Published in final edited form as:

Exp Cell Res. 2013 August 15; 319(14): 2205–2215. doi:10.1016/j.yexcr.2013.06.002.

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

Yoshihiro Ojima^{1,*}, Mark Thompson Duncan², Retno Wahyu Nurhayati¹, Masahito Taya¹, and William Martin Miller^{2,3}

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

²Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA

³Robert H. Lurie Comprehensive Cancer Center of Northwestern University, 303 E. Superior Street, Chicago, IL 60611, USA

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 (H₂O₂) to clarify the function of ROS during MK differentiation. Interestingly, the percentage of high-ploidy (DNA content >4N) cells with H₂O₂ was 34.8±2.3% at day 9, and was 70% larger than that without H₂O₂ (21.5±0.8%). Further, H₂O₂ 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 H₂O₂, the BrdU assay clearly indicated that H₂O₂ 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 H₂O₂ is due to synergistic inhibition of cytokinesis with PMA. Although H₂O₂ 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.

© 2013 Elsevier Inc. All rights reserved.

*Corresponding Author: (Tel; +81-6-6850-6253, Fax; +81-6-6850-6254, ojima@cheng.es.osaka-u.ac.jp).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

K562 cell; megakaryocytic differentiation; polyploidization; hydrogen peroxide; reactive oxygen species; catalase down-regulation

1. 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]

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 MKs 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 H₂O₂ on PMA-induced differentiation, that study did not discuss the effect of exogenous H₂O₂ 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 H₂O₂ was examined to explore the direct relationship between oxidative stress and PMA-mediated differentiation. Finally, based on the profound effects of H₂O₂ 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 effects of H₂O₂ and antioxidants treatments on primary MK cell polyploidization in culture.

2. Materials and methods

2.1 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% CO₂ 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 H₂O₂. The total-cell concentration was determined using a Multisizer 3 (Beckman Coulter, Fullerton, CA, USA) by counting the nuclei released after incubating cell samples with 3% cetrimide (Sigma-Aldrich, St Louis, MO, USA).

2.2 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 thrombopoietin (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% CO₂ and 5% O₂ with the balance N₂ during days 0–5 of culture. At day 5, the O₂ 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 Section 2.3). 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 H₂O₂ 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 Section 2.3).

2.3 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. For intracellular detection of ROS content, K562 cells were cultured with IMDM plus 10% FBS for 1 day. The media was carefully removed and cells were incubated with pre-warmed phosphate-buffered saline (PBS) containing 10 µmol/l DCF-DA (Molecular Probe Eugene, OR, USA) for 30 min at 37 °C in the dark. The treated cells were scraped with cold PBS and then analyzed on an LSRII (BD) or Attune Acoustic (Life Technologies, Tokyo, Japan) flow cytometer. For DNA synthesis, K562 cells were

harvested after incubation with 10 μ mol/l of bromo-deoxy-uridine (BrdU) for 12 h by using the FITC BrdU Flow Kit (559619, BD). Cells were then fixed and permeabilized per the manufacturer's protocol, treated with 10 mg/ml RNase and stained with 7-AAD, and then analyzed by flow cytometry as described [22].

2.4 Real-time PCR and enzyme activity assay

Total RNA was extracted from the cells using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. Reverse transcription was carried out using the PrimeScriptTM RT reagent kit (Takara Bio Inc., Shiga, Japan) or Superscript III RT (Life Technologies) by using random hexamer primers. Gene expression was examined by means of quantitative real-time PCR with a Chromo4TM detector and furnished program (Bio-Rad Laboratories Inc., Hercules, CA). Glyceraldehyde phosphate dehydrogenase (GAPDH) was chosen as a reference gene for normalization of cDNA loading in each reaction. Specific primers were designed using Primer3 software (available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as shown in supplementary Table S1. PCR reactions were performed using the primers and SYBR Premix Ex TaqTM (Takara Bio Inc.) or Power SYBR Green (Applied Biosystems, Life Technologies) under the conditions of 10 s at 95°C, followed by 40 repeats of 5 s at 95°C and 20 s at 60°C. The cycle threshold value (Ct) for each gene was determined as the cycle time when the fluorescence of a given sample became distinct from the basal signal. The Ct value of GAPDH was subtracted from that of the target gene to give the 3Ct value, and the expression level was then calculated from 2^{-3Ct} .

The catalase activity was measured by the conventional method according to the literature [23, 24]. Briefly, the crude protein from whole cell lysates was extracted by treatment with PBS including 0.1% Triton X-100 for 10 min at room temperature. Protein concentration in the extract was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.). One unit of activity was defined as the amount of enzyme converting 1 mol H₂O₂ to H₂O per min at 25°C.

