Targeting peroxiredoxin I potentiates 1,25-dihydroxyvitamin D3-induced cell differentiation in leukemia cells

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Received February 12, 2015; Accepted December 2, 2015

DOI: 10.3892/mmr.2016.4787

Abstract. Although 1,25-dihydroxyvitamin D_3 (VD3) is regarded as a promising inducing agent for leukemia cell differentiation, it is not as effective an agent as all-*trans*-retinoic acid, and its usefulness is also limited by the adverse effects of hypercalcemia. The aim of the present study was to determine whether combining VD3 with adenanthin, a peroxiresoxin I (Prx I)-targeting natural compound, improves the efficacy of VD3. Cell viability was assessed using a trypan blue exclusion assay and flow cytometry was used to evaluate the expression of cell surface markers, CD11b/CD14, and the level of reactive oxygen species (ROS). Wright's staining was used to examine morphological changes and RNA-interference was used to knockdown Prx I and p65 gene expression. Protein expression was determined by western blot analysis. The results demonstrated that adenanthin markedly enhanced VD3-induced cell differentiation of leukemia NB4 cells, as evidenced by the increased percentage of CD11b- and CD14-positive cells, the mature morphology of the monocytes and the increased phagocytic ability. Consistent with these results, knockdown of Prx I, but not nuclear factor-κB (p65), enhanced VD3-induced cell differentiation. The combinatorial effects of adenanthin and VD3 were shown to be associated with the ROS-CCAAT-enhancer-binding protein (C/EBP)β axis, since *N*-acetylcysteine, a ROS scavenger, was able to abrogate the differentiation-enhancing effects of adenanthin, and the knockdown of C/EBPβ also inhibited the combinatorial effects of adenanthin and VD3. In addition, co-treatment with adenanthin and VD3 was able to induce differentiation in other non-acute promyelocytic leukemia cells and primary leukemia cells. In conclusion, the results of the present study revealed a novel role for Prx I in VD3-induced cell differentiation, and suggested that targeting Prx I may represent a novel strategy to enhance VD3-induced leukemia cell differentiation.

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Key words: adenanthin, 1,25-dihydroxyvitamin D3, leukemia, peroxiredoxin I, cell differentiation

Introduction

Acute myeloid leukemia (AML) is characterized by the blood cells being contained within an abnormal state at an early stage of their development, and their failure to differentiate into functional mature cells. The introduction of all-*trans*-retinoic acid (ATRA) into the regime of acute promyelocytic leukemia (APL) therapy verified the concept of differentiation therapy (1), and this has prompted scientists to search for effective differentiation agents. Similar, and promising, differentiation-inducing effects have also been identified for the physiologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 (VD3) (2-4). Encouraging results from clinical trials have suggested that VD3 may be effective as a differentiation agent in the treatment of AML and myelodysplastic syndrome; however, hypercalcemia has limited its usefulness (5-8). Therefore, two approaches have been adopted to solve this problem: One was to develop novel VD3 derivatives that exhibited similar differentiation effects, although without the adverse calcemia-increasing activity; the other was to use VD3 at a lower concentration in combination with other

agents. Indeed, numerous agents have been demonstrated to enhance VD3-induced cell differentiation (9-15).

Reactive oxygen species (ROS) are broadly defined as a group of highly reactive molecules, which are generated in redox reactions in the cells. The representative ROS are superoxide anion, hydroxyl radical, hydrogen peroxide (H_2O_2) and thiyl radical. Since ROS were identified, the predominant focus has been directed at studying the oxidative damage caused to biological macromolecules, including proteins, DNA and lipids. Previous studies have demonstrated that ROS are able to modulate the activation of signal transduction pathways involved in several cellular functions, including cell differentiation and proliferation (16,17). In the mammalian hematopoietic system, it has been reported that modulating the levels of ROS may be important in terms of the control of myeloid precursor differentiation (17,18). With regards to VD3-induced cell differentiation, numerous reports have demonstrated an involvement of ROS. Various antioxidants, including curcumin, ebselen and silibinin, have been shown to potentiate the differentiation of HL-60 cells induced by VD3 (14,19). However, other groups have reported that increasing the levels of ROS by iron chelation or by inhibiting the antioxidant enzyme catalase, could also enhance VD3-induced monocytic differentiation (20,21). Therefore, the functional role of ROS in VD3-induced cell differentiation remains to be fully elucidated.

