Ascorbic acid inhibits TPA-induced HL-60 cell differentiation by decreasing cellular H_2O_2 and ERK phosphorylation

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Abstract. Retinoic acid (RA), vitamin D and 12-O-tetradecanoyl phorbol-13-acetate (TPA) can induce HL-60 cells to differentiate into granulocytes, monocytes and macrophages, respectively. Similar to RA and vitamin D, ascorbic acid also belongs to the vitamin family. High-dose ascorbic acid (>100 μM) induces HL-60 cell apoptosis and induces a small fraction of HL-60 cells to express the granulocyte marker, CD66b. In addition, ascorbic acid exerts an anti-oxidative stress function. Oxidative stress is required for HL-60 cell differentiation following treatment with TPA, however, the effect of ascorbic acid on HL-60 cell differentiation in combination with TPA treatment remains to be fully elucidated. The aim of the present study was to investigate the cellular effects of ascorbic acid treatment on TPA-differentiated HL-60 cells. TPA-differentiated HL-60 cells were used for this investigation, this study and the levels of cellular hydrogen peroxide (H_2O_2), caspase activity and ERK phosphorylation were determined following combined treatment with TPA and ascorbic acid. The results demonstrated that low-dose ascorbic acid (5 μM) reduced the cellular levels of H_2O_2 and inhibited the differentiation of HL-60 cells into macrophages following treatment with TPA. In addition, the results of the present study further demonstrated that low-dose ascorbic acid inactivates the ERK phosphorylation pathway, which inhibited HL-60 cell differentiation following treatment with TPA.

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Introduction

HL-60 cells, belonging to human leukemia cells, have been widely used for differentiation investigations. Previous studies have revealed that HL-60 cells can be induced to differentiate into granulocytes, monocytes and macrophages by treating the cells with various agents, including retinoic acid (RA), dimethyl sulphoxide (DMSO), vitamin D and 12-O-tetradecanoyl phorbol-13-acetate (TPA) (1,2). Several previous studies have indicated that the mitogen-activated protein kinase (MAPK) signaling pathways, c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), are important for HL-60 cell differentiation (3-7). RA and DMSO can induce HL-60 cells to differentiate into granulocytes via the ERK phosphorylation signaling pathway (8-10), vitamin D can induce HL-60 cells to differentiate into monocytes via the EKR, JNK and p38 phosphorylation signaling pathways (4,11,12) and TPA can induce HL-60 cells to differentiate into macrophages via the ERK phosphorylation signaling pathway (13,14). In addition, previous studies have demonstrated that ERK5 is associated with vitamin D-differentiated HL-60 cells, while ERK/1/2 is associated with TPA-differentiated and RA-differentiated HL-60 cells (14-17). These previous reports indicated that ERK5 phosphorylation is required to differentiate HL-60 cells into monocytes, while ERK1/2 is required to differentiate HL-60 cells into granulocytes and macrophages.

The MAPK signaling pathways, protein kinase C (PKC) and oxidative stress may also be associated with

HL-60 differentiation (18-21). The activation of PKC is observed in RA-, vitamin D- and TPA-differentiated HL-60 cells (13,14,22,23). However, oxidative stress can affect HL-60 cell differentiation. A previous study revealed that antioxidants, catalase, superoxide dismutase and N-acetyl cysteine increase the differentiation rate of vitamin D-treated HL-60 cells (24). However, compared with vitamin D-treated HL-60 cells, antioxidant inhibits cell differentiation in TPA-treated HL-60 cells (25). Therefore, oxidative stress exerts a dual role to promote vitamin D-differentiated cells and to inhibit TPA-differentiated cells.