3. Results and discussion

3.1 Gene expression of antioxidant enzymes and intracellular ROS content in PMA-induced K562 cells

Figure 1 shows mRNA expression of selected genes in PMA-induced K562 cells at 24 h. First, expression of catalase (*cat*) was significantly down-regulated by 70% in the presence of PMA compared to that with no treatment. Addition of H₂O₂ did not restore the level of expression. On the other hand, expression of superoxide dismutase (*sod1*) and glutathione peroxidase 1 (*gpx1*) was slightly repressed by PMA or H₂O₂ addition ($p < 0.05$), and adding both PMA and H₂O₂ did not significantly change the expression levels. Thus, among typical antioxidant enzymes, only *cat* was significantly repressed by PMA addition. The repression of catalase by PMA was also confirmed by measuring catalase activity. We found that catalase activities in the whole-cell lysates cultured with or without PMA addition were 15.7 ± 1.5 and 25.3 ± 1.5 units/mg, respectively, indicating that PMA addition significantly decreased the catalase activity of K562 cells. Furthermore, it should be noted that globin transcription factor 1 (*gata1*), a major transcription factor for MK maturation [25], was induced by PMA with a further synergistic increase when H₂O₂ was also added. These results suggest that catalase inhibition and the subsequent increase of intracellular H₂O₂ may be key factors for MK differentiation of K562 cells.

As the following step, the intracellular ROS content of K562 cells was compared among four conditions (no treatment, PMA, H₂O₂, and PMA plus H₂O₂) by using the DCF-DA probe (Fig. 2). Strikingly, the presence of PMA led to about 5-fold increase of intracellular

ROS and the addition of H₂O₂ enhanced the ROS levels by a further 75%. Meanwhile, the addition of H₂O₂ alone did not significantly increase the intracellular ROS, suggesting that exogenous H₂O₂ was consumed by the inherent catalase of K562 cells. These results suggest that down-regulation of the *cat* gene by PMA-differentiation decreased the ability to degrade H₂O₂ and contributed to increased H₂O₂ accumulation in the cells. Therefore, based on these findings, it can be speculated that H₂O₂ has an important role during the polyploidization of PMA-differentiated K562 cells.

3.2 Expansion and polyploidization of PMA-induced K562 cells in the presence of H₂O₂

Figure 3 shows the time courses of total-cell concentration, viability, and the percentage of apoptotic cells during PMA-induced MK differentiation of K562 cells with or without H₂O₂. In the absence of H₂O₂, the total-cell concentration gradually increased to twice the initial concentration at day 11. Meanwhile, in the presence of H₂O₂, the cell concentration reached a maximum value at day 3 and gradually decreased thereafter. The viability at day 1 without H₂O₂ was 91.7%. The viability decreased sharply to 59.8% at day 3 and then recovered to 78.6% by day 7. The viability with H₂O₂ also decreased from 94.7% to 61.1% at day 3, but then remained at about 60% without recovery throughout the culture period. The lower viability with H₂O₂ contributed to the lower total-cell concentration at later days. Meanwhile, the percentage of apoptotic viable cells remained at a low level (<20%) throughout the culture period regardless of H₂O₂ addition. This result is consistent with a previous report that H₂O₂ addition below 200 µmol/l did not induce apoptosis in undifferentiated K562 cells [26].

The ploidy time course was evaluated using flow cytometry. Figure 4A shows typical DNA histograms of PMA-induced K562 cells at days 1 and 9 with or without H₂O₂. The gate shows high-ploidy cells with DNA content > 4N. Interestingly, the percentage of high-ploidy cells with H₂O₂ reached 34.8±2.3% at day 9, and was 1.7 times larger than that without H₂O₂ (21.5±0.8%) (Fig. 4B). Mean ploidy values at day 9 also showed a significant difference (4.51±0.03 without H₂O₂ vs. 5.62±0.16 with H₂O₂). The percentage of high-ploidy cells increased at a similar rate with or without H₂O₂ until day 3 (Fig. 4B). In the absence of H₂O₂, the percentage of high-ploidy cells increased much more gradually after day 3. In contrast, the initial rate of increase was maintained until day 9 in the presence of H₂O₂. While no significant difference was confirmed by day 3, polyploidization was strongly promoted by H₂O₂ from day 5 to day 9 and exhibited a higher level compared to that without H₂O₂.

We further examined the effect of catalase inhibitor, 3-amino-1,2,4-triazole [27], on K562 cell polyploidization. The inhibitor up to 30 mmol/l did not increase the intracellular ROS level at day 1 or the percentage of high-ploidy cells at day 9 (data not shown), suggesting that suppression of *cat* by PMA addition is strong enough to knock out the catalase activity in K562 cells. We conclude that exogenous H₂O₂ could not be digested by catalase in PMA-induced cells and therefore accumulated in the cells, leading to the enhancement of polyploidization.

