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Short communication

PROMOTED MEGAKARYOCYTIC DIFFERENTIATION OF K562 CELLS THROUGH OXIDATIVE STRESS CAUSED BY NEAR ULTRAVIOLET IRRADIATION

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Abstract: Reactive oxygen species (ROS) have been proven to be important activators for various cellular activities, including cell differentiation. Several reports showed the necessity of ROS during cell differentiation of the megakaryocytic (MK) lineage. In this study, we employed near ultraviolet (near-UV) irradiation to generate endogenous oxidative stress in an MK differentiation process of K562 cells with phorbol 12-myristate 13-acetate (PMA) induction. A significant increase in the intracellular ROS level was detected on day 1 after near-UV irradiation. In the initial stage of differentiation, a shifted fraction of G_1 and G_2 phase cells was obtained using near-UV irradiation, giving an increased percentage of G_2 phase cells (up from 31.1 to 68.7%). The near-UV irradiation-induced upregulation of the *p21* gene, which is a cell cycle inhibitor, suggested that the G_2 phase cells were prevented from undergoing cell division. It was found that the percentage of high ploidy (8N and 16N) cells was enhanced significantly at the later stage of the K562 cell culture with near-UV irradiation. Moreover, time-lapse analysis showed that near-UV irradiation encouraged the expression of CD41, a specific surface marker of megakaryocytes. This is the first report that the elevated oxidative stress through the near-UV irradiation promoted the MK differentiation of PMA-induced K562 cells.

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Abbreviations used: EDTA – ethylene diamine tetra-acetic acid; MK – megakaryocytic; PBS – phosphate-buffered saline; PMA – phorbol 12-myristate 13-acetate; ROS – reactive oxygen species

Keywords: Near ultraviolet, Irradiation, K562 cells, Megakaryocytic differentiation, Polyploidization, Reactive oxygen species, Cell cycle, Phorbol 12-myristate 13-acetate

INTRODUCTION

Aerobic metabolisms constantly produce a small quantity of reactive oxygen species (ROS), such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) [1]. Although ROS have been correlated with deleterious effects on the human body (DNA mutation [2] and lipid peroxidation [3]), recent reports have elucidated their importance in the regulation of signal transduction pathways and in gene expression for various cellular activities.

Megakaryocytic (MK) differentiation and maturation, a developmental process that produces platelets from multipotent hematopoietic stem cells, is recognized to require ROS accumulation [4]. The ROS are mainly produced through the induction of cytokines, such as thrombopoietin, and several studies have found that cell differentiation of the MK lineage can be promoted by oxidative stress from the culture environment [5–7]. Thus, in addition to ROS production from cytokine induction, the generation of oxidative stress is expected to encourage the process of MK differentiation.

We reported that oxidative stress through H_2O_2 administration enhanced MK maturation in cells of K562, a human myelogenous cell line [7]. K562 cells have been extensively used as a model in the study of MK differentiation, which can be achieved by exposure to phorbol 12-myristate 13-acetate (PMA) [8–11]. In this study, we treated K562 cell cultures with near ultraviolet (near-UV) irradiation to examine the effect of such stimuli on MK differentiation of K562 cells in terms of endogenous oxidative stress. The MK developmental process was analyzed based on the expression of a specific surface marker of the megakaryocyte, CD41, and based on polyploidization.

MATERIALS AND METHODS

Cell culture and irradiation conditions

The K562 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Hyclone Laboratories Inc.) supplemented with 10% fetal bovine serum (Biofill Australia Pty. Ltd.). The initial cell density was adjusted to 7.0×10^4 cells/ml. A polystyrene culture dish (35 mm ϕ, Sigma-Aldrich) was employed for all of the experiments.

PMA (EMD Millipore) was added to the culture at 10 ng/ml to induce MK differentiation. In the case of irradiated culture, the cells were pre-treated with near-UV irradiation from a black light lamp (FL10 BL-B, Panasonic), with a peak wavelength at 352 nm. The UV intensity on the dish surface was fixed at 3 $\mu W/mm^2$, determined using a quantum sensor (Blak-Ray J-221, UVP). The

cells were irradiated for 2 h on the culture medium, followed by PMA addition to the culture.

All of the cultures were kept at 37ºC in a fully humidified incubator under 5% CO_2 atmosphere. The culture broth was exchanged every 4 days with fresh medium containing 10 ng/ml PMA. The total cell concentration was counted using a hemocytometer under a microscope, and cell viability was evaluated with a dye exclusion test using trypan blue.