Studies from our laboratory demonstrated that adenanthin, a natural diterpenoid, was able to induce differentiation in leukemia cells via targeting the peroxiredoxin I (Prx I)/ROS axis (22,23). Prx I is a small antioxidant protein, which belongs to the peroxiredoxin family and functions primarily to reduce H_2O_2 and other peroxide substrates via conserved cysteine residues using thiol-containing proteins, including glutathione or thioredoxin, as electron donors (24). Currently, no direct evidence has demonstrated whether Prx I exerts a role in VD3-induced cell differentiation. Considering the possible role of ROS in VD3-induced cell differentiation, the present study hypothesized that an increase in the levels of ROS via the targeting of Prx I may regulate VD3-induced cell differentiation.

In the present study, using adenanthin as a chemical tool, it was demonstrated that inhibition of Prx I does enhance VD3-induced cell differentiation. The present study revealed a novel role for Prx I in VD3-induced cell differentiation, and confirmed the hypothesis that targeting Prx I improves the efficacy of VD3.

Materials and methods

Cell culture and agents. The APL cell line, NB4, was obtained from Dr Michel Lanotte (Hospital Saint Louis, Paris, France). Leukemic U937 and HL-60 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in RPMI-1640 medium (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% fetal calf serum (EuroClone S.p.A, Milan, Italy) at 37˚C in a humidified atmosphere containing 5% CO_2. Adenanthin was isolated from the dried aerial parts of *Isodon adenanthus* (Diels) Hara, as described previously (22).

Patient samples. Patients' samples were obtained following the collection of informed consent under a procurement protocol that was approved by the Clinical Investigational Review Board of Shanghai Jiao Tong University School of Medicine (Shanghai, China). Mononuclear cells were isolated according to the manufacturer's instructions (Ficoll-Paque PLUS; 17-1440-03; GE Healthcare, Buckinghamshire, UK) from the bone marrow of one APL patient (female, 29 years old, 46,XX, PML-RARa positive).

Cell differentiation assay. The morphological features of the NB4, HL60,U937 and primary APL cells were examined under a light microscope (BX-51; Olympus, Tokyo, Japan) following Wright's staining (BASO Diagnostic, Inc., Guangdong, China). In addition, cell differentiation was evaluated by the expression of cell surface differentiation antigens, CD11b and CD14. In brief, following treatment with adenanthin and/or VD3, NB4 cells were incubated with monoclonal mouse anti-human PE-conjugated anti-CD14 (cat. no. 557742; BD Biosciences, San Jose, CA, USA; 1:1,000 dilution), monoclonal mouse anti-human FITC-conjugated anti-CD11b (cat. no. 562793; BD Biosciences; 1:1,000 dilution) or isotype-matched control antibody. Following incubation for 15 min at room temperature in the dark, cells were washed twice with PBS and 10,000 cells were analyzed using FACSCalibur (BD Biosciences) and CellQuest software (version 3.0; Becton Dickinson, Mountain View, CA, USA).

Phagocytosis assay. The NB4 cells were treated with adenanthin and/or VD3 for 3 days. Subsequently, 10 μl *Escherichia coli* DH5α (OD_{620}=0.5) cells (Tiangen Biotech, Co., Ltd., Beijing, China) were added to the medium (1 ml), and the culture was incubated for a further 1 h at 37˚C. The cells were washed twice with phosphate-buffered saline (PBS) and spun onto a slide using a Shandon Cytospin cytocentrifuge (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell morphology was examined using Wright's staining.

Trypan blue exclusion assay. NB4 cells were treated with adenanthin and/or VD3 and cell viability was evaluated by trypan blue exclusion assay. In brief, cells were plated in 24-well plates and washed with PBS once, then replaced with PBS, including trypan blue solution at a final concentration of 0.2% (Sigma-Aldrich, St. Louis, MO, USA), and mixed thoroughly. Following standing at room temperature for 5 min, cells were washed with PBS again and then maintained in the well with PBS. The stained and unstained cells in each well were counted under a microscope (Nikon E400; Nikon, Tokyo, Japan). The unstained cells represented viable cells.

