Journal of Radiation Research, 2013, 54, 447–452
doi: 10.1093/jrr/rrs127 Advance Access Publication 7 January 2013

Effects of radiation on the maturation of megakaryocytes

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(Received 15 November 2012; revised 26 November 2012; accepted 29 November 2012)

Megakaryocytes are generated by the differentiation of megakaryocytic progenitors; however, little information has been reported regarding how ionizing radiation affects the differentiation pathway and cellular responses. Human leukemia K562 cells have been used as a model to study megakaryocytic differentiation. In the present study, to investigate the effects of radiation on phorbol 12-myristate 13-acetate (PMA)-induced megakaryocytic differentiation of K562 cells, the cellular processes responsible for the expression of CD41 antigen (GPIIb/IIIa), which is reported to be expressed early in megakaryocyte maturation, were analyzed. The expression of CD41 antigens was significantly increased 72 h after treatment with both 4 Gy X-irradiation and PMA. In this fraction, two populations, $CD41^{low}$ and $CD41^{high}$ cells, were detected by flow cytometry. The $CD41^{high}$ cells sustained intracellular ROS at the initial level for up to 72 h, but $CD41^{low}$ cells had reduced ROS by 48 h. The maximum suppressive effect on CD41 expression was observed when N-acetyl cysteine, which is known to act as a ROS scavenger, was administered 48 h after PMA stimulation. When K562 cells were pretreated with mitogen-activated protein kinase (MAPK) pathway inhibitors, an ERK1/2 inhibitor and a p38 MAPK inhibitor, followed by X-irradiation and PMA stimulation, the reactivity profiles of both inhibitors showed the involvement of MAPK pathway. There is a possibility that the K562 cell population contains at least two types of radiosensitive megakaryocytic progenitors with respect to ROS production mechanisms, and intracellular ROS levels determine the extent of CD41 expression.

Keywords: K562 cells; megakaryocytopoiesis; ROS; MAPK

INTRODUCTION

Megakaryocytopoiesis and thrombopoiesis are unique processes that lead to platelet production and consist of the following events: the commitment of hematopoietic precursors to the megakaryocyte lineage, the differentiation of megakaryocyte progenitors to recognizable megakaryocytes, formation of polyploid cells, cytoplasmic maturation and platelet shedding [1]. There are many uncertain issues regarding the final step of megakaryocytopoiesis and thrombopoiesis, including the identity of the promoting factor(s) for platelet production. Similarly, little information has been reported regarding the effect of ionizing radiation on the terminal stages of megakaryocyte maturation and platelet production from mature megakaryocytes. We have reported previously that the pathway of differentiation from human hematopoietic stem cells through megakaryocytic progenitors to megakaryocytes is extremely sensitive to ionizing radiation [2]. The human K562 erythroleukemia cell line is a multipotent hematopoietic precursor cell line derived from a chronic myelogenous leukemia patient in blast crisis, and thus provides a model system to study molecular mechanisms during the process of differentiation [3–5]. Our previous report has shown that two megakaryocytic progenitor populations with different levels of radiosensitivity are found in steady-state human peripheral blood

[6]. In addition, we have shown that ionizing radiation promotes megakaryocytic differentiation induced by phorbol 12-myristate 13-acetate (PMA) in the K562 leukemia cell line [7].

In the present study, to investigate the effects of radiation on PMA-induced megakaryocytic differentiation of the CML cell line K562, the cellular processes responsible for the expression of the CD41 antigen (GPIIb/IIIa), reported to be expressed early in megakaryocyte maturation and in megakaryoblastic leukemia, were analyzed.

MATERIALS AND METHODS

Materials

RPMI1640 medium, penicillin and streptomycin were acquired from Gibco, Invitrogen (CA, USA). Fetal bovine serum (FBS) was obtained from Bioserum, UBC (Tokyo, Japan). PMA, dimethyl sulfoxide, extracellular signal-regulated protein kinase 1/2 (ERK1/2) inhibitor PD98059, p38 inhibitor SB203580 and N-acetyl cysteine (NAC) were obtained from Sigma-Aldrich (Stockholm, Sweden). 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) was purchased from Invitrogen. Flow cytometry antibody PE-Cy7 (PC7)-conjugated anti-human CD41 (CD41-PC7) and FITC-mouse IgG1 were purchased from Beckman Coulter Immunotech (Marseille, France).

