FLOW CYTOMETRIC ANALYSES ON LINEAGE-SPECIFIC CELL SURFACE ANTIGENS OF RAT BONE MARROW TO SEEK POTENTIAL MYELOTOXIC BIOMARKERS: STATUS AFTER REPEATED DOSE OF 5-FLUOROURACIL

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ABSTRACT — Flow cytometry (FCM) analysis of CD45, CD45R, CD71 and CD90 expression on Crl:CD(SD)Igs rat bone marrow cells was done after 5-fluorouracil (5-FU) administration to examine whether these lineage-specific cell surface antigens could be myelotoxic biomarkers. The expression of CD45 (CD45<sub>low</sub> and CD45<sub>high</sub>, differing in expression intensity), CD45R, CD71 and CD90 on bone marrow cells coincided with previous reports. After repeated administration of 5-FU at 50 mg/kg/day for 1-5 days, a time-dependent decrease in cells expressing CD45<sub>low</sub>, CD71 and CD90 was observed, whereas a decrease in the CD45<sub>high</sub> expressing cells was not observed. Furthermore, the decrease was dose-dependent in CD45<sub>low</sub>, CD71 and CD90 expressing cells after administration of 5-FU between 2 and 50 mg/kg/day for 4 days. After 4-day repeated dose of 5-FU at 50 mg/kg/day followed by a recovery period, the change in number of CD45<sub>low</sub>, CD45R, CD71 and CD90 cells to the bottom and in recovery showed different kinetics. In contrast, the change in number of CD45<sub>high</sub> cells was minimal, and relatively stable after 5-FU administration. The results suggest that CD45, CD45R and CD90 could each be potential myelotoxic biomarkers for a total proportion of common leukocytes including T- and B-lymphocytes, for a total proportion of B-lymphocytes, and for a total proportion of T-lymphocytes plus immature B-lymphocytes and common progenitor cells, respectively. CD71 could be a single myelotoxic biomarker for erythroid cells. Further study is required for isolation of each of the myelo-lymphocytic lineages. However, the present study showed that FCM analysis could be available to assess the lineage or differentiation stage-specific response, such as the different extent and time-course or the kinetics (the time to reach the bottom and to recover to the normal level) of myelotoxic effect in rat bone marrow.

KEY WORDS: Flow cytometry, Cell surface antigen, Myelotoxicity, 5-Fluorouracil, Bone marrow smear

INTRODUCTION

Bone marrow is the principal site of haematopoiesis and maintains homeostatic production of blood cells. Enumeration of bone marrow cells and cytological examination using bone marrow smears have been used mainly as a conventional method for evaluation of the myelotoxic effect of drugs. Mechanistic analysis, i.e., examining the time-course or kinetics (the time to reach the bottom and to recover to the normal level) of response to drugs is considered to be important for understanding lineage or differentiation stage-specific myelotoxic effects. However, the conventional method has disadvantages. For example, it is difficult to estimate lineage or differentiation stage-specific myelotoxic effects by enumeration of bone marrow cells. In addition, observation of bone marrow smears is time-consuming, allowing the evaluation of only relatively few cells, and requiring skill to classify the cells on smears.

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Flow cytometry (FCM) permits the quantitative measurement of a variety of properties on individual cells in a flow stream at rates of thousands of cells per second. For example, FCM has been utilized for analyzing the cell cycle (Kallionemi et al., 1994), intracellular enzyme activity (Malin-Bardel and Valet, 1980; Ruiz et al., 1996), apoptosis (Zhang et al., 1996), intracellular cytokines (Jung et al., 1993; Carter and Swain, 1997) and lineage-specific cell surface antigens of blood cells (Brideau et al., 1980; Williams et al., 1987; Tamatani et al., 1991; Johnson et al., 1998). As for a method of distinguishing human hematopoietic progenitor cells with FCM, a number of studies have been reported (Loken et al., 1987; Wognum et al., 1990; Terstappen et al., 1991; Simmons et al., 1992; Udomsakdi et al., 1992).

In recent years, antibodies to lineage-specific cell surface antigens of rats, such as CD45 (Sunderland, 1979; Woollett et al., 1985), CD45R (Kroese et al., 1986, 1987, 1990; Opstelten et al., 1986; Herrmans et al., 1997), CD71 (Jefferies et al., 1985) and CD90 (Williams, 1976; Hunt et al., 1977; Thierfelder, 1977; Goldschneider et al., 1978), have been available. These lineage-specific cell surface antigens are estimated to be biomarkers or critical endpoints for assessing the toxic effect on myeloid, lymphoid or erythroid hematopoiesis.