Detection of intracellular ROS. The oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen Molecular Probes®; Thermo Fisher Scientific, Inc.), was used to measure the levels of intracellular ROS. H_2DCFDA is deacetylated intracellularly by non-specific esterases, and is further oxidized by cellular peroxides to the fluorescent compound, 2',7'-dichlorofluorescein. Briefly, the NB4 cells were treated or untreated with adenanthin, washed with PBS and incubated with 20 μM H_2DCFDA for 30 min at 37˚C, according to the manufacturer's protocol. The fluorescence signals were detected using a fluorescence-activated cell sorting flow cytometer (FACSCalibur; BD Biosciences). For

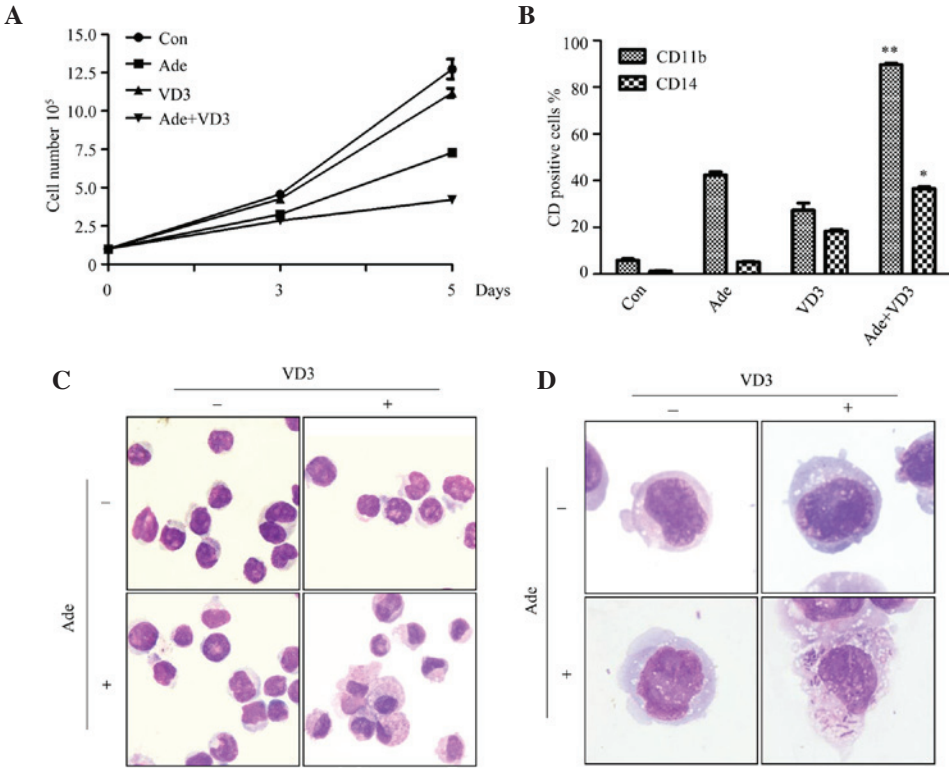

Figure 1. Ade enhances VD3-induced monocyte differentiation of NB4 cells. (A) NB4 cells were treated with 1 μM Ade and/or 1 nM VD3 for the time period indicated, and cell viability was assessed using the trypan blue exclusion assay. (B) Expression of the cell surface markers, CD11b and CD14, was detected by fluorescence-activated cell sorting. All values are presented as the mean ± standard deviation of three independent experiments. *P<0.05; **P<0.01, compared with the single treatment groups (Con, Ade or VD3). (C) Cell morphology was examined using Wright's staining, and the cells were observed under a light microscope on day 3 (magnification, x100). (D) Bacterial phagocytosis assays were performed (magnification, x100). Con, control; Ade, adenanthin; VD3, 1,25-dihydroxyvitamin D_3.

each sample, 5,000 or 10,000 events were collected. The levels of H_2O_2 were expressed in terms of the mean fluorescence intensity.

NAC treatment. NB4 cells were pretreated with 2 mM NAC (A9165; Sigma-Aldrich) for 1 h, then treated with adenanthin in the presence or absence of VD3 for 3 days. The expression of cell surface markers and differentiation-associated proteins was determined.

RNA interference and transfection. Sense sequences against Prx I (5'-AGATATCAGCCTGTCTGAC-3'), nuclear factor-κB (NF-κB; 5'-GATGAGATCTTCCTACTGT-3'), CCAAT/enhancer binding protein β (C/EBPβ; 5'-GCCCTG AGTAATCACTTAAAG-3') and non-target control short hairpin (sh)RNA (NC; 5'-TCCCGTGAATTGGAATCCT-3') followed by the loop sequence (TTCAAGAGA) and the reverse compliment of the targeting sequence, respectively, were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), annealed and ligated into the PSIREN-RetroQ Vector (Clontech Laboratories, Mountainview, CA, USA) according to the manufacturer's instructions of the Knockout™ RNAi Systems User Manual (Clontech Laboratories). The shRNA vectors or negative control pSIREN-RetroQ vector were co-transfected with packaging plasmids VSV-G and Gag-Pol into 293T cells (American Type Culture Collection, Manassas, VA, USA) to produce the retrovirus. Supernatants containing retrovirus were collected 48 h following transfection and were used to infect NB4 cells. After 48 h, 0.5 μg/ml puromycin (Calbiochem, Darmstadt, Germany) was added to the medium for selection and the stable transformants were validated by assessing targeted proteins.

