes ≥5% of the total PGK activity.

Hydroxyurea treatment. Bone marrow cells were incubated for 60 minutes at 37°C in prewarmed serum-free α-medium with or without 200 μg/mL hydroxyurea (Sigma) at a concentration of 10⁸ cells/mL to kill the cells in the DNA synthetic phase (S phase) of the cell cycle. Preliminary experiments revealed that this concentration of hydroxyurea was suitable for killing all cells in S phase.20 After incubation, cells were washed three times in α-medium supplemented with 2% fetal calf serum (FCS) and 10⁵ cells were suspended in 0.2 mL α-medium and injected.

RESULTS

In a previous study, we harvested bone marrow cells of C57BL/6-Pgk-1P/Pgk-1P mice four days after an injection of 5FU (four-day 5FU treated). First, we compared the erythropoietic reconstitution of four-day 5FU-treated mouse cells and nontreated mouse cells. Various numbers of bone marrow cells from four-day 5FU-treated and nontreated C57BL/6-Pgk-1P/Pgk-1P mice were injected into 200-rad-irradiated WBB6F1-W/W+ mice; hemoglobin pattern of recipients was examined at 4-week intervals. When the hemoglobin pattern of WBB6F1-W/W+ recipient mice (HbbS/HbbS) changed to pure HbbP/HbbP, the PGK pattern of erythrocytes was also examined.

Three hundred fifty-two WBB6F1-W/W+ mice survived at

| Table 1. Number of WBB6F1-W/W+ Mice (HbbP/HbbP) in Which Transient or Long-Term Change of Hemoglobin Pattern Was Observed After Injection of Various Numbers of Bone Marrow Cells from C57BL/6-Pgk-1P/Pgk-1P Mice (HbbP/HbbP) Either Injected With 5FU Four Days Previously or Not |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| No. of Cells (× 10⁵) | Transient | Long-term | Total |
| 1 | 0/14 (6%) | 0/26 (0%) | 3/74 (4%) | 1/26 (4%) | 3/74 (4%) | 1/26 (4%) |
| 3 | 3/89 (3%) | 1/9 (11%) | 4/89 (4%) | 0/9 (0%) | 7/89 (7%) | 1/9 (11%) |
| 10 | 2/54 (4%) | 0/26 (0%) | 3/54 (6%) | 5/26 (19%) | 5/54 (10%) | 5/26 (19%) |
| 30 | 1/28 (4%) | 1/23 (4%) | 10/28 (36%) | 6/23 (26%) | 11/28 (40%) | 7/23 (30%) |
| 50 | 2/43 (5%) | 1/8 (13%) | 13/43 (30%) | 1/8 (13%) | 15/43 (35%) | 2/8 (26%) |
| 75 | 0/37 (0%) | 0/19 (0%) | 14/37 (38%) | 11/19 (58%) | 14/37 (38%) | 11/19 (58%) |
| 100 | 0/27 (0%) | 0/16 (0%) | 16/27 (59%) | 10/16 (63%) | 16/27 (59%) | 10/16 (63%) |

*There were no significant differences between WBB6F1-W/W+ mice that received four-day 5FU-treated bone marrow cells and those that received nontreated bone marrow cells (P > .1, by chi-square test).
†Data of 303 of 352 WBB6F1-W/W+ mice injected with 5FU-treated bone marrow cells obtained from ref 3.
EFFECT OF 5-FLUOROURACIL ON STEM CELL

WBB6F₁₋₇/W'' mice survived at least 16 weeks; transient reconstitution occurred in three mice (2%), and long-term reconstitution occurred in 34 mice (27%). Thus, the 5FU treatment did not increase the proportion of WBB6F₁₋₇/W'' mice in which either transient or long-term reconstitution occurred (Table 1).

