Effects of radiation on the maturation of megakaryocytes

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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, CD41low and CD41high cells, were detected by flow cytometry. The CD41high cells sustained intracellular ROS at the initial level for up to 72 h, but CD41low 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

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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% CO2. Briefly, 2.5 x 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 x 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, and analyzed by flow cytometry. 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, CD41low and CD41high cells, which were found as shown in Fig. 1A. The number of CD41low cells decreased with time, while a significant increase in CD41high cells was observed (Fig. 1B). At this time, no significant difference was observed in the number of CD41low and CD41high between 0 h and 72 h after treatment. These results suggest that different radiosensitive megakaryocytic progenitors exist in K562 cell population.

| 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.
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\textsuperscript{high} cells sustained intracellular ROS at the initial level for up to 72 h, but CD41\textsuperscript{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...
reactivity profiles of the two inhibitors were different in each population. Although, CD41\textsuperscript{high} cells sustained intracellular ROS at a higher level for up to 72 h, in CD41\textsuperscript{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, herbinycin, 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...
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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, CD34low and CD34high, have different radiosensitivity and are both found in steady-state human peripheral blood [6]. In this report, G-CSF enhanced the survival of irradiated CD34low megakaryocytic progenitors, while, no enhancement effect was observed in the case of CD34high 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.