Western blot analysis. Cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS and 10% glycerol). Cell lysates were centrifuged at 20,000 x g for 10 min and the supernatants were collected. Protein concentration in the supernatant was determined by the bicinchoninic acid method according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL, USA) following trichloroacetic acid precipitation. Equal amounts of cell lysates (10 μg protein of each sample) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto an Amersham ECL®-nitrocellulose membrane (GE Healthcare). The membranes were stained with 0.2% Ponceau S red to ensure equal protein loading. Following blocking of the membrane with 5% non-fat milk in Tris-buffered saline, the membranes were incubated with monoclonal mouse anti-human Prx I (cat. no. sc-293386; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; dilution 1:1,000), polyclonal rabbit anti-human p21 (cat. no. sc-397; Santa Cruz Biotechnology, Inc.; dilution 1:1,000), polyclonal rabbit anti-human p65 (cat. no. sc-372; Santa Cruz Biotechnology, Inc.; dilution 1:1,000) and polyclonal rabbit anti-human C/EBPβ (cat. no. sc-150; Santa Cruz Biotechnology, Inc.; dilution 1:1,000) overnight at 4˚C. Subsequently, the membrane

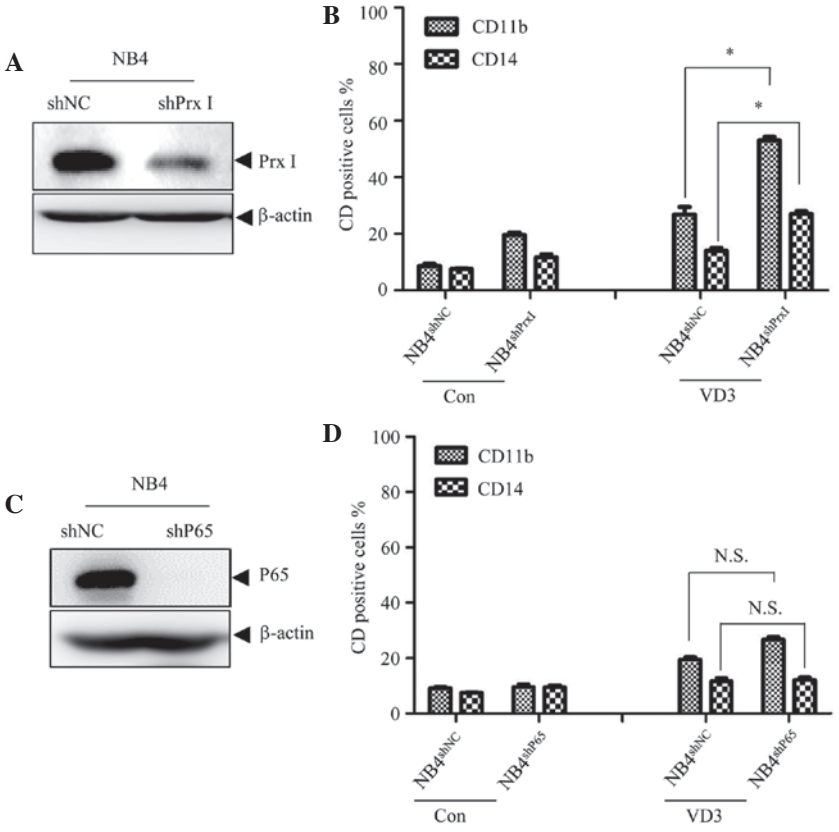

Figure 2. Knockdown of Prx I enhances VD3-induced monocyte differentiation in NB4 cells. (A) NB4 cells were transfected with pSIREN-RetroQ-derived retroviruses carrying Prx I-specific shRNA (shPrx I) or scrambled RNA (shNC), which was used as a control. The expression levels of the indicated proteins were assessed by western blot analysis. (B) Transfected cells were treated with VD3 for 3 days, and the percentage of $CD11b^+$ and $CD14^+$ cells were detected by fluorescence-activated cell sorting. (C) Similarly, p65 was specifically knocked down in NB4 cells, and the protein expression levels were determined by western blotting. (D) Cells were treated with or without VD3, and the percentage of $CD11b^+$ and $CD14^+$ cells were evaluated by fluorescence-activated cell sorting. All data are presented as the mean ± standard deviation of three independent experiments. $^*P<0.05$, compared with the $NB4^{shNC}$ cells. N.S., no significant difference compared with the $NB4^{shp65}$ cells. shRNA, short hairpin RNA; Con, control; Prx I, peroxiredoxin I; VD3, 1,25-dihydroxyvitamin D_3.

