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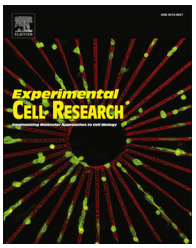

Research Article

Synergistic effect of hydrogen peroxide on polyploidization during the megakaryocytic differentiation of K562 leukemia cells by PMA

Yoshihiro Ojima[a,*], *Mark Thompson Duncan*[b], *Retno Wahyu Nurhayati*[a], *Masahito Taya*[a], *William Martin Miller*[b,c]

[a]*Division of Chemical Engineering, Department of Materials Engineering Science, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama-cho, Toyonaka, Osaka 560-8531, Japan*

[b]*Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA*

[c]*Robert H. Lurie Comprehensive Cancer Center of Northwestern University, 303 E. Superior Street, Chicago, IL 60611, USA*

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ABSTRACT

The human myelogenous cell line, K562 has been extensively used as a model for the study of megakaryocytic (MK) differentiation, which could be achieved by exposure to phorbol 12-myristate 13-acetate (PMA). In this study, real-time PCR analysis revealed that the expression of catalase (*cat*) was significantly repressed during MK differentiation of K562 cells induced by PMA. In addition, PMA increased the intracellular reactive oxygen species (ROS) concentration, suggesting that ROS was a key factor for PMA-induced differentiation. PMA-differentiated K562 cells were exposed to hydrogen peroxide (H_2O_2) to clarify the function of ROS during MK differentiation. Interestingly, the percentage of high-ploidy (DNA content >4N) cells with H_2O_2 was 34.8±2.3% at day 9, and was 70% larger than that without H_2O_2 (21.5±0.8%). Further, H_2O_2 addition during the first 3 days of PMA-induced MK differentiation had the greatest effect on polyploidization. In an effort to elucidate the mechanisms of enhanced polyploidization by H_2O_2, the BrdU assay clearly indicated that H_2O_2 suppressed the division of 4N cells into 2N cells, followed by the increased polyploidization of K562 cells. These findings suggest that the enhancement in polyploidization mediated by H_2O_2 is due to synergistic inhibition of cytokinesis with PMA. Although H_2O_2 did not increase ploidy during the MK differentiation of primary cells, we clearly observed that *cat* expression was repressed in both immature and mature primary MK cells, and that treatment with the antioxidant *N*-acetylcysteine effectively blocked and/or delayed the polyploidization of immature MK cells. Together, these findings suggest that MK cells are more sensitive to ROS levels during earlier stages of maturation.

Introduction

The K562 cell line was derived from a patient with chronic myeloid leukemia (CML) in blast crisis [1]. K562 cells behave as multipotent precursors with marked phenotypic plasticity. Among human myelogenous cell lines, K562 cells have been extensively used as a model for the study of MK differentiation, which could be achieved by exposure to phorbol 12-myristate 13-acetate (PMA) [2,3].

*Corresponding author. Fax: +81 6 6850 6254.
E-mail address: ojima@cheng.es.osaka-u.ac.jp (Y. Ojima).

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MK differentiation of K562 cells by PMA partially mimics the physiologic process that takes place in the bone marrow in response to a variety of stimuli [4]. This differentiation process is characterized by changes in cell morphology and adhesive properties, expression of markers associated with megakaryocytes, and the development of polyploid cells via endomitosis – a modified cell cycle in which several rounds of DNA replication occur without cytokinesis [5–8]. The increase in DNA content is associated with the development of multilobated nuclei and increases in cytoplasmic volume and cell surface area. This characteristic is crucial in the case of primary MK cells and is followed by the extension of proplatelets from which platelets are released [9,10]. Importantly, it has been shown that larger, higher-ploidy MK cells have greater platelet-forming potential [11,12].

It has been established that reactive oxygen species (ROS) may play a physiologically important role in cell growth and probably differentiation [13]. For instance, there is increasing evidence that the oxygen tension in the microenvironment affects MK differentiation, maturation, polyploidization, and proplatelet fragmentation through intracellular ROS generation [14–16]. As for K562 cells, Chénais et al. previously demonstrated the involvement of oxidative stress in erythroid differentiation of K562 cells during exposure to butyric acid (BA) or several anthracycline antitumor drugs such as aclarubicin (ACLA) [17]. Their study showed that the generation of an oxidative stress with treatment of BA and ACLA was due to the repression of antioxidant enzymes, such as glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT), as the first event. However, they did not examine the PMA-induced MK differentiation of K562 cells. Meanwhile, Sardina et al. reported the requirement of ROS generated by NADPH oxidase for MK differentiation in K562 and HEL cells [18]. Although they also briefly noted the positive effect of exogenous H_2O_2 on PMA-induced differentiation, that study did not discuss the effect of exogenous H_2O_2 with experimental data. Therefore, while an importance of oxidative stress has been suggested in the MK differentiation of both primary and K562 cells, the behavior of antioxidant enzymes during PMA-induced differentiation of K562 cells and the effect of exogenous ROS on MK differentiation remain largely unknown.