Cell culture and differentiation

The human CML cell line K562 was obtained from RIKEN Bio-Resource Center (Tsukuba, Japan) and was cultured in RPMI1640 (GIBCO® Invitrogen, Carlsbad, CA, USA) medium supplemented with penicillin, streptomycin, and 10% FBS at 37°C under a humidified atmosphere containing 5% CO_2. Briefly, 2.5×10^5 cells were resuspended in 500 μl of medium supplemented with 10% FBS and seeded in 24-well plates. After 6 h, cells were incubated with 50 nM PMA to induce megakaryocytic differentiation just after 4 Gy X-irradiation. To analyze the megakaryocytic marker CD41, 2.5×10^5 cells were centrifuged, resuspended in 100 μl of PBS, and incubated with the antibody for 30 min at room temperature. Cells were then washed and resuspended in 300 μl of PBS. Samples were analyzed using a Cytomics FC500 unit (Beckman-Coulter, Fullerton, CA, USA). The results were analyzed using the Kaluza 1.1 Program (Beckman-Coulter). The mitogen-activated protein kinase (MAPK) inhibitors, 25 μM PD098059 or 2 μM SB203580 were added to the culture 2 h before PMA treatment.

In vitro irradiation

K562 cells were exposed to radiation (4 Gy, 150 kVp, 20 mA; 0.5-mm Al and 0.3-mm Cu filters) using an X-ray generator (MBR-1520R; Hitachi Medical Co., Tokyo, Japan) with a distance of 44 cm between the focus and target, and a dose rate of approximately 100 cGy/min. During X-ray exposure, the dose intensity of the ionization chamber was evaluated.

Measurement of intracellular ROS levels

The oxidation-sensitive fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) was used to measure intracellular ROS levels. Cells were incubated with 10 μM H2DCFDA in 100 μl of PBS at room temperature for 30 min, and they were stained with PC7-conjugated anti-CD41 antibody when indicated. Cells were then washed, resuspended in 300 μl of PBS, and analyzed by flow cytometry.

Statistical analysis

All experiments were performed at least three times and results are expressed as means ± SE. Statistical significance was estimated using the Mann-Whitney U-test or Student's t-test. Correlation analyses were performed using the software program Origin (OriginLab Corp., Northampton, MA, USA) for Windows. $P < 0.05$ was considered statistically significant.

RESULTS

Phenotypic analysis of the CD41 antigens

The expression of CD41 (GPIIb/IIIa), which is thought to be an early megakaryocytic marker, was analyzed by flow cytometry. As shown in Table 1, the expression of CD41 antigens was significantly increased 72 h after treatment with 4 Gy X-irradiation and PMA, compared with PMA treatment alone. The fraction of $CD41^+$ cells were divided into two populations, $CD41^{low}$ and $CD41^{high}$ cells, which were found as shown in Fig. 1A. The number of $CD41^{low}$ cells decreased with time, while a significant increase in $CD41^{high}$ cells was observed (Fig. 1B). At this time, no significant difference was observed in the number of $CD41^{low}$ and $CD41^{high}$ between 0 h and 72 h after treatment. These results suggest that different radiosensitive megakaryocytic progenitors exist in K562 cell population.

Table 1. Relative value of CD41 expression after each treatment

Time (h)	PMA	4 Gy + PMA
6	0.95 ± 0.04	0.98 ± 0.06
48	3.67 ± 0.09	3.46 ± 0.05
72	5.87 ± 0.14	7.21 ± 0.13*

The values are represented as compared with the untreated condition. * = significant difference ($P < 0.005$) compared with the level of CD41 expression after treatment with PMA alone.