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REFERENCES

1. Monzen S, Osuda K, Miyazaki Y et al. Radiation sensitivities in the terminal stages of megakaryocytic maturation and platelet production. Radiat Res 2009;172:314–20.
2. Kashiwakura I, Kuwabara M, Inanami O et al. Radiation sensitivity of megakaryocyte colony-forming cells in human placentals and umbilical cord blood. Radiat Res 2000;153:144–52.
3. Sutherland JA, Turner AR, Mannoni P et al. Differentiation of K562 leukemia cells along erythroid, macrophage, and megakaryocyte lineages. J Biol Response Mod 1986;5:250–62.
4. Chang YI, Hua WK, Yao CL et al. Protein-arginine methyltransferase 1 suppresses megakaryocytic differentiation via modulation of the p38 MAPK pathway in K562 cells. J Biol Chem 2010;285:20595–606.
5. Huang HM, Chioo HY, Chang JL. Activin A induces erythroid gene expressions and inhibits mitogenic cytokine-mediated K562 colony formation by activating p38 MAPK. J Cell Biochem 2006;98:789–97.
6. Kashiwakura I, Inanami O, Abe Y et al. Different radiosensitive megakaryocytic progenitor cells exist in steady-state human peripheral blood. Radiat Res 2005;164:10–6.
7. Monzen S, Takahashi K, Kashiwakura I. Radiation effects on the induced differentiation of the K562 leukemia cell line. In: The 49th Annual Meeting of The Japan Radiological Society, Sapporo, Japan, 2010. Abstract P30.
8. Rouyes MC, Boucheron C, Gisselbrecht S et al. Control of thrombopoietin-induced megakaryocytic differentiation by the mitogen-activated protein kinase pathway. Mol Cell Biol 1997;17:4991–5000.
9. Whalen AM, Galasinski SC, Shapiro PS et al. Megakaryocytic differentiation induced by constitutive activation of mitogen-activated protein kinase. Mol Cell Biol 1997;17:1947–58.
10. Melemed AS, Ryder JW, Vik TA. Activation of the mitogen-activated protein kinase pathway is involved in and sufficient for megakaryocytic differentiation of CMK cells. Blood 1997;90:3462–70.
11. Herrera R, Hubbell S, Decker S et al. A role for the MEK/MAPK pathway in PMA-induced cell cycle arrest: modulation of megakaryocytic differentiation of K562 cells. Exp Cell Res 1998;238:407–14.
12. Delgado MD, Larga A, Cañelles M et al. Differential regulation of Max and role of c-Myc during erythroid and myelomonocytic differentiation of K562 cells. Oncogene 1995;10:1659–65.
13. Murate T, Kagami Y, Hotta T et al. Terminal differentiation of human erythroleukemia cell line K562 induced by aphidicolin. Exp Cell Res 1990;191:45–50.
14. Rutherford T, Clegg JB, Higgs DR et al. Embryonic erythroid differentiation in the human leukemic cell line K562. Proc Natl Acad Sci U S A 1981;78:348–52.
15. Rutherford TR, Clegg JB, Weatherall DJ. K562 human leukaemic cells synthesise embryonic haemoglobin in response to haemin. Nature 1979;280:164–5.
16. Belhacène N, Maulon L, Guérin S et al. Differential expression of the Kell blood group and CD10 antigens: two related membrane metalloproteases during differentiation of K562 cells by phorbol ester and hemin. FASEB J 1998;12:531–9.
17. Park JI, Choi HS, Jeong JS et al. Involvement of p38 kinase in hydroxyurea-induced differentiation of K562 cells. Cell Growth Differ 2001;12:481–6.
18. Jacquel A, Herrant M, Legros L et al. Imatinib induces mitochondria-dependent apoptosis of the Bcr-Abl-positive K562 cell line and its differentiation toward the erythroid lineage. FASEB J 2003;17:2160–2.
19. Kawano T, Horiguchi-Yamada J, Iwase S et al. Inactivation of ERK accelerates erythroid differentiation of K562 cells induced by herbimycin A and STI571 while activation of MEK1 interferes with it. Mol Cell Biochem 2004;258:25–33.
20. Kawano T, Horiguchi-Yamada J, Saito S et al. Ectopic cyclin D1 expression blocks STI571-induced erythroid differentiation of K562 cells. Leuk Res 2004;28:623–9.
21. Burger SR, Zutter MM, Sturgill-Koszycy S et al. Induced cell surface expression of functional alpha 2 beta 1 integrin during megakaryocytic differentiation of K562 leukemic cells. Exp Cell Res 1992;202:28–35.
22. Tetteroo PA, Massaro F, Mulder A et al. Megakaryoblastic differentiation of proerythroblastic K562 cell-line cells. Leuk Res 1984;8:197–206.
23. Shelly C, Petruzelli L, Herrera R. PMA-induced phenotypic changes in K562 cells: MAPK-dependent and -independent events. Leukemia 1998;12:1951–61.
24. Kim H, Kim IY, Lee SY et al. Extracellular signal-regulated kinase/90-KDA ribosomal S6 kinase/nuclear factor-kappa B pathway mediates phorbol 12-myristate 13-acetate-induced megakaryocytic differentiation of K562 cells. J Biol Chem 2001;276:13186–91.
25. Dorsey JF, Cunnick JM, Mane SM et al. Regulation of the Erk2-Elk1 signaling pathway and megakaryocytic differentiation of Bcr-Abl(+) K562 leukemic cells by Gab2. Blood 2002;99:1388–97.
26. Sutherland JA, Turner AR, Mannoni P et al. Differentiation of K562 leukemia cells along erythroid, macrophage, and megakaryocyte lineages. J Biol Response Mod 1986;5:250–62.
27. Schwenke K, Peterson HP, Wangenheim KH et al. Induction of differentiation in erythroleukemic K562 cells by gamma-irradiation. Leuk Res 1995;19:955–61.
28. Schmidt R, Hecker E. Autoxidation of phorbol esters under normal storage conditions. Cancer Res 1975;35:1375–7.
29. Sardina JL, López-Ruano G, Sánchez-Abarca LI et al. p22phox–dependent NADPH oxidase activity is required for megakaryocytic differentiation. Cell Death Differ 2010;17:1842–54.
30. Iiyama M, Kakihana K, Kurosu T et al. Reactive oxygen species generated by hematopoietic cytokines play roles in activation of receptor-mediated signaling and in cell cycle progression. Cell Signal 2006;18:174–82.
31. Conde I, Pabón D, Jao A et al. Involvement of ERK1/2, p38 and PI3K in megakaryocytic differentiation of K562 cells. Eur J Haematol 2010;84:430–40.
32. Jacquel A, Herrant M, Defamie V et al. A survey of the signaling pathways involved in megakaryocytic differentiation of the human K562 leukemia cell line by molecular and c-DNA array analysis. Oncogene 2006;25:781–94.
33. Ramiro-Cortés Y, Guemez-Gamboa A, Mornán J. Reactive oxygen species participate in the p38-mediated apoptosis induced by potassium deprivation and staurosporine in cerebellar granule neurons. Int J Biochem Cell Biol 2011;43:1373–82.
34. Zhu QS, Xia L, Mills GB et al. G-CSF induced reactive oxygen species involves Lyn-PI3-kinase-Akt and contributes to myeloid cell growth. Blood 2006;107:1847–56.
35. Tesio M, Golan K, Corso S et al. Enhanced c-Met activity promotes G-CSF-induced mobilization of hematopoietic progenitor cells via ROS signaling. Blood 2011;117:419–28.