was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. 7074; dilution 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and horse anti-mouse IgG (cat. no. 7076; dilution 1:2,000; Cell Signaling Technology, Inc.) secondary antibodies for 1 h at room temperature. Detection of the signal was performed using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Inc.), according to the manufacturer's protocol. β-actin (Merck Millipore, Darmstadt, Germany) was used as an internal loading control. The bands were semiquantified using Quantity One image analysis software (version 4.6.3; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Student's t-test was used to evaluate the difference between two different treatments. $P<0.05$ was considered to indicate a statistically significant value.

Results

Adenanthin plus a low concentration of VD3 induces monocyte differentiation of NB4 cells. To determine whether adenanthin was able to enhance VD3-induced cell differentiation, NB4 cells were treated with either VD3 alone (1 nM) or a combination of VD3 and adenanthin (1 μM) for 5 days. As shown in Fig. 1A, the growth inhibitory effect of adenanthin or VD3 on the NB4 cells was greatly strengthened by their co-administration. Compared with treatment with either adenanthin or VD3 alone, the expression of the cell differentiation surface markers, CD11b and CD14, was markedly increased by co-treatment with adenanthin and VD3 (Fig. 1B), which was accompanied by an enlargement of the cytoplasm, the loss of cytoplasmic basophilia and azurophilic granules, and the appearance of cytological modifications of the monocytes (Fig. 1C). In addition, the differentiated cells acquired functional properties of monocytes, including the engulfment of bacteria (Fig. 1D). These results suggest that adenanthin markedly enhances the VD3-induced monocyte differentiation of NB4 cells.

Knockdown of Prx I enhances VD3-induced monocyte differentiation. Since adenanthin induces leukemia cell differentiation by targeting Prx I, the present study postulated that suppressing the expression of Prx I may also enhance VD3-induced cell differentiation. To this end, the NB4 cells were transfected with non-specific shRNA ($NB4^{shNC}$) and Prx I-specific shRNA ($NB4^{shPrx\ I}$; Fig. 2A). Subsequently, the cells were treated with VD3. Following treatment for 72 h, the percentage of $CD11b^+CD14^+$ cells was significantly

