Megakaryocytic differentiation in human chronic myelogenous leukemia K562 cells induced by ionizing radiation in combination with phorbol 12-myristate 13-acetate

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Differentiation-induction therapy is an attractive approach in leukemia treatment. It has been suggested that the accumulation of intracellular reactive oxygen species (ROS) is involved in megakaryocytic differentiation induced by phorbol 12-myristate 13-acetate (PMA) in the K562 leukemia cell line. Therefore, a ROS-inducible technique could be a powerful method of differentiation induction. Accordingly, we hypothesized that ionizing radiation contributes to the acceleration of megakaryocytic differentiation through the accumulation of intracellular ROS in leukemia cells. In the present study, ionizing radiation was shown to promote PMA-induced megakaryocytic differentiation. Cells with high CD41 expression sustained intracellular ROS levels effectively. The enhancement of differentiation by ionizing radiation was found to be regulated through the mitogen-activated protein kinase (MAPK) pathway, involving both extracellular signal-regulated protein kinase 1/2 (ERK1/2) and p38 MAPK. Ionizing radiation also controlled mRNA expression of the oxidative stress response gene heme oxygenase-1 (HO1). Consequently, we concluded that intracellular ROS, increased by ionizing radiation, modulate megakaryocytic differentiation downstream of the MAPK pathway.

Keywords: K562 cells; megakaryocytopoiesis; ROS; MAPK

INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal disorder of hematopoietic stem cells caused by a t(9;22) (q34;q11) chromosomal translocation, which generates the Philadelphia chromosome [1, 2]. The chronic phase of CML, which is characterized by marked accumulation of cells within the granulocytic lineage, progresses inevitably to the advanced stage with blast crisis, caused by the epigenetic and/or genetic changes in cells, which restrain differentiation [3]. Though imatinib mesylate (Glivec) is highly effective in patients in the chronic phase of CML, progression to the blast phase decreases responsiveness to the drug and makes treatment difficult [4]. In this context, differentiation-inducing therapy seems to be a promising treatment technique, especially for patients in poor general condition who cannot tolerate intensive chemotherapy or bone marrow transplantation. Therefore, the discovery of a new and potent differentiation technique in CML treatment has attracted much clinical attention [5].

It has become clear that mitochondrial reactive oxygen species (ROS) act as a physiological redox signal mediator through the modulation of signal molecules and not only through redox stress [6, 7]. It has also been demonstrated that ROS are also involved in the initiation of the cellular differentiation program and cell growth [8, 9]. Intracellular ROS burst, which is produced in the early stage of differentiation,
acts as the initial signal for megakaryocytic differentiation [10]. However, it is unclear which process of the differentiation ROS are involved in.

Low-linear-energy-transfer ionizing radiation, such as X-rays and γ-rays, generates hydroxyl radicals from water molecules by the photoelectric effect, which damages DNA strands, and participates in cell apoptosis and senescence through incompleteness of the DNA repair system, with concentration-dependent effects [11–13]. On the other hand, ionizing radiation enhances cell differentiation in a dose-dependent manner in specific circumstances in certain normal cells [14]. In the present study, to evaluate X-irradiation effects on PMA-induced megakaryocytic differentiation of the CML cell line K562, the cellular processes responsible for the acceleration of megakaryocytic differentiation 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, NADPH oxidase inhibitor diphenylene iodonium (DPI), and N-acetyl cysteine (NAC) were obtained from Sigma-Aldrich (Stockholm, Sweden). 2,7-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) was purchased from Invitrogen. Flow cytometry antibody PE-Cy7 (PC7)-conjugated anti-human CD41 (CD41-PC7), FITC-mouse IgG1, 7-AAD and fluorescein isothiocyanate (FITC)-conjugated Annexin-V were purchased from Beckman Coulter Immunotech (Marseille, France) and Becton Dickinson Biosciences (Franklin Lakes, NJ, USA).

Cell culture and differentiation

The human leukemia cell line K562 was grown in RPMI1640 medium supplemented with penicillin, streptomycin, and 10% FBS at 37 °C under a humidified atmosphere containing 5% CO$_2$.