It is well known that RA and vitamin D can induce HL-60 cells to differentiate into granulocytes and monocytes, respectively. As with RA and vitamin D, ascorbic acid is also a type of vitamin. Previous studies have demonstrated that ascorbic acid can activate the ERK signaling pathway to induce progenitor cell differentiation (26,27). Additionally, several studies have demonstrated that high-doses of ascorbic acid (>100 μM) can activate a caspase cascade to promote radiation-induced and etoposide-induced apoptosis in HL-60 cells (28,29). A previous study also demonstrated that high-doses of ascorbic can induce HL-60 cell apoptosis and induce a fraction of HL-60 cells to express the granulocyte marker, CD 66b (30). By contrast, low-doses of ascorbic acid decreases levels of cellular H_2O_2 and protects HL-60 cells against X ray- and As_2O_3-induced apoptosis (31-34). However, whether ascorbic acid affects TPA-differentiated HL-60 cells remains to be elucidated.

A previous study demonstrated that H_2O_2 may be a secondary messenger associated with cell differentiation (25). Several studies have demonstrated that ascorbic acid exerts anti-oxidative stress functions (35-38) and a previous study demonstrated that ascorbic acid decreases levels of cellular H_2O_2 (39). Previous studies have also reported that the ERK pathway is required for TPA-differentiated HL-60 cells (13,14).

Therefore, the aim of the present study was to determine the cellular effects of treatment with ascorbic acid on TPA-differentiated HL-60 cells.

Materials and methods

Chemicals. An MTT assay kit was purchased from Bio Basic Inc. (Markham, ON, Canada). TPA, ascorbic acid and luminol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ac-DEVD-pNA, a Caspase-3-like substrate, Ac-IETD-pNA, a caspase-8 substrate, and Ac-LEHD-pNA, a caspase-9 substrate, were purchased from Anaspec (San Jose, CA, USA). Fetal bovine serum, RPMI-1640 media, non-essential amino acid, L-glutamine and penicillin/streptomycin were purchased from Gibco Life Technologies (Carlsbad, CA, USA).

Cell line and cell culture. The HL-60 cells were purchased from Bioresources Collection and Research Center (Hsin Chu, Taiwan) and were cultured in Dulbecco's modified Eagle's media, containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 0.1 mM non-essential amino acids. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cell survival rate assay. A total of 3,000 cells were cultured in each well of a 96-well culture dish. The survival rates of the cells in the control group (non-ascorbic acid treated-cells) and the experimental groups (5 μM and 5 mM ascorbic acid-treated cells) were determined for 96 h at 37˚C. Every 24 h, the cells were treated using an MTT assay kit, according to the manufacturer's instructions. Following incubation for 3 h, the absorbance (570 nm) was measured using a multi-well enzyme-linked immunosorbent assay reader (SpectraMax Paradigm Multi-Mode Microplate Reader; Molecular Devices, Sunnyvale, CA, USA). The cell survival rate was calculated using the following formula: $Absorbance_{experimental\ group}$ / $Absorbance_{control\ group}$ x 100%.

Caspase activity assay. Caspase activities in the cells were determined using a substrate cleavage assay, as previous described (40,41). Briefly, the cells were treated with lysis buffer, containing 50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, 1% NP-40 (pH 7.5) and protease inhibitors. The cell pellets were collected by centrifugation at 15,000 x g for 30 min at 4˚C and the quantity of protein was determined using a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, 40 μl of the cell lysates (80 μg total protein) were prepared in 158 μl reaction buffer, containing 20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol, 100 mM HEPES (pH 7.5) and 2 μl fluorogenic caspase substrate (Ac-LEHD-pNA, Ac-DEVD-pNA or Ac-IETD-pNA). The total solution was incubated at 37˚C in the dark. Following incubation for 6 h, fluorogenic substrate cleavage was determined at 405 nm using a FLx800™ fluorescence microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The fold increase of caspase activity was calculated with the following formula: ($Absorbance_{experimental\ group}$ - $Absorbance_{control\ group}$) / $Absorbance_{control\ group}$.