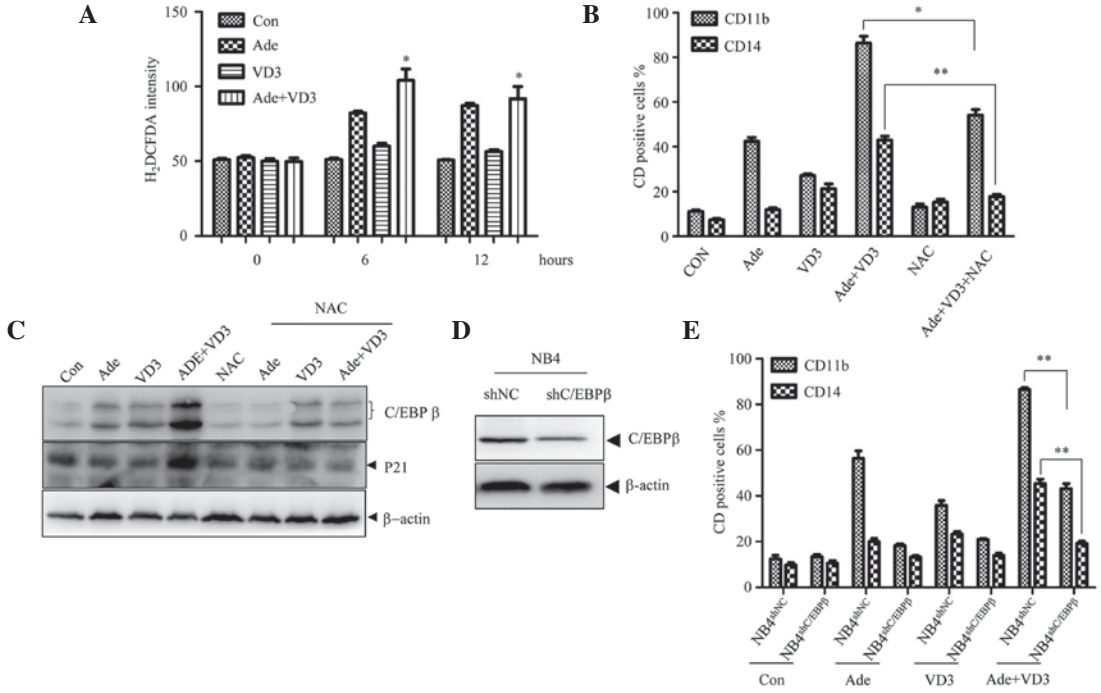

Figure 3. Activation of the ROS-C/EBPβ axis is important in enhancing the effects of Ade on VD3-induced monocyte differentiation. (A) NB4 cells were treated with Ade and/or VD3 for the indicated time periods, and the levels of ROS were determined using the H_2DCFDA staining assay. (B) Following pretreatment with or without 2 mM NAC for 1 h, the NB4 cells were treated with Ade in the presence or absence of VD3, and the percentage of CD11b- and CD14-positive cells was assessed by FACS. (C) Expression levels of the indicated proteins were assessed using western blotting. (D and E) NB4 cells were transfected with pSIREN-RetroQ-derived retroviruses carrying C/EBPβ-specific shRNA ($NB4^{shC/EBP\beta}$) or scrambled shRNA ($NB4^{shNC}$), which was used as a control. (D) Expression levels of the indicated proteins were assessed using western blotting. (E) Subsequently, the $NB4^{shC/EBP\beta}$ cells and $NB4^{shNC}$ cells were treated with Ade and/or VD3 for 3 days, and the percentage of CD11b- and CD14-positive cells was detected by FACS. All data are presented as the mean ± standard deviation of three independent experiments. *P<0.05; **P<0.01, compared with the VD3 and Ade-co-treated $NB4^{shNC}$ cells. Con, control; shRNA, short hairpin RNA; ROS, reactive oxygen species; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; NAC, *N*-acetylcysteine; FACS, fluorescence-activated cell sorting; C/EBPβ, CCAAT/enhancer binding protein β; Ade, adenanthin; VD3, 1,25-dihydroxyvitamin D_3.