5-fluorouracil (5-FU) is known as a myelosuppressive cytostatic agent (Szmigielski and Jeliaszewicz, 1976; Cheng et al., 2000), and more strongly affects cells with a high level of proliferating activity, including hematopoietic progenitor cells. Myelotoxic effect on human bone marrow is one of the serious adverse effects in clinical use (MacDonald, 1999).

In the present study, we performed FCM analysis of CD45, CD45R, CD71 and CD90 expression on cells derived from rat bone marrow after 5-FU administration. In particular, the extent and time-course or the kinetics (the time to reach the bottom and to recover to the normal level) were examined in detail to clarify whether FCM analysis using these lineage-specific cell surface antigens could provide myelotoxic biomarkers or critical endpoints for assessing the lineage or differentiation stage-specific response to myelotoxic drugs in rat bone marrow.

MATERIALS AND METHODS

Animals and animal housing

Six-week-old male Crl:CD(SD)IGS rats were obtained from Charles River Japan Inc. (Tsukuba, Japan) and housed in suspended metal cages. The animals were acclimated for 1 week prior to examination. Age of animals at 5-FU administration was 7-week-old. Solid food (CRF-1, Oriental Yeast Inc., Tokyo) was offered ad libitum in a stainless steel food container. Sterilized well water was supplied ad libitum by an automatic watering device. The animal room was kept at 24 ± 1°C and 55 ± 15% relative humidity. The room air was ventilated 15 times per hour and a 12 hr/12 hr light-dark cycle (lighting, 6:00-18:00) was imposed.

Antibodies

Mouse IgG1 anti-rat CD45, mouse IgG2b anti-rat CD45R, mouse IgG2a anti-rat CD71 and mouse IgG2a anti-rat CD90 were used as antibody. Mouse IgG1, mouse IgG2a and mouse IgG2b were used as isotype control antibody. All antibodies labeled with fluorescein (FITC) were purchased from BD Biosciences (San Jose, CA).

Administration of drugs and sampling bone marrow

5-FU was purchased from Kyowa-Hakko Kogyo (50 mg/mL vial, Tokyo) and administered to rats through the tail vein. The volume administered was 1 mL/kg. Physiological saline was used as the control substance. The bone marrow was collected from left and right femurs. The femurs were taken out under anesthesia with pentobarbital, and cut at both ends. The distance from the osteoepiphysis to the cut site was kept the same as possible. The bone marrow was then flushed out with 1 mL of fetal bovine serum (FBS) using a syringe with a 21G injection needle. Bone pieces or debris were removed by using a Pasteur pipette, and bone marrow cells were then collected by centrifugation (3,000 min⁻¹ for 5 min). The viability of bone marrow cells was examined by trypan blue exclusion assay (range of cell viability: 86-92%).

Enumeration of bone marrow cells using a flow cytometer

To 20 μL of the bone marrow cells, collected by centrifugation, was added 1 mL of a phosphate-buffered saline (PBS). To 100 μL of the resulting cell suspension was added 1 mL of Optilyse® C (Beckman Coulter, Miami, FL) and the mixture incubated at room temperature for 10 min to hemolyze erythrocytes. Then 1 mL of PBS was added and incubation continued for another 15 min to stop the hemolytic treatment. After incubation, cells were collected and washed with PBS.
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by centrifugation. To the cell pellet was added 1 mL of PBS, followed by 20 μL of Flow-Count Fluorospheres (Beckman Coulter) for internal calibration. Analysis was carried out with a flow cytometer (EPICS XL-MCL, Beckman Coulter) using the protocol in which a calibration region of Flow-Count was set according to the manufacturer's instructions.

Analysis of cell surface antigen expression by flow cytometry

To 20 μL of the bone marrow cells collected by centrifugation was added 5 mL of PBS. The cell suspension was filtered with nylon mesh (pore size 35 μm, Becton, Dickinson and Company, Franklin Lakes, NJ), and then 200 μL was added to 2 μg of a specific antibody to the cell surface antigen or isotype control antibody. Incubation was carried out at room temperature for 45 min. Next, 1 mL of Optilyse® C was added and treatment carried out for 10 min to hemolyze erythrocytes. Then 1 mL of PBS was added and the cells incubated for another 15 min to stop the hemolytic treatment. The cells were collected and washed with PBS by centrifugation. After washing, 2 mL of PBS was added to prepare a cell suspension. Analysis was carried out with the EPICS XL-MCL. The analysis was conducted following the procedures described below. Gates were set in the cell populations on forward scattered (FS) light and side scattered (SS) light cytograms as shown in Fig. 1, and the distribution of fluorescence intensity with FITC was displayed for the cells in the gates by histogram. At least 15,000 cells in the gates of FS light-SS light were analyzed. The sensitivity of the flow cytometer was adjusted with the cells treated with the isotype control antibody. To obtain the number of each of the positive cells, the ratio was multiplied by a separately obtained number of bone marrow cells.