Non-irradiated or irradiated K562 cells (2.0–3.0 × 10$^5$ cells/ml) were plated into 24-well plates at 0.5 ml/well with serum-free Iscove’s modified Dulbecco’s medium (Gibco Invitrogen, Grand Island, NY, USA). The cultures were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO$_2$. After 6, 24 and 48 h, total RNA was extracted using the RNeasy® Micro Kit (Qiagen, Bothell, WA, USA); concentration and purity were determined using a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions.

Measurement of intracellular ROS levels

The oxidation-sensitive fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) was used to measure intracellular ROS levels. Cells were incubated with 10 µM H$_2$DCFDA 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.

Total RNA extraction

Non-irradiated or irradiated K562 cells (2.0–3.0 × 10$^5$ cells/ml) were plated into 24-well plates at 0.5 ml/well with serum-free Iscove’s modified Dulbecco’s medium (Gibco Invitrogen, Grand Island, NY, USA). The cultures were incubated at 37°C in a humidified atmosphere containing 95% air/5% CO$_2$. After 6, 24 and 48 h, total RNA was extracted using the RNeasy® Micro Kit (Qiagen, Bothell, WA, USA); concentration and purity were determined using a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

The cells were harvested after 6, 24 and 48 h, and total RNA was extracted using an RNeasy® micro Kit (Qiagen, Valencia, CA, USA) and quantified using a NanoDrop system (Thermo, Wilmington, DE). First-strand cDNAs were synthesized with an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Gene expression was assessed using real-time reverse transcription-polymerase chain reaction (RT-PCR) (Power SYBR® Green and StepOnePlus™; Life Technologies Inc., Carlsbad, CA) with typical amplification parameters (95°C for 10 min followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min). Relative differences were determined by ΔCt values comparing each gene after 6, 24 and 48 h to 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). Cytological changes, including enlarged and lobed nuclei, multiple microvesicles, and vacuolation, were examined by modified Wright-Giemsa staining.

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 at a dose rate of approximately 100 cGy/min. During X-ray exposure, the dose intensity of the ionization chamber was evaluated.
non-irradiated genes treated with PMA at 6 h after normalization with the housekeeping gene GAPDH. The oligonucleotide primer sets used for real-time RT-PCR were purchased from TAKARA Bio Inc. (Otsu, Shiga, Japan) (Table 1).

**Cell viability and apoptosis analysis**

Cell culture and differentiation were performed as described above. Cells cultured in 24-well plates were stained by DPBS containing 0.1% trypan blue. Cell viability was assessed by counting the average number of trypan blue-positive cells in three random fields from at least three separate wells. Cell apoptosis was evaluated by flow cytometry after co-staining with Annexin V-FITC and 7-AAD according to the manufacturer’s instructions.

**Statistical analysis**

All experiments were performed at least three times and results were 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**

**Effect of ionizing radiation on PMA-induced megakaryocytic differentiation**

Consistent with a previous report [15], growth arrest occurred in cells treated with PMA after 24 h. When ionizing radiation was combined with PMA, inhibition of cell growth was found to be no greater than with PMA alone (Fig. 1A). After stimulation with 50 nM PMA, K562 cells exhibited the specific megakaryocyte-like cytological changes, such as enlarged and lobed nuclei, multiple microvesicles, and vacuolation. For quantification, modified Wright-Giemsa staining was performed to examine cell morphology. The number of 4 Gy X-irradiated cells exhibiting these characteristics increased 1.66 ± 0.18-fold more than that of the non-irradiated cells 96 h after PMA stimulation (*P < 0.05*, Fig. 1B). Next, the expression of CD41 (GPIIb/IIIa), which is thought to be an early megakaryocytic marker, was analyzed by flow cytometry. As shown in Fig. 1C, PMA stimulation significantly increased CD41 expression (3.06 ± 1.35%, *P < 0.005*); untreated K562 cells originally were close to zero for CD41 (0.49 ± 0.07%). Notably, X-irradiation (4 Gy), immediately followed by PMA stimulation, enhanced CD41 expression much more (38.85 ± 1.20%, *P < 0.05*) than PMA stimulation alone. Moreover, expression of the megakaryocytopenesis-related genes, platelet glycoprotein IIb (ITGA2B) and Ibα (GP1BA), was enhanced more by a combination of 4 Gy X-irradiation and PMA than by PMA alone (Fig. 1D and 1E). These results suggest that ionizing radiation accelerates PMA-induced megakaryocytic differentiation.

