Manipulation of Cellular Redox State was Essential for Triggering Erythroid Differentiation of K562 Cells by Mycophenolic Acid

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Introduction

In chronic myelogenous leukemia (CML), the typical chromosomal translocation of [1,2] has resulted in uncontrolled and exaggerated growth of undifferentiated cells [1]. Regarding the excessive proliferation of immature cells, most CML chemotherapeutical strategies are mainly based on induction of apoptosis among the cancerous cells. This approach is however associated with numerous toxic side effects [2]. Alternatively, directing immature cells toward differentiation pathway seems to be an effective and ideal approach for treatment of CML [3].

Erythroid differentiation is a highly complex process involving multiple regulatory elements including ROS. Reactive Oxygen Species are free radicals such as superoxide anion (O$_2^-$), hydroxyl radical (.OH) [4]. These intracellular free radicals function as double-edge swords. On one hand, they are involved in signal transduction pathways responsible for cell growth, differentiation and apoptosis [5,6]. On the other hand, the excessive amount of these oxidants is associated with the oxidation of a broad range of cellular components and disruption of various signaling pathways [7].

To remain safe from the deleterious effects of reactive oxygen species, cells utilize highly sophisticated and complex antioxidant protection systems consisting of a variety of enzymatic/non-enzymatic ROS catabolizing components including SOD, catalase, Trx and glutathione system [8,9]. Among these antioxidant elements, Trx, apart from its antioxidant role, implicates in regulation of intracellular redox-dependent processes such as transcriptional activity, cell proliferation and differentiation [10].

Another signaling element involved in modulation of intracellular redox status and implicated in erythroid differentiation process is p27$^{kip1}$, a cyclin-Dependent Kinase inhibitor (CDKI), is a negative regulator of cell cycle progression, which inhibits the kinase activity of cyclin-cdk complexes, specially cyclin E-ckd2 during erythroid differentiation of erythroblast cells [11]. Besides, it has been recently reported that apart from mitogenic stimuli, oxidative stress could also modulate p27$^{kip1}$ level [12,13].

However, there are some disputes on the role of oxidative stress on p27$^{kip1}$ regulation [13]. For instance, several reports have indicated that attenuation of the intracellular ROS content is associated with up-regulation of p27$^{kip1}$ among different cells [12,13]. In contrast, Quintos and colleagues have demonstrated that p27$^{kip1}$ level decreases in human colon carcinoma HCT 116 cells under oxidative stress condition [14].

Regarding the aberrant differentiation pathway and antioxidant system in chronic myelogenous leukemia [1,15], we aimed to examine whether the MPA-induced erythroid differentiation of K562 cells is associated with modulation of the intracellular redox status. Interestingly, our results demonstrated that MPA-dependent erythroid differentiation of K562 cells was associated with augmentation of the intracellular oxidant content, which apparently constitutes the mandatory condition for erythroid differentiation of the exposed cells.
The effect of MPA on intracellular ROS level. K562 cells treated with MPA (0.5 μM) for different times and the intracellular ROS level was measured using DCFH-DA. *Significantly different from control cells (P<0.01).

**Methods**

**Reagents**

The cell culture medium (RPMI-1640), fetal bovine serum (FBS) and penicillin streptomycin were purchased from Gibco BRL (Life Technology, Paisley, Scotland). Cell line was obtained from Pasteur Institute of Iran (Tehran, Iran). GPA antibody was from IQ Products (Groningen, Netherlands). Anti-Trx and anti-p27kip1 antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-Tubulin antibody was from BioSource (Nivelles, Belgium). N-acetyl-L-cysteine (NAC) was purchased from Merk (Frankfurt, Germany). 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probe (Eugene, Oregon, USA). Nitro blue tetrazolium (NBT), 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB), nicotinamide adenine dinucleotide reduced (NADH), phenazine methosulphate (PMS), H2O2, and nicotinamide adenine dinucleotide phosphate reduced (NADPH) were obtained from Merck Co. (Germany). Mycophenolic acid, EDTA and glutathione reductase (GR) were obtained from Sigma–Aldrich Chemical Co. Ltd. (England).