Measurement of H_2O_2. The levels of H_2O_2 in the cells was determined using a lucigenin-amplified method, as described previously (39,42,43). Briefly, the sample (200 μl, containing 10^4 cells) was added to 0.2 mmol/l luminol solution (100 ml). The mixture was then analized using a chemiluminescence analyzing system (CLA-FSI; Tohoku Electronic Industrial Co., Ltd., Sendal, Japan).

Observation of cell morphology and suspension cell counts. Undifferentiated HL-60 cells grow as suspension cells and TPA-differentiated HL-60 cells (macrophages) grow as attached cells (14,44). The morphologies of the suspension cells and attached cells were observed under a phase-contrast microscope (Olympus CK40, Olypmus Corporation, Tokyo, Japan; magnification, x200). The suspended cells located in the media were collected using a pipette, whereas the attached cells remained in the bottom of the culture dish. The cells in suspension were counted using a trypan blue exclusion assay (0.4% in PBS), as described previously (45). Briefly, the media containing the suspended cells were mixed with trypan blue and placed in a CBC Customized Logo Hemocytometer Blood Counting Chamber (VIC Science, Xixiang City, China). The number of cells were then counted under a light microscope (Olympus, CK40; Olympus Corporation).

Western blotting. The cells were treated with lysis buffer and centrifuged at 16,000 x g for 10 min at 4˚C. The proteins

were located in the supernatant layer and were collected, concentrated and determined using a Bradford assay (Bio-Rad Laboratories, Inc.). Equal quantities of protein were separated on a 13.3% SDS-polyacrylamide gel (GHE320 Mini-STD Vertical Gel Electrophoresis Tank; Genepure Technology, Co., Ltd, Taichung, Taiwan). Following separation, the proteins were transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were placed in phosphate-buffered saline (PBS) containing 5% non-fat milk. Following incubation for 2 h at 25˚C, the membranes were washed with PBS. The membranes were further incubated in PBS buffer, containing 5% non-fat milk with primary anti-human monoclonal antibodies to ERK (cat. no. 4965) and p-ERK (cat. no. 4370) (1:400; Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h at 25˚C. Following incubation, the membranes were washed with PBS and incubated with secondary mouse anti-human antibodies (1:2,000; cat. no. 10702-MM01E-50; Sino Biological Inc. Beijing, China) for 1 h at 25˚C. The proteins were detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Waltham, MA, USA)

Statistical analysis. The data were calculated from four independent experiments and are presented as the mean ± standard deviation. Student's t-test was used to analyze the statistical differences. P<0.05 was considered to indicate a statistically significant difference.

Results

High-dose ascorbic acid inhibits HL-60 cell growth, whereas a low-dose does not. Previous studies have demonstrated that a high-dose of ascorbic acid can increase radiation-induced and etoposide-induced apoptosis in HL-60 cells (28,29). Similar to these studies, the present study demonstrated that a high-dose (5 μM) of ascorbic acid inhibited cell growth in the HL-60 cells, whereas a low-dose (5 μM) had no affect on the growth of the HL-60 cells (Fig. 1). As shown in Fig. 1, the cell survival rate was <50% in the high-dose ascorbic acid-treated HL-60 cells at 72 h, however, the survival rate was >90% in the low-dose ascorbic acid-treated HL-60 cells at 96 h. Whether ascorbic acid activates caspase death signals in the HL-60 cells was subsequently investigated. The results demonstrated that activation of caspase-8, caspase-9 and caspase-3 occurred in the high-dose ascorbic acid-treated HL-60 cells, but not in low-dose ascorbic acid-treated HL-60 cells (Fig. 2). These findings indicated that a high-dose of ascorbic acid exerted antitumor activities in the HL-60 cells.