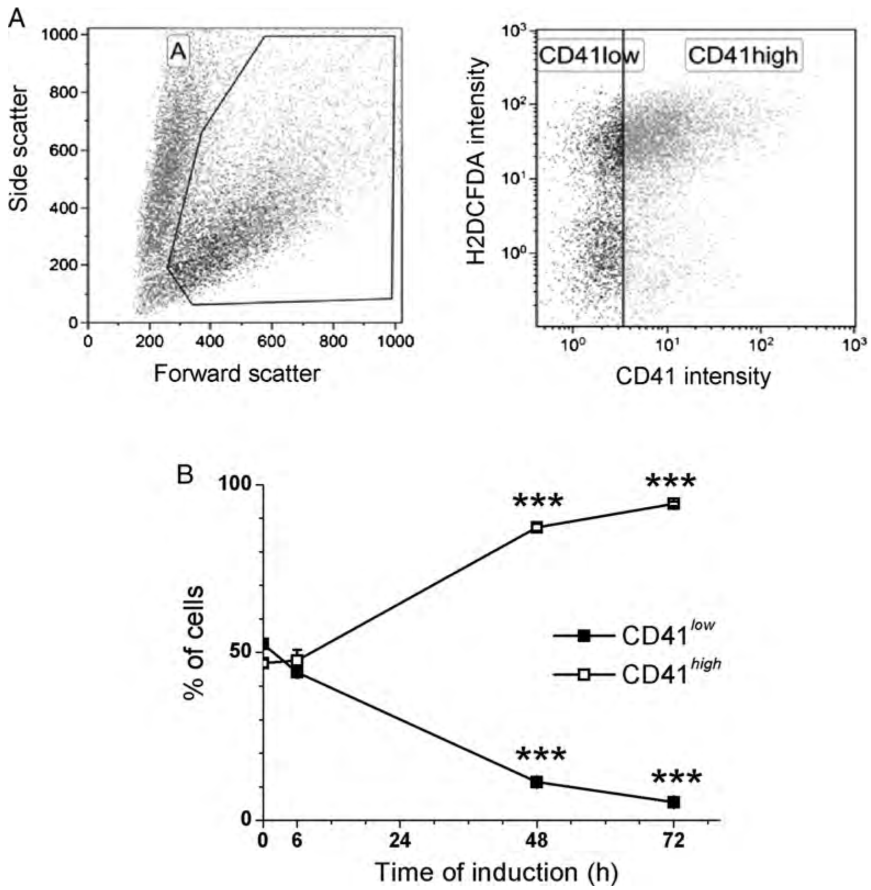

Fig. 1. Flow cytograms of cells harvested from cultures. CD41 cell surface marker expression was analyzed by flow cytometry at 72 h after 4 Gy X-irradiation and treatment with PMA. (A) K562 cells were fractionated into either CD41^{low} or CD41^{high} populations according to mean fluorescence intensity. The gates are depicted in the histogram, and are represented by the irradiated/PMA-stimulated cells at 72 h. (B) The percentages of gated cells are depicted. *** = significant difference ($p < 0.005$) compared with the initial value of the percentage of each cell type at 0 h.

Involvement of sustained ROS levels for CD41 expression

We examined whether CD41 expression is triggered by intracellular ROS accumulation in PMA-induced cells. The CD41^{high} cells sustained intracellular ROS at the initial level for up to 72 h, but CD41^{low} cells had reduced intracellular ROS by 48 h (Fig. 1A). Next, we analyzed the timing of the trigger by increasing intracellular ROS in the process of megakaryocytic differentiation following ionizing radiation. It is known that NAC acts as a ROS scavenger. Radiation combined with PMA treatment sustained a high intracellular ROS level. 4 Gy X-irradiation did not increase the cytotoxicity of PMA at the optimal concentration for differentiation (data not shown). We examined whether the effect of NAC in controlling CD41 expression is dependent on the administration time. Cells were treated with 10 mM NAC, X-irradiated at 4 Gy 2 h later, and then stimulated with PMA. As shown in Fig. 2B, the maximum suppressive effect on CD41 expression was observed when NAC was administered 48 h after PMA stimulation. These results suggest that sustained intracellular ROS levels promote megakaryocytic differentiation.

Modulation of CD41 expression and intracellular ROS levels by inhibition of the mitogen-activated protein kinase (MAPK) pathway

It is considered that the megakaryocytic differentiation of K562 cells treated with PMA is mediated by activation of the MAPK pathway [8–11]. Thus, we examined whether the promoting effect of ionizing radiation through intracellular ROS accumulation in PMA-induced megakaryocytic differentiation is associated with the MAPK pathway. K562 cells were pretreated with MAPK pathway inhibitors (PD98059 as an ERK1/2 inhibitor and SB203580 as a p38 MAPK inhibitor) for 2 h followed by X-irradiation and PMA stimulation. Compared to the control in Fig. 2A, the