In contrast to polyploidization and expansion, the expression level of the MK marker CD41 was not significantly affected by H₂O₂ (Fig. 4C). The mean fluorescence intensity of CD41 increased at a similar rate throughout the culture period regardless of H₂O₂ addition. The intensity value of cells at day 9 with and without H₂O₂ was (21.7±1.7)×10³ and (19.7±1.0)×10³, respectively. This indicates that H₂O₂ does not affect the MK commitment of the K562 cell line. Although the relationship between MK differentiation and endomitosis is complex, it is well-documented that polyploidization can be altered without affecting MK commitment [21, 28, 29].

3.3 Important phase of H₂O₂ addition for increasing polyploidization of K562 cells

To elucidate the important phase of H₂O₂ addition for enhanced polyploidization, we focused on the details of the ploidy time profiles. Although the percentage of high-ploidy cells on day 1 was not significantly different regardless of H₂O₂ addition (Fig. 4B), the detailed classification of lower ploidy cells revealed that H₂O₂ addition greatly decreased the percentage of 2N cells and increased that of 4N cells at day 1 (Fig. 5). In the presence of H₂O₂, the percentage of 2N cells remained about 10% lower than for the control condition throughout the culture period. In contrast, after 5 days, the percentage of 4N cells was essentially the same regardless of H₂O₂ addition. These results suggest that the initial phase is the most important for promoted polyploidization of K562 cells by H₂O₂ administration.

On the basis of these results, we further examined the effect of the timing of H₂O₂ addition on the percentage of high-ploidy cells. In this experiment, H₂O₂ was added in the culture at day 0 and/or day 3 and/or day 6 instead of successive addition. Table 1 summarizes the percentage of high-ploidy cells at day 9 with different timing of H₂O₂ addition. Compared with no addition (condition 1), polyploidization was significantly promoted under all conditions (2, 3, 4, and 5) for which H₂O₂ was added on day 0. The ploidy for H₂O₂ addition on day 0 and again at either day 3 or day 6 (conditions 3 and 4) was almost the same as that with successive addition (condition 2). On the other hand, the percentage of high-ploidy cells was not significantly increased by H₂O₂ addition at day 3 or day 6 alone (conditions 6 and 7). Furthermore, H₂O₂ addition only at day 0 (condition 5) yielded a somewhat greater percentage of high-ploidy cells than did addition at days 3 and day 6 (condition 8). These results show that H₂O₂ addition at day 0 is of great importance to enhanced polyploidization, with further enhancement by continued addition at subsequent days. Interestingly, the percentage of high-ploidy cells was found to be inversely proportional to the total-cell concentration at day 9 when H₂O₂ was added in the culture at day 0 and/or day 3 and/or day 6 (Fig. 6). This suggested the possibility that addition of H₂O₂ inhibited cell division synergistically with PMA, followed by the promotion of polyploidization depending on the degree of exposure to H₂O₂.

3.4 Mechanism of increased polyploidization of PMA-induced K562 cells with H₂O₂

The mechanism underlying the enhancement of polyploidization with H₂O₂ during the initial phase remained unclear. It may be expected that the apparent increase of high-ploidy cells occurred due to selective screening from the toxic effect of H₂O₂. However, cell viability at day 1 (~ 92%) was not different whether H₂O₂ was present or not (Fig. 3). These results indicate that the increase of 4N cells at day 1 (Fig. 5) was not due to selective death of 2N cells by the addition of H₂O₂. Meanwhile, the lower total-cell concentration for higher ploidy led us to the hypothesis that cell division was blocked by H₂O₂.

On the basis of these findings, we further examined DNA synthesis using BrdU in the absence or presence of H₂O₂ to elucidate the mechanism of polyploidization and cell division. Representative dot plots illustrating 7-AAD and BrdU-FITC double staining, as well as the regions used to discriminate between BrdU-negative (BrdU⁻) and BrdU-positive (BrdU⁺) cells and/or 2N and 4N cells, are shown in Figure 7A. The threshold value for BrdU-negative signal was determined from cells not pulsed with BrdU to include 99% of all cells. In addition, populations of 2N and 34N cells were recognized by the difference in intensity of 7-AAD. The plots shifted to a higher intensity of BrdU fluorescence in the presence of BrdU, indicating that BrdU was successfully incorporated during DNA synthesis. Therefore, four regions were defined as BrdU⁻ 2N cells (R1), BrdU⁺ 2N cells (R2), BrdU⁻ 4N cells (R3), and BrdU⁺ 4N cells (R4).