Intracellular ROS assay

After cultivating the cells for a day, the media were carefully removed so that the cells remained in the culture dish. The cells were incubated with pre-warmed phosphate-buffered saline (PBS) containing 5 μmol/l 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Life Technologies) for 30 min at 37ºC in the dark. The cells were harvested by scraping in cold PBS and the fluorescence intensity of the oxidized reagent was measured with a BD Accuri C6 flow cytometer (BD Biosciences). The fluorescence intensity of oxidized reagent from non-irradiated cells without PMA induction was used to calculate the relative value of the ROS content.

Real-time PCR analysis

Irradiated or non-irradiated cells were cultured in the presence or absence of PMA. Adherent cells were detached by trypsinization at 37ºC for 5 min and then the cells were harvested by centrifugation at 1,300 rpm for 5 min. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The reverse transcription and real-time PCR were performed as described elsewhere [7]. Primers for real-time PCR were designed using Primer3 software (available at http://frodo.wi.mit.edu/). The detailed sequences for each primer are shown in Table 1. The *rps18* gene, encoding ribosomal protein S18, was selected as a suitable house-keeping gene from preliminary experiments.

Table 1. Primer sequences used for real-time PCR analysis.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
p21	GTCACCGAGACACCACTGGA	CGTGGGAAGGTAGAGCTTGG
nf-κb1	CCCTGACCTTGCCTATTTGC	GGTCCATCTCCTTGGTCTGC
rps18	GCAGAATCCACGCCAGTACA	TCAGTCGCTCCAGGTCTTCA

Surface antigen and ploidy assays

A specific surface protein marker (CD41) and MK ploidy were analyzed according to literature [12]. Briefly, the cells were washed with PBS containing 2 mmol/l EDTA and 0.5% BSA. The cells were then stained with FITC-conjugated anti-CD41 antibody (Beckman Coulter) and fixed with 0.5% paraformaldehyde for 15 min at room temperature.

The cells were permeabilized with a 70% aqueous solution of methanol at 4ºC for 1 h. After treatment with 10 mg/ml RNase (Sigma-Aldrich) for 30 min, the fixed cells were stained with 50 mg/l propidium iodide (Wako Pure Chemical Industries) and then loaded on a flow cytometer. In this study, total ploidy was defined as follows:

$$\text{total ploidy} = (G_1 \text{ phase cell concentration} \times 2N) + (G_2 \text{ phase cell concentration} \times 4N) \quad (1)$$

where the values of 2N and 4N are the number of chromosome sets per cell (ploidy) of G_1 and G_2 phase cells, respectively. High ploidy cells were determined as cells with 8N or larger.

Statistical analysis

Statistical differences between paired data from two experimental sets were determined with a paired *t*-test. To compare paired data from several experimental sets, one way analysis of variance (ANOVA) followed by Tukey's test was performed using statistical software (Origin 9.1; OriginLab). Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Oxidative stress generation through near-UV irradiation

In this study, near-UV irradiation was found to generate oxidative stress in a K562 cell culture. Based on the results of preliminary experiments, 2 h irradiation at 3 μW/mm^2 was used because of the relatively low toxicity to the cells (data not shown).

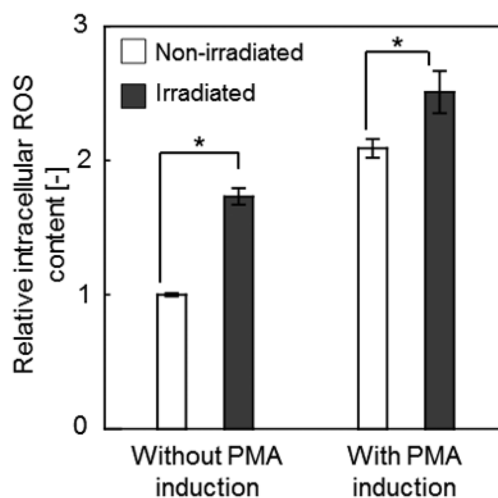

Fig. 1. Relative ROS content in the K562 cells on day 1 after treatment with and without near-UV irradiation. The data are means with standard deviations represented by the vertical bars (n = 3). The asterisks indicate values of $p < 0.05$, based on a paired *t*-test.