Because intracellular ROS are necessary for megakaryocytic differentiation under PMA stimulation [10], we next examined whether the observed enhancement of CD41 expression by X-irradiation depends on intracellular ROS accumulation. X-irradiation (4 Gy) without PMA induction increased intracellular ROS at 48 h, to a level 2.02 ± 0.10-fold greater than that of the control (*P < 0.005*, data not shown). In the cells induced by PMA combined with radiation, higher ROS levels were measured 48 h after PMA stimulation compared to the cells induced by PMA without radiation (*P < 0.005*, Fig. 2A). Administration of the ROS scavenger N-acetyl cysteine (NAC) reduced CD41 expression levels derived from ionizing radiation (Fig. 2B).

| Gene   | Primer sequencea       |
|--------|------------------------|
| ITGA2B | F: 5′-TGCTGCTCACCACCTCGTC-3′ |
|        | R: 5′-AACCAAGCTTTGGAGGCAC-3′ |
| GP1BA  | F: 5′-TCTGTATCGAAAGCCCTGTCTC-3′ |
|        | R: 5′-GCATCGGGAGCTTTGTCTTG-3′ |
| HO-1   | F: 5′-TGCTCAGCCTCAAAATGACG-3′ |
|        | R: 5′-AGGCCCAAGCTGGTAAACA-3′ |
| NQO1   | F: 5′-GGATTGGGACCGCGTGGAA-3′ |
|        | R: 5′-AATTGCAGTGGAAGATGAGCGCAAC-3′ |
| GAPDH  | F: 5′-ACACCCTGGCCTACGCTAAAGAC-3′ |
|        | R: 5′-AGCCCAAGCATCAAAGATGGAG-3′ |

The human oligonucleotide primer sets were purchased from Takara Bio Inc. (Japan). aF = forward primer, R = reverse primer.

**CD41 expression and intracellular ROS levels depend on the MAPK pathway**

As earlier reports have demonstrated that the MAPK pathway plays a role in the megakaryocytic differentiation of K562 cells by PMA stimulation [15–18], we therefore examined whether the MAPK pathway is associated with the promotion of PMA-induced megakaryocytic differentiation by ionizing radiation. As shown in Fig. 3A, PD98059, an inhibitor of ERK1/2, increased CD41 expression. However, SB203580, an inhibitor of p38 MAPK, decreased CD41 expression. Next, we analyzed the effect of MAPK on intracellular redox conditions. Whereas PD98059 increased ROS levels, SB203580 decreased them (Fig. 3B), and a strong correlation between CD41 expression and DCF mean fluorescence intensity was observed ($r^2 = 0.9299, P < 0.001$). These results suggest that ERK1/2 inhibits the megakaryocytic differentiation of K562 cells, whereas p38 MAPK supports this process through
regulating ROS production under irradiation-accelerated K562 maturation.