By limiting dilution analysis, we calculated the concentration of stem cells that may reconstitute the whole erythropoiesis of WBB6F₁₋₇/W'' mice for a long period. From data shown in Table 1, we calculated the proportion of WBB6F₁₋₇/W'' mice in which long-term complete reconstitution of erythropoiesis did not occur after bone marrow transplantation (proportion of unsuccessful transplantation) and plotted against the number of injected cells (Fig 1). In both groups of WBB6F₁₋₇/W'' mice that received either four-day 5FU-treated or nontreated marrow cells, straight lines were obtained that intercepted the ordinate at 0.97 and 1.00, respectively. Therefore, the proportion of unsuccessful transplantations could be used to estimate the fraction of stem cells in both four-day 5FU-treated and nontreated healthy bone marrow cells. The slope of the line is the estimate of the fraction of stem cells. Calculation of \( \phi \) and its 95% confidence limits was carried out according to the method described by Porter and Berry\(^7\) and Breivik.\(^8\) The concentration of such a stem cell that may reconstitute the whole erythropoiesis for a long period was eight (95% confidence limits, seven to 11) per \( 10^6 \) four-day 5FU-treated bone marrow cells and nine (95% confidence limits, seven to 14) per \( 10^6 \) nontreated bone marrow cells; there was no significant difference between these values.

WBB6F₁₋₇/W'' mice reconstituted by bone marrow cells of C57BL/6-Pgk₁⁺/Pgk₁⁺ mice for a long period were divided into three groups by using the PGK pattern of erythrocytes as the criterion.\(^3\) In group 1, erythrocytes showed either A- or B-type PGK throughout the time of reconstitution. In group 2, erythrocytes showed AB-type PGK when the change of hemoglobin pattern to pure Hbb⁺/Hbb⁺ was first detected. Then, the PGK pattern changed to either A or B type. In group 3, erythrocytes showed AB-type PGK throughout the time of reconstitution. Distribution of WBB6F₁₋₇/W'' recipient mice among the abovementioned three groups was apparently influenced by the number of injected bone marrow cells, but not by the four-day 5FU

![Fig 1. Proportion of WBB6F₁₋₇/W'' mice in which hemoglobin pattern changed to the donor type for at least 8 weeks after injection of various numbers of bone marrow cells from C57BL/6-Pgk₁⁺/Pgk₁⁺ mice that had been injected with 5FU (150 \( \mu g/g \) body weight) four days previously.](image)

Table 2. PGK Type of Erythrocytes in WBB6F₁₋₇/W'' Mice (Hbb⁺/Hbb⁺) in Which Hemoglobin Pattern Changed to Donor Type for at Least 8 Weeks After Injection of Various Numbers of Bone Marrow Cells From C57BL/6-Pgk₁⁺/Pgk₁⁺ Mice (Hbb⁺/Hbb⁺) Either Injected With 5FU Four Days Previously or Not

<table>
<thead>
<tr>
<th>No. of Cells (x 10⁶)</th>
<th>No. of Mice at the Following PGK Type After Injection of 5FU-Treated or Nontreated Cells</th>
<th>5FU Treated</th>
<th>Non-treated</th>
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<tr>
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<td>Throughput A or B</td>
<td>AB to A or AB to B</td>
<td>Throughout AB</td>
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The same experiment as shown in Table 1 is represented.
treatment (Table 2). In both groups of WBB6F1-W/W" recipient mice which received either four-day 5FU-treated or nontreated bone marrow cells, all mice belonged to group 1 when they received 10³ or 3 × 10³ cells; the proportion of mice belonging to group 2 was highest when they received from 10 × 10³ to 75 × 10³ cells; the proportion of mice belonging to group 3 was highest when they received 100 × 10³ cells.

Because van Zant reported that the ratio of day 14 to day 8 spleen colonies changed quickly after injection of 5FU, bone marrow cells of C57BL/6-Pgk-1*/Pgk-1" mice were harvested two, four, and six days after 5FU injection and the numbers of CFU-S in those bone marrow cells were examined in our experimental conditions (Fig 2). The number of cells per femur continued to decrease during this period (Fig 2). Concentrations of both day 8 and day 14 CFU-S dropped at day 2 and then started to increase. Since the decrease of day 8 CFU-S was more remarkable than that of day 14 CFU-S, the ratio of day 14 to day 8 spleen colonies showed a 16-fold increase at both day 2 and day 4 after injection of 5FU (Fig 2). Although the ratio was comparable at days 2 and 4, the concentrations of CFU-S were much lower at day 2 than at day 4. However, the number of stem cell that can reconstitute the erythropoiesis may have been greater in bone marrow cells of mice injected with 5FU two days previously (day-2 5FU treated) than in day-4 5FU-treated marrow cells. We injected various numbers of day-2 5FU-treated marrow cells into 200-rad-irradiated WBB6F1-W/W" mice and examined the proportion of long-term reconstitution (Table 3). The concentration of the stem cell that may reconstitute the whole erythropoiesis for a long period was ~30/10⁶ of two-day 5FU-treated bone marrow cells.