Low-dose ascorbic acid reduces cellular levels of H_2O_2 in TPA-treated HL-60 cells. H_2O_2 is important in cell differentiation (25). The present study demonstrated that the levels of H_2O_2 increased in the TPA-differentiated HL-60 cells, compared with the TPA-treated HL-60 cells (P<0.05; Fig. 3), which suggested that H_2O_2 may be associated with HL-60 cells differentiation by TPA. Anti-oxidative functions of ascorbic acid have been demonstrated (35-38), therefore, the present study further determined whether ascorbic acid reduces the levels of H_2O_2 in TPA-treated HL-60 cells. The results revealed that a low-dose of ascorbic acid inhibited the

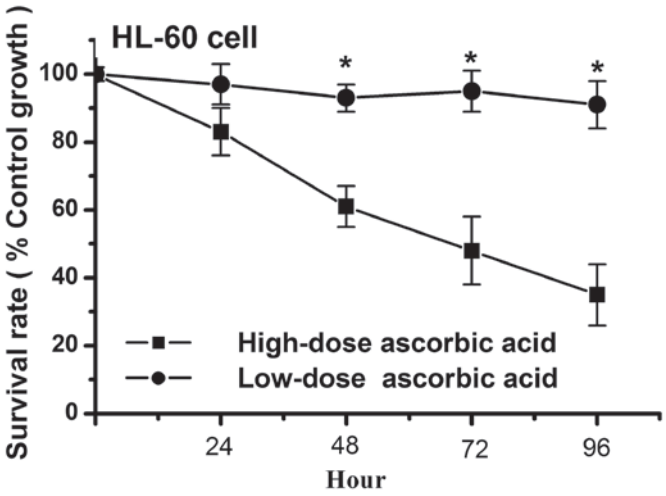

Figure 1. Cell survival rates. The HL-60 cells were treated with a high-dose (5 mM) and a low-dose (5 μM) of ascorbic acid for 96 h. The survival rates were determined using an MTT assay every 24 h. The data were determined from four independent experiments and are presented as the mean ± standard deviation.*P<0.05, compared with the high-dose group.

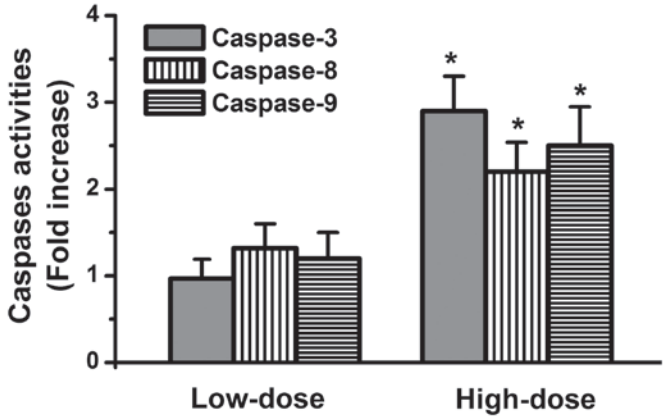

Figure 2. Analysis of caspase activity. The activities of caspase-3, caspase-8 and caspase-9 were examined in the low-dose and high-dose ascorbic acid-treated HL-60 cells at 72 h. The data was measured from four independent experiments and are presented as the mean ± standard deviation.*P<0.05, compared with the low-dose group.

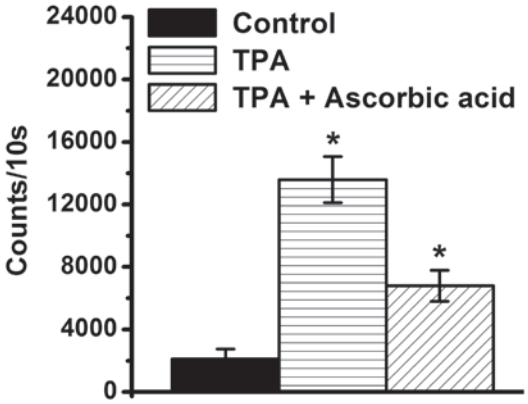

Figure 3. Determination of the levels of H_2O_2. The cellular H_2O_2 levels were counted in the control cells, TPA-treated cells and TPA + ascorbic acid-pretreated cells at 6 h. The H_2O_2 levels were measured using a lucigenin-amplied method. The data was determined from four independent experiments and are presented as the mean ± standard deviation. *P<0.05, compared with the control. TPA, 12-O-tetradecanoyl phorbol-13-acetate.