Sorting of cell surface antigen-expressing cells by flow cytometer

For the sorting of cell surface antigen-expressing cells, a flow cytometer equipped with a sorting system (ALTRA, Beckman Coulter) was used. Cells in the sorted fraction were collected and then suspended in FBS. Cytospin specimens were then prepared, stained with May-Grünewald and Giemsa solution, and subjected to microscopic observation.

Cytological examination using bone marrow smears

Some of the bone marrow samples were suspended in an adequate amount of FBS. An adequate amount of the resulting suspension was then dropped onto a glass slide, and bone marrow smears were prepared by the wedge method. Each smear was stained with May-Grünwald and Giemsa solution for microscopic observation. Five hundred nucleated cells were observed in each smear, and the ratio of the cells was calculated for each lineage and differentiation stage. The resulting ratio was multiplied by the separately obtained number of bone marrow cells to determine the absolute number.

RESULTS

Analysis of expression pattern of cell surface antigens in rat bone marrow cells

Fig. 1 shows the FS/SS light cytogram of rat bone marrow cells. Three main clusters were observed; low FS light/Low SS light (Cluster 1), high FS light/intermediate SS light (Cluster 2) and high FS light/high SS light (Cluster 3). A gate was set to include all three main clusters as shown in Fig. 1, and cells in the gate were analyzed for their intensity of expression of cell surface antigens.

As shown in Fig. 2 (A), untreated normal rat bone marrow cells expressing CD45R, CD71 or CD90 were

Fig. 1. FS/SS cytogram of rat bone marrow cells.
Bone marrow of untreated normal rats was collected and analyzed with EPICS XL-MCL. FCM analysis of cell surface antigens was done for the cell population in the "gate" indicated in the cytogram. FS: Forward scattered light, SS: Side scattered light, 1: Cluster 1, 2: Cluster 2, 3: Cluster 3.
detected as a single peak, whereas two peaks differing in expression intensity were found for CD45 (CD45\textsuperscript{Low} and CD45\textsuperscript{High}). Cytological examination with cytospin smears of sorted fractions revealed that CD45\textsuperscript{Low} were mainly myeloids and granulocytes; CD45\textsuperscript{High}, CD45R and CD90 were mainly lymphocytes; and CD71 were mainly erythroblasts (data not shown).

Fig. 2 (B) shows the histogram of cell surface antigen analysis after 5-FU administration at 50 mg/kg/day for 5 days. CD45\textsuperscript{Low}, CD71 and CD90 peaks decreased markedly. CD45R peak also decreased. However, the extent of the decrease was slight as compared with CD45\textsuperscript{Low}, CD71 and CD90 peaks. In contrast, CD45\textsuperscript{High} did not decrease or rather increase.

Change of cell surface antigen expression assessed by FCM and comparison with cytological examination data after administration of 5-FU

Fig. 3 (A) shows the time-course of change in cell surface antigen expression after repeated administration of 5-FU at 50 mg/kg/day for 1-5 days. The number of CD45\textsuperscript{Low} expressing cells began decreasing on Day 2 and Day 3 of the experiment and decreased to such an extent that scarcely no such cells were observed on Day 5. No decrease in the number of CD45\textsuperscript{High} expressing cells was observed. A slight decrease was observed in the number of CD45R expressing cells on Day 3, and the decrease continued on Day 4 and thereafter. However, the extent of the decrease was slight as compared with that of CD45\textsuperscript{Low} expressing cells. A decrease was observed in the number of CD71 expressing cells from Day 2 to the extent that scarcely no such

![Histogram of CD45, CD45R, CD71, and CD90 analysis of rat bone marrow cells.](image)