Finally, the cycling status of the stem cell that can reconstitute the whole erythropoiesis of a WBB6F1-W/W" mouse was determined by treatment with hydroxyurea, which kills the cells in S phase. Bone marrow cells of nontreated C57BL/6-Pgk-1*/Pgk-1" mice were harvested and incubated with or without hydroxyurea. Then, 10³ hydroxyurea-treated and nontreated bone marrow cells were injected into 200-rad-irradiated WBB6F1-W/W" mice. No
significant differences were observed in the proportions of treated C57BL/6-Pgk1+/Pgk1− mice (Table 4).

**DISCUSSION**

The PGK pattern of erythrocytes was investigated in WBB6F1-W/Wv mice reconstituted by injection of bone marrow cells from either four-day 5FU-treated or nontreated C57BL/6-Pgk1+/Pgk1− mice. Seven of 163 (4%) WBB6F1-W/Wv mice that received 107 or 3 x 107 four-day 5FU-treated bone marrow cells and one of 35 (3%) WBB6F1-W/Wv mice that received the same numbers of nontreated bone marrow cells showed either an A- or B-type PGK pattern. In addition, in 27 of 189 (14%) WBB6F1-W/Wv mice that received 10 x 107 to 100 x 107 four-day 5FU-treated bone marrow cells and in 21 of 92 (23%) WBB6F1-W/Wv mice that received the same numbers of nontreated bone marrow cells, erythrocytes showed AB-type PGK when the change of hemoglobin pattern to pure Hbb′/Hbb′ was first detected. Then the PGK pattern changed to either type A or type B. Since there were no significant differences between nontreated marrow cells and four-day 5FU-treated marrow cells in the proportion of WBB6F1-W/Wv mice that ultimately showed the monoclonal reconstitution, transplantation of a single stem cell with extensive proliferative potential did not change in the first two days after 5FU treatment. In contrast, the numbers of day-14 CFU-S and day-8 CFU-S increased to <10% of those in nontreated marrow. In the next two days, the numbers of both day-14 and day-8 CFU-S began to increase. In contrast, the number of stem cells with extensive proliferative potential decreased to 10% of the value observed on day 2. Therefore, we attributed the decrease of such stem cells to their differentiation into day 14 and day 8 CFU-S; i.e., injection of 5FU enriched the stem cells with extensive proliferative potential when bone marrow cells were harvested two days after injection. The effect of another cytotoxic drug, hydroxyurea, which kills the cells in S phase specifically, was investigated to determine the cycling status of the stem cells with extensive proliferative potential. Table 4 shows that such stem cells in nontreated bone marrow cells were not in S phase.

From the data shown in Tables 1 and 3 and Fig 2, we calculated absolute numbers of the stem cells that reconstitute the whole erythropoiesis of a WBB6F1-W/Wv mouse, day-14 CFU-S, and day-8 CFU-S in the femur of nontreated and 5FU-treated C57BL/6-Pgk1+/Pgk1− mice (Table 5). The absolute number of stem cells with extensive proliferative potential did not change in the first two days after 5FU treatment. In contrast, in the numbers of day-14 CFU-S and day-8 CFU-S decreased to <10% of those in nontreated marrow. In the next two days, the numbers of both day-14 and day-8 CFU-S began to increase. In contrast, the number of the stem cells with extensive proliferative potential decreased to 10% of the value observed on day 2. Therefore, we attributed the decrease of such stem cells to their differentiation into day 14 and day 8 CFU-S; i.e., injection of 5FU enriched the stem cells with extensive proliferative potential when bone marrow cells were harvested two days after injection. The effect of another cytotoxic drug, hydroxyurea, which kills the cells in S phase specifically, was investigated to determine the cycling status of the stem cells with extensive proliferative potential. Table 4 shows that such stem cells in nontreated bone marrow cells were not in S phase.

Recently, we showed that the “primitive” hematopoietic stem cells estimated by the erythropoiesis-reconstituting assay differentiate not only with regard to the erythroid cells but also with regard to other myeloid lineage cells and lymphoid lineage cells (Nakano et al, submitted to Blood).

In conclusion, hematopoietic stem cells that can reconstitute the total erythropoiesis may play an important role in long-term erythropoietic reconstitution even when non-treated healthy marrow cells are used for transplantation, and such stem cells with extensive proliferative potential appear to be dormant in nontreated bone marrow.

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