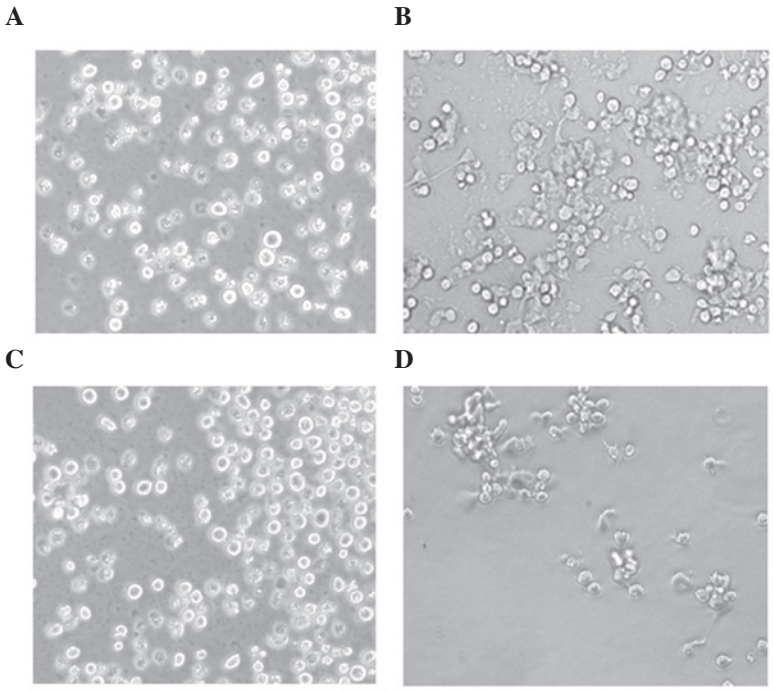

Figure 4. Undifferentiated HL-60 cells and TPA-differentiated HL-60 cells (macrophages). Representative images of the (A) control cells, (B) TPA-treated cells, (C) TPA + ascorbic acid-pretreated cells and (D) TPA + ascorbic acid-post-treated cells (observed at 24 h). The undifferentiated HL-60 cells are suspended cells (A and C) and the differentiated HL-60 cells are attached cells (B and D). Magnification, x200. TPA, 12-O-tetradecanoyl phorbol-13-acetate.

increased levels of H_2O_2 levels the TPA-treated HL-60 cells (Fig. 3).

Pretreatment with ascorbic acid inhibits the differentiation of HL-60 cells into macrophages following TPA treatment. As shown in Fig. 3, a low-dose of ascorbic acid reduced the levels of H_2O_2 in the TPA-treated HL-60 cells. In addition, a previous study demonstrated that H_2O_2 may be an important messenger for cell differentiation (25). Therefore, whether low-dose ascorbic acid inhibits the differentiation of HL-60 cells into macrophages treated with TPA was determined. Previous studies have revealed that TPA-differentiated HL-60 cells (macrophages) are attached cells, whereas the undifferentiated HL-60 cells are suspensed (14,44). The present study assessed the morphology of the cells using a phase contrast microscope, and observed that the control HL-60 cells were in suspension (Fig. 4A) and the TPA-treated HL-60 cells were attached (Fig. 4B). These data suggested that TPA induced the HL-60 cells to differentiate into macrophages. In addition, suspended cells were observed in the TPA-treated HL-60 cells pretreated with ascorbic acid (Fig. 4C), whereas attached cells were observed in the TPA-treated HL-60 cells post-treated with ascorbic acid (Fig. 4D). These data indicated that ascorbic acid pretreatment inhibited the TPA-induced differentiation of HL-60 cells into macrophages, however, post-treatment did not inhibit the ability of TPA to induce HL-60 cell differentiation into macrophages. The number of cells in suspension were also quantified in the present study (Fig. 5). There were ~35,000 suspended cells in the control group and the TPA + ascorbic acid pretreatment group, however, very few suspended cells were observed in the TPA-treated group and the TPA + ascorbic acid post-treatment group, compared with the control group ($P<0.05$; Fig.5) Taken together, these results