**Cell culture**

Human leukemia K562 cell line was grown in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 U/mL). The cells were incubated under 5% CO2-humidified atmosphere at 37°C. Cell numbers and viabilities were assessed using a hemocytometer and the abilities of the cells to exclude trypan blue, respectively.

**Evaluation of hemoglobin in differentiated K562 cells**

Hemoglobin-producing cells were scored by benzidine staining. K562 cells (4 × 10^4 cells/mL) were seeded in a 24-well plate and incubated in RPMI-1640 with 10% FBS. The cells were treated with MPA (0.5 μM in 0.1 mM NaOH) for 3 days. The MPA-treated and untreated cells were collected and the pellets were dissolved in the staining reagent consisted of 100 μL NaCl (0.9% w/v), 50 μL benzidine (0.2% benzidine in 0.5 M acetic acid) and 3% of H2O2 solution (30%). The mixture was incubated for 45 min in the dark at room temperature. Blue cells were counted as hemoglobin-containing cells.

**Flow cytometry analysis of the cell surface marker**

Differentiation of the drug-affected cells to erythrocytes was evaluated based on the expression of glycophorin A (GPA), as a marker of erythroid differentiation. K562 cells (5 × 10^4 cells) were treated with MPA for 72 h, then collected, washed twice with PBS and resuspended in PBS containing 1% FBS and 0.1% sodium azide.

The mixture was incubated for 45 min in the dark at room temperature. Blue cells were counted as hemoglobin-containing cells.

**Figure 1:** The effect of MPA on intracellular ROS level. K562 cells treated with MPA (0.5 μM) for different times and the intracellular ROS level was measured using DCFH-DA. *Significantly different from control cells (P<0.01).

**Figure 2:** The effect of MPA on erythroid differentiation of K562 cells. A: Benzidine staining of MPA-treated K562 cells after 3 days. MPA-untreated control cells (a), cells treated with MPA (0.5 μM, b), cells treated with MPA (0.5 μM) and NAC (2 mM, c) for 72 h. Then, at least 200 cells were counted to determine the extent of differentiation based on benzidine staining of hemoglobin-containing cells relative to untreated control cells (a). B: A quantitative view of the extent of differentiation. After taking photos from each sample, we counted the whole cells; unstained and stained cells in each photo and the results has been shown in quantitative diagram (*P<0.01 compared to untreated cells **P<0.01 compared to MPA-treated cells). C: the effect of MPA on glycophorin A (GPA) expression in K562 cells. Untreated (control) and MPA-treated K562 cells were stained with FITC-conjugated mouse anti-human GPA mAb. A FITC-conjugated irrelevant antibody was used as the negative control for background fluorescence of untreated and/or MPA-treated K562 cells.

Then, five microliters of mouse anti-human FITC conjugated GPA monoclonal antibody was added to 100 μL of the cell suspension and incubated for 30 min at 4°C. After washing, at least 10^4 cells were analyzed by a flow cytometer (Partec PAS, Munich, Germany).

**Determination of intracellular ROS level**

The generation of reactive oxygen radicals was monitored by the cell-permeable redox-sensitive fluorophore 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA). This dye readily...
diffuses into the cells and hydrolyzed by the intracellular esterase to yield DCFH, which is trapped within the cells. It then reacts with intracellular radicals to produce DCF (dichlorofluorescein), which is a fluorophore. Treated and untreated cells were carefully counted and adjusted to a cell density of 4 x 10^5 cells/mL and also monitored for viability by trypan blue exclusion test. Then, the cells were incubated with 10 μM DCFH-DA at 37°C for 30 min and subsequently washed twice with cold phosphate-buffered saline (PBS). Each intracellular ROS level was determined using a Varian spectrophotometer (model Cary Eclipswere) with excitation and emission wavelengths of 485 and 530 nm, respectively.