In the present study, the response of antioxidant enzymes and intracellular ROS content of K562 cells were examined in association with PMA addition. Furthermore, addition of exogenous H_2O_2 was examined to explore the direct relationship between oxidative stress and PMA-mediated differentiation. Finally, based on the profound effects of H_2O_2 on the polyploidization of K562 cells, we further examined the expression of antioxidant enzymes during MK differentiation of $CD34^+$ cells from mobilized peripheral blood, as well as the effect of H_2O_2 and antioxidants treatments on primary MK cell polyploidization in culture.

Materials and methods

Human megakaryoblastic cell line culture

The K562 leukemia cell line was cultured in Iscove's modified Dulbecco's medium (IMDM; HyClone Laboratories Inc., South Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) at 37 °C in a fully humidified incubator under an atmosphere of 5% CO_2 and 95% air. Cells were treated with 10 ng/ml PMA (EMD Millipore, Billerica, MA, USA) to induce MK differentiation. On day 0 for each experiment, cells were seeded at 70,000 cells/ml. Beginning at various time points (days 0, 3, 6, and 9), all medium changes were performed using fresh medium containing 10 ng/ml PMA and either 0 or 60 μmol/l H_2O_2. The total-cell concentration was determined using a Multisizer 3 (Beckman Colter, Fullerton, CA, USA) by counting the nuclei released after incubating cell samples with 3% cetrimide (Sigma-Aldrich, St Louis, MO, USA).

Human primary MK culture

$CD34^+$ cells selected from mobilized peripheral blood (Fred Hutchinson Cancer Research Center) were thawed and cultured in serum-free media as previously described [19], with minor modifications. Briefly, cells were seeded in IMDM with Glutamax-I (Gibco, Life Technologies, Carlsbad, CA, USA) at pH 7.3 supplemented with 20% BIT (Stem Cell Technologies, Vancouver, BC, Canada), 1 μg/ml low density lipoproteins (Calbiochem, San Diego, CA, USA), 100 ng/ml thrombopoeitin (TPO), 100 ng/ml stem cell factor (SCF), 2.5 ng/ml interleukin (IL)-3, 10 ng/ml IL-6 and 10 ng/ml IL-11 (R&D Systems, Minneapolis, MN, USA), in an incubator maintained under an atmosphere of 5% CO_2 and 5% O_2 with the balance N_2 during days 0–5 of culture. At day 5, the O_2 concentration was increased to 20% and the medium was exchanged with a new combination of cytokines: 100 ng/ml TPO, 100 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-9, and 10 ng/ml IL-11. At day 7 or 8, mature ($CD61^+CD42^+$) and immature MK cells ($CD61^+CD42^-$) were sequentially selected using immuno-labeled magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). First, cells were labeled with CD42-FITC (CD42b, Becton Dickinson (BD), San Jose, CA, USA) and subjected to a selection with anti-FITC beads to separate out mature MK cells. Next, the $CD42^-$ fraction underwent a second magnetic selection using beads labeled with antibody against CD61. The purities of the selected fractions were confirmed using flow cytometry analysis (see the next section). Note that since CD61 and CD41 form the GPIIb-IIIa complex found on MK cells and platelets, we used an antibody against CD41a (BD) that recognizes this complex to confirm the purity of the isolated fractions. Post-selection, immature and mature MK cells were returned to culture with TPO and SCF (100 ng/ml each) and treated with various doses of H_2O_2 or *N*-acetylcysteine (NAC; see Results and discussion). Ploidy was analyzed after 1, 2, and 3 days of treatment by staining with propidium iodide or Hoechst 33342 (see the next section).

Flow cytometric detection of surface antigens, ploidy, intracellular ROS content, and DNA synthesis

For viability, apoptosis, CD41 and CD42 expression, and ploidy, cells were prepared for flow cytometry and analyzed as described [19–21]. For analysis of primary MK cell ploidy by Hoechst staining, cells were labeled with 10 μg/mL Hoechst 33342 (Life Technologies, Carlsbad, CA, USA) for 2 h, washed, labeled with CD41 and CD42 antibodies on ice for 1 h, washed, and analyzed on an LSRFortessa (BD) flow cytometer. For intracellular detection of ROS content, K562 cells were cultured with IMDM plus 10% FBS for 1 day. The