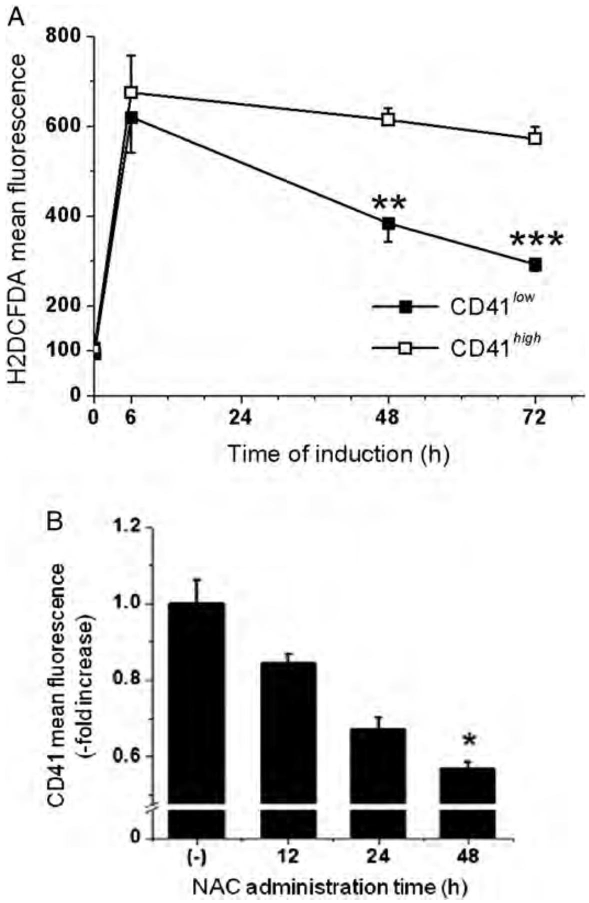

Fig. 2. Relationship between sustained ROS levels and CD41 expression. The expression of CD41 antigens on the surface of K562 cells was analyzed by flow cytometry at 72 h after 4 Gy X-irradiation and treatment with PMA. (A) The intracellular ROS production in two fractionated cell types, CD41^{low} and CD41^{high}, labeled with H_2DCFDA was measured by flow cytometry. These values were normalized to those of untreated cells. $** = P < 0.01$, and $*** = P < 0.005$ (significant difference compared with the value of control cells at 6 h, which is represented by a horizontal line). (B) NAC was administrated to the irradiated cells at different time points, and the total -fold increases of CD41 mean fluorescence were evaluated at 72 h. The values are represented as the ratio of NAC-treated to NAC-untreated cells. $* = P < 0.05$ (significant differences compared with the value of control cells).

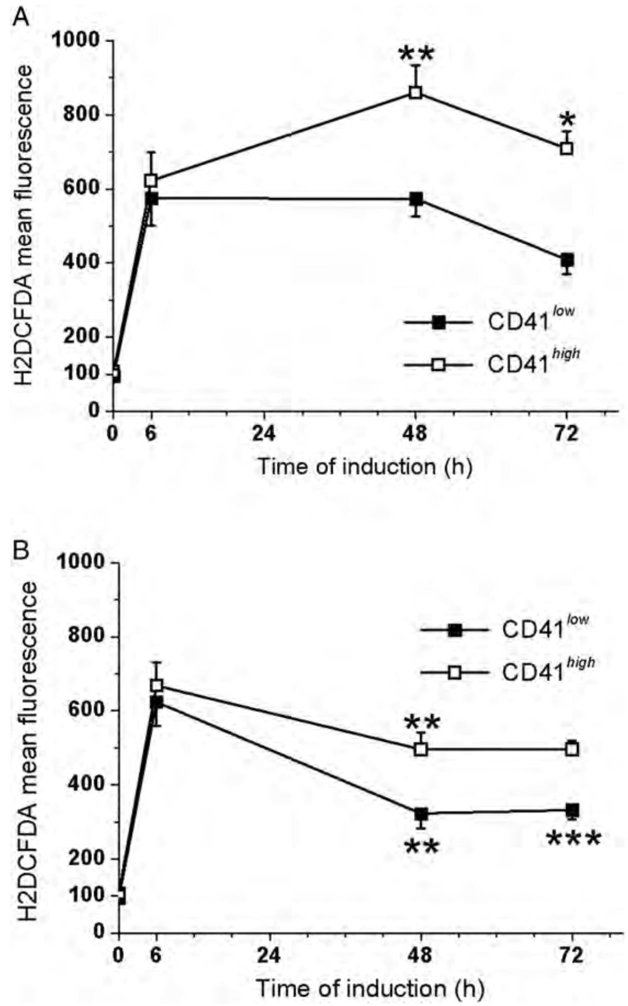

Fig. 3. Modulation of CD41 expression and intracellular ROS levels by inhibition of the mitogen-activated protein kinase (MAPK) pathway. K562 cells were X-irradiated at 4 Gy and then treated with PMA in the presence of PD98059 (PD) or SB203580 (SB), and the following parameters were analyzed by flow cytometry at 72 h after PMA stimulation. K562 cells were fractionated into either CD41^{low} or CD41^{high} populations according to mean fluorescence intensity. Intracellular ROS production was measured in two fractionated cell populations, CD41^{low} or CD41^{high}, and labeled with H_2DCFDA by flow cytometry in the presence of PD98059 (A) or SB203580 (B). $* = P < 0.05$, $** = P < 0.01$, and $*** = P < 0.005$ (significant difference compared to the value of control cells at 6 h, represented by a horizontal line).