**Measuring catalase activity**

Catalase (CAT) activity was determined by the method described by Aebi, which follows the disappearance of hydrogen peroxide at 240 nm for 2 min [16]. In brief, 200 μL of each cell lysate was added to a cuvette containing 1.995 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by addition of 1 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured by a spectrophotometer at 240 nm. Activity of catalase was expressed in K units per mg protein per second (K/mg/s), where K is the rate constant of the first order reaction of CAT.

**Measuring superoxide dismutase (SOD) activity**

SOD activity was measured based on the inhibition extent of amino blue tetrazolium formazan formation in the mixture of Nicotinamide Adenine Dinucleotide, Phenazine Methosulphate and Nitro Blue Tetrizolium (NADH–PMS–NBT) system, according to the method of Kakkar et al. One unit of enzyme activity was defined as the amount of enzyme, which caused 50% inhibition of NBT reduction/mg protein [17].

**Measuring glutathione peroxidase (GPX) activity**

Glutathione peroxidase activity was assayed by the method of Fridovich. In brief, 0.5 mL of cell lysate was precipitated with 1 mL of sulfosalicylic acid (4% w/v). The precipitate was removed by centrifugation. Then, 1 mL of the supernatant was mixed with 0.1 mL DTTNB (4 mg/mL) and 0.9 mL phosphate buffer (0.1 M, pH 7.4). The developed yellow colour was read at 412 nm. Reduced glutathione was expressed as μg/mg of protein.

**Determination of reduced glutathione**

GSH, as the most abundant intracellular acid-soluble thiols, was assayed by the method of Jollow et al. [19]. Briefly, 0.5 mL of cell lysate was precipitated with 1 mL of sulfoalicylic acid (4% w/v). The precipitate was removed by centrifugation. Then, 1 mL of the supernatant was mixed with 0.1 mL DTTNB (4 mg/mL) and 0.9 mL phosphate buffer (0.1 M, pH 7.4). The developed yellow colour was read at 412 nm. Reduced glutathione was expressed as μg/mg of protein.

**Immunoblotting**

After treatment of K562 cells with MPA for different time intervals, the cells were lysed using the lysis buffer containing 1% Triton X-100, 1% SDS, 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM sodium pyrophosphate, 2 mM Na_3VO_4, 1 mM NaF, 0.5% sodium deoxycholate, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/mL leupeptin, 1 μg/mL pepstatin, and 60 μg/mL aprotinin. Protein concentration of each sample was determined using Lowry's method. Equal protein quantity of each lysate (50 μg) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) and/or nitrocellulose membranes (Amersham, Bioscience, UK). The membranes were blocked in Tris-buffered saline pH 7.4 containing 0.1 % Tween-20 and 5 % non-fat milk overnight at 4°C. The blocked blots were incubated with primary antibodies for 2 h at room temperature using antibody dilutions as recommended by the manufacturer in Tris-buffered saline pH 7.4, 0.1% Tween-20, followed by 90 min incubation with anti-rabbit or -mouse horseradish peroxidase-conjugated secondary antibodies (Bio source, Belgium). Each protein band was detected by an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia, Piscataway, NJ) according to the manufacturer’s instructions. The band densities were measured using Image J software.

**Statistical analysis**

Data were expressed as percent of values of untreated control cells, and each value represents the mean ± SD (n=3). The error bars were calculated by the formula error bars= SD/√n, n= the number of repetition of experimental tests [20]. The significant differences between the means of the treated and untreated cells were calculated by unpaired Student's t test and P<0.05 was considered significant.