PMA treatment leads to irreversible cell growth arrest in K562 cells and induces apoptosis [19]. However, it is unclear whether apoptotic cell death in leukemia cells depends on the process of differentiation or occurs spontaneously. Thus, we explored whether apoptosis and megakaryocytic differentiation correlate with each other. Consistent

Fig. 1. Ionizing radiation accelerates PMA-induced megakaryocytic differentiation through ROS. (A) The growth and viability of the cells were assessed by trypan blue dye exclusion after induction. (B) K562 cells were X-irradiated at 4 Gy, and immediately treated with 50 nM PMA. Megakaryocytic differentiation was detected by modified Wright-Giemsa staining for cell morphology. Representative cytological changes at 96 h, such as multiple microvesicles (black arrow) and enlarged nuclei (white arrow) were denoted. (C) CD41 expression on the cell surface was evaluated by flow cytometry. The cells were X-irradiated at 4 Gy and treated with 50 nM PMA for 72 h. Typical cytograms obtained in five separate experiments are shown with arbitrary values for CD41 mean fluorescent intensity. (D, E) The mRNA levels of ITGA2B and GP1BA were examined after 6, 24 and 48 h. Data were calculated relative to control cells after normalization with the housekeeping gene GAPDH. All experiments were performed at least three times, and data are represented as means ± SE. ***P<0.005 reflects significant differences in X-irradiated cells with respect to non-irradiated cells.
with previous reports [15], growth arrest occurred in PMA-treated cells after 24 h. PD98059 partially reversed the cell growth inhibition induced by PMA in non-irradiated cells. When PMA treatment was combined with ionizing radiation, no greater inhibition of cell growth was observed than with PMA treatment alone (data not shown). As shown in Fig. 4A, in the cells induced by the PMA-radiation combination, treatment with PD98059 sustained viability of K562 cells more than non-treatment, whereas treatment with SB203580 had no influence. Next, apoptosis was analyzed by co-staining anti-CD41-PC7 antibody with 7-AAD and annexin-V-FITC. There was no significant difference in apoptotic cell number when PMA treatment alone was compared with PMA treatment combined with irradiation (Fig. 4B and 4C). Moreover, even with MAPK inhibitor, there was no correlation between the percentages of cells with high CD41 expression levels and apoptotic cells 48 or 72 h after induction. In agreement with a previous report [20], these results imply that differentiation and apoptosis are simultaneously induced through independent pathways.

**Increased ROS levels through activation of NADPH oxidase by inhibition of ERK1/2**

Intracellular ROS are mainly generated by NADPH oxidase (NOX), a multi-component enzyme complex. Because it has been suggested that the MAPK pathway controls the expression of NOX component subunits in certain cell lines [21, 22], we analyzed its participation in PMA-induced megakaryocytic differentiation of K562 cells. DPI, an inhibitor of flavoenzymes such as NOX and iNOS, markedly reduced CD41 expression and intracellular ROS levels (Fig. 5A and 5B).
These results suggest the possibility that NOX activity is needed for intracellular ROS production and differentiation. We analyzed the involvement of the oxidative stress genes, heme oxygenase-1 (HO1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Fig. 5C and 5D) in the process of PMA-induced megakaryocytic differentiation promoted by irradiation. Although HO1, which had been controlled with Nrf2, was increased, no change in NQO1 expression was observed after irradiation. These results imply that ionizing radiation modulates the expression of oxidative stress genes.

DISCUSSION

In this study, we examined whether ionizing radiation has an impact on the PMA-induced megakaryocytic differentiation of K562 cells. We demonstrated for the first time that ionizing radiation enhances PMA-induced megakaryocytic differentiation at a relatively late phase, and that the late-phase accelerated differentiation depends on the persistence of the intracellular ROS level.

In the presence of PMA stimulation, irradiated cells sustained higher intracellular ROS levels up to 48 h compared with non-irradiated cells (Fig. 2). It is known that NAC acts as a substrate for the production of glutathione in cells, and also as a ROS scavenger. Having noted that CD41 expression was suppressed by the administration of NAC, we conclude that maintenance of ROS levels is at least partly involved in the promotion of megakaryocytic differentiation. As no correlation between CD41 expression and cell death was confirmed (Fig. 4), the protective effect of NAC against ROS toxicity is deemed to have little influence on megakaryocytic differentiation. Therefore, it is thought that ROS not only trigger differentiation at a very early stage following PMA stimulation [10], but also participate in the promotion of differentiation at a later stage.