To ensure that near-UV irradiation is effective enough to generate oxidative stress in the culture, the intracellular ROS content was measured. Fig. 1 shows the relative values of intracellular ROS contents in the PMA-induced and non-induced K562 cells on day 1. In the absence of PMA, near-UV irradiation elevated the intracellular ROS content 1.7 times, whereas in the presence of PMA, the ROS content increased 1.2 times. It was thus found that ROS generation was encouraged irrespective of the PMA treatment, suggesting that the increase in ROS content through near-UV irradiation is independent of a PMA mechanism.

Cell cycle distribution

On day 1 after PMA treatment, the cell cycle pattern was analyzed using propidium iodide staining. Based on the report that PMA diminishes the S phase cell level [13], we divided the population into G_1 and G_2 phase cells based on their DNA contents. Fig. 2 represents the typical histograms of DNA content analysis via flow cytometry. It is worth noting that near-UV irradiation significantly affected the fractions of G_1 and G_2 phase cells. The G_2 phase cells increased from 31.1 ± 1.3% (non-irradiated) to 68.7 ± 1.3% (irradiated) and consequently the G_1 phase cells decreased in the irradiated cell population. A similar phenomenon was reported for K562 cells [14] without PMA induction in which oxidative stress, produced using a peroxyl radical initiator and hydrogen peroxide, perturbed cell proliferation through G_2–M arrest.

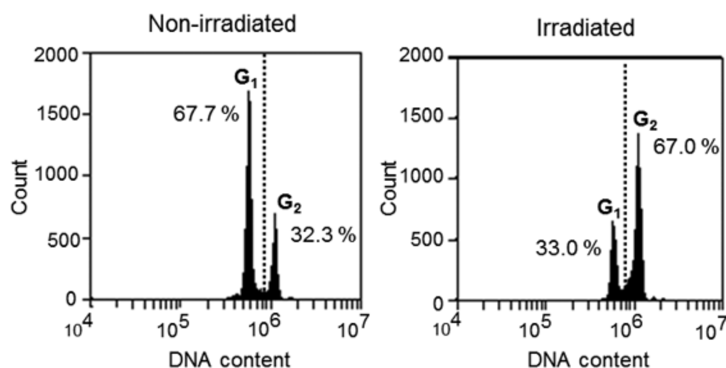

Fig. 2. Typical histograms of ploidy analysis fractionizing G_1 and G_2 phase cells in the PMA-induced K562 cells on day 1 after treatment with and without near-UV irradiation.

We estimated the ploidy of cells by considering the effect of decreasing total cell concentration in the near-UV irradiated population (Table 2). The total ploidy was calculated by assuming that the ploidy values of G_1 and G_2 phase cells are 2N and 4N per cell, respectively, based on Equation (1). In the near-UV irradiated population, the G_1 phase cell concentration reduced from 49.6×10^3 to 18.0×10^3 cells/ml and the G_2 cell concentration increased from 22.3×10^3 to 39.6×10^3 cells/ml. As a result, the total ploidy was not significantly different between the non-irradiated cell population (188.2×10^3 N/ml) and irradiated cell

population (194.4 × 10^3 N/ml). These results clearly demonstrate that rather than having a killing effect against cells with lower ploidy, the irradiation suppressed the division of G_2 phase cells.

Table 2. Effect of near-UV irradiation on the proliferation and ploidization of PMA-induced K562 cells on day 1 after treatment. The data were recorded as means with standard deviations (n = 3).

	Total cell concentration (10^3 cells/ml)	Percentage of viable cells	G_1 phase cell concentration (10^3 cells/ml)	G_2 phase cell concentration (10^3 cells/ml)	Total ploidy (10^3 N/ml)
Non-irradiated	71.9 ± 11.3	94.9 ± 0.8	49.6 ± 8.7	22.3 ± 2.9	188.2 ± 28.2
Irradiated	57.6 ± 12.2	94.8 ± 1.8	18.0 ± 3.8	39.6 ± 8.5	194.4 ± 41.4

Next, real-time PCR analysis was conducted in order to clarify the expression patterns of cell cycle-associated genes. It has been hypothesized that the cell cycle arrest is responsible for the polyploidization process during megakaryocytic differentiation [15]. During this process, p21 (a cyclin-dependent kinase inhibitor) functions as a negative regulator for the cell cycle [16] so that cells are prevented from dividing even though they normally duplicate DNA. Fig. 3 shows the mRNA expressions of selected genes in the cells with or without near-UV irradiation. It was shown that the *p21* gene was expressed at a relatively low level in the K562 cells without PMA induction. *p21* expression was enhanced by PMA induction or near-UV irradiation. Interestingly, the combination of near-UV irradiation and PMA induction strongly elevated its expression level.