**Results**

**Alteration in intracellular ROS level in MPA-treated K562 cells**

In CML, it is believed that p210 bcr-abl fusion protein tyrosine kinase oncogene is responsible for uncontrolled ROS production and aberrant differentiation of the cells [1,11]. By this knowledge, we attempted to examine whether MPA (0.5 μM) would affect the intracellular redox status. The intracellular ROS contents of the MPA-treated and untreated cells were investigated in a time dependent manner using DCFH-DA staining approach (Figure 1). Despite its advantages, the DCFH-DA technique is often criticized. Photoreduction of DCF results in artificial production of a semiquinone radical that in turn can reduce oxygen to superoxide [21]. In addition, the oxidation of DCFH to the DCF can be self-catalyzed by peroxidases. As it is evident from this figure, in MPA-treated cells, the intracellular ROS level decreased by 36.5% and 49% after 3 and 8 h relative to untreated cells, respectively. However, at longer exposure times, the level of ROS gradually increased and reached to a max level after 24 h followed by a second drop to 49%, of its original level at 72 h of exposure.

**Involvement of oxidative stress in MPA-erythroid differentiation**

According to numerous reports, oxidative stress is implicated in erythroid differentiation [22]. To explore whether hemoglobinization of the treated cells depends on the intracellular ROS content, we simultaneously exposed the cells to MPA and NAC (2 mM) treatment. As shown in (Figures 2A and 2B), erythroid differentiation induced by
MPA (43%), was attenuated in the presence of NAC by 23% relative to MPA-treated cells. This confirms the involvement of oxidative stress in erythroid differentiation of K562 cells upon treatment with MPA (0.5 μM). Besides, we evaluated the effect of oxidative stress inhibition on the extent of cell surface GPA, as an erythroid differentiation marker [23]. Based on flow cytometry analyses, cell surface level of GPA also decreased by about 11% among the cells exposed to the combined MPA and NAC treatment relative to MPA-treated cells (Figure 2C).

Modulation of antioxidant enzymes activities in MPA-treated K562 cells

Variation in intracellular reactive oxygen species is frequently associated with alteration in the activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase [6]. Hence, we planned to assess the activities of these enzymes in MPA-treated K562 cells. As it is evident from (Figure 3), the SOD activity was decreased by 51% and 69% after 24 and 48 h exposure to MPA, respectively. However, its activity increased by almost 2 folds following 72 h of exposure to MPA relative to untreated control cells. The assessment of catalase activity showed that its activity was augmented by 54% after 72 h of exposure to MPA (0.5 μM) in comparison to the control cells. In addition, GPX activity was increased by almost 2 folds after 8 h of treatment with MPA (0.5 μM) and returned to the basal level following 72 h exposure to MPA (0.5 μM).

![Figure 3](image-url)  
**Figure 3:** The effect of MPA on the intracellular activities of CAT, SOD and GPX in K562 cells. Cells were treated with MPA (0.5 μM) for different times. The activities of superoxide dismutase (unit/mg protein), catalase (K/Sec/mg) and glutathione peroxidase (nmol/min/mg protein) were measured spectrophotometrically. Each value represents the mean ± SD (n=3). *Significantly different from untreated control cells (P<0.001).

Alteration in intracellular thiol-based antioxidants in MPA-treated K562 cells

GSH and Trx are the most important endogenous thiol-based non-enzymatic free radical scavengers which play essential roles in the maintenance of intracellular redox homeostasis [19,20]. According to our data, the intracellular GSH content of K562 cells increased by 54% and 66% relative to untreated cells after 3 and 8 h of exposure to MPA (0.5 μM), respectively. Then, its level returned to the basal level by 24 to 48 h. However, GSH level again increased by 21% after 72 h of treatment with MPA (0.5 μM) in comparison to the untreated control cells (Figure 4). Immunoblotting analyses of Trx expression revealed that the Trx level was increased by 22% in MPA-treated K562 cells after 72 h of exposure (Figure 5).

![Figure 4](image-url)  
**Figure 4:** Evaluation of Intracellular reduced glutathione (GSH). Cells were treated with MPA (0.5 μM) for different times. The GSH content of the untreated cells was expressed as 100%.*Significantly different from untreated control cells (P<0.01).