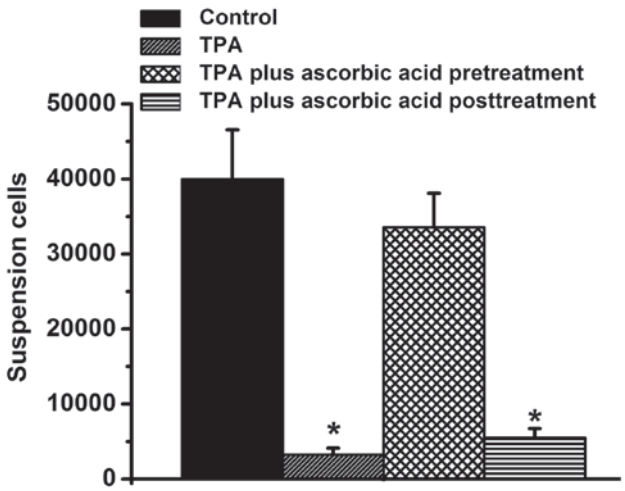

Figure 5. Numbers of cells in suspension. The number of (A) control cells, (B) TPA-treated cells, (C) TPA + ascorbic acid-pretreated cells and (D) TPA + ascorbic acid-post-treated cells were quantified. Undifferentiated cells were considered suspended cells. The data were determined from four independent experiments and are presented as the mean ± standard deviation. *$P<0.05$, compared with the control group.

suggested that pretreatment with ascorbic acid inhibited the ability of TPA to induce the differentiation of HL-60 cells into macrophages.

Ascorbic acid inhibits TPA-induced HL-60 cell differentiation via ERK phosphorylation. Previous studies have revealed that the induction of HL-60 cells to differentiate into macrophages by TPA requires ERK phosphorylation (13,14). These studies demonstrated that the inhibition of p-ERK inhibits TPA-induced HL-60 cell differentiation. In the present study,

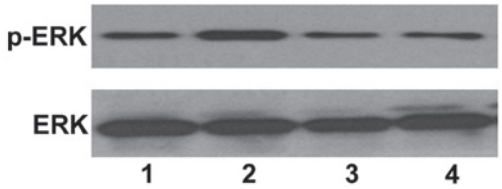

Figure 6. Western blot analysis to determine the phosphorylation of ERK. The protein expression levels of ERK and p-ERK were observed at 30 min in the control (lane 1), TPA-treated (lane 2), TPA + ascorbic acid-pretreated (lane 3) and ascorbic acid-treated cells (lane 4). The levels of p-ERK were increased in the TPA-treated cells, and ascorbic acid-pretreated cells inhibited TPA-induced increases in p-ERK. p-, phosphorylated; TPA, 12-O-tetradecanoyl phorbol-13-acetate; ERK, extracellular signal-regulated kinase.

as shown in Figs. 4 and 5, pretreatment with ascorbic acid inhibited the differentiation of the HL-60 cells into macrophages following TPA treatment. Whether pretreatment with ascorbic acid inhibited TPA-differentiated HL-60 cells via ERK phosphorylation was subsequently investigated, and western blotting revealed that TPA induced an increase in the protein expression of p-ERK (Fig. 6; lane 2). In addition, pretreatment with ascorbic acid reduced the expression of p-ERK in the TPA-treated HL-60 cells (Fig. 6; lane 3). This data suggested that ascorbic acid inhibited the ability of TPA to induce HL-60 cell differentiation via ERK phosphorylation.