reactivity profiles of the two inhibitors were different in each population. Although, CD41^{high} cells sustained intracellular ROS at a higher level for up to 72 h, in CD41^{low} cells intracellular ROS decreased significantly after 48 h (Fig. 3A and 3B), indicating the involvement of the MAPK pathway. These results suggest that intracellular ROS levels determine the extent of CD41 expression.

DISCUSSION

The human K562 erythroleukemia cell line has subsequently been shown to possess phenotypic markers typical of erythroid and myeloid cells, to differentiate into the erythroid cell line (induced by hydroxyurea, herbimycin, hemin, butyrate, and Ara-C) [12–20], and into the myeloid cell line (induced by phorbol esters such as PMA) [21–26]. Furthermore, a previous report suggests that γ-irradiation induces cytological changes that are characteristic of differentiating red blood cell precursors [27]. Although it is known that cytotoxicity after PMA treatment is dependent on oxidant production [28], the molecular mechanisms underlying PMA-induced megakaryocytic differentiation are still not clearly understood. In the present study, we showed that irradiation promotes the megakaryocytic differentiation induced by PMA through intracellular ROS accumulation. Although previous reports suggest that the rapid burst of ROS after PMA stimulation is required for megakaryocytic differentiation [29, 30], the present study revealed that sustained intracellular ROS in the later phase accelerate differentiation. It is known that NAC acts as a substrate for the production of glutathione in cells and as a ROS scavenger. Having noted that CD41 expression was suppressed by the administration of NAC, it is considered that maintenance of ROS levels is at least partly involved in the promotion of megakaryocytic differentiation. In addition, the present study results suggest that ERK1/2 inhibits and p38 MAPK promotes the maintenance of intracellular ROS. It has been shown that PMA-induced megakaryocytic differentiation in K562 cells chiefly depends on the activity of the ERK/MAPK pathway [9–11], and it is accelerated by inhibition of the p38 MAPK pathway [31, 32]. Furthermore, it is suggested that the MAPK pathway regulates ROS generation but also that ROS could be responsible for the late activation of the MAPK pathway [33]. These studies may reflect the influence of MAPK on the intracellular ROS control system, which predominantly regulates CD41 expression under irradiation. Taken together, these results suggest that K562 cells self-adjust megakaryocytic differentiation by controlling intracellular ROS levels through the MAPK pathway, especially in the late phase following PMA stimulation. Two populations, classified by the level of CD41 expression, revealed varying reactivity to the MAPK inhibitor, as well as different levels of retention of intracellular ROS. In other words, there exists a possibility that the K562 cell population contains at least two types of cells with respect to ROS production mechanisms. Although the present study suggests the potential role of ERK1/2 and p38 MAPK in the control of intracellular ROS, further studies are needed to define the involvement of the MAPK pathway in the later accumulation of ROS. Our previous study showed that two megakaryocytic progenitor populations, CD34^{low} and CD34^{high}, have different radiosensitivity and are both found in steady-state human peripheral blood [6]. In this report, G-CSF enhanced the survival of irradiated CD34^{low} megakaryocytic progenitors, while, no enhancement effect was observed in the case of CD34^{high} progenitors. It is known that G-CSF stimulation shows a time- and dose-dependent increase in ROS production [34, 35]. Although the ROS generation in the cells exposed to X-irradiation was not analyzed, the present results suggest a possibility of ROS involvement.

In conclusion, the present results have indicated the possibility that the K562 cell population contains at least two types of megakaryocytic progenitors, with respect to radiosensitivity and ROS production mechanisms. In addition, intracellular ROS levels determine the extent of CD41 expression. Our findings may offer a novel strategy model for leukemia therapy.

FUNDING

This work was supported by JSPS KAKENHI Grant Number 24791266 (KT) and by a Grant from the Co-medical Education Program in Radiation Emergency Medicine by the Ministry of Education, Culture, Sports, Science and Technology, Japan (2012).

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