Modulation of p27kip1 level as an oxidative stress biomarker

P27kip1 cyclin-dependent inhibitory kinase (p27CDKI) is a negative regulator of cell cycle progression which inhibits the kinase activity of cyclin E-cdk2 and cyclin D-cdk4. It has been demonstrated that, p27kip1 is also involved in other biological functions unrelated to cell cycle such as differentiation [23]. Given that our data in this study has confirmed the involvement of oxidative stress in MPA-induced erythroid differentiation and the proven role of p27kip1 in erythropoiesis, we planned to examine the expression of p27kip1 in MPA treated cells.

As shown in (Figure 6A), p27Kip1 protein level was decreased by 41% parallel to the transient elevation in intracellular ROS content. In addition, in order to explore whether p27Kip1 expression is under the influence of oxidative stress, K562 cells were pre-treated with NAC (2 mM) as a free radical scavenger. According to data shown in (Figure 6B), attenuation of the intracellular ROS level led to an increase in the expression level of p27Kip1, implying that expression of p27Kip1 is dependent on the redox status of the cells under MPA treatment condition.

**Discussion**

It has been postulated that p210 bcr-abl fusion protein tyrosine kinase oncogene is responsible for uncontrolled ROS production and the aberrant differentiation of CML cells [23,24]. Excess accumulation of ROS causes oxidative stress, which is implicated in proliferation of CML cells. Thus, targeting ROS manipulation would constitute a strategy with high potential for fighting back leukemia.
In addition, the implication of intracellular ROS content, generated by the process has been shown [28]. Butyric acid (BA) or anthracyclines, in the irreversible process of erythroid differentiating effects such as FoxO3 [27]. Thus, it was presumed that the observed transient elevation of the intracellular ROS could be attributed to hemoglobinization of the MPA-treated cells. Application of NAC considerably suppressed the expression of hemoglobin and GPA among the treated cells, suggesting that ROS are involved in MPA-induced erythroid differentiation process. Collectively, it could be concluded that although MPA requires free radicals to trigger erythroid differentiation however, it precludes the deleterious effect of these oxidants through attenuation of the intracellular ROS content at the late stage of differentiation.

Regarding the recent evidence that ROS can function either as signaling molecules in the normal process of HSC differentiation to the erythroid cells [25], or as cellular toxicants in hematopoietic malignancy such as CML [23,24] the effect of MPA, a well-known differentiating agent [26], on the intracellular ROS level during the process of erythroid differentiation of CML cells was evaluated. Our results showed that although MPA-induced erythroid differentiation was accompanied by a final reduction of intracellular ROS level, but it provided a temporary augmentation in intracellular ROS contents followed by suppression of the intracellular ROS toward completion of erythroid differentiation. Production of ROS in normal erythroid precursor cells is believed to be coupled with hemoglobin accumulation and down-regulation of the inhibitory effect on the essential erythroid differentiation signaling factors such as FoxO3 [27]. In addition, the implication of intracellular ROS content, generated by butyric acid (BA) or anthracyclines, in the irreversible differentiation process has been shown [28]. Thus, it was presumed that the observed transient elevation of the intracellular ROS could be attributed to hemoglobinization of the MPA-treated cells. Application of NAC considerably suppressed the expression of hemoglobin and GPA among the treated cells, suggesting that ROS are involved in MPA-induced erythroid differentiation process. Collectively, it could be concluded that although MPA requires free radicals to trigger erythroid differentiation however, it precludes the deleterious effect of these oxidants through attenuation of the intracellular ROS content at the late stage of differentiation.