Discussion

Previous studies have demonstrated that TPA induces ERK phosphorylation, which in turn causes HL-60 cells to differentiate into macrophages (13,14). In addition, a previous study indicated that H_2O_2 accumulation is important for macrophage differentiation following TPA treatment (25). Similar to previous findings, the present study demonstrated that TPA induced an increase in the levels of H_2O_2 and induced ERK phosphorylation (Figs. 3 and 6). These results suggested that TPA induced HL-60 cells to differentiate into macrophages via the accumulation of H_2O_2 and phosphorylation of ERK. However, the association between H_2O_2 and the phosphorylation of ERK remains to be elucidated. The present study demonstrated that pretreatment with ascorbic acid reduced TPA-induced H_2O_2 accumulation (Fig. 3) and inhibited TPA-induced HL-60 cell differentiation into macrophages (Figs. 4 and 5). However, the data also revealed that post-treatment with ascorbic acid did not have an inhibitory effect of TPA (Figs. 4 and 5). The results of the present study indicated H_2O_2 accumulation as an upstream signal, affecting HL-60 cell differentiation by TPA at an early stage. In addition, several previous studies have demonstrated that H_2O_2 induces the phosphorylation of EKR in various types of cell (46-48). Therefore, the present study indicated that TPA induced an increase in the levels of H_2O_2 initially, and subsequently induced the phosphorylation of ERK, leading to HL-60 cell differentiation. However, pretreatment with ascorbic acid inhibited TPA-induced H_2O_2 accumulation at an early stage, preventing HL-60 cell differentiation.

The dual role of ascorbic acid in promoting cell death and preventing cell damage have been previously reported. Generally, a high-dose of ascorbic acid induces cell cytotoxicity (28,29), whereas a low-dose of ascorbic acid protects cells against oxidative stress-induced damage (32-34). Similar to these studies, the present study demonstrated that a high-dose of ascorbic acid inhibited cell growth and activated the caspase-death pathway in the HL-60 cells (Figs. 1 and 2). However, a low-dose of ascorbic acid reduced TPA-induced increases in H_2O_2 levels (Fig. 3). Therefore, high-dose and low-dose ascorbic acids exerted different mechanisms to affect cell growth. Previous studies have also reported that ascorbic acid induces ERK phosphorylation in various types of cell, including acute myeloid leukemia cells and human endothelial cells (49,50). By contrast, ascorbic acid inhibits ERK phosphorylation in human dermal fibroblasts (51). The present study demonstrated that a low-dose of ascorbic acid inhibited the TPA-induced phosphorylation of ERK (Fig. 6; lane 3). Therefore, it was hypothesized that ascorbic acid induces different signaling pathways to affect cell growth in a dose-dependent and cell-dependent manner.

Regarding the association between ascorbic acid and cell differentiation, several studies have demonstrated that ascorbic acid can promote cell differentiation in various types of cell, including periodontal ligament progenitor cells, osteoblastic cells and embryonic stem cells (26,27,52-56). However, the present study demonstrated that low-doses of ascorbic acid inhibited the HL-60 cells from differentiating into macrophages following TPA treatment. The possible reason may be that TPA-induced cell differentiation requires increases in cellular oxidative stress (25), while ascorbic acid can reduce cellular H_2O_2 levels to inhibit TPA-treated cells. Another possible reason is that ascorbic acid induces a small fraction of HL-60 cells to express the granulocyte marker, CD66b (30) and induces a small fraction of HL-60 cells to differentiate into granulocytes, therefore, inhibiting the differentiation of HL-60 cells into macrophages, induced by TPA.

In conclusion, the present study demonstrated for the first time, to the best of our knowledge, that low-doses of ascorbic acid inhibited TPA-treated HL-60 cells from differentiating into macrophages by decreasing TPA-induced levels of H_2O_2 and ERK phosphorylation.

Acknowledgements

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