**Fig. 2.** Histogram of CD45, CD45R, CD71, and CD90 analysis of rat bone marrow cells. Bone marrow of untreated normal or 5-FU administered (50 mg/kg/day; 5 days) rats was collected and analyzed for CD45, CD45R, CD71 or CD90 expressing cells with EPICS XL-MCL. Antibodies labeled with fluorescein (FITC). At least 15,000 cells were analyzed for each lineage-specific cell surface antigen. Sorting (data not shown) and cell counts for each of the cell surface antigen expressing cells (Fig. 3, 4 and 5) were obtained from an analysis of cell populations in the linear region shown by arrows in each histogram. The horizontal axis shows FITC fluorescence intensity (expression intensity of each cell surface antigen). (A): Untreated normal rats. (B): 5-FU administered rats.
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Dose-dependent change of cell surface antigen expression assessed by FCM after administration of 5-FU

Fig. 4 shows the dose-response of myelosuppression after repeated administration of 5-FU between 2 and 50 mg/kg/day for 4 days. As for cells expressing CD45^{low}, CD45R, CD71 and CD90, a dose-dependent decrease in number was observed between 2 and 50 mg/kg/day. No decrease was observed for CD45^{high} expressing cells up to 50 mg/kg/day.

Kinetics of decrease and recovery of cell surface antigen expression assessed by FCM after administration of 5-FU followed by withdrawal period

Fig. 5 shows the decrease and recovery of CD45, CD45R, CD71 and CD90 expressing cells after

cells were observed on Day 4 or thereafter. A slight decrease was observed in the number of CD90 expressing cells on Day 3, and the number decreased markedly from Day 4.

Fig. 3 (B) and Photo 1 show the results of microscopic examination of smears using bone marrow cells derived from the same animals on which FCM was carried out. A decrease was observed in myeloid lineage cells (promyelocytes/myelocytes/metamyelocytes) and erythroblasts from Day 2, and the number dropped to such an extent that scarcely no such cells were observed on Day 4 or thereafter. The number of band or segmented neutrophils decreased from Day 5. On the other hand, little change was observed in lymphocytes up to Day 3, but the number decreased from Day 4.

Day

Fig. 3. Results of cell surface antigen analysis with FCM and cytological examination of bone marrow smears after 5-FU administration.

Rat bone marrow was collected after 1-5 days administration of 5-FU (50 mg/kg/day), and the expression of cell surface antigens was analyzed with FCM (A). At least 15,000 cells were analyzed with EPICS XL-MCL to obtain the ratio of each lineage-specific cell surface antigen expressing cells. The ratio was then multiplied by the separately obtained number of bone marrow cells with EPICS XL-MCL using Flow-Count Fluorospheres for internal calibration. Bone marrow smears stained with May-Grunwald and Giemsa solution were also prepared at the same time, and a cytological examination was performed (B). Five hundred nucleated cells were observed in each smear, and the ratio of the cells was calculated for each lineage and differentiation stage. The resulting ratio was multiplied by the separately obtained number of bone marrow cells to determine the absolute number. Values are the means and SD (n=3). ■: Control group (Physiological saline administered), ●: 5-FU group, ▼: Administration of 5-FU (50 mg/kg/day).
repeated administration of 5-FU at 50 mg/kg/day for 4 days followed by a withdrawal period. The number of CD45<sup>low</sup> expressing cells showed a trough between Day 5 and 8, and then recovered after Day 9 to reach the control level on Day 12. No marked change in the number of CD45<sup>high</sup> expressing cells was observed in spite of a very slight decrease between Day 7 and 11. CD45R expressing cells continued to decrease from Day 3 to 10, but then recovered after Day 11. The number of CD71 expressing cells rapidly decreased on Day 3 and reached a minimum during Day 3 and 8. A recovery of CD71 expressing cells was observed from Day 9 with the control level reached on Day 12. CD90 expressing cells continued to decrease markedly from Day 3 to 8, but recovered after Day 9 to reach the control level on Day 12.

**DISCUSSION**

In previous reports, the expression and distribution of lineage-specific cell surface antigens including CD45 (Sunderland, 1979; Woollett et al., 1985), CD45R (Kroese et al., 1986, 1987, 1990; Opstelten et al., 1986; Hermans et al., 1997), CD71 (Jefferies et al., 1985) and CD90 (Williams, 1976; Hunt et al., 1977; Thierfelder, 1977; Goldschneider et al., 1978) were reported, and antibodies to these cell specific markers have been available in recent years. In the present study, FCM analysis of CD45, CD45R, CD71 and CD90 expressing cells in rat bone marrow after 5-FU administration was done to examine whether these lineage-specific cell surface antigens could be myelotoxic biomarkers.