Cells adjust their redox state by a complex and inducible antioxidant system consisted of enzymatic elements including superoxide dismutases, which convert superoxide to oxygen peroxide, catalase and glutathione peroxidase, which convert hydrogen peroxide to water, and non-enzymatic scavengers such as glutathione [4]. Our data indicated that the deduction and/or enhancement in superoxide dismutase activity were parallel to the transient elevation and subsequent reduction in intracellular ROS contents, respectively. In agreement with these findings, it could be suggested that MPA provides the requisite intracellular ROS level for hemoglobinization via targeting the antioxidant enzymatic activity of SOD and then, the activity of superoxide dismutase was augmented probably to protect cells from the deleterious effects of this temporal elevated ROS level toward the late stage of erythroid differentiation. Similarly, examination of the catalase activity showed the distinct role of CAT enzyme in protection of K562 cells against the oxidative stress in late stage of erythroid differentiation. Based on our results, glutathione peroxidase, another antioxidant enzyme, also scavenged hydrogen peroxide at the initial times of MPA-triggered erythroid differentiation. Collectively, it could be concluded that although MPA is capable of regulating the activity of the antioxidant enzymes (GPX, CAT and SOD); however it inhibited the activity of superoxide dismutase to provide the appropriate and adequate oxidative environment for hemoglobinization.

Thiol-based antioxidants (GSH, Trx) are a sub group of non-enzymatic endogenous antioxidants, which are implicated in adjusting the intracellular ROS level. The reduced forms of GSH and Trx provide a large portion of the intracellular reducing power available to the cell [19,20]. In this study, we examined the GSH content during erythroid differentiation and observed that the increase in GSH content was accompanied with reduction in the intracellular ROS level at initial time and late stage of MPA-induced erythroid differentiation. Regarding our results indicating significant enhancement in the GSH

**Figure 5:** Evolution of Trx in Erythroid Differentiation. Cells were treated with MPA (0.5 μM) and the protein content of Trx was determined at different time intervals by immunoblotting, using the relevant monoclonal antibody.

**Figure 6:** Modulation cellular p27 content during erythroid differentiation. Cells were treated with MPA (0.5 μM) and the protein content of p27 (A) was determined after 3, 8, 24, 48 and 72 h of exposure to MPA using immunoblotting technique. (B), The intracellular protein levels of p27 among the cells pre-treated for 3h with NAC followed by exposure to MPA for the indicated times using immunoblotting technique.
content and GPX activity in the early time of MPA-induced differentiation process, it could be concluded that the glutathione system plays a key role in manipulation of the intracellular redox status at initial time of erythroid differentiation. The probable reasons for the involvement of GSH at initial time of MPA-induced erythroid differentiation might be due to the fact that GSH is the most abundant endogenous antioxidant [29] and secondly, the fact that its synthesis is not regulated at transcription level [30]. Furthermore, examination of the TRX content revealed that in addition to GSH, CAT and SOD, TRX is also implicated in protection against oxidative stress in the late stage of MPA-triggered erythroid differentiation.

The p27\(^{kip1}\) inhibitor arrests cells in G1 phase partly through inhibition of CDK2 and CDK4 kinase activities which are required for transition from G1 phase to the S phase. In malignant situation such as hematopoietic malignancies, p27\(^{kip1}\) is generally inactivated leading to potentiation of tumor progression [11]. This view is further supported by Leon et al. has shown that induction of p27\(^{kip1}\) in K562 cells has resulted in erythroid differentiation [31]. Based on these facts, we assessed the level of p27\(^{kip1}\) during MPA-triggered erythroid differentiation. As our results showed, p27\(^{kip1}\) expression level decreased in correlation with temporal elevation in ROS content while reduction of the ROS level by NAC resulted in higher levels of p27\(^{kip1}\). Thus, it could be concluded that within the cells, p27\(^{kip1}\) acts as a sensor of oxidative stress during MPA-triggered erythroid differentiation. In HT29 cells, the expression of p27\(^{kip1}\) has been reported to be regulated by hypoxia-inducible factor 1a (HIF-1a) [14].

Conclusion

In conclusion, our results revealed that MPA-triggered erythroid differentiation is highly dependent and manipulated by the redox state of the cells, which is controlled mainly by both the cellular SOD and glutathione contents. Furthermore, while confirming the implication of p27\(^{kip1}\) in erythroid differentiation, our results indicated that p27\(^{kip1}\) acts as a sensor of the intracellular ROS content supporting the initiation and completion of the differentiation process in K562 cells under the influence of MPA [32].

Competing Interests

The authors declare that they have no competing interests.

Acknowledgements

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