Inhibition of the MAPK pathway decreased the high intracellular ROS levels that were maintained by X-irradiation (Fig. 3). In these circumstances, the regulated intracellular ROS determined the degree of megakaryocytic differentiation. Intracellular ROS were produced, accumulating at later phase, by ionizing radiation. It is thought that the expression of proteins, such as NOX, that produce and control intracellular ROS, is involved in the radiation-induced change in intracellular ROS levels, rather than radical production from water molecules by the photoelectric effect. Recent studies suggest that intracellular ROS levels are modified by the MAPK pathway. Furthermore, the expression of NOX, which produces a large proportion of intracellular ROS, appears to be controlled by the MAPK pathway [21, 22]. Consistent with these reports, the present results suggest that the MAPK pathway is the mechanism for the adjustment of ROS levels and the regulation of megakaryocytic differentiation in K562 cells. In the present study, DPI was used as a NOX inhibitor to confirm the involvement of NOX in the process of intracellular ROS production. As DPI also inhibits iNOS and eNOS, which generate reactive nitrogen intermediates, detailed investigation is needed to reveal whether these
enzymes are involved in this process. It has been shown that PMA-induced megakaryocytic differentiation in K562 cells chiefly depends on the activity of the ERK/MAPK pathway \[15, 17, 18\], and it is accelerated by inhibition of the p38 MAPK pathway \[20, 23\]. Unexpectedly, under the present experimental conditions, megakaryocytic differentiation induced by PMA and X-irradiation was enhanced by ERK1/2 inhibition and suppressed by p38 inhibition. The concentration of PD98059 used in previous studies appears to have partially inhibited ERK1/2 \[18\]. For this reason, ERK1/2 may have been partially activated by PMA under the influence of PD98059. One report suggested that the MAPK pathway regulates ROS generation, and that ROS could be responsible for the late activation of the MAPK pathway \[22\]. In addition, there is another pathway independent of the MAPK pathway relating to the induction of megakaryocytic differentiation of K562 cells by PMA stimulation \[24\]. The aforementioned mechanisms may have influenced the results of the present experiment, which was focused on radiation-induced changes in intracellular ROS. These results 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.

In addition, the oxidative stress genes HO1 increased compared to the control. We previously reported that 2 Gy X-irradiation enhanced mRNA expression of these genes in the proliferation and differentiation pathway from human hematopoietic stem cells to mature megakaryocytes \[25, 26\], suggesting the possibility of involvement of the oxidative stress genes in the differentiation. Further work is needed to determine whether direct cross-talk exists between the differentiation and oxidative stress responses modulated by ionizing radiation.

It is unclear whether apoptotic cell death in leukemia cells depends on the process of differentiation or occurs spontaneously. In the present study, PMA treatment alone increased the percentage of apoptotic cells, but in combination with ionizing radiation, although it promoted CD41
expression and the ratio of megakaryocyte-like cells, it did not increase the percentage of apoptotic cells. Furthermore, no correlation was found between the percentages of cells with high CD41 expression and apoptotic cells in the presence of MAPK inhibitors. Thus, in agreement with a previous report [20], apoptosis and differentiation do not appear to be simultaneously induced through the same pathway.

**CONCLUSION**

In the present study, we successfully increased the number of cells with megakaryocyte-like features and CD41 expression using 4 Gy X-irradiation. We also showed for the first time that intracellular ROS sustained into the late phase after stimulation accelerates megakaryocytic differentiation. The MAPK pathway and NOX activity is responsible for ROS accumulation leading to the differentiation process. These findings imply that megakaryocyte differentiation induction in leukemia cells may be promoted by some kind of upregulation of intracellular ROS levels and of signals of the MAPK pathway, such as ERK1/2 and p38. Certainly, there is little possibility of any clinical application for PMA, which has marked cytotoxicity and carcinogenicity. However, further studies are needed to develop methods for the safe utilization of inducers in combination with ionizing radiation. Our findings provide a new scientific insight into differentiation induction and ionizing radiation as a technique for upregulating intracellular ROS, and may suggest a novel strategy model for leukemia therapy.

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