Without H₂O₂, 2N cells were clearly separated into populations of BrdU⁻ and BrdU⁺ cells. 4N cells did not separate clearly into two populations, but the threshold value determined above was used to distinguish between BrdU⁻ and BrdU⁺ cells. In contrast, it was surprising that a population of BrdU⁺ 2N cells was not observed in the R2 region with H₂O₂. Figure 7B rearranges the plotted data into percentages of BrdU⁻ or BrdU⁺ cell in terms of ploidy. Without H₂O₂, BrdU⁺ 4N and 2N cells were approximately 60% and 10%, respectively. On the other hand, in the presence of H₂O₂, BrdU⁺ 4N cells was approximately 70% and the population of BrdU⁺ 2N cells was less than 1%. The total percentage of BrdU⁺ cells in the whole population was approximately 70% with or without H₂O₂. From the aspect of cell cycle, these results strongly suggest that DNA was synthesized during S phase regardless of H₂O₂, and then H₂O₂ completely inhibited the cell division from BrdU⁺ 4N cells to new BrdU⁺ 2N cells at G₂/M phase. Consistent with our finding, it was reported that protein synthesis and cell cycle progression of K562 cells were inhibited by the addition of 150 μmol/l H₂O₂ in the normal culture of K562 cells without PMA [30]. This inhibition of cell division was due to the inactivation of a cell cycle regulatory protein, p34^{cdc2} kinase, causing G₂/M arrest. These results suggest that the promotion of polyploidization by H₂O₂ is due to the synergistic inhibition of cell division with PMA.

3.5 Antioxidant gene expression in immature and mature primary human MK cells

Based on the results in K562 cells, we further investigated the role of oxidative stress in the differentiation of primary human MK cells derived from mobilized peripheral blood CD34⁺ cells in culture. As a first step, we considered the expression of antioxidant enzymes during the MK differentiation process. After 7–8 days of culture, we separated MK cells by their maturity level based on expression of the antigens CD41 and CD42. While MK cells express CD41 throughout the differentiation process, only more mature MK cells express CD42, which is acquired prior to undergoing endomitosis [31]. Thus the CD41⁺CD42⁻ and CD41⁺CD42⁺ fractions isolated as shown in Figure 8 represent immature and mature MK cells, respectively. For comparison, we also isolated predominantly non-MK cells (CD41⁻CD42⁻). The expression of *cat*, *gpx1* and *sod1* genes was analyzed using mRNA extracted from cells in each fraction. As a control, we further compared the expression levels to those in cells harvested after only 3 days of culture, when >90% of cells displayed the hematopoietic stem/progenitor cell marker CD34 (Data not shown).

Figure 9 shows the gene expression results. Notably, the expression of *cat* was repressed in CD41⁺CD42⁻ and CD41⁺CD42⁺ cells compared to day 3 progenitor cells from both donors tested. This decrease was not observed in the CD41⁻CD42⁻ fraction, indicating that catalase repression is a key characteristic of both K562 and primary MK differentiation. In addition, the *cat* mRNA level was lower in CD41⁺CD42⁺ cells than in CD41⁺CD42⁻ cells, indicating that the repression of catalase was increased with MK maturation. On the other hand, *gpx1* expression was strongly up-regulated in CD41⁺CD42⁻ cells, and even more so in CD41⁺CD42⁺ cells, while *sod1* was up-regulated much less extensively. With respect to the different tendency of *gpx1* expression between K562 and primary cells, Hussain et al. reported that expression of *gpx1* was dependent on p53 activity [32]. Therefore, in the case of primary cells, it is likely that increasing repression of *cat* leads to accumulated ROS which then triggers expression of *gpx1* through induced p53 activity. However, this effect would not be expected in K562 cells despite PMA-induced ROS accumulation, because K562 cells are p53-null [33]. Thus, these results suggest that *cat* repression may be a mechanism by which MK cells regulate ROS levels during differentiation.

3.6 Effects of oxidative stress on the polyploidization of immature and mature primary human MK cells

Based on the finding that H₂O₂ synergistically promoted the polyploidization of K562 cells, we used the immature and mature MK cell fractions shown in Figure 8 to evaluate whether modulation of oxidative stress would alter the ploidy of culture-derived primary human MK cells. Considering the mature MK cell fraction allowed us to directly examine whether modulation of oxidative stress could affect polyploidization independently of potential effects at earlier stages of commitment and differentiation. Considering the less mature CD42⁻ MK cells allowed us to assess whether similar modulation could affect progression to the endomitotic phase of differentiation. Thus, we treated cells from both fractions with varying doses of H₂O₂, and subsequently monitored polyploidization for 3 days. For comparison, we also examined the effects of the antioxidant NAC on ploidy since previous studies have demonstrated that NAC treatment inhibits human MK differentiation *in vitro*. In particular, Sardina et al. showed that 0.1 mM NAC reduced PMA-mediated ROS and differentiation of MK cell lines, and inhibited acquisition of the MK antigens CD41, CD42, and CD61 in primary MK cells derived from Tpo-stimulated CD34⁺ cells [19]. A separate study demonstrated that NAC treatment reduced the expression of platelet-related genes in culture-derived MK cells [17]. Thus, NAC appears to inhibit changes in gene expression associated with both early and late MK differentiation. However, the specific effects of NAC on the MK endomitotic cell cycle have not been previously described.