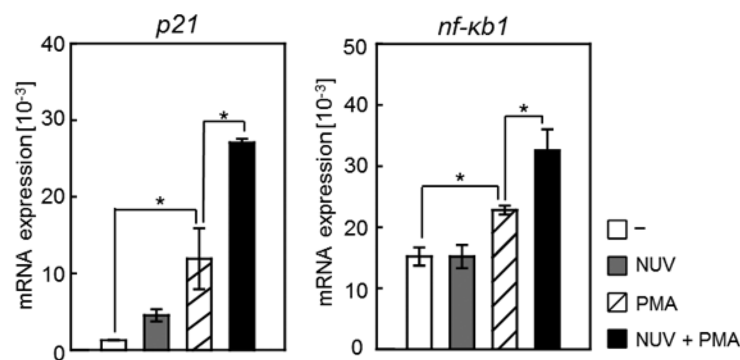

Fig. 3. Relative mRNA expression of cell cycle-associated genes in K562 cells on day 1 after treatment. Each condition for the treatments is represented as no treatment (-), near-UV irradiation (NUV), PMA induction (PMA), or near-UV irradiation and PMA induction (NUV+PMA). The data are means with standard deviations represented by the vertical bars (n = 3). The asterisks indicate values of $p < 0.05$, based on Tukey's test. Abbreviations: *p21* – cyclin-dependent kinase inhibitor; *nf-κb1* – nuclear factor-κ-B1.

As reported by Wuerzberger-Davis et al. [17], the induction of the *nf-κb*-dependent *p21* gene by gamma ray irradiation resulted in G_2–M arrest. In this study, we found a distinctive expression pattern of *nf-κb1* (nuclear factor-κ-B1), an important transcription factor in MK differentiation [18], was affected by near-UV irradiation (Fig. 3). Near-UV irradiation was able to enhance *nf-κb1* expression under the PMA induction. In non-induced K562 cells, near-UV irradiation did not affect the expression level of *nf-κb1*. Therefore, it seems that *nf-κb1* expression is associated with PMA induction. It is most likely that along with the *p21* upregulation, the enhanced expression of *nf-κb1* by the near-UV irradiation prevents the cells from entering into a G_2 phase at an early stage of differentiation. Moreover, it was considered that the increased expressions of both *nf-κb1* and *p21* genes with near-UV irradiation were attributable to the change in intracellular oxidative stress level, since similar increases were confirmed when hydrogen peroxide was introduced into the cultures (data not shown).

Effect of near-UV irradiation on MK differentiation

In order to clarify the effect of near-UV irradiation on MK maturation, we carried out an extended culture of K562 cells for 8 days. Fig. 4 shows the time courses of the total cell concentration, viability and percentage of high ploidy (8N and 16N) cells. In the absence of irradiation, the total cell concentration

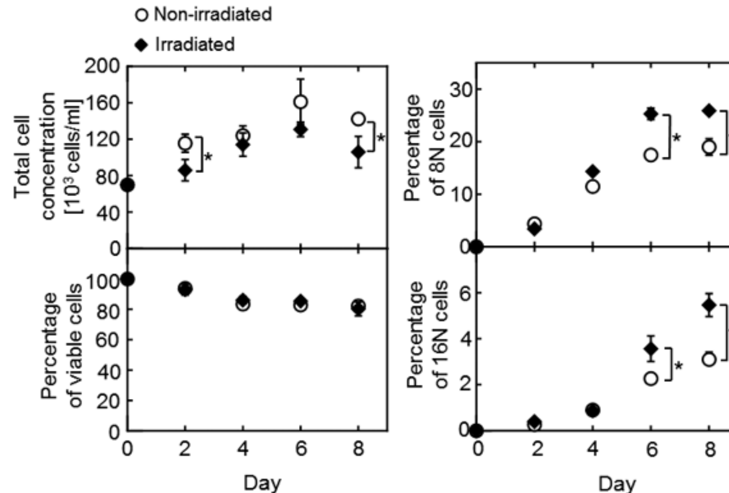

Fig. 4. Time courses of total cell concentration, cell viability and percentage of high ploidy (≥ 8N) cells during cultures of PMA-induced K562 cells with and without near-UV irradiation. The data are means with standard deviations represented by the vertical bars (n = 3). The asterisks indicate values of $p < 0.05$, based on a paired *t*-test.