**Photo 1.** Bone marrow smears of rats administered 50 mg/kg/day of 5-FU.

Bone marrow of 5-FU-administered rats was collected on the indicated day (1-6) of the experiment, and smears were prepared by the wedge method and stained with May-Grünwald and Giemsa solution. (A): Original magnification is 100×. (B): Original magnification is 250×.
As shown in Fig. 3 (A) and Fig. 5, the number of cells expressing CD45R or CD90 clearly decreased in a time- and dose-dependent manner. In contrast, CD45\textsuperscript{High} expressing cells did not decrease after 5-day administration of 5-FU. Cytological examination with cytospin smears of sorted fractions of lymphocytes in rat bone marrow sample could be distinguished as CD45\textsuperscript{High} expressing, CD45R expressing or CD90 expressing cells (data not shown). In previous reports, CD45R was found on most B lymphocytes including developing B cells in the bone marrow and peripheral B cells (Kroese et al., 1986, 1987; Opstelen et al., 1986; Hermans et al., 1997). CD90 is a Thy-1 membrane glycoprotein found on bone marrow cells including immature B lymphocytes and hematopoietic stem cells (Hunt et al., 1977; Thierfelder, 1977; Goldschneider et al., 1978). In general, such developing B cells or stem cells possess strong proliferating activity. 5-FU is known as a myelosuppressive cyto- static agent (Szmigielski and Jeliaszewicz, 1976; Cheng et al., 2000), and 5-FU would more strongly affect cells with a high level of proliferating activity. Based on these previous reports, it is estimated that the CD45R or CD90 expressing cells detected in our study mainly consisted of undifferentiated developing B cells or hematopoietic progenitors, and the decrease after 5-FU treatment would be due to their proliferative activity. In contrast, it is suggested that CD45\textsuperscript{High} expressing cells would be terminally differentiated lymphocytes, which are possibly in a quiescent state, rather than lymphoid progenitors in bone marrow.

Assessing the different sensitivities to 5-FU among the various differentiation stages of lymphocytes is difficult to achieve by the conventional method, enumeration of bone marrow cells or observation of bone marrow smears. Data in this study suggested that CD45R and CD90 could each be potential myelotoxic biomarkers, especially for examining the
toxic effect on total proportion of B-Lymphocytes and total proportion of T-lymphocytes plus immature B-lymphocytes and common progenitor cells, respectively.

CD71 is known as a transferrin receptor and good marker of cell proliferating activity (Jefferies et al., 1985). The decrease of CD71 expressing cells after 5-FU treatment would be due to their proliferative activity. Cytological examination with cytospin smears of sorted fractions in our study indicated that CD71 expressing cells mainly consisted of erythroblasts (data not shown). Promyelocytes/myelocytes/metamyelocytes and lymphocytes were also observed in CD71 peak fraction, while the proportions of these cells were much less than that of erythroblasts. Based on these data, CD71 could be a potential myelotoxic biomarker, mainly for examining the toxic effect on erythroid cells.

Changing of CD45, CD45R, CD71 and CD90 expressing cell numbers after 5-FU administration showed the different extent and time-course (Fig. 3) or the kinetics (the time to reach the bottom and to recover to the normal level) (Fig. 5). These data suggested that FCM analysis would be available to assess the lineage or differentiation stage-specific response to myelotoxic drugs. Obtaining such information is difficult based only on enumeration of bone marrow cells. In addition, in terms of the time taken to obtain such data, FCM was much quicker than the conventional method of cytological examination of bone marrow smears. As for the number of cells assessed, FCM allows for the evaluation of a huge number (15,000 or more possibly) of cells in a very short time.

In the present study, three main clusters observed in FS/SS light cytogram (Fig. 1) were analyzed for their cell surface antigen expression. While sorting data was not obtained in this study, based on their light intensity, clusters 1, 2 and 3 were considered to be lym-

**Fig. 5.** Decrease and recovery of CD45, CD45R, CD71, and CD90 expressing cells after 5-FU administration followed by a withdrawal period.

Rat bone marrow was collected during administration of 5-FU or withdrawal, and the expression of cell surface antigens was analyzed with FCM. The dose of 5-FU was 50 mg/kg/day. Bone marrow cell number (BMNC) was obtained with EPICS XL-MCL using Flow-Count Fluorospheres for internal calibration. Values are the means and SD (n=3). ▼: Administration of 5-FU.
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