We found that NAC delayed polyploidization, but H₂O₂ did not affect MK ploidy. Treatment with 15, 60, or 120 μ M H₂O₂ did not appear to alter the polyploidization of the isolated CD41⁺CD42⁺ MK cells (Fig. 10A). However, treatment with 5 mM NAC delayed polyploidization for at least one day in both donors tested, while lower doses did not have a significant effect. In the case of the immature MK cell fraction (Fig. 10B), NAC resulted in a clear dose-dependent repression of polyploidization by the third day of treatment. In this case as well, H₂O₂ did not appear to have a significant effect on ploidy.

Since the immature MK cell fraction was affected by even the lower doses of NAC (Fig. 10B), our results suggest that progression through the earlier stages of MK maturation are more sensitive to modulation of ROS levels. It seems likely that there is a threshold level of ROS required for optimal maturation of committed MK cells, and that further increase of ROS level (e.g., by exogenous H₂O₂ addition) does not enhance or accelerate polyploidization. On the other hand, the polyploidization process itself appears to be less sensitive to ROS modulation. On this point, there is a clear difference between primary MK cells and PMA-stimulated K562 cells. However, since K562 cells inherently possess erythroid features and do not express CD41 or CD42 prior to PMA addition, they closely resemble uncommitted megakaryocyte-erythroid progenitor cells. It is possible that increasing ROS levels either prior to CD41 acquisition (i.e., identifiable commitment to the MK lineage) or soon thereafter has more potent effects on the expression of MK-specific genes and/or the cell cycle. Alternatively, we cannot rule out the possibility that the observed difference is related to K562 cells' Bcr-Abl⁺ status, which imparts a high level of constitutive tyrosine kinase activity [34] and is followed by increases of ERK1/2 and JNK activation [35, 36].

4. Conclusions

It was found that PMA-mediated MK differentiation significantly repressed the expression of *cat*, reduced *cat* activity, and led to an increase of intracellular ROS concentration. The repression of *cat* expression was also observed during the differentiation of primary human MK cells, progressed during the maturation process, and was specific for the MK lineage.

Thus, reduced *cat* levels may contribute to the generation of ROS, which are known to play a key role in promoting MK gene expression in TPO-stimulated CD34⁺ cells [18].

Furthermore, it was shown that increasing oxidative stress by exogenous H₂O₂ addition significantly promoted the polyploidization of PMA-induced K562 cells. This beneficial effect depended on addition at an early phase of the differentiation, and was due in part to synergistic inhibition of cell division with PMA. In primary MK cells, it was found that the polyploidization of mature (CD41⁺CD42⁺) MK cells was insensitive to ROS modulation by H₂O₂ or the antioxidant NAC, except at the highest NAC dose considered (5 mM). However, in less mature (CD41⁺CD42⁻) MK cells, there was a definitive and dose-dependent block and/or delay in polyploidization. Thus, the progression of committed MK cells to the endomitotic phase of differentiation likely depends on the ROS level. Taken together, these results provide additional evidence of the particular importance of ROS to MK differentiation. In future studies, it will be interesting to further investigate whether regulation of antioxidant enzymes is an important aspect of the normal MK differentiation program.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by JSPS Institutional Program 'Development of International Network for Training of Young Researchers Exploring Multidisciplinary Fields', J091113016, and U.S. National Institutes of Health (NIH) grant R01HL93083 (WMM). MD was supported in part by NIH predoctoral Biotechnology Training Grant T32GM008449. This work was also supported by a Grant-in-Aids for Scientific Researches 22360344 from the Ministry of Education, Culture, Sports, Science and Technology in Japan, and received financial support from the Multidisciplinary Research Laboratory System for Future Developments of the Graduate School of Engineering Science, Osaka University.

References

1. Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood*. 1975; 45:321–334. [PubMed: 163658]
2. Alitalo R. Induced differentiation of K562 leukemia cells: A model for studies of gene expression in early megakaryoblasts. *Leukemia Res*. 1990; 14:501–514. [PubMed: 2197510]
3. Tabilio A, Pelicci PG, Vinci G, Mannoni P, Civin CI, Vainchenker W, Testa U, Lipinski M, Rochant H, Breton-Gorius J. Myeloid and megakaryocytic properties of K-562 cell lines. *Cancer Res*. 1983; 43:4569–4574. [PubMed: 6349795]
4. Long MW, Heffner CH, Williams JL, Peters C, Prochownik EV. Regulation of megakaryocyte phenotype in human erythroleukemia cells. *J Clin Invest*. 1990; 85:1072–1084. [PubMed: 2318965]
5. Butler TM, Ziemiecki A, Friis RR. Megakaryocytic differentiation of K562 cells is associated with changes in the cytoskeletal organization and the pattern of chromatographically distinct forms of phosphotyrosyl-specific protein phosphatases. *Cancer Res*. 1990; 50:6323–6329. [PubMed: 2169344]
6. Colamonici OR, Trepel JB, Vidal CA, Neckers LM. Phorbol ester induces c-sis gene transcription in stem cell line K-562. *Mol Cell Biol*. 1986; 6:1847–1850. [PubMed: 3466024]
7. Tetteroo PA, Massaro F, Mulder A, Schreuder-van Gelder R, von dem Borne AEG. Megakaryoblastic differentiation of proerythroblastic K562 cell-line cells. *Leukemia Res*. 1984; 8:197–206. [PubMed: 6232432]
8. Fukuda M. Tumor-promoting phorbol diester-induced specific changes in cell surface glycoprotein profile of K562 human leukemic cells. *Cancer Res*. 1981; 41:4621–4628. [PubMed: 7306980]