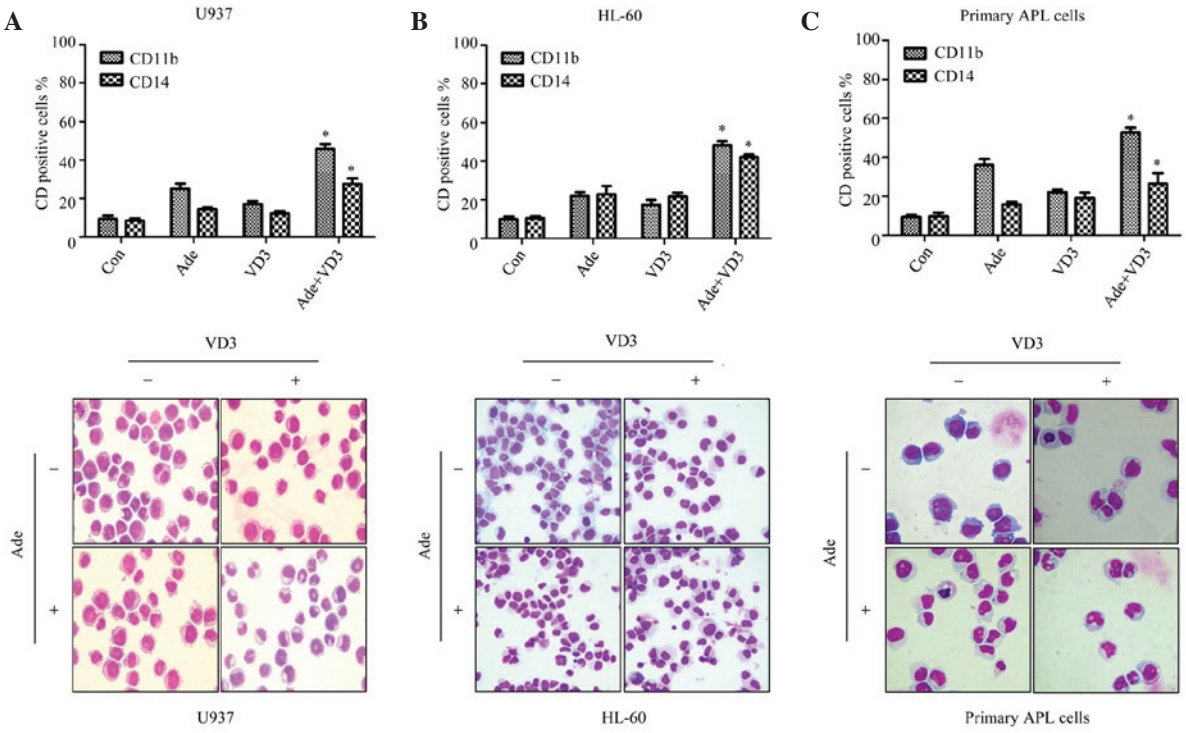

Figure 4. Ade enhances VD3-induced monocyte differentiation in U937, HL-60 and primary APL cells. The (A) U937, (B) HL-60 and (C) primary APL cells were treated with Ade (1 μM) with or without VD3 (1 nM) for 3 days. (A-C) Upper panels: The percentage of $CD11b^{+}$ and $CD14^{+}$ cells was assessed using fluorescence-activated cell sorting. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, compared with the single treatments (Con, Ade and VD3). (A-C) Lower panels: Cell morphology was examined following Wright's staining by observing the cells under a light microscope (magnification, x100). Ade, adenanthin; APL, acute promyelocytic leukemia; VD3, 1,25-dihydroxyvitamin D_3.

increased in the $NB4^{shPrx\ I}$ cells, as compared with the $NB4^{shNC}$ cells (Fig. 2B; $P<0.05$). Since NF-κB has also been demonstrated to be a target of adenanthin, whether inhibition of the NF-κB (p65) pathway also contributed to the cell differentiation-enhancing effects of adenanthin was examined. However, the specific knockdown of NF-κB (p65) expression in NB4 cells (Fig. 2C) did not significantly enhance VD3-induced cell differentiation (Fig. 2D, $P>0.05$). These results indicate that targeting Prx I enhances VD3-induced cell differentiation.

Adenanthin potentiates VD3-induced differentiation via activation of the ROS-C/EBPβ pathway. Since Prx I functions as a H_2O_2 scavenger (22), the present study determined whether ROS may have an important role in the differentiation-enhancing effects of adenanthin on VD3. As shown in Fig. 3A, adenanthin treatment alone led to an increase in the levels of ROS in the NB4 cells, and the levels were further increased by co-treatment with VD3. These results suggest the ROS is involved in the combinatorial effects of adenanthin and VD3. In support of this hypothesis, *N*-acetylcysteine (NAC), a ROS scavenger, significantly inhibited the adenanthin plus VD3-induced upregulation of CD11b and CD14 (Fig. 3B). Since C/EBPβ has been shown to exert an important role in monocyte differentiation, the expression levels of C/EBPβ were detected following co-treatment with adenanthin and VD3. Treatment of the cells with adenanthin or VD3 alone increased the expression of C/EBPβ, whereas co-treatment of the cells with adenanthin and VD3 led to a further increase in the expression levels of C/EBPβ (Fig. 3C). The expression levels of p21, a downstream target of C/EBPβ, were also upregulated. Notably, in the presence of NAC, the effects of co-treatment with adenanthin and VD3 on C/EBPβ expression were abrogated (Fig. 3C). In addition, the knockdown of C/EBPβ abrogated adenanthin plus VD3-induced cell differentiation (Figs. 3D and E). These results suggest that adenanthin may enhance VD3-induced cell differentiation via the ROS-C/EBPβ axis.

Adenanthin potentiates VD3-induced differentiation in U937, HL-60 and primary leukemia cells. To examine whether the combinatorial effects of adenanthin and VD3 were cell-type-specific, U937, HL-60 and primary APL cells were treated with adenanthin and/or VD3. Adenanthin plus VD3 also enhanced monocyte differentiation of the U937, HL-60 and primary APL cell lines, as determined by the increase in the number of CD11b- and CD14-positive cells (Fig. 4A-C, the upper panels) and the appearance of the monocytes' morphology (Fig. 4A-C, the lower panels). These results suggest that the combinatorial effects of adenanthin and VD3 are not limited to NB4 cells.