increased during the first 2 days. In the same period, a limited increase in the total cell concentration was detected in the irradiated cells. From day 2 to day 6, the cell proliferation was restricted in both cultures, showing no substantial difference of total cell concentration. This process was caused by the PMA, which affected cell

proliferation and subsequently allowed polyploidization to occur. There was a tendency that total cell concentration decreased from day 6 to day 8 in both non-irradiated and irradiated cultures. The cell viability of non-irradiated and irradiated cultures remained more than 80% throughout the culture periods.

Considering that the non-PMA-induced K562 cells were composed of 2N and 4N cell populations, we fractionized high ploidy cells into 8N and 16N cells. The cells with ploidy more than 16N were not detected until day 8. The percentages of 8N and 16N cells in the two cultures increased gradually throughout the culture time regardless of irradiation, confirming that polyploidization occurred under the employed conditions. After 6 days, the irradiated cells produced higher percentages of 8N and 16N cells than the non-irradiated cells. On day 8, the percentages of 8N and 16N cells of irradiated cells reached 25.9% and 5.5%, respectively. These values were significantly higher than the percentages for non-irradiated cells: 19.0% and 3.1%, respectively. These results suggest that the near-UV irradiation was able to accelerate the formation of high ploidy cells in the PMA-induced K562 culture.

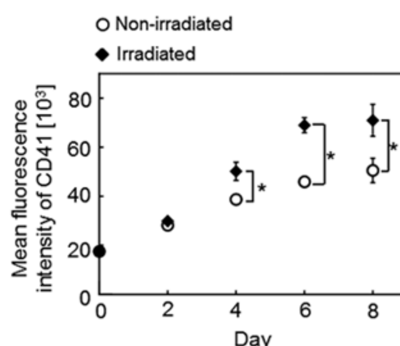

Fig. 5. Time courses of mean fluorescence intensity of CD41 during the cultures of PMA-induced K562 cells with and without near-UV irradiation. The data are means with standard deviations represented by the vertical bars (n = 3). The asterisks indicate values of $p < 0.05$, based on a paired *t*-test.

A trans-membrane glycoprotein, CD41, is a specific marker of megakaryopoiesis during an early stage of cell differentiation [19]. In this study, the CD41 expression was analyzed every two days during the cultivation. As seen in Fig. 5, the CD41 expression increased during the differentiation process with or without the near-UV irradiation. It is worth noting that the irradiated cells maintained higher CD41 levels than the non-irradiated cells, and on day 8, the level reached 71.0×10^3, the value of which was 1.4 times higher than that of the non-irradiated cells (50.5×10^3). These results indicated that the near-UV irradiation encouraged MK commitment in the PMA-induced K562 cells. Thus, the near-UV irradiation promoted MK differentiation of PMA-induced K562 cells as confirmed from the polyploidization and CD41 expression analyses.

Hirose *et al*. [6] reported an irradiation effect on the promotion MK differentiation of a K562 cell line. They employed high energy X-ray exposure to PMA-induced K562 cells and found that the CD41 expression increased in response to ROS generation, whereas polyploidization was not mentioned in their report.

In conclusion, it has been shown that 2 h pre-treatment with near-UV irradiation elevated the intracellular ROS content in K562 cells. Increased ROS in PMA-induced K562 cells affected the expression levels of cell cycle regulator genes, *p21* (a cell cycle inhibitor) and *nf-κb1* (a nuclear factor κ-B1). The upregulation of *p21* gene along with *nf-κb1* gene resulted in the inhibition of G_2 phase cell division, so the G_2 phase cells accumulated on day 1 after irradiation.

Inhibition of cell division in an early stage of differentiation through near-UV irradiation led to an increase in the high ploidy cell population at the later stage. The fractions of 8N and 16N cells produced under near-UV irradiation increased appreciably, as compared with those in the non-irradiated cell population. In addition, the MK commitment, as confirmed by CD41 expression, was elevated throughout the culture of the irradiated cells. This study reports first that the near-UV irradiation can be used as a useful tool to enhance the MK differentiation process of K562 cells through oxidative stress generation.

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