9. Italiano JE Jr, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol.* 1999; 147:1299–1312. [PubMed: 10601342]
10. Patel SR, Richardson JL, Schulze H, Kahle E, Galjart N, Drabek K, Shivdasani RA, Hartwig JH, Italiano JE Jr. Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. *Blood.* 2005; 106:4076–4085. [PubMed: 16118321]
11. Trowbridge EA, Martin JF, Slater DN, Kishk YT, Warren CW, Harley PJ, Woodcock B. The origin of platelet count and volume. *Clin Phys Physiol Meas.* 1984; 5:145–170. [PubMed: 6488722]
12. Mattia G, Vulcano F, Milazzo L, Barca A, Macioce G, Giampaolo A, Hassan HJ. Different ploidy levels of megakaryocytes generated from peripheral or cord blood CD34⁺ cells are correlated with different levels of platelet release. *Blood.* 2002; 99:888–897. [PubMed: 11806991]
13. Zs-Nagy I. A proposal for reconsideration of the role of oxygen free radicals in cell differentiation and aging. *Ann NY Acad Sci.* 1992; 673:142–150. [PubMed: 1485711]
14. Eliades A, Matsuura S, Ravid K. Oxidase and reactive oxygen species during hematopoiesis: a focus on megakaryocytes. *J Cell Physiol.* 2012; 227:3355–3362. [PubMed: 22331622]
15. Mostafa SS, Papoutsakis ET, Miller WM. Oxygen tension modulates the expression of cytokine receptors, transcription factors, and lineage-specific markers in cultured human megakaryocytes. *Exp Hematol.* 2001; 29:873–883. [PubMed: 11438210]
16. Motohashi H, Kimura M, Fujita R, Inoue A, Pan X, Takayama M, Katsuoka F, Aburatani H, Bresnick EH, Yamamoto M. NF-E2 domination over Nrf2 promotes ROS accumulation and megakaryocytic maturation. *Blood.* 2010; 115:677–686. [PubMed: 19901266]
17. Chénais B, Andriollo M, Guiraud P, Belhoussine R, Jeannesson P. Oxidative stress involvement in chemically induced differentiation of K562 cells. *Free Radic Biol Med.* 2000; 28:18–27. [PubMed: 10656287]
18. Sardina JL, López-Ruano G, Sánchez-Abarca LI, Pérez-Simón JA, Gaztelumendi A, Trigueros C, Llanillo M, Sánchez-Yagüe J, Hernández-Hernández A. p22phox-dependent NADPH oxidase activity is required for megakaryocytic differentiation. *Cell Death Diff.* 2010; 17:1842–1854.
19. Panuganti S, Schlinker AC, Lindholm PF, Papoutsakis ET, Miller WM. Three-stage *ex vivo* expansion of high-ploidy megakaryocytic cells: toward large-scale platelet production. *Tissue eng Part A.* 2013; 19:998–1014. [PubMed: 23190353]
20. Giammona LM, Fuhrken PG, Paoutsakis ET, Miller WM. Nicotinamide (vitamin B3) increases the polyploidisation and proplatelet formation of cultured primary human megakaryocytes. *Br J Haematol.* 2006; 135:554–566. [PubMed: 17054670]
21. Giammona LM, Panuganti S, Kemper JM, Apostolidis PA, Lindsey S, Paoutsakis ET, Miller WM. Mechanistic studies on the effects of nicotinamide on megakaryocytic polyploidization and the roles of NAD⁺ levels and SIRT inhibition. *Exp Hematol.* 2009; 37:1341–1352.
22. Apostolidis PA, Woulfe DS, Chavez M, Miller WM, Papoutsakis ET. Role of tumor suppressor p53 in megakaryopoiesis and platelet function. *Exp Hematol.* 2012; 40:131–142. [PubMed: 22024107]
23. Aebi H. Catalase *in vitro*. *Meth Enzymol.* 1984; 105:121–126. [PubMed: 6727660]
24. Holmes RS, Masters CJ. Epigenetic interconversions of the multiple forms of mouse liver catalase. *FEBS Lett.* 1970; 11:45–48. [PubMed: 11945446]
25. Vyas BP, Adult K, Jackson CW, Orkin SH, Shivdasani RA. Consequences of GATA-1 deficiency in megakaryocytes and platelets. *Blood.* 1999; 93:2867–2875. [PubMed: 10216081]
26. Wagner BA, Buettner GR, Oberley LW, Darby CJ, Burns CP. Myeloperoxidase is involved in H₂O₂-induced apoptosis of HL-60 human leukemia cells. *J Biol Chem.* 2000; 275:22461–22469. [PubMed: 10801811]
27. Price M, Terlecky SR, Kessel D. A role for hydrogen peroxide in the pro-apoptotic effects of photodynamic therapy. *Photochem Photobiol.* 2009; 85:1491–1496. [PubMed: 19659920]
28. Huang Z, Richmond TD, Muntean AG, Barber DL, Weiss MJ, Crispino JD. STAT1 promotes megakaryopoiesis downstream of GATA-1 in mice. *J Clin Invest.* 2007; 117:3890–3899. [PubMed: 18060035]