Discussion

Improving the efficacy and decreasing the side effects of VD3 has recently garnered a lot of attention in the scientific community. The present study demonstrated that adenanthin was able to promote low dose (1 nM) VD3-induced cell differentiation via the Prx I-ROS-C/EBPβ axis. Therefore, the present study provides a basis for targeting Prx I to improve the efficacy of VD3 in leukemia cells.

In mammalian cells, the homeostasis of ROS is controlled by a diverse set of peroxidases, including catalase, glutathione peroxidases and peroxiredoxins (25). The levels of ROS change according to the differentiation status of hematopoietic cells (26); therefore, modulating the expression or activity of these enzymes may regulate cell differentiation. For example, in inducing the monocytic differentiation of U937 cells, treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) was shown to decrease the expression of catalase and increase the levels of ROS, whereas overexpression of catalase inhibited TPA-induced differentiation (18). Conversely, Ding *et al* (27) reported that catalase co-operates with ATRA to induce the differentiation of human THP-1 monocytes into macrophages via a reduction in the generation of H_2O_2. Therefore, these results suggested that the functional role of ROS in cell differentiation may be dependent on the nature of the differentiation induction. In terms of VD3, Callens *et al* (21) demonstrated that an increase in ROS enhanced VD3-induced cell differentiation, whereas Bondza-Kibangou *et al* (19) reported that antioxidants, including catalase, enhanced VD3-induced cell differentiation. Therefore, further studies are required to fully delineate the role of ROS in VD3-induced cell differentiation.

Peroxiredoxins comprise a family of six ubiquitous peroxidases that reduce peroxides; their major functions include protection against oxidative stress, and the induction of cell signaling and proliferation (28). Compared with catalase, peroxiredoxins are more abundant, and have a higher degree of affinity for H_2O_2. An aberrantly high expression of peroxiredoxins has been detected in various types of cancer, which may contribute towards resistance to chemotherapy or radiotherapy (24,29). A previous study in our laboratory demonstrated that adenanthin is a Prx I inhibitor that may induce partial differentiation of APL cells in a ROS-dependent manner (22). However, whether the modulation of Prx I may alter VD3-induced cell differentiation has yet to be elucidated. The results of the present study demonstrated that targeting Prx I by adenanthin or RNA interference enhanced VD3-induced cell differentiation. On the basis of these findings, it is proposed that an increase in the levels of ROS by targeting antioxidant enzymes, such as Prx I, may represent a novel method for enhancing the VD3-induced monocyte differentiation of leukemia cells.

C/EBPβ, which is a basic leucine zipper transcription factor, is a member of the C/EBP family (30). C/EBPβ is known to have an important role in the control of monocytic differentiation (31). It has previously been reported that VD3 enhances the mRNA and protein expression levels of C/EBPβ via activation of Raf, mitogen-activated kinase kinase/extracellular signal-regulated kinase and c-Jun N-terminal kinase (32,33). In the present study, adenanthin plus VD3 was shown to markedly increase the expression levels of C/EBPβ, and knocking down C/EBPβ expression inhibited adenanthin plus VD3-induced cell differentiation, thus suggesting that C/EBPβ fulfills a critical role in the combinatorial effects of adenanthin and VD3. However, the precise mechanisms underlying C/EBPβ upregulation remain to be fully elucidated. The present study demonstrated that increases in ROS were associated with the upregulation of C/EBPβ, since NAC, a well-known ROS scavenger, markedly inhibited the combinatorial effects of adenanthin and VD3 on C/EBPβ expression. Therefore, the

activation of the ROS-C/EBPβ axis has been demonstrated to fulfill an important role in monocytic differentiation, which may provide the basis for further studies to combine low doses of VD3 with other ROS-increasing agents in order to induce leukemia cell differentiation.

In conclusion, the present study demonstrated that adenanthin was able to enhance VD3-induced cell differentiation in leukemia cells via activation of the Prx I-ROS-C/EBPβ axis. These results suggested that targeting Prx I may be considered a novel strategy to enhance the efficacy of VD3. The development of novel Prx I-targeting agents warrants further investigation.

Acknowledgements

The present study was supported in part by grants from the National Basic Research Program of China (973 Program) (grant nos. 2015CB910403 and 2013CB910903), the National Natural Science Foundation of China (grant nos. 91313303, 81272886 and 31100980), the Science and Technology Committee of Shanghai (grant nos. 11JC1406500, 13431900501 and 13ZR1456900), the Shanghai Talent Development Project of Shanghai Human Resource and the Social Security Bureau.

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