29. Jung YJ, Chae HC, Seoh JY, Ryu KH, Park HK, Kim YJ, Woo SY. Pim-1 induced polyploidy but did not affect megakaryocytic differentiation of K562 cells and CD34⁺ cells from cord blood. *Eur J Haematol.* 2006; 78:131–138. [PubMed: 17313559]
30. Kulkarni AP, Mittal SPK, Devasagayam TPA, Pal JK. Oxidative stress perturbs cell proliferation in human K562 cells by modulating protein synthesis and cell cycle. *Free Rad Res.* 2009; 43:1090–1100.
31. Debili N, Issaad C, Masse J, Guichard J, Katz A, Breton-Gorius J, Vainchenker W. Expression of CD34 and platelet glycoproteins during human megakaryocytic differentiation. *Blood.* 1992; 80:3022–3035. [PubMed: 1281685]
32. Hussain SP, Amstad P, He P, Robles A, Lupold S, Kaneko I, Ichimiya M, Sengupta S, Mechanic L, Okamura S, Hofseth LJ, Moake M, Nagashima M, Forrester KS, Harris CC. p53-Induced up-regulation of MnSOD and GPx but not catalase increases oxidative stress and apoptosis. *Cancer Res.* 2004; 64:2350–2356. [PubMed: 15059885]
33. Durland-Busbice S, Reisman D. Lack of p53 expression in human myeloid leukemias is not due to mutations in transcriptional regulatory regions of the gene. *Leukemia.* 2002; 16:2165–2167. [PubMed: 12357376]
34. Roger R, Issaad C, Pallardy M, Leglise MC, Turhan AG, Bertoglio J, Breard J. BCR-ABL does not prevent apoptotic death induced by human natural killer or lymphokine-activated killer cells. *Blood.* 1996; 87:1113–1122. [PubMed: 8562937]
35. Jacquel A, Herrant M, Defamie V, Belhacene N, Colosetti P, Marchetti S, Legros L, Deckert M, Mari B, Cassuto JP, Hofman P, Auberger P. 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; 25:781–794. [PubMed: 16186797]
36. Jacquel A, Herrant M, Legros L, Belhacene N, Luciano F, Pages G, Hofman P, Auberger P. Imatinib induces mitochondria-dependent apoptosis of the Bcr-Abl positive K562 cell line and its differentiation towards the erythroid lineage. *FASEB J.* 2003; 17:2160–2162. [PubMed: 14597677]

Highlights

1. Catalase is repressed during PMA-induced K562 cell megakaryocytic differentiation (82 characters including space)
2. H₂O₂ addition enhances PMA-induced K562 cell polyploidization (62 characters)
3. H₂O₂ synergistically inhibits the cytokinesis of K562 cells with PMA (69 characters)
4. Catalase is down-regulated in both immature and mature human primary CD41⁺ cells (81 characters)
5. *N*-acetylcysteine effectively delays the polyploidization of immature CD41⁺ cells (82 characters)

Fig. 1.

Catalase expression is repressed by PMA. mRNA expression levels of selected genes at day 1 in the culture of PMA-induced or control K562 cells in the presence or absence of 60 $\mu\text{mol/l}$ H_2O_2 . The error bars represent standard deviation. Based on a paired *t*-test, values of $p < 0.05$ (*) are indicated. Abbreviations: *cat* catalase, *sod1* superoxide dismutase 1, *gpx1* glutathione peroxidase 1, *gata1* globin transcription factor 1.

Fig. 2.

Intracellular ROS content of K562 cells is increased by PMA and further increased by H₂O₂. ROS content was measured using oxidized DCF-DA at day 1 in the culture of PMA-induced or control K562 cells in the presence or absence of 60 μ mol/l H₂O₂. The error bars represent standard deviation. Based on a paired *t*-test, values of *p* < 0.05 (*) are indicated.

Fig. 3. H₂O₂ greatly decreases expansion of PMA-treated K562 cells. Time courses of total cell concentration, percentage of viable cells, and percentage of apoptotic cells during the PMA-induced differentiation of K562 cells in the presence or absence of 60 μ mol/l H₂O₂. The error bars represent standard deviation. Based on a paired *t*-test, values of *p* < 0.05 (*) are indicated for the various time points in comparison to the PMA-only culture.

Fig. 4. H₂O₂ increases ploidy of PMA-induced K562 cells. (A) DNA histograms of K562 cells in the culture with PMA at day 1 and day 9 with or without 60 μ mol/l H₂O₂. The gate shows high-ploidy K562 cells with DNA content >4N. (B) Time courses of the percentage of high-ploidy K562 cells throughout the culture period with or without H₂O₂. (C) Time courses of the mean fluorescence intensity of CD41 of K562 cells in the absence or presence of H₂O₂. The error bars represent standard deviation. Based on a paired *t*-test, values of *p* < 0.05 (*) are indicated for the various time points in comparison to the PMA-only culture.

Fig. 5.

H₂O₂ decreases the percentage of 2N cells and transiently increases the percentage of 4N cells. Time courses of the percentage of 2N and 4N cells throughout the culture period with or without 60 μ mol/l H₂O₂. The error bars represent standard deviation. Based on a paired *t*-test, values of *p* < 0.05 (*) are indicated for the various time points in comparison to the PMA-only culture.

Fig. 6.

The percentage of high-ploidy cells is inversely proportional to the total-cell concentration. Correlation between total-cell concentration and percentage of high-ploidy cells at day 9 for the experiments shown in Table 1, in which $60 \mu\text{mol/l}$ H_2O_2 was added in the culture at day 0 and/or day 3 and/or day 6. The solid line represents the linear regression curve. In this experiment, total-cell concentrations were determined using a hemocytometer.

Fig. 7. H₂O₂ blocks the division of 4N K562 cells after PMA addition. (A) Representative dot plot showing BrdU-FITC vs. 7-AAD fluorescence intensities at day 1 with or without 60 μ mol/l H₂O₂ after incubation with BrdU for 12 h. The indicated regions were used to discriminate the BrdU-negative (BrdU⁻) 2N cells (R1), BrdU-positive (BrdU⁺) 2N cells (R2), BrdU⁻ 4N cells (R3), and BrdU⁺ 4N cells (R4). (B) Percentages of BrdU⁺ and BrdU⁻ cell populations in terms of ploidy. The data were from three independent cultures. The error bars represent standard deviation.

Donor 1

Donor 2

Fig. 8.

Primary human MK cells derived from mobilized peripheral blood CD34⁺ cells in culture were sorted to isolate immature and mature populations. Immature CD41⁺CD42⁻, and mature CD41⁺CD42⁺ MK cells were selected by immuno-labeled magnetic beads. Purity of the selected populations was verified by flow cytometry. This analysis was performed for two independent donors (donor 1 selected at day 7, donor 2 selected at day 8).

Fig. 9.

Catalase expression is repressed by MK maturation. A fraction of each selected population was used for RNA collection and subsequent analysis by real-time PCR. Expression of the antioxidant genes in the 3 selected fractions (CD41⁺CD42⁻, CD41⁺CD42⁺, CD41⁻CD42⁻) was compared to the expression in CD34⁺ progenitor cells after 3 days of culture. This analysis was performed for two independent donors (donor 1 selected at day 7, donor 2 selected at day 8).

Fig. 10.

The antioxidant NAC effectively delayed the polyploidization of immature human MK cells. Culture-derived (A) CD41⁺CD42⁺ and (B) CD41⁺CD42⁻ MK cells were selected (see Figure 8) and returned to culture with various doses of NAC or H₂O₂. Subsequent polyploidization of (A) the mature and (B) immature fractions were monitored for 3 days. This analysis was performed for two independent donors (donor 1 selected at day 7, donor 2 selected at day 8).

Table 1

The effect of timing of H₂O₂ addition on the percentage of high-ploidy cells at day 9.

Condition	H ₂ O ₂ added(60 μ mol/l)	Percentage of high-ploidy cells
1	No addition	24.0 $\pm$ 3.0
2	Day 0, 3, 6	40.5 $\pm$ 2.8*
3	Day 0, 3	40.0 $\pm$ 1.2*
4	Day 0, 6	38.0 $\pm$ 3.6*
5	Day 0	33.9 $\pm$ 2.0*
6	Day 3	29.3 $\pm$ 1.1
7	Day 6	26.8 $\pm$ 4.5
8	Day 3, 6	32.0 $\pm$ 2.7*

Base on a paired *t*-test, values of *p* < 0.05 (*) are indicated in comparison to the PMA-only culture (no addition).